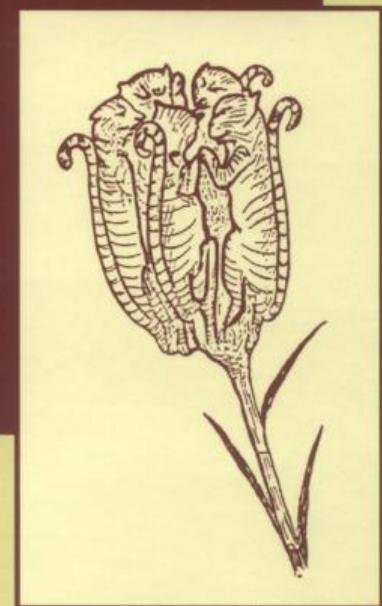




AGRICULTURAL BIOTECHNOLOGY

EDITED
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This edition published in the Taylor & Francis e-Library, 2006.

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ISBN 0-203-07071-2 Master e-book ISBN

ISBN 0-203-22306-3 (Adobe eReader Format)
ISBN: 0-8247-9439-7 (Print Edition)

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the address below.

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To my family,
Yehudith, Orly, and Ron

Preface

The expansion of biotechnology in the medical arena often overshadows the emerging role of agricultural biotechnology. However, many more people around the world die from famine and malnutrition than from "Western" diseases. With the human population expected to reach 10 billion by the year 2050, the challenges facing agriculture and food research are enormous. Therefore, there is an urgent need to intensify our development of agricultural biotechnologies.

While established agricultural techniques, such as breeding and growth control have resulted in remarkable achievements since the dawn of plant and animal domestication, new methodologies are clearly required. Emerging biotechnologies have seen dramatic developments during the last decade and should be able to overcome and complement the limits of former standard procedures.

This handbook contains 36 chapters that are grouped within the four major areas of modern agricultural biotechnology (excluding food biotechnology):

1. *Biotechnology of plants and specific crops*, including genetic engineering for yield and quality traits, metabolite production, rapid clonal propagation, and germplasm conservation.
2. *Microbial agro-biotechnology*, including new approaches to plant pest control, biofertilization, bioremediation, and fermentation of agricultural products.
3. *Livestock biotechnology*, including genetic manipulations for improving fertility and reproduction, growth, milk quality, egg production, disease control, and gene-pharming.
4. *Marine biotechnology*, including the use and improvement of fish, macro- and micro-algae for food and biochemical production and environmental uses.

All these domains are concerned with food production and the conservation of our environment. They share a search into the regulation of genes that are responsible for specific qualitative or quantitative traits. As with any new revolutionary technique, agricultural biotechnology should be continuously monitored and regulated as to its effect on humanity.

and the environment. Therefore, an opening chapter on developmental trends of agricultural biotechnology towards the 21st century, as well as specific chapters on the legal, public, moral, and environmental effects of agricultural biotechnology, are also included in the book.

The chapters of this book are intended to provide a cross-section of the up-to-date accomplishments of agricultural biotechnologies and its envisaged directions into the future. Some of the topics were given more weight than others, because of technical limitations. The book deals comprehensively with most of the major aspects of agricultural biotechnology. As such, it is addressed mainly to graduate level students and to professional scientists in each of the mentioned disciplines who seek to expand their knowledge to other related biotechnologies. It is my hope that this volume will contribute to the needs and future prospects of all agricultural biotechnologies that are so critical to supporting the ever-increasing population on this planet, while keeping our environment clean and safe.

Biotechnology is a most powerful tool to further advance the various fields of agriculture. However, it can work practically only if it can be combined with established breeding strategies and with common agricultural practices. In fact, all agricultural biotechnologies should be looked upon as an extension and integral part of traditional breeding and agriculture, contributing successfully to shortening the breeding cycle, and to production of quality agricultural commodities.

The basic biotechnological methodologies are available. They need to be adapted and oriented to the specific needs of agriculture. They should be integrated with a better comprehension of the physiology and metabolism of the organism in question, and standard agricultural practices.

I wish to express my appreciation to all contributing authors. Each chapter was written by distinguished scientists who have made significant contributions and are pioneers in the field of agricultural biotechnology. The various articles present the opinion of the authors and their points of views. While every effort was made to reach uniformity in style, the presented results and the expressed ideas and final shape of the chapters remain the sole responsibility of the respective authors.

I am especially grateful to Rita Colwell, Neal First, Jeff Schell, and Indra Vasil, members of the International Advisory Editorial Board, for their critical and unequaled help in selecting the authors and organizing this book. Thanks are also due to many of my colleagues and friends for their advice and collaboration, and to Marcel Dekker, Inc. for technical assistance.

Finally, with love and affection, I am indebted to my family for their patience and understanding.

Arie Altman

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AGRICULTURAL

BIOTECHNOLOGY

Agriculture and Agricultural Biotechnology: Development Trends Toward the 21st Century

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I. AGRICULTURAL BIOTECHNOLOGIES AND BREEDING: A HISTORICAL PERSPECTIVE

Agriculture, has evolved since the dawn of human civilization, first as a means to guarantee food supply and, then, as a source of family income and improved profitability [1]. Domestication of plants and animals that were found in the wild, combined with gradual, long-term changes in their quality and quantity, were the first signs of what is now collectively termed "agriculture." At that time, agriculture replaced the former nomadic habit of food collection for immediate consumption [2]. A continuous effort to improve plant and animal yield was already documented in the ancient scriptures of many nations of the Old and New World. The documented history of all cradles of civilizations—Mesopotamia and the Lands of the Bible, Egypt and China, as well as in Africa, South and Central America, and Greece—contain detailed records of agricultural activities and improvement of plant and animal productivity [3]. The experiments carried by Jacob, the father, provided strong evidence that he succeeded in selecting for spotted, colored sheep against brown sheep by using classic breeding. The spotted, colored sheep made him rich because he could specify them as his own herd, distinct from that of his father-in-law, Lavan (Gen. 30:32–43). Noah's ark was perhaps the first documented "breeding institution," on which males and females of all known living creatures were given the chance to survive, breed, and produce the necessary number of offspring for selection of future generations (Gen. 6:18–20).

Domestication of plants and animals, followed by food storage, coincided, most probably unintentionally, with the growth of microorganisms [4]. Thus, was born classical food fermentation, the earliest known application of biotechnology, focusing on the use of microorganisms to produce food products. The technique is well documented for beer brewing, wine production, and bread baking, in various archeological relics and scriptures of the ancient civilizations of Egypt, China, and the Lands of the Bible. Wine consumption was already

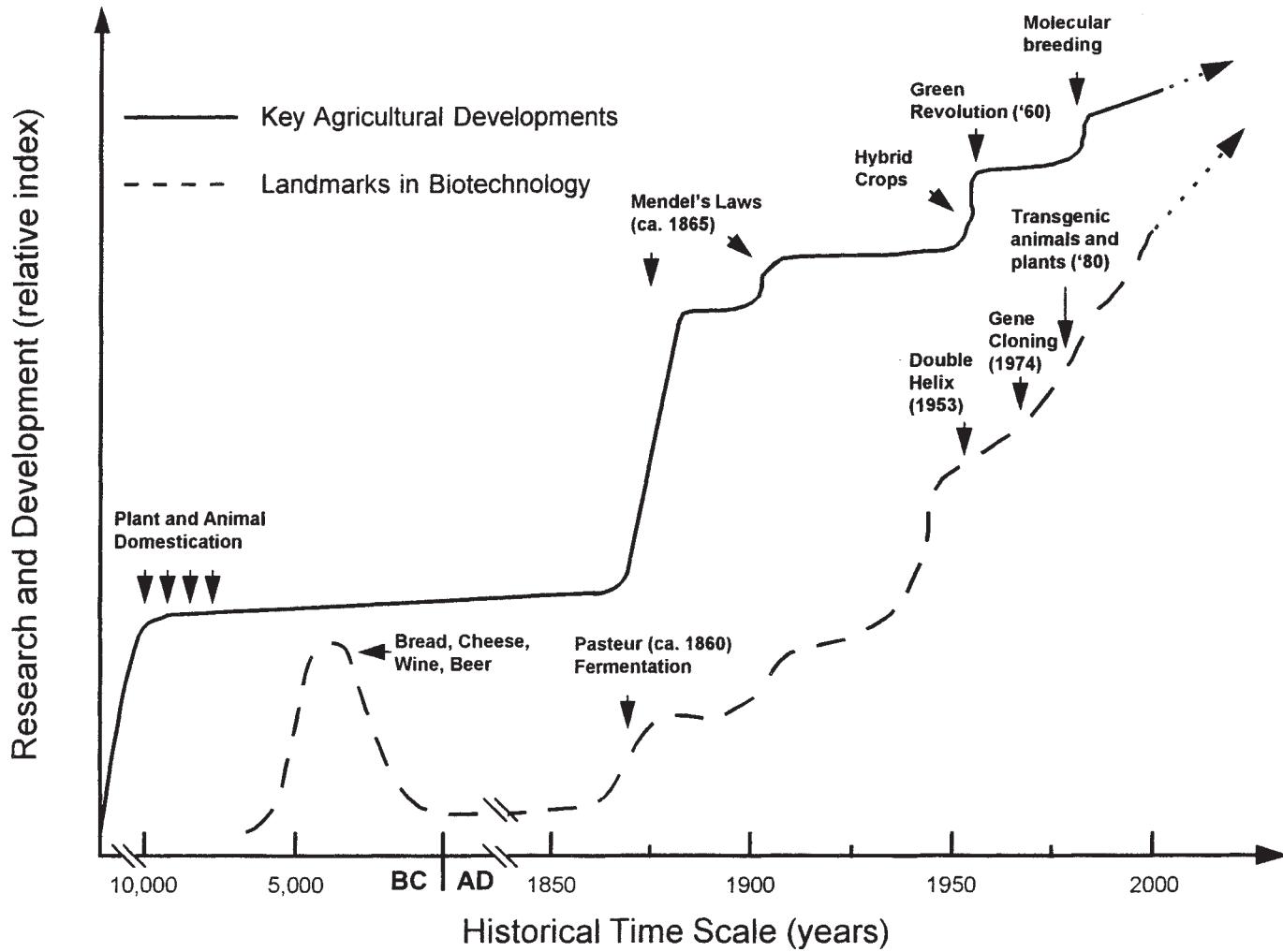
suggested, thousands of years ago, by Lot's daughters to "make our father drunk" (Gen. 19:30–38). Cheese, yogurt, vinegar, soy sauce, and bean curd, are additional examples of traditional uses of microorganisms in biotechnology for both food and industrial applications. A long road was crossed in terms of the use of microorganisms in biotechnology by the time the U.S. Supreme Court ruled in 1980 in favor of patented microorganisms in the case of *Diamond v. Chakrabarty* [5,6]. This verdict opened a new commercial avenue for fermentation as a major tool in genetic engineering [1,2,6].

Gradual improvements in agricultural techniques, domestication of additional plant and animal species from the wild, step-by-step selection of better-performing and more-adapted genotypes, along with intuitive breeding, continued at a slow pace. Mendel's discoveries and laws, in the period of the 1860s, revolutionized genetics, and led at the beginning of the 20th century, to planned, controlled breeding experiments [1]. Many achievements during this century and toward the end of the 1970s have been successfully implemented since then [2–4,6]. Breeding, or the "old" biotechnology, was now harnessed as a most successful tool, resulting in better crops and farm animals. This rapid scientific development has significantly improved agricultural yields and product quality, to better support the increasing human demands for a variety of foods. The selection of cereal subspecies with shorter stalks and the isolation of high-yield rice with better growth capacity are just two examples of successful achievements of the "green revolution" of the 1960s [1–4,6,7]. More productive genotypes of corn, cereals, rice, and legumes, as well as cattle and poultry, enabled individual farmers and agricultural enterprises alike to provide more food. In addition they received better financial returns for their agricultural activities [1–4,6–8]. Soon, this old biotechnology was revolutionized again, when DNA was cloned in 1973 [1,3]. Toward the end of the 1970s and during the early 1980s, recombinant DNA technologies resulted in the first development of transgenic microorganisms and, subsequently—animals and plants. The novel scientific discoveries and techniques thus paved the way for the "new" agricultural biotechnology [9]. This historical development is schematically illustrated in [Figure 1](#).

II. THE LIMITATION OF TRADITIONAL AGRICULTURE IN MEETING LAND, ENVIRONMENTAL, AND ECONOMIC CONSTRAINTS

Traditional agriculture suffers from several serious limitations in facing current changes in international markets. The world transition to a global village, and the increased flow of information, are changing the market conditions [6–9]. Local pricing policies and growers'

Fig. 1 An historical perspective of "old" and "new" agricultural biotechnologies. Both key agricultural developments (referring mainly to "traditional" agriculture) and landmarks in biotechnology (relevant to all living organisms) are depicted on the historical time scale. Only major achievements are indicated, and many other important scientific and technological contributions have been omitted. The three major developments in traditional agricultural start with plant and animal domestication over a long period of time, continues with the era of scientific breeding (Mendel's laws and their rediscovery and implementation at the beginning of the 20th century), until the production of commercial hybrid crops and the green revolution. From there began the era of molecular breeding. The three major landmarks in biotechnology include ancient fermentation for food production, the introduction by Pasteur of modern scientific fermentation and the subsequent related processes, and the "burst" of molecular breeding and gene cloning. Commercial growing of transgenic plants became a reality in 1993–1995, following field experiments since 1985. The discovery of new continents during 1500–1600 AD brought about extensive exchange and introduction of new agricultural crops and animals, another important landmark in agriculture.



profitability are no longer effective, except in certain "closed" communities, and they are increasingly affected by the volume of international trade and international prices of food and other agricultural products [9,10]. This is in addition to the limitations of classic breeding (see later).

Agriculture, being strongly dependent on the availability of natural resources, is also facing new conditions of reduced land and water availability. This is aggravated by increased soil, water, and air quality deterioration, owing to global climatic changes, desertification, pollution, and industrialization. In the more developed countries, but also in many developing countries, rapid industrialization and urbanization already cause a severe competition over land. Regions best suited to agriculture, are turning into real-estate and are becoming targets of "developers." The problem is international and affects Europe, America, the Far East, and the Middle East, [2,6,10,13]. Partial solution is offered by the policy of European countries that issued rules and allocated financing for saving "green belts" around urban centers [9].

Another way of circumventing some of the problems is technological development of greenhouses, use of solar energy, development of innovative irrigation techniques, and novel applications of water desalination and recycling. The greenhouse technology, otherwise known as "protected agriculture," was developed originally as a means to improve plant quality and to extend crop harvest and marketing beyond the traditional regional seasons [10,13]. Today, it is also an increasingly important tool for utilizing previously unsuitable land for agriculture. Similar trends are evident for cattle and poultry husbandry in climate-controlled structures and shelters, enabling their productivity in regions that were previously unfavorable. Classic breeding provided improved plant and animal species suitable for growing under unfavorable environmental conditions, such as saline and arid lands [2,3,6,10,13]. These stress-tolerant genotypes can be exploited to their utmost when grown in controlled structures and supplied with desalinized or recycled water, or with improved feedstuff, thereby expanding agriculture in marginal regions [14]. This avenue of research dramatically improved the ability of modern agriculture to face the challenges of industrialization and environmental changes [2,6,7,10]. The research and development of greenhouses for plants (mostly vegetables and ornamentals), and protected structures for farm animals and poultry, are continuously progressing: advanced greenhouses are fully computerized, and various environmental sensors are capable of sustaining the optimal conditions of humidity, light, temperature, pH, irrigation, and planting [10,13]. At this stage, the investments required for such developments are still skyrocketing. The resulting production costs endanger their continuous development. In addition, advanced controlled agriculture relies on highly educated growers [1,13].

Food production to sustain the expected large increase in world population in the 21st century in developing countries around the globe cannot, however, rely solely on protected agriculture [10,13]. Pastures, most cereals and legumes, and forests cannot be maintained as environmentally protected agriculture. Their survival, and indeed their continuously increasing productivity, rely on classic and new breeding methods (i.e., on plant and animal biotechnology) [4]. This is in addition to alternative sources of food and products that will become increasingly important in the 21st century, such as aquaculture and marine biotechnology [2].

III. COMBINING THE NEW BIOTECHNOLOGY WITH CLASSIC BREEDING IS A MEANS OF PROMOTING AGRICULTURE BEYOND THE YEAR 2000

Definitions of biotechnology differ among individuals. The term *new biotechnology* usually applies to the use of recombinant DNA technology. *Biotechnology* itself has a broader definition,

covering any technique that involves the use of biological species and biomass, or their derivatives, for the generation of beneficial products [6,7]. In this respect, classic breeding can be considered synonymous with “old biotechnology.” The comprehensive definition of biotechnology, both old and new, as defined by the U.S. Congress in 1984, indeed states that it is “any technique that uses living organisms, or part of organisms, to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses” [6,7–9].

Classic breeding has not yet exploited its potential to improve plant and animal productivity nor their proper use in agriculture, for production of food and various commodities. Classic breeding and selection for pest resistance has resulted in better yields. Selection for genotypes that are more tolerant to extreme climates, water, and soil conditions, has just begun [1,3,7]. Moreover, even classic breeding of plants and animals now relies on the newly developed DNA biotechnologies that permit one to detect successful genotypes, and enable better selection of favorable traits [2,8,15]. In fact, molecular biology resulted in the generation of revolutionary powerful and precise breeding tools. There are many examples of successful integration of these new biotechnologies in classic breeding. Among these we may list the microscopic visualization of chromosomes in tissue culture, the fluorescent *in situ* staining by hybridization (FISH) for fluorescent labeling of chromosome markers in cells, restriction fragment length polymorphism (RFLP) for the isolation of DNA markers, development of the simple-sequence repeat (SSR)/DNA markers for isolation of important plant traits, and the use of Southern and western blot techniques to validate successful trait isolation. All of these genetic techniques constitute an integral part of classic breeding, contributing successfully to shortening the breeding and selection cycle, and significantly accelerating breeding of plants and animals [7–9,15].

The new biotechnology, as discussed in this book, refers to the use of recombinant DNA and *in vitro* biological techniques in three major areas: (1) as powerful tools in classic breeding, (2) as means for generating transgenic plants, animals, and other organisms, and (3) as a means of integrating microorganisms into various agricultural production systems [3,4,6–10,13,14]. This new biotechnology was adapted to agriculture during the last two decades, and indeed opened new vistas along the way. The achievements so far have already exceeded previous expectations, but continued success in this direction depends not only on successful research and development, but also on economic growth, favorable regulatory “climate,” and public acceptance [3,16].

The progress of agricultural research and the use of molecular biology techniques facilitated successful breeding [3]. It is expected to continuously result in better agricultural crops. However, the current changes in the international market for agricultural products are limiting this progress. The world turning into a global village, along with the increases in the flow of information, are changing the market conditions. Because of the increasing volume of international trade, and following the establishment of the international trade organization in Morocco in 1994, the global market follows the rules of “free market” more closely. This is expressed by the emergence of international prices for agricultural goods and a decreased ability of governments and local interests to protect growers’ profitability by local pricing policies [8,10–12]. Agricultural biotechnology (agbiotech) has thus become essential to the success of the growers, who are now more affected by competitors from far away [13].

Despite many difficulties, the agbiotech industry continues to grow ([Table 1](#)). According to annual reports published by Ernst and Young in 1995 and 1996 [12,17], the compelling aspect of agbiotech is that agriculture is the world’s largest business, and biotechnology can both reduce costs and produce plants with more valuable characteristics. To improve their product competitive edge, Monsanto Corp. (USA) invested 1 billion US dollars in agbiotech research and development over the last decade, a significant part of which has been directed to Posilac, a version of recombinant bovine growth hormone which is now used for 15% of

Table 1 Financial Highlights of the Biotech Industry in 1996

Field	Sales	R&D	Investments
Agbiotech	12.82 (25%)	4.39 (24%)	6.23 (31%)
Therapeutics	28.24 (25%)	21.02 (19%)	36.39 (12%)
Diagnostics	12.65 (12%)	4.66 (10%)	8.43 (09%)
Food, chemical, and environmental	25.87 (22%)	15.77 (12%)	27.27 (13%)
Total	79.58 (21%)	45.84 (16%)	78.32 (16%)

Source: Ref. 17.

US dairy cows. Monsanto also developed a transgenic soybean, resistant to the premier herbicide Roundup, the application of which yields larger crops. Herbicide-resistant corn, cotton, and potato came next [17]. Monsanto is not alone in this endeavor. China is marketing a virus-resistant tomato [13], while virus resistant potatoes are being tested in Mexico [12]. Examples of some leading agbiotech companies are given in Table 2. Calgene Inc. (USA), one of the world leaders in agbiotech, was valued at 158 million US dollars by Wall Street, and its revenues reached 117 million US dollars. At the same time, companies, such as Mycogen Inc. (USA), had revenues of 12 million US dollars and a market value of 61 million US dollars. The total market values of US agbiotech companies traded in the US stock market, as summarized in Table 2, is 1014 million US dollars. The list includes American biotechnology companies, such as DNA Plant Technology Corp., Embrex Inc., Ecogen Inc., Consep Inc., Syntro, biosis Inc., Synbiotics Inc., and EcoScience, entirely focusing on novel biotechnological techniques to develop new agricultural products, means of growth regulation, and other agricultural inputs. Total revenues in North America of publicly traded agbiotech companies (see Table 2) reached close to 0.5 billion US dollars in 1995. The Ernst and Young report emphasizes the 25% annual growth in sales of agbiotech-based products, as compared with 21% annual growth of the industry at large. This also represents a major increase from the mean 18% annual increase calculated for the last decade. The increase in revenues reflects the impression that investments in research and development-based agbiotech companies are finally paying off [12,17].

Table 2 Agbiotech Market Valuation^a

Company	Market value (\$ millions)	Revenues (\$ millions)
Genzyme	622	167
Calgene	158	117
Mycogen	61	12
DNA Plant Technology	37	11
Embrex	36	9
Ecogen	26	25
Consep	24	7
Syntro	19	24
biosys	19	24
Synbiotics	18	14
EcoScience	13	15
Total	1,014	479

^aValues are in millions US dollars. All companies are publicly traded in the stock markets.
Source: Refs. 4, 17, and 23.

The regulation barrier has recently reached a favorable climate in the United States, Canada, and a few, but not all, European countries. In January 1995, the US Department of Agriculture (USDA) gave a nonregulated status to DNA plant technology for their "Endless Summer" tomato, designed to delay ripening. However, crossing the regulatory barrier was insufficient for the company to move forward in the US market. Ironically, the company chose to delay market penetration, assuming that their entire sale of classic products will be reduced, because people may believe that all their products are "genetic." In the United States, nonregulated status was also given to the virus-resistant ZW-20 squash of Asgrow Seeds Corp., and to the genetically enhanced corn hybrid seeds of Mycogen Inc. The latter two are at their early marketing phase. A major success was achieved with the deregulation of genetically modified cotton of Calgen Inc. (USA) [17,18]. Public fears of genetically engineered cotton seem to be much lower than that of genetically engineered food plants or animals [3,16]. The US Environmental Protection Agency (EPA) took a major step forward in 1995 when, for the first time, it granted limited premarket approval for herbicide-resistant transgenic plants to Monsanto, Mycogen Plant Science, and Ciba Seeds (all US-based companies) [17]. According to the Crop Protection Conference held in Brighton in 1995, the herbicide-resistant plants successfully penetrated the market in 1995 [19]. Among them were herbicide-resistant maize and soybean. Herbicide-resistant potatoes, corn, and cotton entered the market in 1996 [8–10]. The European Commission unveiled proposals to amend the directive on contained use of genetically modified organisms (GMOs) by its ruling 90/219/EEC. The possibility of amending the deliberate release of genetically modified organisms is approved in the directive 90/220/EEC [12].

A very broad and sympathetic view of world markets and scientific acceptance and progress are required if agriculture is to satisfy the world needs for food into the 21st century [6,8–10]. The rapid technological development enables the production of larger yields and improved quality of crop plants and farm animals. Because of the use of bovine growth hormone and transgenic plants that are pest or herbicide resistant, products became more competitive in the market. This is clearly a result of large investments in agbiotech research and development, in general, and in recombinant DNA technologies for agriculture, in particular [2,3,8–11].

Public acceptance of agbiotech and engineered organisms is very flexible, and changes continuously [3,16]. Thus, for example, the European Council of Ministers agreed to limit the labeling of genetically modified food products. Britain approved a tomato paste based on modified tomato (Zeneca Corp., UK), rapeseed oil from Plant Genetic System Ltd. (Belgium), and soybean products from Monsanto Corp. (USA) [12]. However, during the summer and fall of 1996, Monsanto Corp. and Unilever Ltd. (UK) faced a strong public resistance, and, there were demonstrations in Germany and The Netherlands against the use of transgenic plants in Europe, based on fears of the unknown and the presumed long-term effects. Germany is currently blocking the import of transgenic food products. Several other European countries also have their reservations. Agbiotech is evidently different from biopharmaceuticals. Thus, because the demand for biopharmaceuticals is so high, companies such as Amgen Inc. (USA), Genentec Inc. (USA), and other pharmaceutical companies enjoy a significant commercial success, with little public objection [16,17]. The demand for agbiotech products is, on the other hand, very changeable [8–10,12]. The consumers in industrialized countries are more affected by changing prices, food sources (classic versus transgenic), and the availability of substitutes [8,10–13]. These became critical factors for market penetration of agbiotech products. The success is, in fact, determined by the consumers. It seems that science can produce more than what the public is willing to consume. The situation is no better in the East and in developing countries [13]. Although agbiotech can significantly improve food availability for the continuously growing population, many of the developing countries, in which there is a food shortage, cannot afford expensive biotechnology-based products [4,12,17].

A partial solution to some of these difficulties of public acceptance and prices is additional time. During this time, agbiotech research and development will permit an evaluation of the real consequences of genetically modified organisms, and product prices will eventually decrease. Education, clever explanations, and desensitization may foster future public acceptance [13]. Along these lines, the European Commission plans to invest large sums of money in programs focusing on the development of public awareness and on promotion of biotechnology in Europe [12].

IV. BIOTECHNOLOGY IN AGRICULTURE HAS MULTIPLE FACES

Biotechnology in agriculture has diversified applications, multiple technological approaches, and a large repertoire of products. Some of these, which are discussed in the following chapters of this book, will be briefly mentioned here.

A. Biopharming: Higher Plants and Farm Animals for the Pharmaceutical Industry

One of the most successful fields of modern biotechnology is biopharmaceutical production [21–23]. *Escherichia coli*, and subsequently yeasts, have been successfully employed for drug development and production. Success in this direction yielded several billion US dollars in sales of erythropoietin, human insulin, and other products [8,12,17]. A major new direction is biopharming: the use of transgenic technologies to produce human-valuable proteins in genetically modified plants or farm animals. The biopharming approach is designed to expand the capacity for drug production in a larger repertoire of plants and animals that can act as drug manufacturers, thereby increasing the versatility of “production lines and facilities.” Transforming transgenic plants and animals into pharmaceutical production lines may create conditions to optimize production [21].

Cytokines and nutritional proteins can be manufactured in tobacco (a project under development in North Carolina and Israel). The latter may substitute the problematic use of tobacco for smoking. Consequently, the USDA tobacco program funds research on production of bioactive compounds in transgenic tobacco [2,15,22]. Human serum albumin, a major protein constituent of the blood (which is used to treat emergency blood losses and chronic blood deficiency), and factor VIII (an essential constituent of the body response to repair injuries of blood vessels), can be produced in goat milk to replace the blood-derived products that are currently threatened by risks of acquired immunodeficiency syndrome (AIDS) and other diseases. In addition, mammals can produce glycosylated proteins that are required for the tertiary structure of most human proteins, which cannot be provided by *E. coli*. Genzym Transgenic Corp. (USA) uses this technology to develop antithrombin III, recombinant tissue plasminogen activator (tPA), and its market value on Wall Street is very high. Human growth hormone is produced in goat milk by Serumtech Ltd. (Israel), and Pharmaceutical Proteins Ltd. (UK) uses this technology to produce anti- α_1 -transferrin antibody in sheep. Additional examples are Gene Pharming Ltd. (The Netherlands) that uses cows for production of lactoferrin, collagen, and erythropoietin, and the Red Cross is using swine to develop protein C for blood coagulation [21–23].

Biopharming combines the advantages of agriculture with those of the pharmaceutical industry, to generate a larger production capacity for novel products to meet the increasing world demand [24]. With these techniques, the profit margins for the growers of farm animals are expected to increase at least twofold over that for their traditional use for meat and milk

[21]. The “Cleaning in Process” Act, recently issued by the European Community and by the US Food and Drug Administration (FDA) [17,21] will help increase the safety of the commercial products. It will provide growers of farm animals with specific guidelines for animal growth and product processing in production lines. If successful, this novel trend will add to accelerating agrobusiness and its manufacturing capabilities [21,22,24].

B. Plant In Vitro Technologies: Micropropagation, Somatic Cell Genetics, and Transgenic Plants

1. *Micropropagation*

The promise of plant in vitro technologies in three major areas (micropropagation, somatic cell genetics, and generation of transgenic plants) has not as yet been exploited to its full potential. Plant propagation in tissue culture (micropropagation) is used to develop high-quality clonal standard plants [6,7,25]. These plants are selected for unique horticultural traits, pest resistance, crop quality, or suitability for environmental stress conditions. Micropropagation has many advantages over traditional plant propagation. The main advantages are attributed to the potential of combining rapid, large-scale propagation of new genotypes, the use of small amount of original germ plasm (particularly at the early breeding stage, when only few plants are available), and generation of pathogen-free propagules (e.g., virus- or bacteria-free) [25]. Enhanced production of pyrethrum (a natural insecticide, extracted from dried *Chrysanthemum* sp. flowers), for example, is assisted by micropropagation [2,13]. This forms the basis for a significant export from countries such as Kenya, Tanzania, Ecuador, Rwanda, and Tasmania. Ironically, if pyrethrum should become available through biopharming from genetically engineered plants, Kenya's export, valued at 75 million US dollars, could be destroyed [2]. The real bottleneck in further commercialization of micropropagation are the relatively high production costs, because of the involved manual labor, [25]. The lack of automation delays the anticipated market increase of plant propagation in tissue culture. Further commercialization is dependent on automation, currently under development, and on technological solutions to large-scale micropropagation, combined with novel products (e.g., artificial seeds and semiautotrophic cultures) and quality control [25].

2. *Somatic Cell Genetics*

The contribution of in vitro methods to plant breeding (i.e., somatic cell genetics) is most significant, especially in terms of haploid production and somatic hybridization. Regeneration of haploid cell lines and plants from microspores is highly important for production of homozygous offspring for further breeding. This was realized in barley, rice, rapeseed, potato, asparagus, and other plants. Somatic hybridization by protoplast fusion is an elegant solution to overcome the interspecific crossing barriers. It has already resulted in introgression of useful traits (e.g., cytoplasmic male sterility) in genomes of several cultivated plants, especially Solanaceae and Cruciferae. The embryo rescue technique is another alternative to overcome some crossing barriers [7–9].

3. *Transgenic Plants*

A recent global review of field testing and commercialization of transgenic plants during 1986–1995 was published by James and Krattiger, who have listed more than 3500 field trials of transgenic crops in more than 15,000 individual sites in 34 countries [26]. This achievement is already beyond the earlier expectations [6,11], and reflects the dramatic and effective contribution of molecular biology to the production of transgenic plants with improved agricultural traits.

This survey gives details of 56 crop plants, mostly in North America and the European Union, that have been engineered for a large number of traits. Moreover, by the end of 1995, 35 applications had been granted to commercially grow nine transgenic crops involving eight traits in the European Union and additional six countries. The major transgenic crops approved for commercial production (in the United States) include tomato (delayed ripening), cotton (insect- and herbicide-resistance), soybean (herbicide-resistance), corn (herbicide- and insect-resistance, male sterility), canola (modified oil quality), and others [26–28]. This reflects the continued impressive increase in using transgenic techniques to breed better plants.

C. Germplasm of the Future

Germplasm (i.e., the self-contained units of propagation in plants [seed and vegetative propagules] and reproduction in animals [embryos]) is, in fact, a “concentrated package of genes.” It is thus obvious why biotechnological techniques are especially relevant to commercialization of unique germplasms. This includes biotechnology of hybrid and artificial seeds, germ plasm banks and cryopreservation, in vitro fertilization, and embryo implantation [23,25,27].

Hybrid seed production in plants is developing rapidly. Implementation of novel biotechnologies that involve gene manipulation, somatic hybridization, and mutagenesis has led to the identification of the roles of specific mitochondrial, chloroplast, and nuclear DNA elements in cell growth, fertility, and control of blossom [19,27]. Understanding their roles can lead to the design of tools for regulation of commercially important traits, such as male sterility [7,19]. Moreover, the production of improved zygotic seeds can now be complemented by plant in vitro techniques to induce a high rate of somatic embryogenesis [19,28]. This can result in development of new miniature propagulus that combine favorable traits, such as high yields, synchronized plant development, and disease resistance [25]. Encapsulation and coating of the somatic embryos results in production of artificial—“synthetic”—seeds. These seeds can become suitable for regular seed technologies, including their automated planting [25]. Sales of artificial seeds produced by this technology is estimated to reach 10.8 million US dollars annually. This is only a fraction of the hugh 37 billion US dollars in sales of hybrid seeds in the world market annually [19,28]. The potentially large market for alternative seed sources indeed prompted the Dutch government, for example, to strongly support research programs directed at the commercial production of artificial seeds [25]. The emphasis on seeds and embryos is common to the entire field of agriculture. The artificial seeds described in the foregoing are just one direction in which a rapid development has followed world shortage of quality products. Seed inputs represent only 5–10% of the total grower investment. Thus, the reward for improved seeds is highly profitable. The market value of 1 kg of elite greenhouse tomato seeds can reach 20,000 US dollars, compared with 4–7 US dollars per kilogram of fresh tomato in season [6,10]. Similarly important are efforts for improving reproduction of farm animals. Novel in vitro biotechnologies, including superovulation, in vitro fertilization, and embryo implantation in surrogate mothers, allow breeders to produce multiple embryos with the most desired qualities [28]. This, together with successful gender selection and embryo manipulation, is a major asset for livestock agriculture. The results are increased meat and milk production that is currently six to seven times larger than 30 years ago. Germplasm banks and germ-plasm cryopreservation of plant seeds, vegetative tissues, sperm, oocytes, and embryos, are another important biotechnological development [2]. The resulting potentially marketable products are of great value in view of the need to save and protect germplasms. This development provides the basis for breeding programs of germplasm, that otherwise may become extinct (see later) [8]. Breeding of pet fish for coloration and shape, for example, can be strongly assisted by sperm cryopreservation of favorable subspecies [2,11,12].

D. Agbiotech Disease Control, Diagnostics, and Biological Control of Plant Pests

Biotechnology has an increasingly important role in improving the health of farm animals. New biotechnology-based vaccines against brucellosis, encephalitis, and hepatitis [10,13], for example, became crucial for proper maintenance of farm animals and poultry. Increased regulation is required for approval of germ-free fish and meat products, and of pesticide-free fruits and vegetables. Diagnostic biotechnology of animals and plants, for early monitoring and detection of diseases, pests, and chemical residues, becomes extremely important, for both improving health care and life expectancy of plants and animals, and for the marketability of the products [4,6,8].

Biological control of plant pests has now come of age, along with growing public acceptance and increasing revenues [8,18,20]. According to Hoechst AG, the global market for chemical pesticides is estimated at 36.8 billion Deutsche mark in 1993 [18]. The market is now saturated, and no market growth was detected in the last 5 years. On the other hand, the sales of biopesticides, which currently consist of only 10% of the total aforementioned pesticide market, increased 7–14% annually during the last 5 years. Fears of biological control are associated with the assumption that resistance to biopesticides may develop with time, and that it may destroy the fragile interbalance of the environment [20,29]. This fear is, however, counterbalanced by the danger of continuous exposure to organophosphates, bromo- and chloro-derivatives, and others, that have a detrimental environmental effect and pose cumulative health risks [8,29]. In addition, the effectiveness of many chemical pesticides has been lost owing to resistance build-up [8,18]. The market effects of “organic” vegetables and fruits, which avoid the use of any chemicals, has not taken a large share of the sales, as their yield is at least 30% lower [11].

Biopesticides are considered to be more pest-specific, with less negative effects on humans, farm animals, and the environment. Sales of *Bacillus thuringiensis* (Bt) alone amounted to 100 million US dollars in 1995 [12,29]. The anticipated global market of baculoviruses for protection of vegetables and cotton is estimated at 2 billion US dollars. Biosys Inc., is developing beneficial nematodes, especially for garden pest control and citrus protection. Experiments in Egypt have shown the usefulness of pheromones to destroy pink ballworm in large-scale field experiments [29]. W.R.Grace Corp. is using azadirachtin, extracted from the oil of the Neem tree seeds, as an insecticide [2]. Ecoscience Inc. developed anticockroach fungi [29], and Ecogen Inc. is producing biofungicides to protect postharvest spoilage of citrus, apples, and other pome fruits [8]. The biocontrol industry has been pushed forward through merging of several small American companies (Agridyne Inc., AgriSense Inc., biosys Inc., and others) to produce a required critical mass [12,17]. The formation of such a consortium was listed by several industry analysts as one of the most important events of the biotechnology industry in 1996 [12,17]. Biofertilizers had entered the market as well. Considering the pros and cons, biopesticides and bioherbicides show a positive balance and an annual market-value increase of 7–14% over the past 5 years [17]. The EPA and USDA approval and clearance for biocontrol products of nematodes, phytopathogenic fungi, and insect pests, as well as bacterial biofertilizers, imply an expected substantial growth in sales [17,29].

E. Phytoremediation and Bioremediation

Biotechnology holds major promise for control and protection of many environmental problems. The ability of plants and seaweeds (algae) to absorb heavy metals is considered a useful tool for developing “biofilters” for effluent detoxification, water desalination, and sewage treatment. The technology is referred to as phytoremediation [2,8].

Bioremediation, using microorganisms for similar purposes, is currently a favored approach for cleaning water of oil contamination, as tested in the spoilage of water at the Exxon oil company disaster site, or in the Persian Gulf during the Gulf war [31]. It is used for cleaning soil at evacuated army base camps in Germany. The sale of bioremediation products and services in the United States has reached several billion dollars in 1995, of which 500 million dollars were for environmental cleanups [8,17]. Another successful trend involves the addition of nutrients, such as nitrogen and phosphorus, to augment the efficacy of microorganisms in digesting environmental waste [30]. Fungi are used to degrade DDT, PCB, cyanide, TNT, and other soil pollutants [29]. The ability of bacteria and fungi to decompose organic material has been successfully employed to clean oil-contaminated shorelines [2].

V. EMERGING NEW MARKETS FOR BIOTECHNOLOGY

In addition to the aforementioned, biotechnological techniques are being incorporated into several promising emerging markets. Among them are marine biotechnology and production of nutraceuticals, cosmeceuticals, and, for example, cholesterol-free food additives. These new market trends create significant opportunities.

A. Marine Biotechnology: Supply for a Growing Demand

In 1991 the mariculture industry produced 14 million tons of fish, with a market value of 28 billion US dollars. Since 1996, the supply of fish from open seas and lakes is lagging behind the demand [2]. The latter is projected to increase by 65% toward 2020. The shortage in fish supply from mariculture is aggravated by difficulties in fish egg-laying, egg-spawning, and proper development of young fish during their adaptation to growth in captivity [10]. Methods for slow release of hormone were developed for application in fish. Recent genetic manipulation elucidated some of the molecular-hormonal aspects of egg laying and reproduction, resulting in better understanding and hopes for major progress [2]. Chile, Hawaii, Maryland, South America, Norway, Cyprus, Turkey, Gaza, Portugal, and Israel, among others, are working intensively to produce the required supply [13]. Water and environmental conservation are likely to enhance the development of mariculture. Technologies were developed for growing fish in integrative systems, in which organisms, behaving as a food chain cascade, are placed in a single pond. The food chain saves costs on food supplements and maintains the system as a balanced ecosystem that controls cleaning of the required water [2,13]. Other environmentally friendly closed systems for growing fish are supplied with filtered, recycled water. Fish cultivation in growing cages in deep water in seas and oceans is another recent, novel method. All together, biotechnology has a repertoire of tools for the success of mariculture, including large-scale enhanced growth of fish, marine invertebrates, and macro and microalgae, both to supplement world food supply and for new, marketable products [2,10,13].

B. Cosmeceuticals and Nutraceuticals: Back to Natural Products

The world demand for cosmeceuticals (natural cosmetics) and nutraceuticals (food products enriched with nutrients derived from self-enhanced biochemical production) has increased by 20% annually over the past 7 years [13].

1. Nutraceuticals

Synthetically designed plant storage proteins can radically improve human and animal nutrition [4]. Eight of the 20 essential amino acids, not synthesized in humans and farm animals, are made by plants (especially soybean). Storage proteins constitute the bulk of seeds, and production of transgenic plants that are engineered for elevated biosynthesis of specific physiologically stable storage proteins in seeds of cereals and legumes improves their nutritional quality [6,23]. Furthermore, chemically produced vitamins are not as efficient as their natural counterparts, and alternative sources are required, suggesting the growth of fresh fruits and vegetables that have been engineered to contain high levels of vitamins [31]. This may become important, according to some sources, for delaying aging, degenerative neurological diseases, and cancer [2,8,22].

The possible role of antioxidants in improving human health and life expectancy, and for rejuvenating facial skin is slowly revealed. The demand for antioxidants from natural pigments such as *Dunaliella*-enriched carotene complex and tomato lycopene, is reflected in a market of about 1 billion US dollars [2,13]. This is a remarkable increase from the 100 million-dollar market only 5 years ago. Physiological optimization for enhanced light harvesting, classic breeding, and novel genetic manipulations, along with an elucidation of lycopene biosynthesis in tomato and of 9-cis-β-carotene biosynthesis in the microalga *Dunaliella*, have led to significant pigment enrichment in these species and others. High-yield operations and high-quality products are being continuously developed in the "sun belt" areas of countries, including Israel, California, Spain, Australia, and the Far East [13]. However, the market increase has slowed since the confusing data that were published during 1996. Although a large study in China reported on the benefit of β-carotene and lycopene, a study in Finland showed that, in long-term consumers, lung cancer frequency is further elevated among the smokers, beyond the smoking risk. The understanding today is that the carotene complex, extracted from sources such as enriched microalgae, is more valuable than pure β-carotene [28]. Another promising direction is the use of transgenic algae to develop vaccines against gastrointestinal infections. Algae may also become an important source of novel potent antiviral compounds and pesticides [31].

2. Cosmeceuticals

Natural cosmetics are in demand by consumers who are frightened by reports of artificial chemicals in cosmetics that are known to have cell transformation activity, that could increase the risk of developing cancer [8]. Although FDA has increased regulation of new cosmetics, the consumers are looking for natural cosmetics [13]. Initially, suppliers of cosmeceuticals distributed natural eye and face makeup derived from plant extracts. These were followed by the use of algal extracts, hyaluronic acid, and liposome technologies to deliver a new generation of effective, longlasting, and safer cosmetics [10].

C. Biochemicals for the Food and Chemical Industries

Plant processing in the food industry represents a significant market trend worldwide. Traditional uses of plant products in the food industry (e.g., the use of cocoa and coffee beans for chocolate and for coffee production) relies heavily on plant breeding and could thus gain significantly from new plant biotechnologies [4]. In addition, there is a growing recognition of the importance of natural polysaccharides, for which the market has grown from annual sales of 150 million US dollars in the 1980s to over 500 million dollars today [10,13]. These include, for example, carbohydrates with heat hysteresis properties, such as algal (seaweed) extracts for production

of agar-agar, carrageenan, and agarose for food and microbiological markets, that account for this major market change. Natural polysaccharides are important for developing novel applications, such as the use of bioaffinity beads for advanced bioaffinity purification, replenishing liquids during eye operations, providing a protective coating for better control of germination, offering coating of fertilizers for slow release, and others [22,23]. Coating of food products, such as agar coating of chocolate chips for increased crispiness, is becoming a popular trend in the food industry [4].

Equally important is the production, from plants and animals, of important enzymes that are basic constituents of the chemical and biotechnological industry [7]. Enzymes isolated from thermophilic bacteria, are required for DNA amplification by the polymerase chain reaction (PCR), thereby accelerating DNA sequencing and research [32]. The enzymes are also used in the paper industry for extracting pulp, cellulose, and lignin. Amylase is used for the food industry [4]. Additional examples of the biotechnological prospects for novel enzymes production include recombinant rennin, which is used to produce cheese, thereby increasing the efficiency of the dairy industry [12], and temperature- and salt-resistant enzymes which make up another important fraction of today's world agbiotech food market [8,10].

VI. THE AGBIOTECH INDUSTRY: TRENDS, REGIONAL DIFFERENCES, AND MARKET VALUES

Agricultural biotechnology already represents a substantial proportion of biotechnology in developed countries, and is expected to hold an equally important position in developing countries. Transgenic plants, biological pest control, tissue culture techniques for plant propagation, microbial products for nutrient cycling, pathogen diagnosis for crops, and genetic mapping of tropical crops are of major concern in many countries in Africa, South America, Asia, and the Middle East. The emphasis in developing countries is on providing sufficient food, especially in countries that are continually suffering from famine and poverty [13]. In industrialized countries however, crop productivity is no longer the major emphasis. Profitability is the major evaluation factor in a country whose economy follows the rules of a free market. This can be demonstrated in the example of recombinant bovine growth hormone applied to dairy cows, where milk production increased but milk prices decreased [12,17], thus farmers profitability remained the same. Therefore, in free markets of developed countries, where demand and supply behave according to market rules, agricultural biotechnology aims to provide the growers with a larger added value [10]. Accordingly, agricultural biotechnology in developed countries is geared toward two major purposes: (1) improving the quality and elevating the added value of food products and agricultural commodities [13], and (2) developing practices designed to be environmentally friendly [20,30].

There are major differences among nations in the proportion of biotechnological activity devoted to agbiotech pursuits ([Table 3](#)). In the United States, agricultural biotechnology consists of only 8% (104 companies) of total United States biotechnology sales [17]. In Europe, agricultural biotechnology is 20% of total biotech sales (80 companies) [12], and in Argentina it is 16% of total biotechnology sales [10]. In Canada, 28% of total biotechnology sales is agbiotech [8] and similar values were reported for Japan (not shown) [13], whereas, in Israel, agbiotech is estimated at being 34% of its total biotechnology industry sales (47 companies), 87% of it for export (personal information). In fact, if we consider agbiotech, together with chemical and environmental products and marine biotechnology that are related to agbiotech,

Table 3 Share of Different Biotech Activities to the Total Industry^a

Field	United States	Canada	Europe	Israel	Argentina
Therapeutics and diagnostics	68%	43%	43%	42%	45%
Agbiotech	8%	28%	20%	34%	16%
Chemical and environmental products	9%	10%	10%	10%	13%
Marine biotech	1%		1%	8%	3%
Others ^b	14%	19%	26%	7%	13%

^aValues are in percentage of total industry revenues.

^bBiomaterials, Biosensors

Sources: Refs. 8 for the US and Canada; 10 for Europe, North and South America; (1995 COBIOTECH symposium) for Argentine; and Israel's National Committee of Biotechnology for Israel.

the level of direct and related agbiotech activity reaches 18% in the United States, 38% in Canada, 31% in Europe, 52% in Israel, and 32% in Argentina (see Table 3). Table 3 thus represents intensive global efforts related to agricultural biotechnology.

VII. BIODIVERSITY AND THE ENVIRONMENT: THE PROMISE OF BIOTECHNOLOGY TOWARD THE 21ST CENTURY

Environmental concerns and biodiversity are now the central guidelines in agricultural biotechnology. Besides the basic need to save natural germplasm variability and to guarantee it against possible extinction, the economic value of biodiversity has attracted the attention of industrial and financial organizations [13]. At the World Congress on Biodiversity held in Buenos Aires, Argentina (1996), the World Monetary Fund (WMF) has announced its allocation of 100 million US dollars for 3 years, toward preserving the biodiversity in environmentally important regions, such as the Amazon. This is one outcome of the 1992 Rio Convention, where 162 states signed an agreement to save the biodiversity of germplasms, species, and ecological systems [10]. Central issues were the importance of biodiversity as a natural resource of the countries involved, and its use for new product development. Oceans and specific ecological regions, such as the Amazon, represent the frontier of discovery of new genotypes, products, medicines, and alternative food sources [3,10,13].

Biodiversity gives developing countries an opportunity to establish profitable industries, based on newly discovered compounds and rare species, that are unique for those countries. The Neem tree was used in India for many years for fertility control and for agriculture, and it is now industrially processed by developed countries for the same applications. As a result, the people of India appealed to the World Court to recognize their intellectual proprietary rights over potential uses of the new compounds from the Neem tree [2]. Paclitaxel (Taxol) [33], extracted from trees of the rain forest in South America, and used as an anticancer drug, is another example of the potential benefit of biodiversity to human health. The Brazilian government has passed an industrial proprietary bill to determine compensatory agreements between the sources of biodiversity and the accessors that develop these sources into a profitable product [10,13]. This is an important legal issue touching on how to sustain the rights of both developing and developed countries. Continuous progress and cooperation in this direction will benefit all involved parties. Developing countries will thus be acknowledged for the natural resources of their people, and industrial companies will establish, in the developing countries, new ventures for turning the basic knowledge into a commercially viable product.

VIII. CONCLUDING REMARKS

Domestication of plants and animals that were found in the wild, combined with gradual long-term changes in their qualitative and quantitative traits, are the first attributes of agriculture. Domestication, followed by food storage, coincided with the growth of microorganisms. Thus was born classic food fermentation, the earliest known application of biotechnology (i.e., the use of microorganisms to produce wine, bread, cheese, and the like) [1,4].

Traditional agriculture has several serious limitations (e.g., local pricing policies and farmers profitability are no longer effective in the current global market). Added to this are the limitations of lengthy classic breeding methods, reduced availability of land and water, and increased deterioration of soil, water, and air quality. Thus, food production to sustain the expected large increase in world population in the 21st century, in developing countries around the globe, cannot rely solely on classic agriculture. In fact, human survival, vis-a-vis a continuous increase in agricultural productivity, depends on the effective merging of classic and alternative breeding methods (i.e., plant and animal biotechnology). This is in addition to alternative sources of food and agricultural commodities, such as aquaculture and marine biotechnology. The potential to improve plant and animal productivity and their proper use in agriculture relies largely on the newly developed DNA biotechnologies and molecular markers. These techniques allow one to select successful genotypes, enable better isolation and cloning of favorable traits, and permit the creation of transgenic organisms of importance to agriculture. All of these genetic techniques thus constitute an extension, but nevertheless an integral part of classic breeding, contributing successfully to shortening the breeding and selection cycle. The "new" biotechnology refers to the use of recombinant DNA and in vitro biology in three major areas: (1) as an aid to classic breeding; (2) for the generation of engineered plants, animals, and microorganisms; and (3) for the integration of microorganisms into various biotic production systems. This new biotechnology was adapted over the last two decades to agricultural practices and opened new vistas for agriculture.

Agricultural biotechnology already occupies a predominant fraction of biotechnology, at large, in developed countries, and is expected to become so in the developing countries. The major emphasis in many developing countries is on improving the quality and added value of food products, and on agricultural practices that are more environmentally friendly. In industrialized countries however, in which crop and animal productivity is no longer a major focus, agbiotech markets are geared to the production of new commodities and special products. This is complemented by environmental concerns and biodiversity issues, including saving natural germplasm variability and guaranteeing it against possible extinction.

The achievements of agricultural biotechnology have already surpassed previous expectations. However, its continued success depends not only on successful and innovative research and development, but also on a favorable regulatory climate and public acceptance. Although agbiotech can significantly improve food availability to the ever-growing world population, many of the developing countries cannot currently afford expensive biotechnology-based agricultural products. The solution to this requires a revolutionary new order of global priorities. A critical mass of investments is required. A more balanced emphasis on agricultural biotechnology versus medical biotechnology, along with full integration of agricultural and environmental biotechnologies are considered essential. The challenges for agricultural biotechnology, as we approach the 21st century, are overwhelming. The world's envisaged tripled demands for food, agricultural commodities, and natural products cannot be met without environmentally friendly and effective novel agricultural biotechnologies.

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Micropropagation: Clonal Plant Propagation In Vitro

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I. INTRODUCTION

Clonal, true-to-type propagation of plants by a variety of tissue and cell culture methods, better known as *micropropagation*, is now the most commercially efficient and practically oriented plant biotechnology. Most other plant biotechnologies (e.g., genetic engineering and production of transgenic plants harboring agriculturally important traits) hold a greater share of the investment capital in research and development, both at practical and basic levels. However, they have not yet been commercialized to the same extent as micropropagation. Moreover, successful and efficient production of improved transgenic plants relies heavily, if not exclusively, on the ability to regenerate whole plants from those cells, tissues, or organs in which foreign DNA has been inserted and expressed. The ultimate need for large-scale regeneration of transgenic plants by in vitro techniques is obvious for those plant species that are normally propagated by vegetative methods (cuttings, grafting, division, and separation; i.e., clonal propagation). It is, however, also required for seed-propagated plants, although on a relatively smaller scale, for the production of the first, parent, fertile plants that will be crossed and selected for eventual seed production.

The possibility of plant cloning using in vitro methods (i.e., regeneration of whole plants from cells and cell clusters, tissue, and organ explants) has been known since the 1940s [1], mainly under experimental, small-scale laboratory conditions. In vitro propagation of some agriculturally important plants, primarily ornamentals, by mass production of clonal propagules, became practical in the early 1970s [2,3]. The diversity of plant species that can be propagated in vitro has dramatically increased, and it is now practiced on a commercial scale worldwide, resulting in over 500 million plants annually; 50–75% of them flowers and ornamental plants [4–8]. The science and practice of plant micropropagation have been dealt with extensively in several books and reviews [4,8–14]. In the following, the principles and various procedures of plant micropropagation and their applications will be reviewed. Examples of in vitro propagation of selected crops will be presented, and surveys of world distribution and trends, with special emphasis on applied, economic, and commercial

considerations will be presented and evaluated. The use of plant tissue culture techniques for regeneration of new plants with improved agricultural traits (e.g., production of pathogen-free plants and of genetically engineered plants) are dealt with separately in other chapters of this book.

II. WHY IN VITRO CLONAL PROPAGATION?

A. Generative (Sexual) and Vegetative Propagation (Cloning)

Plant reproduction by seeds (sexual propagation) is routinely used, is usually inexpensive, and is easy for most field crops and vegetables. This is possible in homozygous plants and in annual plants for which pure seeds can be produced by backcrossing, in a relatively short time. However, most perennial heterozygous cultivated plants, including many ornamental plants, plantation crops, and fruit trees, are propagated vegetatively by a variety of techniques (i.e., cuttings, layering, separation, division, grafting, and budding), resulting in true-to-type clonal plant material. In this respect, in vitro propagation is synonymous with vegetative propagation, the main differences between cuttings and micropropagation being that (1) a very small plant part (explant), usually in the range of a few millimeters or less, is used as starting material for in vitro propagation; (2) the explant is maintained in vials, in a defined and balanced culture medium; (3) micropropagation is carried out under aseptic conditions; and (4) micropropagation usually results in numerous clonal propagules per unit of initial (stock) plant material; many times over that by other means of vegetative propagation, and in a much shorter time. Therefore, a dramatic increase of the propagation coefficient is obtained.

B. Advantages and Disadvantages of In Vitro Propagation

Micropropagation offers significant advantages in quality, quantity, and economics over conventional vegetative propagation for many species. However, inherent in micropropagation are several disadvantages that cannot be ignored if the maximum potential of tissue culture technologies is to be achieved.

1. Advantages:

a. *Production of a Very Large Number of Clonal Propagules Within a Short Time Span.* The techniques of plant tissue culture give an exponential increase of the propagation coefficient. Depending on the multiplication rate, thousands and millions of plants can be rapidly produced in vitro from relatively few selected source plants.

b. *Production of Disease-free Plant Material with the Possibility of Eliminating Viral, Bacterial, and Fungal Contamination.* Diseases, and the contamination of source plant material are significant limiting factors in conventional plant propagation [15]. Micropropagation and in vitro techniques address these problems to minimize infection and its negative ramifications (see [Chap. 3](#)). This is especially significant for many important agricultural and horticultural crops.

c. *Production of a Large Stock of True-to-Type Clonal Propagation Material.* A basic premise in commercial micropropagation, as in all mass production, is the guarantee of a consistently high degree of likeness between the template, here the source plant, and the product. Methods have been developed for each plant type, and they are designed to

provide the maximum number of true-to-type plants from a minimum number of selected source (mother) plants.

d. The Ability to Air-Ship Large Quantities of Plant Material Quickly, Efficiently, and Relatively Inexpensively. As many as 30,000–50,000 in vitro (in closed vials or small containers), or 3,000–10,000 in vivo (hardened) plants can be packed into a cubic meter of shipping space.

e. The Possibility of Bringing Newly Bred Plants and Selections to Market Quickly and in Large Quantities. Breeding and selection of commercial plant products have been lengthy processes. With tissue culture techniques and new modalities in molecular biology, tracking and testing new genotypes or field selections of plants can be achieved in several months or a few years. Plant micropopagation techniques have provided quicker and often more qualitative resolution of many questions in horticultural, agricultural, chemical, and medical and pharmaceutical sciences. Additionally, molecular biology methods now offer the possibility of patenting cultivars for the mass market [8].

2. Disadvantages

a. Contamination. Some of the greatest economic losses, direct and indirect, in commercial micropropagation, are caused by both endogenous and environmentally induced contamination of plant cultures [15].

b. Higher Than Acceptable Levels of Somatic Variation. Plant variants, genetic or epigenetic, can be the result of poor source plant selection or incomplete monitoring of production methods and plant material during micropropagation [14,16].

c. Losses Incurred During Transfer of Plant Material from In Vitro Conditions to the Acclimatization Stage. Those in vitro plants that are not in optimal condition upon leaving the laboratory, or the use of poor and inadequate acclimatization methods, can account for significant wastage and resultant failure.

d. High Production Costs. Expensive technology and facilities, and the labor-intensive nature of tissue culture, often render micropropagation economically unfeasible and engender costs that are untenable for certain plants, varieties, or markets.

C. Other Implications of In Vitro Propagation

In addition to the major use of in vitro culture and plant regeneration for commercial rapid clonal propagation, this technique is highly important for several other purposes, as discussed in other chapters of this volume:

1. Production and maintenance of pathogen-free stock plants
2. Long-term in vitro conservation of germ plasm
3. Selection and generation of transgenic plants

III. MICROPROPAGATION: PRINCIPLES AND PRACTICES

A. Plant Regeneration in Tissue Culture

In the following, the basic aspects of plant regeneration in vitro, which are common to, and form the basis of, all micropropagation procedures, will be discussed. Specific references to culture media and methods will be detailed later, as relevant to the various plant species.

1. Totipotency and Regeneration

All *in vitro* methods of plant propagation rely on the unique totipotency of plant cells (i.e., the regeneration of whole plants from individual cells, or groups of cells from within tissue or an organ, expressing the full plant genome). This is usually achieved after the tissue or organ has been excised and placed on culture medium (an exception being young seedlings, which can be cultured intact without prior excision) and involves the following consecutive events: (1) Dedifferentiation of the source tissue or organ; this results in the activation of physiological mechanisms that lead, under the appropriate endogenous and exogenous conditions, to cell division. (2) Active cell division in the entire cut surface, or localized meristematic activity in specific regions of the explant, or both; this often results, but not always, in proliferation of callus tissue. (3) Organization of defined meristems; this occurs within the zones of active cell division and results in formation of shoot or root meristems, or both. (4) Regeneration and differentiation of new organs: namely, *organogenesis* (formation of new shoot buds or new roots), or *somatic embryogenesis* (the bipolar differentiation of somatic embryos). Organogenesis and somatic embryogenesis may be direct, coming from the explant and not involving callus formation, or indirect, from a callus tissue. The difference between direct and indirect regeneration is important mainly where it relates to the genetic stability of the resulting plantlets. A callus stage, and meristem organization from callus cells, usually lead to more genetic aberrations (resulting from mutations) than direct regeneration. The entire micropropagation process must be carefully controlled and monitored, otherwise it may result in generation of plantlets that are not true to type. Regeneration (both organogenesis and somatic embryogenesis), in contrast with the proliferation of axillary buds (see later), may also be a disadvantage for clonal propagation of chimeric varieties. The organization of new shoot buds usually occurs from a few cells that do not necessarily contain the diverse types of cells or tissues that formed the chimera in the source plant, thereby resulting in loss of the chimera. Some micropropagation stages do not necessarily involve a regeneration process. Culture of shoot tips may lead to proliferation of already-existing axillary buds, but their further development into plantlets involves a regeneration process (i.e., root formation).

2. Axillary Bud Proliferation

Axillary bud proliferation *in vitro* is usually considered a convenient route for micropropagation. Because it does not include a callus stage, it is considered "safer" for the preservation of clonal characteristics (see foregoing). Bud meristems already exist in the axils of leaves, but because of apical control, they normally do not develop in planta until the stem elongates and grows. Thus, the short stem tip contains many axillary buds at different stages of development, condensed in a small explant. A very large number of otherwise quiescent axillary shoot buds grow extensively when shoot tips, or even smaller apical meristems are cultured in an appropriate medium, usually containing high concentrations of cytokinins. Both the excision of the explant and the cytokinin-rich medium "activate" the bud, leading to proliferation of many side shoots. After induction, these shoots are separated for further culture.

3. Organogenesis: Shoots, Roots, and Specialized Structures

Organogenesis from explants results in *de novo* formation of both shoots and roots. These two events may take place simultaneously during culture, but frequently either shoots or roots (usually shoots) are formed first. Regeneration of the complementary organ occurs only

later, either in the same medium or after subculture to another medium and under different environmental conditions. Organogenesis starts with the distinct organization of a shoot or a root meristem within the explant or from the callus. This is controlled by the type of explant, composition of the culture medium (especially the balance of growth regulators), and the environmental conditions during culture. Once shoot or root meristems have been organized, they start developing, forming small shoots and roots. Proper organization and differentiation, including the formation of functional vascular connections between the developing shoots and roots, finally give rise to plantlets in vitro. These result in new plants that are acclimatized and grown under greenhouse or field conditions.

In some cases organogenesis brings about the formation of modified shoots and roots, usually storage organs such as bulbs and tubers. Here, the shoot or the root meristem forms as described previously, but instead of further growth and elongation, minibulbs, minitubers, or minicorms, depending on the specific plant genotype, soon develop. Culture conditions determine whether the resulting bulbs, tubers, or corms grow further or go into dormancy. Similarly, minibulbs, minitubers, and minicorms can also develop from axillary buds (see foregoing), especially when basal plates of bulbs, or certain corm and tuber explants are used as primary explants.

4. Somatic Embryogenesis

Somatic embryogenesis is different from organogenesis in that regeneration and organization are bipolar: the shoot and root meristems are formed simultaneously from progenitor cells that give rise to a group of cells known as proembryonic masses. As with organogenesis, differentiation and organization of a somatic embryo take place directly in the explant or from the callus, depending on the type of explant, composition of the culture medium, and the subculture regimen. Usually, a two-stage culture is involved: first, induction of proembryonic masses, which is frequently favored by an initial exposure of the tissue to 2,4-D or other auxin-type growth regulators; and second, by transfer of the proembryonic cultures to a medium with a modified composition and the removal of the potent auxin, in which the somatic embryos fully differentiate. Somatic embryogenesis is often favored by culture in an agitated liquid medium. The patterns of differentiation are very conserved. Proceeding from the formation of globular embryonic structures, to the "heart" stage at which the shoot and root meristems can be clearly distinguished at the two poles, to the "torpedo" stage at which elongation of the shoot, and especially the root, takes place, and vascular connections between the two are already established. This coordinated development results, after some time, in the formation of well-organized embryoids or plantlets. Careful control of medium composition and subculture regimens is required to obtain synchronized development of the somatic embryos.

a. Artificial (Synthetic) Seeds. The possibility for large-scale formation of somatic embryos has set the stage for developing the concept and practice of "artificial seeds," also referred to as "synthetic seeds" [17,18]. "Conventional" seeds have been used routinely in agriculture and horticulture and, additionally, are the basis of traditional breeding and selection programs. Commercial micropropagation has proved itself useful when seeds are either unavailable or inadequate, and production of artificial seeds may become an alternative to traditional seeds, offering a unique combination of traits to the market. When large-scale production of somatic embryos became feasible, methods and materials for encapsulation and transplantation were developed [19–21], with the goal of providing sufficient survival and germination rates. However, for commercial mass production, this goal has yet to be reached.

B. Establishing and Operating a Micropagation Laboratory

There are many reasons, and varied purposes (i.e., challenging plant species, available technologies, and economic bases) for using tissue culture to carry out micropagation. However, all of these, at all production levels, require adherence to certain basic principles and practices.

1. Facilities

a. Direct Production Facilities and Activities.

1. Initial plant preparation, before culture, is carried out in an area located between an outside entrance and buffered access to clean room spaces (see Sec. III. B. 2, Asepsis).
2. Media preparation area: Although not a “clean” room, this space has easy access to all parts of the laboratory and provides the basic nutrition for all laboratory products.
3. Plant production center: This “clean” room is the most aseptic area of the laboratory and is central to all production. It contains the laminar flow hoods in which most plant manipulation and replanting on new growth media take place.
4. Plant-growing rooms: Compared with other industrial processes, plant micropagation has a lengthy production time, usually lasting from 1 to 2 years. Most of this time is spent in a clean growing room. After each manipulation, plantlets must grow and develop for 1–2 months. To maintain growth, maximal environmental conditions are employed. An average-sized room may hold up to 500,000 plantlets.

b. *Production Support Facilities and Activities.* In addition to direct plant production activities, 30–40% of time, space, and personnel in a commercial laboratory are devoted to providing components and support. The standard infrastructure includes the following:

QUALITY ASSURANCE FACILITIES. Commercialized, high-technology propagation requires mass production of uniform plantlets delivered on time. Assuring that each worker maintains defined quality standards is almost impossible. As a result, the level of wastage in viable commercial micropagation often reaches 20–30%. As the 21st century approaches, quality is a central element in manufacturing. Bringing plant micropagation and biotechnology firms in line with total quality management (TQM) [22] and the International Standards Organization (ISO 9000) [23], requires professional staff, space, and facilities that are appropriate for bacteriological, virological, chemical, and horticultural testing. In the event of problems, usually spontaneous, troubleshooting and solution development are carried out. A trained staff is responsible for maintenance and control systems in plant-growing rooms. They visually examine all media and plant material regularly and serve as a link between production and quality assurance staffs. Despite this, quality control and uniformity have been difficult to achieve in commercial micropagation. Standard agricultural issues, such as plant source diversity, pests and disease, and the weather, have been complicated by the unique problems of placing a living plant into the production line. There is an uncertainty principle in plant micropagation that is exacerbated by issues ranging from the use of potentially dangerous chemicals in plant media to the natural damage plant material undergoes in transit. The success of the industry in the coming decade will be heavily dependent on quality and efficiency.

INFORMATION SYSTEMS. The computer-aided information systems required to monitor personnel, long production processes with multiple manipulations, and inventory control

also require space, equipment, and personnel. The biological nature of the production system usually requires customized software for inventory identification and quality control.

PERSONNEL SUPPORT. The current labor-intensive nature of commercial micropropagation assures the presence of many workers and individual tasks performed by them. Ancillary areas, including dressing rooms, eating and rest areas, and office space, must be provided.

PACKING AND SHIPPING. When in vitro plants are ready for shipment, growth containers are moved to an area designed for sorting, final quality checking, and packing. As an in vitro product, asepsis and environmental control are essential for the entire process, including transit by air freight. Survival on arrival to the customer, followed by successful acclimatization and planting out are essential.

2. Asepsis

In vitro micropropagation and storage of plant material requires axenic cultures and an aseptic environment. This is achieved by combining surface-sterilization of the plant material from which primary explants are removed, with planting and subculture under aseptic conditions. The use of presterilized culture media and vials and continuous culture and maintenance in a clean environment is employed. Aseptic work spaces must be buffered and isolated from other areas of the laboratory. This usually involves a gradient of spaces that are progressively cleaner and require more stringent behavior and operations as the central, clean transfer room is approached. The range of techniques for achieving asepsis in plant micropropagation is large. All facilities, however, will require a plan that defines and separates distinct work areas according to function and degree of asepsis. Most commercial companies employ the following principles:

a. Clean Room Technology. To provide workspaces with a minimal level of particulate matter, including spores, air under positive pressure is directed through high-efficiency particulate air (HEPA) filters and discharged into the laboratory at central points. An outflowing gradient of clean air prevents the passive entrance of contaminated, "fresh" air and particulate matter. Plant material is manipulated in laminar flow tables located in a clean transfer room and stored in similarly designed plant-growing rooms. Filters and levels of asepsis must be examined regularly, using internationally accepted methods and equipment.

b. Disinfection and Sterilization. Everything that comes in contact with workers and plant material is a potential contaminant. Sterilization and disinfection, therefore, are an integral factor in creating an aseptic environment and a contamination-free product. Commercial laboratories are equipped with autoclaves that are used for most media, container, and tool sterilization. Additional large-scale sterilization can be carried out by gamma irradiation or by ethylene oxide gas treatment. Glass bead sterilizers, electric incinerators, and Bunsen burners are used to disinfect tools during plant manipulations, and chemical disinfectants are used throughout the facility for cleaning work surfaces, floors, and equipment.

c. Surface-Sterilization of Plant Material. The plant parts are first cleansed, usually after peeling off outer layers of tissues, with combinations of scrubbing and the use of detergents, agitation, and water rinses, followed by surface sterilization. Chemicals, concentrations, and duration of surface sterilization procedures differ according to type, size, and origin of plant material. When required, additional treatments with fungicides, fungistats, or antibiotics can be applied.

3. Environmental Conditions

Maintenance of optimal environmental conditions for the handling and development of in vitro plant cultures is a key factor in all operational steps. A constant balance between complex and expensive electrical, air-conditioning, and filtration systems is required. The most critical environmental balance should be maintained in plant-growing rooms.

a. Temperature. In vitro growth conditions for most plants require ambient temperatures between 22° and 27°C. Some species, growth stages, or storage conditions may require different or variable temperature regimens.

b. Relative Humidity. The microhumidity in the plant growth container is the most significant humidity factor. It is generally accepted that the relative humidity (RH) in the container is approximately 98–100%. However, recent studies indicate that, for some plants, it might be more effective to maintain RH at 88–94% [24]. Growth room ambient humidity is much lower, usually 50–80%. Room RH lower than 40% can result in medium desiccation, increased salt concentration, and drying out of plant material. Room RH higher than 85% might often increase the incidence of microbial contamination. In extreme climates or areas with significant variations in weather conditions, a humidity control system may be necessary.

c. Light. Conventional in vitro growth media provide cultures with a carbon source. Although there is some photosynthetic activity in vitro, the plantlets do not rely on photosynthetic carbon fixation for growth. Fluorescent lamps have been the primary light source used in micropropagation [25,26]. Photon density is between 20 and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ [27], with a standard photoperiod of 16 h. Different plants, species, micropropagation technologies, and stages of growth often demand exceptional environmental conditions. Laboratories may be equipped with multiple growing rooms and options for altering standard parameters. In recent years, much research has dealt with environmental factors in the growth and development of in vitro plants, and there is experimental data showing that many of the standard methods described here are not optimal [28].

4. Culture Media

A sterile nutrient source in appropriate growth containers must be provided to initiate and sustain in vitro plant growth. An appreciation and a knowledge of the nutritional requirements and of the metabolic needs of the cultured cells and tissues is invaluable, not only in a decision on the type of media to use, but also in their preparation [29]. Since 1934, when P.R. White [1] demonstrated the continuous growth of tomato roots in vitro, there has been constant improvement in the understanding and effectiveness of the use of synthetic plant growth media [14]. Today, synthetic growth medium is the primary source of nutrition for plants and plant tissues in vitro. Basal media components are water; sugar(s) as a primary carbon source; inorganic salts, providing macro- and microelements; vitamins (some essential, others beneficial); and hormones or growth regulators. The discovery, isolation, and synthesis of the plant growth hormones (e.g., auxins, cytokinins, and gibberellins) have provided the ability to achieve hormonal control of plant growth, development, and regeneration. Additional factors, including complex, undefined materials, such as coconut milk, yeast extract, or protein hydrolysates, may be beneficial for certain plants. Gelling of media solutions is usually achieved with agar agar or agar substitutes such as Gelrite [4]. The most widely used standard medium formulation is that of Murashige and Skoog, MS [30]. Today, micropropagationists have a vast catalog of synthetic, analytical-grade media and media components at their disposal. In addition to sterilization techniques mentioned earlier, microwave or filtration for

nonautoclavable materials is routine for smaller quantities. Media preparation requires a laboratory equipped with a pH meter, analytical scales, and measuring containers; cooking, stirring, and pouring equipment; refrigeration; a water distillation and deionization system; storage facilities; and a washing up area for tools and equipment.

5. Vials, Containers, and Closures

A multitude of configurations and materials for containers and stoppers are available for micropropagation. The growth and development of a plantlet in vitro is affected by light transference, relative humidity, temperature, and gas exchange. The microenvironment may, in turn, be influenced not only by the generation and absorption of gases by the plantlet and the culture medium, but also by the gas exchange between the room and the vessel [4,25,31; P.Debergh, personal communication]. Additional specifications universally sought are availability, low unit cost, uniformity, nonphytotoxicity, and ease in sterilization and handling. Standard containers have been glass test tubes, flasks, and jars, but in recent years, the material of choice has been plastic, usually polypropylene or polycarbonate, often disposable, including petri dishes, tubs, boxes, and even flexible wall, disposable, bioreactors [G.Tanny, personal communications, 1996]. Limitations in production methods still require that most containers be small, usually holding from 1 to 100 plantlets. Storage space for containers at various stages of use is a significant issue in commercial laboratories. New techniques in liquid culture often use bioreactors, which may hold from 1 to 2000 L of growth medium and plant material and save a significant amount of space (see under Sec. V.C). As is often true, equipment, instruments, and machinery were adapted from other industries (food, chemical, pharmaceutical, and others) to the needs of plant micropropagation.

C. Stages Involved: From Theory to Practice

Plant micropropagation in vitro is an integrated process in which cells, tissues, or organs of selected plants are isolated, surface-sterilized, and incubated in a growth-promoting, sterile environment to produce many clonal plantlets. At least five critical and ordered operational stages are involved, three of them in vitro: selection and preparation of the explant source, establishment of viable explants in culture, rapid regeneration of numerous propagules (multiplication), establishment of complete plants or other propagation units, and acclimatization ex vitro [4,9,32].

1. Stage 0: Explant Source and Mother Plants

The success of micropropagation is largely dependent on the quality of the source (mother) plant. Effective selection and maintenance of source plants should provide assurance that the plant is

1. A certified, horticultural true-to-type representative of the desired species and cultivar
2. Free from infections and disease, or can become pathogen-free using specific, in vitro procedures, as verified by routine examinations in accordance with international standards
3. Viable and vigorous (i.e., potentially able to respond to culture conditions that induce intensive cell division and regeneration)

To comply with these requirements, selected mother plants are often "preconditioned" by a variety of specific growth regimens and horticultural procedures. These may include nutrition and irrigation; optimization of day length, light quality, and temperature; treatments with

growth regulators; pruning; and pest control. Plant organs, from which explants for culture are surface-sterilized and prepared, can range in size from large corms of bananas and dates, to seeds or fragile stems and leaves.

2. Stage 1: Explant Establishment in Culture

The initial explant may range in size from 0.1 mm (e.g., meristems used for establishing virus-free plants) to about 1 cm (e.g., bulb scales, stems). During this stage, lasting from 1 week to 2–3 months, or even longer, the explant is established in culture, resulting in tissue activation and multiplication. This stage is usually carried out on agar-based media, but liquid media can also be employed. The choice of basal media and growth regulators at this stage is of special importance, and may vary according to plant and tissue type and to the desired multiplication method. With continual visual monitoring, this stage is also used to screen for microbial contamination and horticultural fitness. Specific indexing and treatment of microbial and viral contamination may also be undertaken. The relatively few primary explants, and their size, render this the most cost-effective and efficient stage for evaluation and treatment of plant material.

3. Stage 2: Rapid Multiplication

Primary explants that have successfully passed through stage 1 are transferred aseptically to stage 2 for generation of numerous clonal propagules. Masses of tissues are repeatedly manipulated by subculturing onto new culture media that encourage propagule proliferation. The types of regeneration and proliferation are largely dependent on growth regulator combinations. A high proportion of cytokinins usually stimulates continued multiplication of axillary or adventitious shoots, and a higher proportion of specific auxins is required for callus proliferation vis-a-vis somatic embryogenesis. The combined and balanced adjustment of growth regulators, basal media composition, and environmental conditions are optimized to achieve maximal proliferation of quality, new, plant propagules. Although basic media formulations tend to remain constant, extensive experimentation may be necessary to reach commercially efficient multiplication with specific cultivars or varieties. The duration of this stage is potentially unlimited, but usually lasts from several months to 1 to 2 years. At the chosen endpoint, the stock culture is renewed to prevent possible accumulation of mutations and the loss of vigor and regeneration potential.

4. Stage 3: Plantlet Establishment, Elongation, and Rooting

After repeated subcultures and screening, the resulting plantlets are transferred to the final in vitro stage. This stage is designed to arrest rapid multiplication and to induce the establishment of a fully developed plantlet: shoot elongation, root formation, and when required, formation of storage organs that serve as independent propagation units (bulbs, corms, and tubers). This stage also provides the conditions for stimulation of photosynthesis and other physiological changes that are required for autotrophic, ex vitro growth in the acclimatization stage. This is achieved by culture media modifications (e.g., reduction of cytokinin concentrations or their total elimination, sometimes increased auxin levels, reduced sugar levels, and often, increased concentration of gelling agents), and by modifying environmental conditions (e.g., increased light intensity). To improve the efficiency of this stage and to reduce production costs, systems that shorten the process or allow much of it to take place ex vitro, or in a less aseptic, in vitro environment, have been proposed [4,25,28].

5. Stage 4: Acclimatization

The healthy plantlet that has emerged from the laboratory is usually incapable of existence in “natural” field or greenhouse conditions: 4–8 weeks in an acclimatization greenhouse provide a weaning process. The first few days in the greenhouse are spent under low-light, high-temperatures, and high-humidity conditions, often provided by fogging or an incubator for ex vitro plants [32]. As cuticular waxes, stomatal function, and new, functional roots develop: photosynthetic activity is increased; and plants become autotrophic. Over the following few weeks, light intensity is raised, and ambient temperature and humidity are regulated to natural-growing conditions. Rooting hormones may be used to additionally stimulate root development. Most laboratories and nurseries transplant into a uniform medium that adequately supports the plant, has a suitable pH, is well buffered, is reproducible, and is sufficiently porous to allow adequate drainage and aeration [4]. In vitro plants that are unable to survive this stage must be considered unsuccessful.

IV. CROP ORIENTED, COMMERCIAL MICROPROPAGATION

The potential of rapid in vitro clonal propagation of a given plant genotype does not necessarily mean that this technique is also practical, or is economically feasible. Moreover, the number of plant species that can be regenerated in tissue cultures far exceeds the number of plants that are actually being propagated in vitro on a commercial scale. The practical application of in vitro techniques to plant propagation is dependent on two major considerations: (1) the current status of a particular plant in agriculture or horticulture relative to market value and critical issues associated with production and marketing; and (2) the cost-effectiveness of micropropagation relative to alternative conventional methods of multiplication; namely, is there technology available for micropropagation, and is micropropagation expected to provide solutions to questions or problems that have arisen in conventional propagation? In the following, the application of micropropagation to economically important crops and plants will be discussed.

A. Field Crops and Vegetables

Traditional field and vegetable crops (i.e., wheat, corn, and tomato) have been successfully grown and improved for generations using conventional seed technology. However, there are some crops and situations that have successfully used micropropagation technologies. These include varieties of vegetables that have been specially bred or genetically altered, crops that demand exact and uniform harvesting times to meet industrial demands and those for which in vitro culture methods are required for production of virus- or bacteria-free propagation material. In the following section we will discuss potatoes, which serve as an effective model for a variety of micropropagation methods and technologies.

1. Potato (*Solanum tuberosum L.*)

Potato, an annual plant that belongs to the family Solanaceae, had its center of origin in the Andean region of Peru, Bolivia, Ecuador, and Columbia, at altitudes of 2000 ms. Potato tuber is a source of food in almost every nation of the world. Among the major world crops, potato ranks fourth in terms of total food production [33] and accounts for 90% of micropropagation of vegetable crops in the United States, with more than 11 million

propagation units produced annually [5]. Potatoes are traditionally propagated by planting tubers gathered during the previous season. However, many tubers become infected by various virus diseases while still in the field and, when replanted, pass the infection to the following generations—a problem especially severe in tropical climates [34]. Potato is one of the crop plants on which most in vitro culture techniques have been applied with success [33]. Tissue culture methods have been applied to overcome the problems of time and quantity associated with conventional breeding schemes, for the selection and propagation of virus-free planting material, the propagation of large quantities of planting material resulting in seed tubers, and the establishment of in vitro storage banks and cryopreservation programs for germ plasm.

With the advent of virus-free stocks of potato germ plasm [35], mass propagation schemes for plantlets and seed tubers have been developed [36–39]. As a result, the potato-growing sector has significantly changed in the past 20 years. Use of in vitro plants and tubers has been researched and reported in Denmark [40], China [41], Vietnam [42], Taiwan [43], Korea [44], Ireland [35], Israel [33,45], Philippines [46], Japan [47], Peru [48], and in almost every country where potato is a valued crop. Tubers and plants are produced in vitro as nuclear material and, subsequently, are grown for multiple seasons in disease-free, monitored, plots. The resulting certified seed stock is then distributed to farmers for planting out. Some descriptions of programs and results are discussed in the following.

a. International Research and Field Studies. A field study in Taiwan has shown that the disease rate for a virus-free clone was 0.66%, and for standard, virus-infected farmers' seed stocks, it was 16.8%. Some farmers' stocks showed a 100% disease rate. The yield of the virus-free clone was 26% higher than that of the farmers' seed stock [43]. A major research effort has been launched at the International Potato Center (CIP) in Lima, Peru, to develop an effective means for producing potatoes from "true potato seeds" (TPS), which are infection-free. The crop yield from 50 g of TPS is equivalent to that obtained from 3 tons of tubers [34]. A new program for the production of pathogen-free seed potatoes was inaugurated in Denmark in 1977. It is generally accepted that all potatoes grown in Denmark from the late 1980s should have originated from meristem culture [40]. Experience in Vietnam in the early 1980s showed that farmers with little or no experience in laboratory techniques or "modern" agriculture could satisfy their own seed potato needs. Thus, one satellite farm can produce about 300,000 plants from 200–250 test tubes, for planting on about 10 ha. This produces at least 70 tons of small seed tubers for the next season. In the Dalat area of Vietnam, 6% of the growing area is providing enough seed tubers to satisfy all farmers [42]. Similar programs using tissue culture, green house propagation, and field growth are used in countries such as Philippines [46], Korea [44], and Peru [48,49].

b. Advantages and Disadvantages for Potatoes. It would seem that the advantages of in vitro propagation of potatoes (tubers and plants) far outweigh the disadvantages. However, as demands for mass quantities and a higher quality planting material rise, issues of somaclonal variation [50,51] and the costs of maintaining and running appropriate laboratory and greenhouse facilities will become more significant. There may be conditions or areas in which tissue culture techniques—or products—will prove too expensive or complicated when compared with local conventional methods [50]. The use of new in vitro and molecular technologies in potatoes has resulted in significantly increased yields [33] owing to freedom from disease and selection for quality traits; establishment of multiple, international storage banks for valuable germ plasm; increased research opportunities; and wider distribution of this important food source. The basis of this success with a major food crop has been the use of a variety of tissue culture techniques.

B. Flowers and Ornamental Species

Initial ventures in commercial micropropagation concentrated on flowers and ornamental crops. As traditional consumer products with great horticultural and commercial value, they lent themselves to the new technology. Small laboratories, associated with established nurseries, began experimenting with and producing standard cut flower species, ornamental house plants, and foliage crops. These plants and others have been the production models for micropropagation methods and problem-definition and problem-solving techniques. Much progress in all aspects of plant micropropagation has come from work done on somaclonal variation [10,16,52,53], endogenous contamination, and disease detection and prevention [3,15]. That work has set international standards for the establishment of "mother block nurseries" with elite, tested, disease-free plants [54]; the use of enzyme-linked immunosorbent assay (ELISA) testing for the detection of plant disease; and the establishment of germ plasm banks for the maintenance, breeding, and protection of selected, disease-free varieties. To this day, plants for cut flowers and ornamentals account for the great majority of commercial micropropagation endeavors [5,7–9].

The advantages of tissue culture methods and micropropagation for flowers and ornamentals include a relatively low frequency of off-types for many species, a higher level of uniformity than with conventional methods, the ability to bring plant material to market year-round, the possibility for eliminating or significantly reducing disease, and the ability to bring new varieties and cultivars to market quickly and in relatively large quantities.

Among the disadvantages are the inability to lower high frequencies of off-types in certain cultivars, or to deal effectively with endogenous bacterial and bacteria-like contaminants that contribute to significant losses in, and immediately after, culture [15]. These problems remain limiting factors for the conventional and tissue culture production of some species and varieties.

1. Carnation (*Dianthus caryophyllus*)

An excellent example of the use of tissue culture techniques for commercial purposes is the carnation, a highly popular cut flower. The carnation industry of the 1960s and 1970s was suffering increasingly from disease and poor results from commercial growing [55,56]. As a result of progress in plant science during this period, it was determined that a combination of new technologies could significantly alleviate and, sometimes, eliminate viruses and disease symptoms, as well as provide higher yields and levels of uniformity from large-scale, commercial nurseries. The following scheme, developed in the 1970s, is used today with some modifications. It consists of the following [32,55]:

1. Meristem culture is carried out on selected cultivars, and the resulting individual plants are grown *in vitro*.
2. Clones of successful plants grown *in vitro* are tested with ELISA, to determine the presence or absence of viruses.
3. Those clones that are virus-free by testing are transferred as prenuclear stock plants to an approved, insect-proof, quarantined greenhouse for further growth and testing.
4. Plants from this group that are further proved to be virus-free are used as a source of cuttings for similarly maintained and tested nuclear stock.
5. Cuttings from virus-free nuclear stock are transferred to a nursery where plants are maintained and tested to provide cuttings for use as mother plants by commercial producers of carnation flowers.

This method, the *mother block system*, uses plant tissue culture for pathogen elimination and for cloning initial plant material. Less expensive, protected cultivation, and conventional methods (cuttings) are used for propagation. This has served as a model for other horticultural and agricultural crops, including gypsophila, phlox, strawberry, and sugarcane [32].

2. *Syngonium* and *Spathiphyllum*

Syngonium and *Spathiphyllum* species (Araceae), are among the world's most popular house plants. By the 1970s, use of conventional methods for separating and planting-out daughter plants could not supply market demands. Quality and uniformity were undependable, and disease remained a problem [57,58]. By using meristem culture of selected mother plants and micropropagation to produce millions of plants annually, the market today offers a wide variety of high-quality house plants, year-round, at affordable prices [59].

C. Fruit Trees and Plantation Crops

Fruit trees (i.e., citrus, olives, apples, pears, and stone fruits, such as peach) have not been a high-volume product of plant micropropagation. However, the ability to produce micrografts, rooted cuttings, and rooted plants for rootstock or grafts from selected, disease-free mother plants with the use of plant tissue culture has created a market. Today, there are selected laboratories that specialize in woody plants and fruit trees. Plantation crops (e.g., pineapples, sugarcane, palms, and bananas) require large quantities of planting material on a regular basis. Most of these crops have been cultivated for generations by conventional methods. Rising demand for both quantity and quality in planting material and final products has highlighted several issues worldwide. Among these are the spread of disease and lower yields, which have created the necessity to replant more frequently, often in new locations. More and more growers are attempting to solve problems and increase quality, yield, and efficiency with micropopagated planting material.

1. Banana (*Musaceae*)

Dessert bananas (*Musa acuminata* A and/or *M. balbisiana* B) have a triploid genome. Cavendish, AAA, varieties have been produced extensively for commercial markets using micropropagation technologies. An international cash crop since the late 1800s, dessert bananas are grown on tropical and subtropical plantations to provide fruit for the tables of developed nations. Bananas, *Musa* spp., are also a primary food crop and feature prominently in domestic production and consumption in Africa, South America, the South Asian Pacific rim, and the Indian subcontinent. In the second half of the 20th century, commercial production of bananas, as well as other plantation crops has been plagued by diseases, pests, and depleted and contaminated soils [60]; rising prices; increasing market demands for quality and uniformity; and world politics [32,54,61,62]. Costly, long-term breeding programs have been underway for decades, but their results have had a minimal effect on commercial production. Field selection remains the primary source for potential new varieties.

a. Development of Dessert Banana Tissue Culture Products for International Trade. The first report of the in vitro culture of banana meristems was in 1972 [63]. Since then, large-scale, commercial production of in vitro banana plants has developed, particularly in Taiwan, France [16]. South Africa [J.Parsely, personal communication, 1991], and Israel [16,32]. Micropropagation of banana plantlets is carried out by using standard shoot tip tissue culture procedures, as described earlier. In vitro, or hardened plantlets are sent to their destination,

where they are acclimatized, placed in shade nurseries, and planted in prepared fields. By the 1990s, companies were providing millions of plantlets annually for commercial plantations in South America, Africa, and Southeast Asia. In South America alone, nearly 100 million micropropagated banana plants have been planted since the early 1980s [32]. Israel, a country that produces bananas for domestic consumption, has not been afflicted with debilitating banana diseases. With a highly developed micropropagation industry, an academic infrastructure, and experienced nursery and field agronomists, Israeli companies have been in the forefront of providing disease-free, selected clones of in vitro dessert banana plantlets to the international market.

b. Advantages and Disadvantages. As with other crops, the ability to provide quantities (millions per year) of uniform, disease-free banana plantlets from selected clones is the primary advantage. Deliveries are timed to fit predetermined planting regimens which, in turn, are determined by the climate and market forces.

The primary disadvantage associated with in vitro culture of banana is somaclonal variation [16,52,53] (i.e., genotypic or epigenetic changes that are inconsistent with commercial production). Micropropagated bananas have shown a high propensity for variation, and considerable research in multiplication schedules, plant hormone quantities, and proportions has been performed to minimize this problem. Additional issues affecting production success and ultimate marketing strength are selection and quality of source plants, recognition and control of endogenous (nonpathogenic) bacteria, and adherence to acceptable commercial principles.

D. Forest Trees

As with other groups of plants, many studies show that micropropagation is also feasible in forest trees [64–66]; however, with some exceptions, traditional in vitro methods are not as yet practical or economically viable for most forest trees. Therefore, improvement of current procedures and their scaling-up are required. A major constraint on the use of in vitro procedures in forestry is the common belief that, from the environmental perspective, the genetic diversity of forests should be conserved; hence, the traditional use of mixed populations of seedlings for forestation. Three major exceptions, for which application of micropropagation becomes most relevant, do exist: (1) In vitro propagation methods are of crucial importance in breeding work, both to accelerate the process of provenance selection, and to establish selected mother trees for further breeding (2) Uniformity of the trees (i.e., clonal propagation) is desired where forestation is practiced for industrial applications, such as for pulp or specialized timber production (e.g., certain poplar and eucalyptus genotypes). In these cases, micropropagation can compete with traditional methods of vegetative propagation; (3) Whenever a need exists for rapid, large-scale production of selected trees for forestation.

Forest trees are no exception as far as the basic techniques are concerned, but proper adaptation of existing procedures should be worked out for each plant genotype. In the following, the application of micropropagation to some conifers will be reviewed.

1. Conifers

Micropropagation has been applied over the last 20 years to an increasing number of conifer species including Norway spruce, white and black spruces, radiata pine, and *Pinus pinaster* [65–68]. Considerable studies have been done on Southern pines, including loblolly and slash pines, longleaf and Virginia pines, and others. In some cases, these methods have generated numerous transplanted plants. Studies on other pines, including Mediterranean species,

such as Aleppo and brutia pines, are very limited, and practical success has not yet been reported. Four major types of micropropagation have been employed for conifers:

a. *Direct Organogenesis of Adventitious Buds from Organized Explants.* In general, the more juvenile the tissue, the better it will respond to treatments leading to organogenesis. Thus, mature isolated embryos are frequently used as the explants of choice, as well as cotyledons and epicotyls. Shoot tips, immature needles, needle fascicles, and short shoots (brachyblasts) were used in other cases. Adventitious bud formation offers a greater potential for shoot production than axillary bud breaking, because it involves the induction of localized meristematic tissues, and results in many adventitious buds at sites other than axillary bud meristems. Another potential approach (i.e., "meristematic nodule multiplication"), has been successful with radiata pine [69]. In this example, over 5000 pieces of meristematic tissues were obtained from one embryo of radiata pine in 13.5 months, resulting in numerous shoots. By using this method, shoots are multiplied so that the potential for large-scale clonal propagation can be realized.

b. *Fascicular and Axillary Bud Breaking.* Micropropagation of pines by this process usually begins with initially stimulating axillary or fascicular meristems on field-grown or potted trees by hedging and/or repeated cytokinin applications. The resulting shoots are then excised, and restimulated in culture to produce additional axillary or fascicular shoots. Sequential elongation and restimulation can produce many shoots. Although not as frequently used as induction of adventitious buds, the process of axillary bud breaking has become useful for multiplying dormant and nondormant buds of some mature conifers.

c. *Organogenesis by Callus.* Indirect adventitious bud formation, from callus, has shown only limited success in conifers. A few instances of shoot formation in conifer callus have been reported, and regeneration from long-term subcultured callus of *Pinus eldarica* has only more recently been established.

d. *Somatic Embryogenesis and Artificial Seeds.* Somatic embryogenesis in many woody species is less successful than other methods of micropropagation. The earliest report of somatic embryogenesis in a coniferous tree is that for spruce *Picea abies* [70]. Since then, somatic embryogenesis has been reported in several other *Picea* species. Mainly immature, and occasionally mature embryos, were used successfully in conifers, yielding somatic embryos and plantlets. Although the number of species that can be propagated through this method has been increasing, the percentage of recovery for plantlets remains relatively low. Studies of somatic embryo formation in pines show that they can arise either in the conventional manner, as for spruce, or by a process termed somatic polyembryogenesis, as found with sugar and loblolly pines [71]. The resulting embryos elongated and formed numerous cotyledons in a repetitive and true-to-type polyembryonic process. In the loblolly pine, the somatic embryos thus produced developed transplantable plants. The rate of somatic embryo formation has been improved recently, making it commercially viable for some species. Procedures for induction of somatic embryogenesis and subsequent conversion of the embryos to artificial seeds have been developed in a limited number of spruce and pine genotypes, such as Norway spruce and loblolly pine, with treated embryos being stored for further use as artificial seeds.

Long-term field performance of conifers that were micropropagated, as described in the foregoing, has been evaluated in some species, showing no major detrimental changes relative to traditional seed-propagated conifers.

V. COMMERCIALIZATION AND ECONOMIC CONSIDERATIONS

A. A Survey of World Production and Distribution

Production of in vitro plantlets continues to expand worldwide. In the early 1980s, the Netherlands, a founding center for commercial micropropagation, produced 20 million plants annually. By the close of the 1980s, they were producing 50 million [9]. From its beginnings in the 1970s, the commercial micropropagation industry has been loosely documented. The 1980s saw a particularly sharp rise in the number of laboratories in the United States, Europe, and Israel. They were soon producing beyond market demand, and additionally, by the mid to late 1980s, Eastern Europe, Southeast Asia, and South America, with cheaper manpower and overhead, had entered the market and were providing significant competition in the production of orchids, cut flowers, and house plants [8], heretofore, mainstays of traditional European and American producers.

In 1992, the European Community [7] conducted a survey of professional and commercial laboratories from 21 European countries. They presented data from 501 responding laboratories, 172 of them commercial. This survey indicates that commercial production in Europe, Great Britain, and Ireland was approximately 80–100 million, or more, plants annually. In 1996, Zimmerman conducted a survey of 113 commercial laboratories in the United States [5]. This survey reported the current level of output, approximately 120 million plants annually, will almost certainly continue to grow in the next decade. In 1996, Israel, a small country with a very active micropropagation industry, had approximately ten commercial laboratories producing between 20 and 25 million plantlets per year [R. Levin, personal communication, 1996]. During the 1990s, South Africa has consolidated an active academic and commercial plant propagation industry [O. Reuveni and J. Parsley, personal communication, 1996]. It must be noted that commercial plant micropropagation is a highly competitive and dynamic industry. Therefore, exact figures are difficult to acquire, and the information provided here reflects the most reliable information available.

B. The Financial Aspects of Commercial Micropopagation

Biotechnology research and development presents financial risks. Scientists, encouraged by the quality and advantages offered by new methods, have been creating commercial ventures for plant biotechnology since the 1970s. Most of these companies, however, have been unable to continuously meet the demands of a market-driven economy, and many have failed. Remaining companies have continued, often with some government or corporate subsidies, and few have become financially independent. This has been true for all sizes, types, and locations of micropropagation companies [32]. The reasons for this and some possibilities for overcoming problems will now be addressed.

1. Economic Potential

In the 1970s, plant biotechnology workers, looking for markets for their new and potential products, recognized that there were over 15 million ha planted with basic food and forest crops worldwide. Many of these crops and the areas in which they grow were, and are, suffering from disease, poor cultivation techniques, conditions of abiotic stress, and resulting lower yields. Accordingly, they could benefit both from crop improvement programs that use in vitro technologies and from mass production of quality propagation material. This could,

and still can, become a reality only if efficient, relatively inexpensive production processes are developed.

2. Costs

The establishment of one micropropagation facility and the agricultural, marketing, and management systems necessary to support it, may cost 1 million US dollars or more. Additionally, conservative, agriculturally based populations are reluctant to abandon traditional methods and to invest in new ideas. Micropropagation companies often must develop expensive, long-term-marketing programs, including pilot farms, education, and technical, after-sales-service networks. The labor-intensive nature of the plant micropropagation industry is high-cost-intensive. A large-sized, high-volume company may employ 50–100 workers for in vitro subculture and transplantation alone. In industrially developed countries and in an increasing number of developing countries, labor, often accounting for 40–60% of production costs, remains a limiting factor in the production and marketing of micropropagated plants [32]. Given that most production work is relatively simple and repetitive, automatic or robotic solutions have been sought for some years. Concomitantly, export-oriented commercial tissue culture companies have been set up in many developing countries to exploit cheap labor [72]. Such countries as China, India, or Indonesia, are looking for labor-intensive industries to employ ever-enlarging masses of workers. The issue of automation versus inexpensive foreign labor creates a potential dichotomy within the industrialized sector of micropropagation.

3. Additional Limiting Factors

Despite scientific advances and available technology, micropropagated plants are subject to the unknowns associated with agriculture. Sophisticated methods are used in production processes, but “unexpected” and, often, inexplicable problems occur that affect growth and development or microbial contamination. As a result, significant production may be lost, accompanied by the related economic and marketing ramifications. Micropropagation companies are often based on export; consequently, they must be equipped to deal with international marketing, monetary issues, and protectionist policies. Political situations often limit marketing efforts and reduce profitability. Only consistent, quality products and services, and dedicated customers, can deal with these complications.

C. Industrial Micropropagation

The three defining characteristics of industrialized plant micropropagation follow:

1. A company that has developed a specialty in certain plants or product types and markets more than 2 million plants annually
2. A company that has become relatively self-sufficient in research and development, production, professional manpower, facilities, and sources of plant material
3. A company that engages in broadscaled, vertically integrated agrotechnology projects

A successful industrialized plant technology company will have achieved expertise in most aspects of developing and growing a particular product. When this is coupled with a business-like approach to marketing, management, and customer service, there is a greater likelihood of economic success.

1. Scaling-Up

As the industry has enlarged, expanding companies have encountered a legion of new situations. The dynamics of scaling-up and cost-effectiveness must be harnessed to stringently engineered inventory control and quality assurance programs. With expansion, there is often an initial loss of ability to identify subtle changes or problems in production, and timely solutions are not initiated. Expanded inventories with short shelf lives are much more difficult to manage in an export-based company.

Because most crops are not planted or harvested on a year-round basis, there are peaks in production schedules. However, commercially viable, year-round employment of staff and facilities is a necessity, and industrialized companies often have the advantage of maintaining stocks of start-up cultures that allow them to minimize the time between order and delivery.

2. Large-Scale Production and Automation

The demand for high-quality transplants has recently been increasing rapidly worldwide for reforestation, food and forage production, landscape and indoor horticulture, and global environmental protection [4]. However, widespread use of micropropagation for major crops in agriculture and forestry is still restricted because of relatively high production costs [73,74]. In recent years, much effort has been devoted to developing automated, robotized, and more efficient transplant production methods [75]. Developing an automated mass-propagation system for producing in vitro plantlets at lower costs will become more and more important in horticulture, agriculture, and forestry. When analyzing current standard methods for commercial micropropagation, several critical issues can be discerned, as discussed in the following.

3. Automated Systems

Several systems are in various stages of development and testing. Among them is an automated system in Holland, based on image analysis, computer-controlled laser cutting and robotized planting of explants [76]. Liquid culture, using large containers (1–2000 L) with plant material immersed in liquid media, is the most cost-effective form of growing in vitro cultures. It also lends itself to automation more easily than culture on semisolid media. Various commercial and research laboratories, including Biological Industries in Israel and Kirin Breweries in Japan, have production lines and develop prototypes based on liquid culture [77–80; M. Aharoni, personal communication, 1994; R. Levin, personal communication, 1993; M. Ziv, personal communication, 1994]. Research and development of automated systems can be found in many countries, with good examples in Spain [81] the United States [82,83], and Australia [D. Schonstein, CIG, personal communication, 1991]. “A novel approach to multiplication of in vitro plantlets” [26] claimed that a considerable reduction in production costs could be attained by means of a deliberate multifactorial system, including robotics.

4. Issues Facing Automated Micropropagation Mass Production Systems

The issues fall into three categories: technical, horticultural, and economic.

a. Liquid Culture. Despite its many advantages, liquid culture is still a subject of considerable research, and there are several physiological limitations to propagating many plant types in immersed culture. Among these are vitrification, deformation, and somaclonal variation because of sometimes uncontrolled multiplication [84,85]. Although these conditions are

known to appear in agar-based micropropagation schemes, the small number of plants in a given batch or container usually minimizes the damage. In the event of contamination, where the entire liquid culture system might be contaminated or damaged, the technical problems and economic losses are potentially severe.

b. Complex Plantlets. Systems using semiautomated, automated, or robotized cutting tools are hampered by at least two problems: (1) Tools are often engineered for one standard cutting mode (i.e., stem sections), making them inappropriate for plants that are manipulated differently; and (2) the use of computer-aided cutting systems and visual analysis is complicated. These systems tend to be less successful and often unsuccessful for plant types that are manipulated in three dimensions. For many, if not most, commercial laboratories, the combination of these two issues makes the systems undesirable.

c. Costs. In addition to the basic costs of establishing and operating a plant micropropagation laboratory, the high expenses for research and development and for purchase of automated systems limit their use for most commercial laboratories. Only high-volume, single-type product, established companies usually consider such an option. Additionally, as is often true in the use of robotics, if the machine ceases to be necessary, it continues to occupy space and must be paid for.

d. Practical applications. Many pitfalls and unsolved problems remain in the scale-up and full automation of plant micropropagation. As a result, many commercial laboratories have successfully implemented partial or semiautomation; for example, development of a prototype for the automated manipulation of growth containers with *in vitro* plants. This robotic system carries out the sterile exchange of liquid medium at an accelerated rate, with minimal worker participation [86]. There are processing systems for which media components are measured, mixed, processed, and poured into containers, based on computer programming and minimal human support [personal observation]. Other examples include systems for inventory and production task control, computed environmental control systems that monitor, store, and report regularly on environmental data and set off alarms when standards are not met. In every medium- or large-scale laboratory, creative workers and maintenance support people have engineered many original, in-house solutions to the problems of time, space, and costs.

e. Advantages and Research Goals. Commercial micropropagationists seek any cost-effective, reliable, system that can be used routinely and that minimizes human labor while, at the same time, guaranteeing quality and uniformity. There is no doubt that a high-quality, moderately priced, maximally engineered and automated system for selecting, manipulating or planting *in vitro* plantlets could provide solutions to production problems that currently prevent laboratories from reaching high, predictable, and uniform levels of quality.

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In Vitro Production of Pathogen- and Contaminant-Free Plants

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I. INTRODUCTION

Disease-free planting material is the essential first step in holistic plant health management [1]. It is a generalization that most pathogens affecting a plant are eliminated at seed formation, and provided the seed is surface decontaminated, seed-derived crops start disease-free. The field problem is thus disease exclusion until an economic yield has been achieved. For vegetatively propagated crops the converse is generally true. Infection is transmitted by the propagation material to the young crop [1,2].

Historically, the production of disease-free or, more correctly, symptomless planting material of vegetatively propagated crops depended on the selection of disease-escapes and the multiplication of these individuals under isolation conditions [3]. Both indexing of individual plants for latent contamination [4], and attempts at disease elimination [5], were subsequently introduced. Indexing took the form of attempts to isolate cultivable pathogens on a range of media, including selective media; and of mechanical and vector transmission of virus and other noncultivable or fastidious organisms to indicator species [6]. Elimination of bacteria, fungi, and viruses was attempted using antibiotics [7], heat [5], or cold therapy [8]. When the latter strategies were successful, there remained the problem of maintaining the high health status of this elite stock during mass clonal propagation.

The dual problems of efficient pathogen elimination and of clonal propagation of clean stock were resolved, in principle, by developments in plant tissue culture. Morel and Martin [9] demonstrated that meristem-tip culture could result in the elimination of pathogens from infected plants, and Murashige [10] and others [11] pioneered the development of in vitro cloning systems.

Several alternative in vivo and in vitro, or hybrid, options are now available, both for the elimination of pathogens and for the multiplication of elite stock ([Fig. 1](#)). The choice depends on the technical and economic feasibility for the particular crop and circumstances. Here the bias is toward in vitro techniques, although it is acknowledged that much practical benefit may

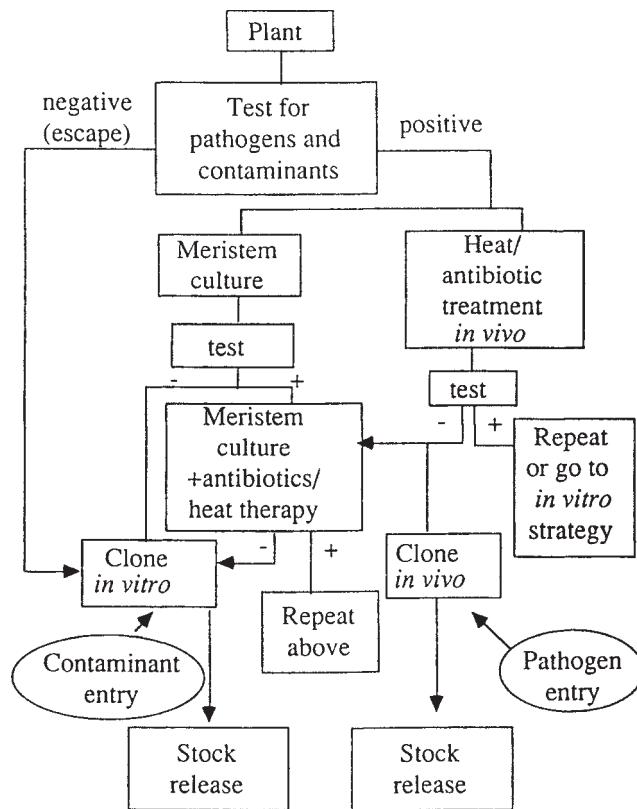


Fig. 1 In vivo, in vitro, and hybrid strategies for the production of pathogen- and contaminant-free plants.

be gained from investigation and treatment of the tissue-donor plant before using in vitro procedures for disease elimination. It is also recognized that, although with in vitro cloning there is a lower risk of pathogen reinfection than in field multiplication, owing to the low probability of pathogen inoculum availability in the aseptic laboratory environment, nevertheless, there is a risk of laboratory (nonpathogenic) contamination.

II. THE NATURE OF MICROBIAL CONTAMINATION OF PLANTS IN VIVO AND IN VITRO

Over the past decade our understanding of the nature of the microbial contamination of plants *in vivo* and *in vitro* has developed [12–16]. Primary contaminants can be categorized as known pathogens of the crop; plant surface-inhabiting bacteria and fungi, especially yeasts, that randomly colonize plant tissues; viruses; and vector-transmitted microorganisms. The latter include intercellular or intracellular bacteria, bacteria-like organisms, viruses, and viroids. Secondary contaminants, that is, those that enter clean cultures during *in vitro* cloning, include common airborne bacteria and fungi, and microorganisms associated with the operatives, for example, skin-associated microorganisms [14]. These contaminants may grow on the media or grow endophytically in the plant tissues.

The primary contaminants may include organisms that are pathogenic to the crop in specific geographic areas or that are pathogens of other crops. In the latter example, cultivation of the microplants may introduce a reservoir of pathogen inoculum into the environment [17].

Of more immediate consequence is the effect that the expression of secondary contaminants may have during micropropagation. These range from contaminants outgrowing the plant tissues and possibly affecting growth by nutrient depletion, production of toxic by-products (e.g., acids that make the pH of the medium unsuitable for plant growth) or toxins [11]. In the absence of controls, the effects of contaminants on productivity cannot be estimated; in some cases contaminant expression may occur in stage 1, resulting in the loss of the cultures; other times, expression is delayed until the explants are transferred to a different medium, as for example, at the rooting stage, when 100% loss may occur; subliminal losses in productivity have also been reported [11]. Consequently, it is essential that plants cultures and progeny plants be clean. Unfortunately, it is practically impossible to confirm that cultures are axenic, that is, free of all contaminants, but certification is possible. Previously [12] the following health status categories were suggested:

- Class 1: Free of all cultivable contaminants and free of all known diseases of the crop.
- Class 2: Free of all cultivable contaminants and free of specified diseased of the crop.
- Class 3: Free of cultivable contaminants.
- Class 4: Uncertified.

Monitoring for primary and secondary contamination will be discussed here in relation to the stages of micropropagation, as originally defined by Murashige [10], and modified by Debergh and Maene [18] (see also Cassells for an expanded perspective on Stage 0 [14]), namely:

- Stage 0: Selection and preparation of the plant for culture
- Stage 1: Establishment of explant in vitro
- Stage 2: In vitro cloning
- Stage 3: Preparation for return of the in vitro propagule to the environment
- Stage 4: Establishment in vivo

It is considered good-working practice that all cultures be confirmed to be of pathogen- and contaminant-free status before cloning and that cultures be monitored during cloning for secondary contamination [15]. Contaminants are considered under the following categories:

1. Cultivable bacteria and fungi
2. Characterized pathogens of the crop
3. Noncultivable and fastidious contaminants
4. Low titer, noncultivable and fastidious contaminants

III. DETECTION OF PATHOGENS AND CONTAMINANTS IN PLANTS AND CULTURES

A. General Considerations

In the context of pathogen elimination and contamination management using in vitro procedures, the detection of pathogens and contaminants should be at a meaningful level (e.g., the tissues of annual, and especially perennial plants, are frequently colonized to varying degrees by pathogenic and surface-inhabiting bacteria and fungi) [17,19]. In general, this has no significance if the contaminant-free tip of the plant is to be introduced into culture. Virus titer, on the other hand, may be low in in vitro tissues, making detection difficult; hence,

screening to determine pathogen presence may be more accurate when carried out on the donor plant [20]. As a generalization, it may be strategically useful to screen donor plants for intracellular (i.e., "systemic") contaminants before stage 1 and to defer screening for cultivable contaminants until explant establishment in stage 1.

1. Detection of Cultivable Bacteria and Fungi

a. Detection of Cultivable Bacteria. Detection of bacteria in plant tissues has been discussed extensively [e.g., 21]. It is important to appreciate that less highly domesticated plants (i.e., nonfood plants) may contain inhibitory levels of tannins and such, thus, culture on bacteriological media of explants, rather than expressed sap, may be advisable for the detection of low-titer contaminants [6,21]. A form of enrichment may occur when the contaminated tissues are cultured on appropriate media allowing low levels of bacteria to multiple above the threshold for detection. It is also advisable to use a range of bacteriological media, rather than just one, or a few media, as some bacteria (e.g., *Xanthomonas* spp.) have specific nutrient requirements [21–23]. Selective media may also be employed when screening for bacterial pathogens, but it should be recognized that avirulent forms of many pathogens abound and that virulence testing is the only certain way of confirming an isolate's pathogenicity [22].

In culture, bacterial contaminants may be detected visually where they grow on the culture medium. Clear geling agents have been advocated to detect bacteria, which may be revealed as "halos" around the explant. To encourage bacterial expression in the plant tissue culture media, the incorporation of components of bacteriological media, such as casein hydrolysate, has been recommended [24]. In any event, it is essential that in vitro tissues be sampled and cultured in stage 1 to confirm freedom from bacterial contamination before proceeding to stage 2.

Laboratory contamination of clean cultures is a serious, but poorly documented, problem in commercial micropropagation: the causes are due to poor working practice [25,26]. Because the bacteria concerned are selected from the environment for their ability to grow on plant tissue culture media or in plant tissue, they can be detected on general bacteriological media.

b. Detection of Cultivable Fungi. Macrofungi can be readily detected visually, although occasionally tissue incubation may be required for expression [6]. Fungal contamination of cultures is generally avoided by using the shoot tip as explant. Yeasts are a more frequent problem than filamentous fungi. Yeasts grow on plant culture media, but few detailed studies have been carried out on their occurrence as tissue culture contaminants [27].

B. Detection of Characterized Pathogens

There is a vast amount of literature covering the detection of plant pathogens [e.g., 6,20,22,28–30]. For the major pathogens of important crops, commercial diagnostics are available [28]; for lesser pathogens or minor crops, protocols for identification have been published [6]. The task of pathogen identification, of which detection is the first step, can be summarized as confirming the identification of a known pathogen. A caveat is that the virulence of an isolate cannot be assumed and must be confirmed by test plant inoculation in fulfillment of the requirements of Koch's postulates [6]. Modern diagnostics, based on detection of the determinants of pathogenicity, may obviate this requirement when the determinant of pathogen virulence is a characterized factor [31].

C. Detection of Noncultivable and Fastidious Contaminants

The distinction in this category is between potentially cultivable (fastidious) and noncultivable, intracellular organisms.

1. Detection of Fastidious Microorganisms

The ease of detection of fastidious, potentially cultivable, bacteria and bacteria-like organisms is dependent on titer and on tissue distribution. These organisms can be detected *in situ* in tissue sections by nucleic acid fluorescent dye-staining techniques [32]. The bacteria appear as multiple, additional centers of fluorescence when compared with control-stained sections.

2. Detection of Noncultivable Microorganisms

Intractable bacteria and bacteria-like agents can be detected by fluorescent staining, as described in the foregoing. Viruses, again depending on titer, and recognizing the problems of nonuniform tissue distribution, may be detected by leaf-dip electron microscopy [33]. Viruses and viroids may also be detected by polyacrylamide gel electrophoresis of their nucleic acids following nuclease treatment [34–36]. This method is based on the resistance to nucleases of viroid double-stranded (ds)RNA genomes and replicative viral dsRNAs.

D. Detection of Low-Titer, Noncultivable or Fastidious Microorganisms

The first step in the detection of low-titer noncultivable or fastidious organisms is attempted transmission to a high-titer host to facilitate detection. This may be possible using dodder, mechanical, graft, or vector transmission [6]. If it is not possible to transmit the organisms to a high-titer host, *in situ* tests may be used in an effort to place the organism in a pathogen class. These tests can be employed sequentially, starting with nucleic acid fluorescence staining to detect bacteria and bacterial-like organisms [32]; followed by nucleic acid analysis on polyacrylamide gels to detect viroids and replicative forms of viral RNA [34], and leaf-dip electron microscopy to detect virus particles [33]. If the virus titer is too low to detect by leaf-dip electron microscopy, then attempts at virus purification or partial purification may be carried out and the leaf-dip technique repeated [33]. This latter step may increase virus titer enough to enable particles to be detected which, in turn, may allow attribution to a virus group and facilitate further characterization [37]. The technique of last resort is thin-section electron microscopy, which is demanding of expensive capital equipment, skilled labor, and can be time-consuming. It has the potential, however, to detect contaminants of all classes [6].

IV. IDENTIFICATION OF MICROORGANISMS

It is not appropriate or possible here to discuss the taxonomy of microorganisms other than at a basic and applied level. However, those approaching the problem of detection, identification, and elimination of pathogens and contaminants should appreciate that historically and to date, there has been confusion, controversy, and instability in the grouping of microorganisms based, in large measure, on the nonphylogenetic classification schemes used; this is particularly so in the classification of bacteria.

Microorganisms, whether pathogens or contaminants, as defined in Section II, are considered by plant pathologists under three classes, (1) fungi; (2) prokaryotes (bacteria and bacteria-like organisms; i.e., mycoplasma- and rickettsia-like organisms), and (3) viruses and virus-like organisms (i.e., viroids) [2]. Historically, the classification of the fungi has been based on morphological characteristics, visible under the light microscope, with emphasis on spore and spore-bearing structures. The presence or absence of cross walls (septae) in the mycelium and the composition of the cell walls are also important. The recognition of these

characteristics allows identification to the species level. Pathovars are further characterized by inoculation of test plants [6]. Fungal taxonomy has recently been revised [38].

The classification of bacteria is more problematic, for reasons of their simple cell morphology and the general instability or variability that they exhibit [39]. The conventional classification of bacteria has been based on the grouping of specific morphological, biochemical, cultural, and pathological characteristics. In plant pathology, identification to genus (of pathogenic species) has been based, for example on the Gram stain and cultural response on defined media [40], with arbitrary, biochemical and cultural tests to determine species, and specific tests to identify pathovars [40,41]. The identification of mycoplasma- and rickettsia-like organisms, MLOs and RLOs, respectively, is based on their localization in the host tissue and, when cultivable, on their growth on defined media [43].

In medical and environmental bacteriology, common bacteria are identified with biochemical test kits or by fatty acid profiling or other chemical analyses [44]. A consequence of the instability of bacteria, however, is that prokaryotes that do not fall into the category of plant pathogens, clinical or "commercial" bacteria (bacteria of economic importance; e.g., in the food industry), may be difficult to characterize to species level.

The classification of viruses has been based primarily on the form of the nucleic acid in the genome and on particle morphology [43]; division into subgroups being based on biological properties, such as mode of transmission and so on. The essential feature of viroids is the nonencapsulated dsRNA structure of the genome [37,43].

Current developments in molecular biology have led to a major reappraisal of the taxonomy of viruses. Studies of nucleic acid base sequence homology have shown that conventional classification, based on particle morphology, masks more fundamental relations seen at the level of the genome replication strategy. This is leading to a regrouping on a phylogenetic basis, which also shows relations with vertebrate and invertebrate virus groups [37]. Similarly, it is anticipated that sequence characterization of RNA in prokaryotes will also lead to a phylogenetic scheme for bacterial classification [44]. It is anticipated that these molecular schemes will lead to practical applications, particularly in the detection, identification, and elimination of the many rare and exotic microorganisms that plant propagators encounter.

A. Identification of Pathogens

Pathogens fall into two distinct categories: first, those for which their identity is known, or suspect, and requires confirmation; and second, those for which their identity is unknown [2,6].

1. Methods for the Confirmation of Pathogen Identity

The requirements of tests for the confirmation of the identity of a pathogen depend on fulfilling Koch's postulates [6]. In practice these requirements are usually not met. Most molecular "diagnostic" methods are based on the sensitive detection of antigens or DNA base sequences at the species, or less often, at race level [31]. They include serological tests, such as enzyme-linked immunosorbent assay (ELISA) and the use of nucleic acid probes [28]. An important consideration is that these kits do not generally distinguish between pathogenic races (pathovars) and nonpathogenic races [22,31]. Thus, it is essential that the test includes detection of the determinants of pathogenicity. Molecular diagnostics, for this purpose, for example for the detection of pathogen toxins [31], are under development but, as is usually the case, they are likely to be available only for pathogens of major crops. Those working with minor crops may have to rely on more classic methods for confirmation of identity, or material can be sent to specialists.

B. Characterization and Identification of Contaminants

Although certification of cultures as being free of specific pathogens requires that this category of contaminant be fully characterized, that is, their detection and identification must involve specific diagnostic tests; the same argument does not necessarily apply to nonpathogenic contaminants, nor may it be economic, nor practical, to attempt identification of the latter. It is, however, advisable to characterize cultivable bacterial contaminants to group level to facilitate the choice of appropriate antibiotics [16]; and also fastidious and noncultivable organisms to the class level to facilitate testing for confirmation of elimination.

1. Characterization and Identification of Cultivable Bacteria

Three types of bacterial contaminants are associated with plants and tissue cultures; namely, plant pathogenic bacteria, and plant-associated bacteria in the tissue donor plant; and in cultures, bacteria carried over in the explants, and laboratory contaminants, including operative-associated bacteria. The distinction between the degree of difficulty in identifying plant-associated nonpathogenic bacteria, as opposed to laboratory contaminants, should be emphasized. The characteristics of plant pathogenic bacteria, environmental bacteria, and human-associated bacteria exist in databases, and isolates can be identified by reference to such databases using standard methods; for example fatty acid profiles [41], or biochemical profiles, using the biochemical test systems (e.g., from API-bio-Merieux, France) [25]. For the latter, it is advisable to carryout preliminary tests to group the organisms for the appropriate test kit [16].

The identification of nonpathogenic plant-associated bacteria poses special difficulties because fatty acid profiles and biochemical test kits depend on the appropriate profile being in the database, experience has shown that such databases are incomplete in this respect. A range of more specialized tests are available for the grouping and identification of bacteria. These are beyond the scope of this review. The reader is referred to Goodfellow and O'Donnell [44] and references therein.

2. Characterization and Identification of Fastidious or Noncultivable Microorganisms

The task of identifying any unknown microorganism is formidable and rarely justified on cost grounds in this applied context. However, if identification is to be attempted, the first stage is as for detection; namely, determination of the class to which the microorganism belongs. The scheme in [Figure 2](#) is suggested as a pragmatic identification strategy for fastidious or noncultivable organisms. When the pathogen class has been elucidated, tests to determine the microorganism's affinities with characterized microorganisms within the class can be carried out. It is not appropriate to go into these procedures here in detail. The reader is referred to Fox [6] and references therein.

C. Fungi

Macroscopic fungi can be identified by reference to the appropriate keys [6]. Increasingly, serological [42] and DNA-based [28] diagnostics are becoming available for this group, but again, with the caveat that identification will be incomplete unless determinant(s) of pathogenicity are included [31].

Biochemical test kits are available for clinical and environmental yeasts, but these may not identify many plant-associated yeasts [27].

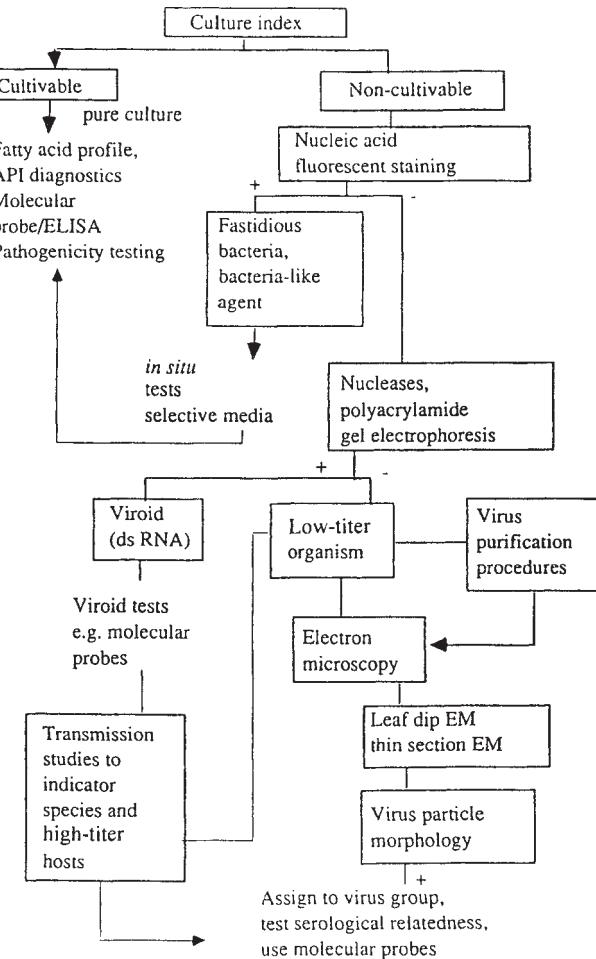


Fig. 2 Tests for the assignment of a pathogen or contaminant to a microorganism group.

V. ELIMINATION OF PATHOGENS AND CONTAMINANTS

It is generally accepted that the meristematic region escapes infection by most pathogens and contaminants affecting the soma of the plant [37]. Thus, meristem culture gives a high probability, in many cases, of establishing pathogen- and contaminant-free cultures. When contaminant- and pathogen-free status is confirmed, the production of clean plants depends on monitoring for, and controlling, laboratory infection [14,16]. Such infection is unlikely to result in pathogen reentry, as sources of inoculum should be excluded. There may, however, be cases for which it is not possible to ensure the survival of a small enough meristem explant to satisfy the exclusion conditions, or indeed specific cases (e.g. beneficially infected cultivars) where meristem culture may be precluded, as this would alter the characteristics of the cultivar [12]. In these circumstances, appropriate elimination strategies are essential. Here, for the reasons given in Section I, strategies for pathogen elimination are considered in the context of the process of micropropagation, with consideration of the need to detect, characterize, and identify pathogens and contaminants during the respective tissue culture stages.

A. Action to Be Taken in Stage 0

Symptom expression in the donor plant may indicate the presence of a specific pathogen; however, the absence of symptoms should not be taken as proof of pathogen absence [2,6]. The main factors in stage 0 are the avoidance of pathogen spread by using overhead water and the use of nutrient regimens that harden the plant, and thereby, may restrict the entry and movement of bacteria. The treatments may result in suppression of symptoms. For a discussion of symptomatology see Agrios [2].

A diversity of cultivable bacterial and fungal contaminants have been isolated from plant tissues [11,13,17,25]. The host genotype may be a selective niche for pathogenic strains, but plants in general, and perennials in particular, may be colonized endophytically, opportunistically, by a range of common phylloplane- and rhizosphere-inhabiting bacteria. This colonization may be localized (e.g., at a site of wound damage) or "systemic" when the bacterium or bacteria are widely distributed. In general, such contaminants may not reach the plant apex and thus meristem tip culture can be used to eliminate the majority. Even if they are transmitted in the explant, it may be more effective to take action in stage 1 than in stage 0; however, the bacterial titer may be reduced by the application of antibiotics to the donor plant in stage 0 [7,45]. Use of antibiotics on plants *in vivo* must conform to local restrictions that may apply to the nonclinical uses of antibiotics, and the contaminant should be screened for antibiotic sensitivity before chemotherapy [46].

Although the application of antiviral chemicals to plants has been the subject of much research, it has been difficult to maintain an effective concentration of the materials that appear to be mainly viristatic [43]. Physical treatments, such as heat or cold therapy, are effective means of eliminating pathogens, especially viruses [43]. When a virus is not eliminated in meristem culture in stage 1 (see following section), it may be cost-effective to attempt heat therapy of the donor plant. A limitation of this approach is the time taken, which may run into weeks, normally 6–12 weeks at 30°–40°C [43].

B. Action to Be Taken in Stage 1

A tenet of good-working practice in micropropagation should be the establishment of clean cultures in stage 1. The action to be taken to ensure freedom from contamination follows on from the action taken in stage 0. If the donor plant has been disease-indexed positive for one or more viruses, viroids, fastidious bacteria, or bacteria-like agents, cultures from the progeny of each established meristem must be exhaustively screened and shown to be pathogen- and contaminant-free before progressing to stage 2. Comprehensive indexing for cultivable bacterial contaminants is necessary, using a range of bacteriological media [21]. If the donor plant has not been indexed, pathogen testing *in vitro* is essential, but the results should be interpreted with caution, as the pathogen titer in *in vitro* tissues may be below the threshold for detection. Establishment and testing of progeny plants is advisable in crops for which there is a serious pathogen risk.

If pathogens or contaminants are detected, clean donor plants can be sought, or stage 0 donor plant treatment considered (e.g., the use of antibiotics or heat therapy *in vivo* for bacteria and viruses, respectively). Alternatively, *in vitro* heat or chemotherapy can be applied [43]. For bacterial contaminants, the bacterium or bacteria should be isolated in pure culture and their antibiotic sensitivity determined using correct procedure [46]. The antibiotic should then be included in the culture medium, at the appropriate concentration, and new growth excised, replated, and rescreened before proceeding. For viruses and viroids, heat therapy, or the use of the broad-spectrum, nonphytotoxic, viristatic antiviral agent ribavirin (Virazole), or other

Table 1 Sensitivity of Representatives of the ssRNA Genome Plan Virus Families/Groups to Ribavirin

Plant virus family/group	Representative virus eliminated in vitro	Representative virus inhibited in vivo	Ref.
Superfamily A (Sindbisvirus-like viruses)			
Subgroup A1	Potato virus X		47
Subgroup A2	Cucumber mosaic virus		48
Superfamily B (Luteovirus-like viruses)			
Subgroup B1	Carnation mottle virus		49
Subgroup B2	Potato leaf-roll virus		50
Superfamily C (Picornavirus-like viruses)			
Subgroup C1	Grapevine fan leaf virus		51
Subgroup C2	Potato virus Y		47
Rhabdovirus group	—	(Fish Infectious hematopoietic necrosis virus) ^a	52
Bunyavirus group	—	Tomato spotted wilt virus	53
Viroid	—	Citrus exocortis viroid	54

^aNo example of inhibition of a plant rhabdovirus has yet been published.

Source: Ref. 37.

antiviral chemicals, should be evaluated [43]. It is essential that the ribavirin is maintained at an inhibitory concentration in the culture medium during the culture period, and that only new growth is excised for growing on and testing [47]. The spectrum of activity of ribavirin against virus representative of the main groups of plant viruses is summarized in Table 1. Ribavirin is active against all groups of single-stranded (ss)RNA genome viruses which includes the vast majority of plant pathogenic viruses [37]. It is also reported to inhibit viroids [54]. The efficacy in vitro of other antiviral chemicals merits investigation.

C. Action to Be Taken in Stage 2

Recontamination during stage 2 cloning is a cause of significant loss in the micropropagation industry. The contaminants are generally ruderal bacterial and fungal species that can overrun the culture before action can be taken. This type of contamination is easy to detect visually and can be controlled by discarding contaminated cultures.

A second and more problematic form of contamination is that by contaminants that are weakly expressed or remain latent in the plant tissues. Contaminant build up in this instance can be prevented by good-working practice [26], an important aspect of which is the rigorous monitoring of production based on random sampling. It is important in sampling to recognize that contamination may not follow a Poisson distribution, but may occur in foci at which, for example, an operator has introduced contamination through incompletely sterilized instruments. In this event, cultures up- and downstream from the detected contaminated culture should also be screened to determine the limits of the foci [15,55].

Although ruderal species may be too fast growing to allow control by antibiotics, slower-growing contaminants (e.g., those restricted to the plant tissues) may be controllable by subculture onto media containing appropriate antibiotics.

D. Action in Stage 3

Although labor costs have tended to encourage cloning protocols in which stage 3 is bypassed [11], if included, this stage should be subject to the same monitoring as stage 2. Previous studies have indicated that reducing the concentration or altering the composition of the medium at this stage may result in the massive expression of latent or subliminal contamination [45]. This emphasizes the need to exercise good quality control over contamination at all stages in micropropagation.

E. Action in Stage 4

It is advisable to screen samples of production at microplant establishment and growing-on to ensure that no pathogens have escaped detection in the earlier stages of the process. It is also necessary to ensure that the microplants establish without succumbing to damping-off diseases. To this end, production of biomass of good physiological status should be the objective of stages 2 and 3 [56] to avoid damping-off [2].

VI. CONCLUSIONS

Disease-free planting material is a prerequisite for good crop growth and high yield. Mass clonal propagation of plants *in vivo* or *in vitro* poses great risks for the spread of pathogens in planting material. It is essential that these risks be minimized. A series of options exists for the elimination of pathogens from planting material (see Fig. 1) that, when combined with efficient pathogen detection and identification procedures, ensure high mother stock quality. However, disease-free mother stock has to be multiplied to ensure an adequate supply of propagules for crop planting. During multiplication in the environment, the material is always at risk for reinfection. Cloning *in vitro* of disease-free individuals avoids this risk, but cultures are vulnerable to laboratory contamination. Good laboratory practice can minimize losses. An advantage of *in vitro* strategies for the production of disease-free material is that pathogen elimination is a once-off process, clean cultures can be maintained under slow-growth conditions [57] with minimal risk of reinfection by pathogens.

In the major vegetatively propagated crops, such as potatoes and other tuber crops, or bananas and other plantation crops, it may be advisable to initiate cultures for cloning only from officially certified (in the EU the term "classified" is used to denote such material) disease-free mother cultures. In the absence of such cultures, it may be advisable to send cultures to specialized laboratories for pathogen testing and elimination. Given, however, the diversity and limited scale of production of many micropropagated crops, in-house pathogen and contaminant detection, characterization, and elimination may be the only economic option. Again, it is recommended that as much as possible of this work be subcontracted to specialist laboratories.

The routine use of bacteriostatic chemical is not recommended because of the risks of resistant microorganisms developing.

Laboratory contamination of cultures is a matter for the individual laboratory and is largely crop nonspecific. Good working practice and staff training are the key elements in control. Indeed, the problem of production losses from laboratory contamination in heterotrophic micropropagation is a major factor in the move toward autotrophic tissue culture [58]. If adopting autotrophic micropropagation, it is advisable to consider the contamination problems encountered in hydroponic (soil-less) culture [59]. *In vitro* plants

represent a biological vacuum, and if not colonized by beneficial organisms, they may be a niche for potential pathogens.

As understanding of the nature of bacterial contamination of plants and plant tissue cultures has increased, it is disquieting to note that some of the contaminants may be potential pathogens of other crops [17], or operator-associated microorganisms [25]. Further data is required to evaluate the significance and implications of these preliminary findings.

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In Vitro Conservation of Plant Genetic Resources

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I. INTRODUCTION

The conservation of biodiversity is widely recognized as a high priority area for attention in the ongoing debate linking environment and development [1,2]. In particular, the conservation of plant genetic resources for food and agriculture, one sector of biodiversity, is considered to be a major element of any strategy to achieve sustainable agricultural development, along with the conservation of other natural resources. Diverse conservation methods are pursued according to the situation at hand. These methods can be divided broadly into ex situ and in situ. The latter cover conservation in wilderness areas, reserves, protected areas, and within traditional farming systems (so-called, on-farm conservation). Ex situ conservation involves removing the plant genetic resources from their natural habitat and placing them under artificial storage conditions. The following sections examine the different ex situ options available.

A. Classic Approaches to Ex Situ Conservation

The most familiar approach to ex situ conservation is seed storage. A large proportion of agricultural crops produce seeds that can be dried to a sufficiently low moisture content that they can be stored at low temperatures. There is an interaction between moisture content, temperature, and survival in storage and longevity, so that drying to lower moisture contents permits storage at relatively higher temperatures [3]. This principle underlies current research into ultradry seed storage that should greatly reduce the constraints imposed by difficulties in maintaining sufficiently cold seed stores [4,5]. This can be an especially serious problem in developing countries and, worldwide, is a factor in the cost of operating seed stores. Nevertheless, for crops that produce seeds amenable to drying and cold storage (i.e., "orthodox" seeds), this approach to conservation is convenient, is easily adopted, and is secure. Its drawbacks relate to biological, rather than practical, features that prevent its wider application beyond orthodox seeds.

Three categories of crop present problems for seed storage. First, there are those that do not produce seeds at all, and are propagated vegetatively, for example, banana and plantain (*Musa* spp.). Second, there are crops, including potato (*Solanum tuberosum*); other root and tuber crops, such as yams (*Dioscorea* spp.), cassava (*Manihot esculenta*), and sweet potato (*Ipomoea batatas*); and sugarcane (*Saccharum* spp.), that have some sterile genotypes and some that produce orthodox seed. However, similar to temperate fruits, including apple (*Malus* spp.), these seeds are highly heterozygous and, therefore, of limited usefulness for the conservation of gene combinations. These crops are usually propagated vegetatively to maintain clonal genotypes [7]. Then, third, there are those crops that produce what are known as "recalcitrant" seeds. Several tropical fruits and timber species fall into this category, including coconut (*Cocos nucifera*), avocado (*Persea americana*), mango (*Mangifera indica*), cacao (*Theobroma cacao*), and members of the Dipterocarpaceae family [8–10]. Recalcitrant seeds cannot tolerate desiccation to moisture contents that would permit exposure to low temperatures. They are often large, with considerable quantities of fleshy endosperm. Although there are clear groups of species that can be classified categorically as orthodox or recalcitrant, there are also intermediate types for which seed storage is problematic [5,6].

Traditionally, the field genebank has been the ex situ storage method of choice for these problem materials. In some ways, it offers a satisfactory approach to conservation. The genetic resources under conservation are readily accessed and observed, permitting detailed evaluation. However, there are certain drawbacks that limit its efficiency and threaten its security [11,12]. The genetic resources are exposed to pests, diseases, and other natural hazards, such as drought, weather damage, human error, and vandalism. Nor are they in a condition that is readily conducive to germplasm exchange. Field gene banks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance, and even their very survival in times of economic stringency. Even under the best circumstances, field genebanks require considerable input in the form of land (often needing multiple sites to permit rotation), labor, management, and materials.

In the light of the problems presented by the three categories of crops outlined in the foregoing, it is not surprising that efforts have been made to improve the quality and security of conservation offered by field gene banks, and to understand and overcome seed recalcitrance to make seed storage more widely available. However, it is clear that alternative approaches to genetic conservation are needed for these problem materials and, since the early 1970s, attention has turned to the possibilities offered by biotechnology, specifically in vitro or tissue culture [13–19].

B. New Approaches to Conservation

In vitro techniques feature in the conservation strategies of animals, microbes, and plants [19–21], but it is probably fair to say that the potential for exploiting these techniques and integrating them into wider practices, including genetic improvement, is greatest for higher plants. This relates largely to the ease with which plant material can be manipulated in vitro, in particular the phenomenon of totipotency (capacity to regenerate whole plants from single cells). Early efforts in the development of new approaches to conservation focused on storage *per se*, but applications of biotechnology have been demonstrated in all aspects of conservation and use, from germ plasm collecting and exchange, to multiplication, disease indexing and eradication, characterization and evaluation, storage, stability monitoring, distribution, and utilization [19]. The main body of this chapter will deal with collecting and storage, the latter involving slow-growth storage for short- to medium-term conservation, and cryopreservation

for the long-term. However, before so doing, it is instructive to explore the broader context of conservation and use of plant genetic resources.

The interdependence of nations and regions of the world in terms of access to genetic resources [22,23] reveals a crucial role for any means that can be used to facilitate the collecting and exchange of germ plasm. However, there is an important rider to place on that; the facilitation of germ plasm exchange should not lead to any increased risk of exchanging pests and pathogens. Fortunately, biotechnology in the form of in vitro and biochemical or molecular techniques can offer ways of both eradicating and indexing for diseases. Typical illustrative examples can be found in the root and tuber crops, such as potato, sweet potato, and cassava [24,25]. Because of the predominant use of vegetative propagation techniques in these crops, there is a tendency to accumulate pathogens through successive clonal generations without the "filter" provided by seed production. Meristem-tip culture used alone or in combination with thermotherapy can effectively eliminate viral pathogens, and a combination of symptomatology, the use of grafting or inoculation onto indicator plants, enzyme-linked immunosorbent assay (ELISA), and molecular techniques, such as double-stranded (ds)RNA detection can confirm the success or otherwise of eradication [e.g., 26,27]. Precise details should be sought on a species-by-species basis from the wider literature. Overall, an important caution to apply in this area is that transfer to in vitro culture does not confer disease-free status. Indexing is the only sure way of making this judgment.

One of the most important aspects of in vitro culture, particularly for the problem crops identified earlier, is mass propagation. In vitro propagation not only facilitates the agricultural production of the crop [e.g., 28–30], but also underpins the use of all other biotechnologies in conservation and use. The advantages of being able to multiply a given genotype with relative ease, with a low risk of introducing or reintroducing pathogens, and with a low risk of genetic instability, need not be emphasized. However, the latter point of genetic instability bears further examination. Genetic stability in culture is not a given. There are clear links between the culture system in use and risk of instability through somaclonal variation [31,32]. As a broad generalization in the context of genetic conservation, the more instability-prone culture systems, such as protoplast and cell cultures, should be avoided in favor of more highly organized systems, such as shoot cultures. Somatic embryos present some attractive options here, being both relatively amenable to storage by cryopreservation and manipulable as synthetic seeds [33]. Genetic stability under conditions of in vitro conservation is dealt with later, as is the amenability of different culture systems to in vitro storage.

The application of biotechnological approaches to the genetic improvement of plants is the subject of many other sections of this book and need not be expanded on here. Suffice it to say that the generation of an awareness of the scope for applying new techniques for conservation as well as those for the use of plant genetic resources among practitioners of the respective techniques can only be beneficial. Molecular technologies based on DNA extraction and storage offer new ways of conserving as well as of using genetic information [34–36]. Although routine DNA storage for genetic conservation may be some way off in the future, it is possible to envisage applications for the storage of specific gene sequences within a broad complementary conservation strategy. Similarly, in vitro conservation techniques can facilitate the application of genetic manipulation procedures by, for example, providing a simple way of storing experimental material in the form of in vitro cultures. More importantly, perhaps, new storage techniques can relieve the burden placed on all in vitro-based procedures imposed by the need to maintain stock cultures. Applications can be envisaged in aspects ranging from basic physiological studies through to secondary product synthesis on an industrial scale, with potential savings on costs, and reduced risks of loss through human error and genetic instability.

II. COLLECTING AND EXCHANGE OF GERM PLASM

A. Problems with Conventional Methods

The problems outlined in the previous sections for the conservation of genetic resources of particular crops are mirrored, and sometimes amplified, in their collecting and exchange. Looking first at clonally propagated crops, the material of choice for collecting is often a vegetative propagule, such as a stake, piece of bud-wood, a tuber, corm, or sucker. Only in some cases are these materials adapted to survival once excised from the parent plant and, almost invariably, they present a plant health risk owing to their vegetative nature and contamination with soilborne organisms [see Refs. 37,38]. The collector can compensate for these problems, to some extent, by good planning, careful selection of material, and observation of basic plant health precautions. Nevertheless, fundamental and unavoidable risks remain.

For recalcitrant seeds, there is a dual problem. Not only are they prone to microbial attack or deterioration if exposed to unsuitable environmental conditions, or if held too long in transit to the gene bank [see Ref. 39], there are also very limited options in how to handle them once they do arrive at their destination. Seed storage under conventional conditions is not available, and successful germination to produce a seedling for the field gene bank will require high-quality seed. A further practical problem encountered with many recalcitrant seeds is their sheer weight and bulk. If a satisfactory population sample is to be gathered, this can represent a dauntingly large mass of material to transport, with attendant high costs.

Collecting the germ plasm of orthodox, seed-producing species can also be problematic. Sometimes the window of opportunity for a collecting mission does not coincide with the ideal stage of development of the plant. The material available for collecting may be sparse, immature, past its optimal state of maturity, shed from the plant, or even eaten by grazing animals [38,40,41]. Collecting expeditions in general are a logistic challenge, but when they involve exploration in remote or politically sensitive areas, or when there are factors, such as climatic aberrations, to contend with, the challenge is increased and the collector needs to keep open as many options as possible. In this context, the adaptation of in vitro techniques to the collecting environment, as described in the next section, illustrates one of the simplest, but most effective, applications of biotechnology to plant genetic resources work.

With the exception of timing, where the curator has more control over the deciding when to exchange material, all of the comments made earlier in relation to problems in collecting germ plasm apply to its exchange. Therefore, there is scope for using techniques that help maintain germ plasm in as healthy a general condition as possible and reduce the risk of introducing pathogens.

B. In Vitro Field Collecting

Some sporadic work had been carried out in the past on developing in vitro collecting methods but the first coherent and comprehensive examination of its potential was made by the International Board for Plant Genetic Resources (IBPGR). This took the form of a meeting of scientists with expertise in specific crops and in vitro techniques in general [41], followed by sponsorship and encouragement of focused research [42–45]. Four crop models serve to illustrate the general potential and flexibility of in vitro collecting. These are coconut, cacao, forage grasses, and *Musa* spp.

The problems inherent in collecting coconut germ plasm are obvious. The species produces large, recalcitrant seeds. However, the key to finding a solution lies in recognizing that only the small embryo is needed to propagate a coconut palm, given adequate handling techniques.

Research over the last 8 years has demonstrated not only the feasibility of collecting isolated embryos, but also the great flexibility that can be exercised within the basic concept.

Research by Assy-Bah and colleagues [42,43] tackled the field-collecting technique itself and the follow-up procedures needed to nurture the embryo and generate an independently growing plant. The principle used in the field collecting was the idea that, with minimal, but dexterous aseptic precautions, embryos could be isolated from nuts in the field, surface sterilized, and then either dissected at the field location or transported in endosperm plugs held in sterile salt solution for subsequent dissection in the laboratory. The basic principle demonstrated by this research is taken in the direction of greater complexity by Sossou et al. [46], who closely simulated laboratory conditions in the field, and in the direction of greater simplicity by Rillo and colleagues [47,48]. **Table 1** provides details of the different approaches that have been taken with coconut and the other model crops examined herein.

The success that has been demonstrated with coconut can be repeated with other recalcitrant seeds that are physiologically similar but structurally very different, such as cacao, avocado, and *Citrus* spp. Embryos of these species have fleshy cotyledons that can be dissected away to reveal the embryo axis. In these cases, it is easier to keep the embryo axis sterile, and it can suffice to flame sterilize the fruit and, by using frequently sterilized instruments, maintain the inherent sterility of the interior of the seed [49].

A very different challenge is presented by collecting vegetative tissues. Within this broad category are a wide variety of types of material, woody and herbaceous, more or less succulent, and with great structural variations. One example presenting serious collecting problems is cacao. The seeds of cacao are recalcitrant. Accordingly, bud wood is often the target for collecting. Yet this is vulnerable owing to the potentially long transit periods involved in collecting from, say, the Amazon basin, which is the center of origin of cacao and a valuable source of genetic resources (39).

Experimentation to develop an in vitro collecting method for cacao bud wood sought to minimize the materials and equipment to be carried to the collecting site. It was based on the premise that absolute sterility would be difficult to achieve in the field and would not necessarily be essential for robust, woody material. The technique eventually developed for cacao is detailed in Table 1 [50–55]. It involves the use of drinking water-sterilizing tablets and culture medium supplemented with fungicides. Antibiotics can also be used, but this must be weighed against the inherent disadvantages in their use from the point of view of hazards to the user and the maintenance of low levels of persistent infection [52,53]. The general approach used for cacao has been used successfully for other similar materials, such as woody shoots of coffee (*Coffea* spp.), *Prunus* spp., and grape *Vitis* spp. [49,54].

Forage grasses present problems in their collecting because often the only material available is the herbaceous shoot or tiller. Ruredzo [44] developed a successful procedure for collecting such material as detailed in Table 1. This was used to acquire material for the forage germ plasm collection at the International Livestock Centre for Africa (ILCA).

The final example of stem tissue to be considered is exemplified by *Musa* spp. that is collected in the form of stem suckers, which are large, fleshy, and likely to be covered in soil. The strategy that has proved successful with this material involves surface sterilization, combined with extensive dissection to reach naturally aseptic inner tissues. A sucker of some 30×8 cm is reduced to a shoot tip less than 1×1 cm [49,55].

These examples illustrate the flexibility of in vitro collecting. There is no one formula to be followed, nor need there be. The approach to be taken should be based on prior knowledge of the requirements of the species and explant in question, combined with the collective experience gained with diverse species in different collecting environments. As in any germ plasm transfer operation, particular attention should be given to phytosanitary considerations. In vitro collected

Table 1 Summary of Conditions Used in the In Vitro Collecting of Diverse Specimens

Species	Explant	Surface sterilization	Initial handling	Laboratory treatment	Ref.
Coconut (<i>Cocos nucifera</i>)	Embryo in endosperm plug, extracted with cork borer	Calcium hypochlorite at 45 gL ⁻¹	Endosperm plug inoculated into sterile solution of KCl at 16.2 gL ⁻¹	Repeat sterilization if necessary. Embryo dissected, inoculated onto semisolid medium, cultured under standard conditions, transferred to the nursery.	42
Coconut (<i>Cocos nucifera</i>)	Embryo in endosperm plug, extracted with cork borer	Commercial bleach (8%Cl)	Embryo dissected at field work bench and inoculated onto semisolid medium	Embryo cultured under standard conditions, transferred to the nursery.	50
Coconut (<i>Cocos nucifera</i>)	Embryo in endosperm plug, extracted with cork borer	None	Endosperm plugs placed in a bag of freshly gathered coconut milk, held in a cool box	Endosperm plug surface sterilized, embryo dissected and inoculated onto standard culture medium	47
Coconut (<i>Cocos nucifera</i>)	Embryo in endosperm plug, extracted with cork borer	Inoculation is carried out in an inflatable glove box sterilized with alcohol. Sterilization with 5% calcium hypochlorite for 15–20 min. Embryo is then excised and sterilized with 2% calcium hypochlorite for 2–5 min, followed by washing in sterile water.	Embryos are placed on standard culture medium in screw-top flasks	Standard culture procedure	46
Cacao (<i>Theobroma cacao</i>)	Stem nodal cutting	Drinking water sterilizing tablets containing "Halozone" (<i>p</i> -carboxybenzene-sulfondichloroamide), 4 mg/tablet: 10 tablets dissolved in 100 mL of boiled water, plus 0.05% FBC protectant fungicide	Inoculation onto semisolid medium containing fungicide Tilt MBC at 0.1% with or without antibiotics rifamycin at 30 mgL ⁻¹ and trimethoprim at 30 mgL ⁻¹	Continued culture or resterilization using standard treatments, or grafting	45

Table 1 Continued

Species	Explant	Surface sterilization	Initial handling	Laboratory treatment	Ref.
<i>Digitaria decumbens</i> (forage grass)	Stem cutting	Drinking water-sterilizing tablets containing "Halozone" (<i>p</i> -carboxybenzene-sulfondichloroamide) at 1 g tablets/L of boiled water	Inoculation onto culture medium containing 1.5 gL ⁻¹ benlate and 0.1 mgL ⁻¹ rifamycin	Standard culture conditions; transfer to soil after 14 weeks	44
Cotton (<i>Gossypium</i> sp.)	Stem nodal cutting	20% commercial bleach in 30% ethanol for 45 s; no washing	Inoculation onto solid culture medium containing half-strength salts, 1% glucose, antibiotics rifamycin at 15 mgL ⁻¹ and trimethoprim at 15 mgL ⁻¹ , fungicide Tilt MBC at 1 gL ⁻¹ , NAA at 1 mgL ⁻¹ and casein hydrolysate at 0.5 gL ⁻¹	Resterilize with 4% bleach, treat with rooting hormone and plant in soil/sand/vermiculite mix with lime and slow-release nutrients	51

explants should be treated with the same care and observance of regulations as any other type of collected material.

C. In Vitro Germ Plasm Exchange

In vitro techniques have been used widely for several years for the international distribution of plant genetic resources. Notable examples that have been tried and tested, yielding routine procedures include potato, cassava, yam, and *Musa* spp. The techniques used are based on standard mass propagation procedures, with minor but important modifications of detail to increase structural stability in transit. For example, the concentration of agar or other gelling medium used for preparing the culture medium may be raised to increase its firmness. Also, plantlets may be transferred in sterile heat-sealable polyethylene bags, rather than the more fragile glass or plastic containers [56,57]. For species that produce storage propagules, such as stem tubers, capable of regenerating plants, a further option is available. This approach has been used successfully in potato and yam, the tubers being more resilient with the result of producing a more robust system for germ plasm exchange [58].

III. SLOW-GROWTH STORAGE

A. Classic Techniques

Standard culture conditions can be used only for medium-term storage of naturally slowly growing species. For example, plantlets of *Coffea arabica* can be conserved on standard medium

at 27°C for 1 year without subculturing [59]. However, such examples are in the minority. Accordingly, techniques have been developed for reducing the growth rate of cultures. Classic slow-growth storage techniques involve modification of the physical environmental conditions or culture medium, or both. The most successful and widely applied technique is temperature reduction. A decrease in light intensity or culture in the dark is often used in combination with temperature reduction. Strawberry (*Fragaria ananassa*) plantlets have been conserved in the dark at 4°C [59], Regular addition of a few drops of liquid medium to the cultures maintained the plantlets viable for up to 6 years.

Apple (*Malus domestica*) and *Prunus* shoots survived 52 weeks at 2°C [61]. Temperatures in the range of 0°–5°C can be employed with cold-tolerant species, but higher temperatures have to be used with tropical species that are often cold-sensitive. Roca et al. [62] indicated that cassava shoot cultures have to be stored at temperatures higher than 20°C. Oil palm (*Elaeis guineensis* Jacq.) somatic embryos and plantlets cannot withstand even short-term exposure to temperatures lower than 18°C [63]. In contrast, banana in vitro plantlets can be stored at 15°C without transfer for up to 15 months [64,65].

It is also possible to limit growth by modifying the culture medium. Reduction in the concentration of mineral elements and elimination of sugar allowed the conservation of *C. arabica* plantlets for 2 years [66]. Addition of osmotic growth inhibitors (e.g., mannitol) or hormonal growth inhibitors (e.g., abscisic acid) is also an efficient way to achieve growth reduction [67–72].

The type of culture vessel, its volume and the volume of medium, and the closure of the culture vessel influence the survival of stored cultures [18,73]. Roca et al. [62] indicated that storing cassava shoot cultures in larger vessels improved their condition and maintenance of viability during storage. Replacing cotton plugs by polypropylene caps, thereby reducing the evaporation rate of the culture medium, increased the survival of *Rauvolfia serpentina* during storage [74]. As well as standard glass and plastic vessels, the use of heat-sealable polypropylene bags has been reported [57].

At the end of a storage period, cultures are usually transferred onto fresh medium and placed in optimal culture conditions for a short period to stimulate regrowth before entering the next storage cycle. (But note treatment of strawberry plantlets discussed earlier [60]).

B. Alternative Techniques

Alternative slow-growth techniques include modification of the gaseous environment and desiccation or encapsulation of explants. Growth reduction can be achieved by lowering the quantity of oxygen available to the cultures. The simplest method consists of covering the tissues with paraffin oil, mineral oil, or liquid medium. This technique was first developed by Caplin [75], who stored carrot (*Daucus carota*) callus under paraffin oil for 5 months. It was employed more recently by Augereau et al. [76] with *Catharanthus* calluses and by Moriguchi et al. [77] with grape calluses. Florin [78] showed that 86 and 50% of a collection of 313 different callus lines could be stored with the same technique for 6 and 12 months, respectively. Similarly, 13 of 20 cell suspensions from eight different species survived after 6 months of storage under liquid medium without shaking (78).

Attempts to store microcuttings under mineral oil have been performed with pear (*Pyrus communis*) [79], coffee [80], and several ginger (*Zingiber officinale*) genotypes [81]. Growth reduction was achieved in all cases, but hyperhydration of explants was often observed during storage. After return to standard conditions following 4 months in storage, regrowth of surviving cultures was very slow for coffee, and partial or complete necrosis

of explants was noted with pear. However, this storage technique was very efficient with some ginger genotypes, which could be conserved under mineral oil with high viability for up to 2 years [81].

Reduction in the level of available oxygen can also be achieved by decreasing the atmospheric pressure of the culture chamber or by using a controlled atmosphere. Tobacco (*Nicotiana tabacum*) and chrysanthemum (*Chrysanthemum morifolium*) plantlets were stored under low atmospheric pressure (with 1.3% O₂) for 6 weeks [82]. Oil palm somatic embryos could be conserved for 4 months at room temperature in a controlled atmosphere with 1% O₂, and proliferated rapidly after subsequent transfer to standard conditions [83].

Desiccation as a means of storage of embryogenic cultures was first described by Jones [84]. Embryogenic cultures of carrot were left on semisolid medium for up to 2 years at 25°C. Supply of a sucrose solution to the cultures resulted in "germination" of the embryos that, on planting out, produced healthy individual plants. More recently, Senaratna et al. [85] have shown that alfalfa (*Medicago sativa*) somatic embryos, dehydrated progressively using saturated salt solutions, could be conserved with 10–15% moisture content for 1 year at room temperature. They displayed only a 5% decrease in their conversion rate after storage. Similarly, Lecouteux et al. [86] stored carrot somatic embryos for 8 months at 4°C without viability loss.

Large-scale propagation by means of somatic embryogenesis is being developed for elite genotypes of numerous crop species, leading to the production of large numbers of synchronously developing embryos. These embryos can be encapsulated in a bead of gel (e.g., calcium alginate), containing nutrients and fungicides, thereby forming synthetic seeds which, theoretically, can be stored and sown directly *in vivo* similar to true seeds. The production of synthetic seeds has been developed for many plant species [33,87,88]. The application of synthetic seed technology to somatic embryos or shoot tips also appears of interest in a germ plasm conservation context. However, only a limited number of short- to medium-term storage experiments have been performed with encapsulated material (but see [Section IV](#) for long-term storage by cryopreservation).

Encapsulated axillary buds of mulberry (*Morus indica* L.) and somatic embryos of sandalwood (*Santalum album*) can be stored for 45 days at 4°C [89,90] and somatic embryos of interior spruce (*Picea glauca*) for only 1 month [91]. Storage for longer periods was achieved if beads were placed in liquid medium at low temperature. Under these conditions, Machii [92] conserved mulberry apices for 80 days and Shigeta et al. [93] carrot somatic embryos for 3 months. Mathur et al. [94] reported that encapsulated shoot tips of *Valeriana wallichii* could be conserved over 6 months at 4°–6°C without affecting viability, but no detailed results were provided. Redenbaugh et al. [33] mention that the rapid survival loss of encapsulated material that is generally observed is mainly due to the encapsulating matrix, which dehydrates rapidly and limits the respiration of the embedded embryos.

IV. CRYOPRESERVATION

A. History

Cryopreservation (i.e., storage at ultralow temperatures in a cryogenic medium, such as liquid nitrogen) has the potential to achieve the goal of suspending metabolism and, to all intents and purposes, suspending time. Cryopreservation has a relatively long history in microbiology for the storage of stock cultures, and in livestock husbandry for the storage of semen of elite male cattle [20]. Research into the response of higher plant systems to cooling to ultralow temperatures has been carried out over the past 40 years, following two main themes: (1) to

gain an understanding of the physiological and biochemical processes involved in the transitions to and from the frozen state, including cold acclimation; and (2) to preserve plant material in a viable state [15,95–98]. Although the two themes have rather different motivations, the respective lines of research have much of mutual interest, and some of the most successful cryopreservation work has involved attention to the underlying processes of cryoinjury and cryoprotection, rather than an empirical approach alone.

Some sporadic reports of successful cryopreservation of in vitro systems appeared in the late 1960s, but the first report of exposure of cultured plant material to the temperature of liquid nitrogen was made by Quatrano in 1968 [99], using cultured cells of flax (*Linum usitatissimum*). This research was explicitly conducted with a genetic conservation motive, highlighting the early awareness and convergence of interests of scientists in the genetic conservation and in vitro culture communities. The methods adopted closely followed the classic procedures found to be successful with other living systems [20,95,100]; namely, chemical cryoprotection, slow, dehydrative cooling, storage in liquid nitrogen, rapid thawing, washing, and recovery. As will be described in the following section, subsequent studies did much to optimize and elucidate the flexibility of this approach to plant cryopreservation, to extend it to other culture systems, and importantly, to illustrate its limitations.

Recent years have seen a diversification of cryopreservation techniques, providing the interested scientist with a portfolio of general and specific options from which to choose. These options seek to match both varying biological requirements and varying infrastructural situations, from the highly sophisticated to the minimally equipped laboratory. They thereby extend cryopreservation to a wide range of users.

B. Classic Techniques

Most of the early work on the cryopreservation of in vitro plant cultures focused on a method based on chemical cryoprotection and dehydrative cooling. This was particularly successful with cell suspension cultures, which is not surprising when the underlying biophysical events are explored. The vast majority of higher plant somatic cells, be they *in vivo* or *in vitro*, are not inherently freeze-tolerant. The transition of extra- and intracellular water into ice causes damage of a physical or biochemical nature [101–105].

The dynamics of the freezing process are particularly important. Extracellular freezing commonly occurs first, causing a flow of water from the cytoplasm and vacuole to the extracellular space where it freezes [106–108]. Depending on the rate of cooling, different amounts of water will leave the cell before the intracellular contents solidify [109]. Rapid cooling will result in more water remaining within the cell and causing potentially damaging ice than in slow cooling. Ice causes damage when formed in the freezing process *per se*. It can also cause damage during rewarming owing to the phenomenon of recrystallization, in which ice melts and reforms at a thermodynamically favorable, larger, and more damaging crystal size. This can be mitigated by rapid thawing [110,111]. Slow cooling reduces this risk, but can incur different damaging events owing to the concentration of intracellular salts and changes in the cell membrane [112–114]. Shrinkage of the protoplast and loss of surface area in the plasmalemma can render the protoplast incapable of resuming its original volume and surface area after thawing, resulting in rupture [115,116].

Light and electronmicroscopic studies of cell suspension cultures and isolated protoplast systems have helped clarify the nature of damage under different cooling regimens [e.g., 105,117–120]. They have also revealed the mitigating effects of cryoprotectants [105,118]. Cryoprotectants facilitate the flow of water across the cell membrane, and protect both

molecular and gross structures through a range of modes of action, including colligative effects and free radical scavenging [121–123].

Studies of the effect of cooling rates on survival in cryopreserved cell suspension culture systems clearly illustrate the existence of an optimum cooling rate, commonly in the region of -1°C or $-2^{\circ}\text{C min}^{-1}$, providing the central strategy of the classic approach to cryopreservation [see Refs. 15,16]. A strong body of research followed the initial elucidation of this approach, to explore other attendant factors, including the effect of culture conditions before cryoprotection, the age of the cells at the time of harvest for cryopreservation, immediate postthaw treatment, and recovery growth conditions, as well as looking more closely at the temperature excursions [e.g. 124–128]. Linear cooling and warming are not the only or necessarily the most successful options and may, in fact, prove more difficult to achieve, other than in complex, costly equipment [129]. Withers and King [128–130] describe improvised and simpler apparatus that can offer reproducible, but nonlinear slow cooling.

Some key findings are as follows: The age of cells at the time of harvesting for storage can affect their survival. This is linked to cell size and water content. Rapidly growing cells are small and have a relatively low water content. Modification of the pregrowth medium used for the passage before cryopreservation by, for example, the addition of osmotically active compounds, such as mannitol and sorbitol, can lead to reduction in cell size and an increase in freeze tolerance. For cell suspension cultures, in particular, mixtures of cryoprotectants are much more effective than single cryoprotectants, and preparation in culture medium is usually beneficial. Removal of cryoprotectants after thawing has not been demonstrated to be essential, and there is clear evidence for a detrimental effect of washing. Similarly, recovery growth on solid medium is generally much more effective than dilution in liquid medium [128,130,131]. If toxicity is suspected, precautions can be taken, such as moving cells on a supporting filter paper through a series of dishes of solid culture medium [132].

Table 2a provide examples of the application of the classic approach to cryopreservation for a range of in vitro plant cultures systems. Its flexibility is evident, but it is also clear from the studies carried out to date that the approach is most successful with culture systems that consist of small units of uniform morphology, such as would be found in a protoplast culture, an exponentially growing cell suspension culture, or fragmented callus culture. The approach is less successful with culture systems that consist of larger units comprising a mixture of cell sizes and types, such as shoot tips or relatively mature somatic embryo cultures.

A clue to an alternative approach that might be taken with organized cultures can be found in some data in which wide ranges of cooling rates are explored and an upturn in survival is seen at the fastest rates. This is interpreted as being due to the formation of microscopically small ice crystals that develop without damaging cell structures. As long as these can be thawed again without recrystallization, by applying an adequately rapid warming rate, the specimen can survive. By this approach, problems in achieving uniform dehydration in a large, dense mass of tissue, such as a shoot tip, and the differential requirements of small, highly cytoplasmic meristem cells and large, more vacuolated cells elsewhere in the shoot tip that limit slow cooling's success are bypassed. Details of several successful reports of ultrarapid cooling are given in Table 2b. In general, although this approach is practically uncomplicated and inexpensive to carry out, reservations must be expressed over its practicality in the context of genetic conservation. It often entails the use of naked specimens, specimens in open containers, and specimens in droplets of medium on a sheet of aluminium (e.g., 136–139). In all of these cases, there is a risk of microbial contamination either during the cooling and warming stages or during storage, where the specimens do not easily lend themselves to organized management.

Table 2 Examples of (a) the Classic, Slow, Cooling-Based Approach to Cryopreservation of In Vitro Plant Cultures, and (b) Rapid Cooling

Species	Culture system	Pregrowth	Cryoprotection	Cooling, storage, warming	Recovery	Ref.
(a)						
Sycamore (<i>Acer pseudoplatanus</i>)	Cell suspension	Culture for 3–4 days in medium containing 6% mannitol	0.5 M DMSO + 0.5 M glycerol + 1 M sucrose	-1°C min ⁻¹ to -35°C, hold for 30 mins; transfer to liquid nitrogen; thaw in water bath at +40°C	Layer cells in suspending liquid over semisolid medium	131
Soyabean (<i>Glycine max</i>)	Protoplasts	Protoplasts isolated from exponentially growing cells	5% DMSO + 10% glucose	-10°C min ⁻¹ to -35°C; transfer to liquid nitrogen; thaw in water bath at +40°C	Wash in liquid medium; transfer to standard medium	133
Potato (<i>Solanum tuberosum</i>)	Shoot-tip	Shoot-tip dissected from glasshouse or in vitro plants; incubate overnight in standard liquid medium	10% DMSO	-0.20°C min ⁻¹ to -35°C; transfer to liquid nitrogen; thaw in water bath at +37°C	Wash twice with liquid medium; transfer to semisolid medium	134
Pear (<i>Pyrus spp.</i>)	Shoot-tip	Culture in vitro plantlets at 22°C/16 h day, -1°C/8 h night for 7 days; dissect shoot-tips and pregrow for 48 h in medium containing 5% DMSO	10% polyethylene glycol + 10% glucose + 10% DMSO	-1°C min ⁻¹ to -40°C; transfer to liquid nitrogen; thaw in water bath at +40°C for 1 min; transfer to +23°C	Wash in liquid medium; drain; transfer to semisolid medium	135

(b)

Carnation (<i>Dianthus caryophyllus</i>)	Shoot-tip	Dissect shoot-tip from cold-hardened plant	5% DMSO	Place in ampule and plunge into liquid nitrogen.	Culture under standard conditions	136
Potato (<i>Solanum tuberosum</i>)	Shoot-tip	Dissect shoot-tip from plantlet and culture on filter paper floating on liquid medium for 2 days.	10% DMSO	Collect shoot-tip on hypodermic needle and plunge into liquid nitrogen; thaw by plunging into liquid medium at 34°–40°C	Culture on filter paper bridge over liquid medium	137
Oilseed rape (<i>Brassica napus</i>)	Shoot-tip	Dissect shoot-tip from in vitro plantlet; incubate for 24 h in medium containing 5% DMSO	15% DMSO	Collect shoot-tip on hypodermic needle and plunge into liquid nitrogen; thaw by plunging into liquid medium at room temperature (+40°C)	Transfer without washing to semisolid shoot-induction medium	138

C. New Techniques

1. Principle

In classic cryopreservation techniques, the removal of cellular water and the behavior of remaining cellular water during the freezing and thawing processes are critical to success. In contrast with these freezing-based techniques, new cryopreservation techniques are based on the phenomenon of vitrification. *Vitrification* can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, while avoiding the formation of crystalline ice [140]. (Note: Vitrification in the present context should not be confused with the phenomenon of "hyperhydration," which sometimes goes by the same name.)

In vitrification-based procedures, cell dehydration is performed before freezing by exposure of samples to concentrated cryoprotective media or air desiccation. This is followed by rapid cooling. As a result, all factors that affect intracellular ice formation are avoided. Glass transitions (changes in the structural conformation of the glass) during cooling and rewarming have been recorded with various materials using thermal analysis [141–144]. Dumet et al. [145] showed that increased survival rates for cryopreserved oil palm somatic embryos were correlated with the progressive disappearance of ice crystallization peaks and their replacement by glass transitions.

Vitrification-based procedures offer practical advantages in comparison with classic freezing techniques [146]. Similar to ultrarapid freezing, they are more appropriate for complex organs (shoot tips or embryos) which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration. By precluding ice formation in the system, vitrification-based procedures are operationally less complex than classic ones (e.g., they do not require the use of controlled freezers) and have greater potential for broad applicability, requiring only minor modifications for different cell types.

Luyet [147] was the first to envisage the use of vitrification for cryopreserving biological specimens, but it is only somewhat recently that numerous reports on cryopreservation of plant material using vitrification-based procedures have appeared in the literature [146–149]. Four different procedures based on the phenomenon of vitrification can be identified: encapsulation-dehydration, desiccation, pregrowth-desiccation, and a procedure that actually goes by the name of vitrification.

2. Encapsulation-Dehydration

The encapsulation-dehydration technique is based on the technology developed for the production of synthetic seeds, by which embryos are encapsulated in a bead of calcium alginate gel [33]. Cryopreservation using encapsulation-dehydration has been applied mainly to shoot apices of various species, and also to carrot, walnut (*Juglans regia*), and coffee somatic embryos, and to oil seed rape (*Brassica napus* L.) microspore embryos (Table 3).

The encapsulation-dehydration technique permits freezing explants of large dimensions; pear shoot tips up to 5 mm in length [153], and heart or torpedo stage embryos (2–3 mm in length) have been successfully cryopreserved [162,164].

Before the cryopreservation procedure itself, plant material is often submitted to various treatments that increase survival potential. For cold-tolerant species, such as pear, apple, or mulberry, mother plants [152,165] or apices [151] can be placed at a low temperature (0–5°C) for several weeks. Scottez [153] showed that this cold treatment resulted in an increased quantity of unsaturated fatty acids in the pear apices. Before encapsulation, apices of mulberry are transferred daily onto solid media with progressively increased sucrose concentrations to initiate dehydration [151].

Table 3 List of Plant Species and Specimens (Apices, Somatic and Microspore Embryos) That Have Been Successfully Cryopreserved Using the Encapsulation-Desiccation Technique

Specimen	Species	Ref.
Shoot apex	Potato	150
	Apple	152,151
	Pear	142,153
	Mulberry	151
	Carnation	154
	Grape	155
	Chicory	156
	Eucalyptus	157
	Coffee	158
	Cassava	159
Somatic embryo	Sugarcane	160
	Carrot	161
	Coffee	162
Microspore embryos	Walnut	163
	Oilseed rape	164

The stages of the process after encapsulation are pregrowth, desiccation, cooling, warming, and recovery growth. Pregrowth is performed in liquid medium enriched with sucrose (0.3–1 M) for periods of between 16 h [151] and 7 days [150]. Partial replacement of sucrose with other sugars (raffinose, maltose, glucose, or trehalose) did not improve the survival of cryopreserved grape (*Vitis vinifera*) shoot tips [155]. For plant species that are sensitive to direct exposure to high sucrose levels, a progressive increase in sucrose concentration is used [157,158,166].

Encapsulated samples are desiccated either in the air current of a laminar airflow cabinet or by using silica gel. The latter method is preferred because it provides more precise and reproducible desiccation rates [160]. The optimal water content of desiccated beads is about 20% (fresh weight basis), ranging from 13% with coffee somatic embryos [162] to 30–35% with apple, grape, mulberry [151], and cassava apices [158].

Cooling is usually carried out rapidly, by direct immersion of samples in liquid nitrogen. However, controlled slow cooling, down to -100°C, led to improved survival of grape apices [166]. In contrast, the survival rate of sugarcane (*Saccharum* spp.) apices was higher after rapid than after slow cooling [167,168]. These results suggest continued dependence on control of the residual water content in the specimen.

Storage is usually performed at -196°C. Scottez [153] showed that survival of pear apices was not modified after 2 years of storage in liquid nitrogen. The same author demonstrated that samples could also be conserved for 1 year at a higher temperature (-75°C) which, nevertheless, is below the temperature of ice recrystallization (-50 to -70°C). Similarly, apices of apple, pear, and mulberry have been stored for 5 months in a deep-freezer at -135°C [151]. In these cases, the use of liquid nitrogen is not necessary for storing the plant material, giving obvious advantages in situations where its regular supply is unreliable. Importantly, survival of explants after thawing is independent of the rewarming rate.

For recovery, samples are usually placed directly under standard culture conditions. However, survival of cryopreserved apices of sugarcane was improved if they were placed for 1 week in the dark, on a medium supplemented with growth regulators [160]. Extraction of apices from the alginate beads was necessary to allow regrowth in the case of grape and pear [165,166].

Recovery growth of cryopreserved material is usually direct and rapid, without callus formation. Histological studies performed with apices of several plant species revealed that the structural integrity of most meristematic cells is preserved after cryopreservation by encapsulation-dehydration [158]. Therefore, recovery growth originates from the entire meristematic zone. This is contrary to what is generally observed after classic cryopreservation, during which many cells are destroyed, frequently leading to callus formation during recovery. With sugarcane, apices withstood freezing as a whole and cell divisions could be observed within 2 days after thawing [167].

Successful extension of encapsulation-dehydration protocols has been performed with 11 varieties of pear [153], 9 varieties of apple [151,152], and 14 varieties of sugarcane [158]. With all three species, even though genotypic variations were noted, survival rates were sufficiently high to envisage large-scale routine application of the cryopreservation protocols developed.

3. Desiccation

Cryopreservation using a desiccation procedure is very simple because it consists of dehydrating the plant material, then freezing it rapidly by direct immersion in liquid nitrogen. Desiccation has been applied mainly to zygotic embryos of a large number of species [see Ref. 169 for a review]. Experimentation has been carried out with only one other type of material, shoot tips of mulberry [170].

The physiological state of the starting material is an important parameter. In *Coffea arabica*, mature embryos (1 week before harvest) showed higher survival rates than immature ones (2 months before harvest) [171]. High variability in the survival rates of embryos extracted from seeds of some recalcitrant seed-producing trees (*Aesculus*, *Castanea*, and *Quercus*) harvested at different periods was noted by Pence [172].

Desiccation is usually performed by placing the embryos or embryonic axes in the air current of a laminar airflow cabinet. However, more precise and reproducible desiccation can be achieved by placing plant material in a stream of compressed air [173] or in an air-tight container with silica gel [144]. The duration of desiccation varies with the size of the embryos and their initial water content. Optimal survival rates are generally noted when embryos are dehydrated down to 10–20% moisture content (fresh weight) [169]. Dehydration must be sufficient to ensure survival after freezing, but not so intense to induce extended desiccation injury. In optimal cases, no significant difference is observed in the survival rates of desiccated control and cryopreserved embryos, as noted with tea (*Camellia sinensis*), banana, and hazelnut (*Corylus* spp.) [171,174,175].

Regrowth of plant material after warming is usually direct, but modified regrowth patterns are occasionally observed. Chin et al. [176] noted the nondevelopment of the haustorium and more rapid leaf expansion of cryopreserved embryos of *Veitchia* and *Howea*, in comparison with unfrozen controls. Abnormal regrowth of a fraction of cryopreserved embryos in the form of callusing or incomplete development occurred with *Castanea* and *Quercus* [172], *Hevea brasiliensis* [177], and oil palm [178].

Modified recovery conditions, notably of the hormonal balance of the culture medium, can significantly improve the survival rate of the cryopreserved material, as observed with coffee embryos [171,179].

4. Pregrowth-Desiccation

Cryopreservation using a pregrowth-desiccation procedure comprises the following steps: pregrowth treatment with cryoprotectants, desiccation, rapid cooling, storage, and rapid warming. This technique has been applied to only a limited number of specimens: stem segments

of in vitro plantlets of asparagus (*Asparagus officinalis* L.) [180], somatic embryos of melon (*Cucumis melo*) and oil palm [181,182], microspore embryos of rapeseed [164], and zygotic embryos of coconut [183].

The application of cryoprotectants is usually performed before desiccation. However, in the coconut embryos, dehydration was carried out before preculture with cryoprotectants [183]. The duration of treatment with cryoprotectants varies between 20 h for coconut [183] and 7 days with oil palm somatic embryos [182]. Sugars (sucrose or glucose) are generally employed for preculture. However, abscisic acid only was used for the pretreatment of melon somatic embryos [181].

Various methods have been employed for desiccation: coconut embryos were placed either in the air current of a laminar airflow cabinet [183], Asparagus stem segments, rapeseed and oil palm embryos were placed in an air-tight chamber containing silica gel [164,180,182], and melon somatic embryos were placed over a salt solution, ensuring a constant relative humidity [181]. Optimal water contents (fresh weight) for storage range between 11.8% for melon somatic embryos [181] and 25–30% with oil palm somatic embryos [182].

All materials cryopreserved using pregrowth-desiccation are cooled rapidly by direct immersion in liquid nitrogen. Storage is usually performed in liquid nitrogen. Experiments with oil palm somatic embryos have shown no modification in the recovery rate after 1 or 52 months of storage at -196°C [169]. More recently, Dumet et al. [184] have been able to conserve oil palm somatic embryos for 6 months at -80°C (i.e., below the glass transition temperature) without any modification in recovery rate compared with embryos stored at -196°C.

Warming is generally carried out rapidly except for stem segments of asparagus and rapeseed microspore embryos, which were rewarmed slowly at room temperature. Specimens are usually transferred directly onto standard medium for recovery. However, oil palm somatic embryos were cultured on media with a progressively reduced sucrose concentration and transitory supplement of 2,4-dichlorophenoxyacetic acid (2,4-D), to stimulate proliferation [182].

Cryopreservation using pregrowth-desiccation has ensured satisfactory survival rates with all materials tested, and recovery is usually rapid and direct. Alterations in regrowth pattern have been observed only with coconut embryos and oilseed rape microspore embryos. The haustorium of frozen coconut embryos browned rapidly and did not develop further [183]. Even though up to 93% of oilseed rape embryos withstood freezing, only 43% of them developed directly into plantlets. The remaining 50% produced calluses or secondary embryos [180].

Pregrowth-desiccation has been tested with four varieties of coconut, giving recovery rates of between 33 and 93% [183]. Large-scale application of this technique has been performed in the case of oil palm somatic embryos. Eighty clones are now routinely stored at -196°C [185].

5. Vitrification

Vitrification procedures consist of the following steps: treatment (“loading”) of samples with cryoprotective substances, dehydration with a highly concentrated vitrification solution, rapid cooling and warming, removal (“unloading”) of the vitrification solution. Vitrification solutions are complex mixtures of cryoprotective substances that have been selected in view of their ability to vitrify (i.e., form an amorphous glassy structure) during cooling. The most commonly employed are derived from the solution proposed by Sakai’s group which comprises 22% glycerol, 15% ethylene glycol, 15% propylene glycol, 7% dimethyl sulfoxide (DMSO), and 0.5 M sorbitol [141] and by Steponkus’ group [186], which consists of 40% ethylene glycol, 15% sorbitol, and 6% bovine serum albumin.

Vitrification procedures have been developed for about 20 species, using protoplasts, cell suspensions, shoot apices, and somatic embryos [see Ref. 149 for a review]. The plant material is often submitted to various treatments before the cryopreservation procedure itself, to increase its survival potential. In the cold-tolerant species, the *in vitro* mother plants can be cultured at low temperature for several weeks [144,187]. Explants have been placed for 1 or 2 days on a medium supplemented with a high sugar concentration or cryoprotective agents. Mint (*Mentha* spp.) shoot tips have been cultured thus for 2 days on a medium containing 0.75 M sucrose and 4% DMSO [188].

Explants are then loaded (i.e., suspended) in a medium containing cryoprotective substances (ethylene glycol, glycerol, sucrose) for a short period (5–90 min, depending on the material). This reduces their sensitivity to the vitrification solutions. Survival of rye (*Secale cereale*) protoplasts after exposure to a vitrification solution increased from 4% without loading with 1.5 M ethylene glycol to 65% with loading [189].

The duration of contact between the plant material and the vitrification solutions is a critical parameter owing to their high toxicity. The period generally increases with the size of explants treated. Rye protoplasts have to be dehydrated for only 60 s [189], whereas the optimal dehydration period is 80 min for apple and pear shoot tips [187]. Encapsulated carnation (*Dianthus caryophyllus* L.) apices are treated for up to 5 h with a vitrification solution comprising 38% sucrose and 35% ethylene glycol [154].

Dehydration of samples at 0°C instead of room temperature reduces the toxicity of the vitrification solutions and increases the potential period of exposure to vitrification solution, thereby giving more flexibility for handling the plant material during this critical phase of a vitrification protocol. Survival of asparagus cell suspensions dropped rapidly after 5 min of dehydration at 25°C, whereas if it were performed at 0°C, dehydration for between 5 and 60 min ensured satisfactory survival rates, with an optimum at 20 min [190].

Once dehydrated, samples are cooled rapidly by direct immersion in liquid nitrogen to achieve vitrification of intracellular solutes. Reduction in the quantity of suspending cryoprotectant solution and the use of containers of a small volume (e.g., 500 µL plastic straws) led to increased cooling rates. Asparagus cell suspensions have been enclosed in 50 µL of medium in 500 µL plastic straws, thus giving a cooling rate of -990°C min⁻¹ [191]. Mint and sweet potato shoot apices were cooled ultrarapidly (-4800°C min⁻¹) without cryoprotective medium [188,192].

Potato and carnation apices cryopreserved using a vitrification procedure have been stored at -196°C for 1 and 2 years, respectively, without any modification in their survival rate [154,193].

Rewarming of samples has to be performed as rapidly as possible to avoid devitrification processes, which would lead to the formation of ice crystals that would be detrimental to cellular integrity. Thus, samples are immersed in a water bath or liquid medium held at 20°–40°C. However, Steponkus et al. [146] have advised holding vitrified samples in air for a few seconds before plunging them in a thermostated water bath. This is to achieve slow rewarming through the glass transition region (ca. -130°C) to minimize mechanical fracturing of the glass caused by excessive thermal gradients [194].

After warming, the highly concentrated vitrification solution must be removed progressively to minimize osmotic shock. This is usually performed by diluting the vitrification solution in liquid medium supplemented with 1.2 M sucrose or sorbitol, before transferring the explants to standard medium.

Vitrification procedures generally lead to high survival rates, and direct and rapid recovery is usually observed. However, Towill [188] mentioned callus formation and abnormal development of some mint apices after vitrification.

Vitrification experiments involving a large range of genotypes are still infrequent. In the case of mulberry apices, experiments performed with 13 cultivars or species gave survival rates ranging between 40 and 80% [144]. More recently, 45–47% and 40–72.5% survivals have been noted with apices of five varieties of apple and eight cultivars of pear, respectively [187].

V. GENETIC STABILITY OF IN VITRO CONSERVED GERM PLASM

It is implicit in the genetic conservation context that genetic stability should be a very high priority. The facility of cloning in vitro does then offer, superficially, a very attractive means of perpetuating given genotypes, particularly for traditionally vegetatively propagated material. However, assumptions of clonal integrity in the in vitro situation may be unsafe. It is more pragmatic to consider supposed clones to be very tight populations with potential for deviation from the original distribution of genotypes. This then leads to a consideration of the factors that might contribute to such a deviation, namely creation and selection. Genetic variation may arise by somaclonal variation, with obvious implications for the choice of culture system used for genetic conservation. It may be intrinsic in the cultured material, possibly linked to its genetic structure, such as in sugarcane or banana, for which polyploids are more prone to instability than diploids [195]. Selection may occur under conditions that either cause differential damage of a lethal nature or that favor the growth of one genotype over others in a mixture. The issues to be taken into consideration and the information now available on genetic instability in material conserved in vitro are rather different for slow growth and cryopreservation, as described in the following sections.

A. Slow Growth

It has long been documented that in cultures comprising a mixture of genotypes, the different components of the mixture may not grow at the same rate [e.g., 196]. Under the stressed conditions implicit in slow growth, the risk of selection must be considered to be greater than under standard growth conditions. Accordingly, it is important to minimize the initial risk of instability and take measures to minimize additional risks and monitor cultures at intervals to detect variation. One of the most effective ways of minimizing risks of instability both at the outset and during slow growth storage is through control of the culture system. If the cultures are maintained in a highly organized state, as shoots, plantlets, or embryos, the risk of somaclonal variation is much lower than if they were in the form of cells or calluses. This will apply when there is an effective choice available, but sometimes it will be unavoidable to use cells or calluses, in which event, the choice of slow-growth storage must be questioned.

There have been few controlled experiments to monitor the genetic stability of cultures over time in slow-growth storage. However, such evidence as is available would suggest that organized cultures need not incur unacceptable risks. The genetic integrity of cassava cultures maintained in slow growth at CIAT over a period of 10 years, was confirmed when tested by isozyme analysis, DNA analysis, and by monitoring their morphology when returned to the field [26]. A slight suggestion of some acclimatization to slow-growth conditions [197] was not confirmed by any of the other analytic criteria.

There are differences from species to species in their susceptibility to somaclonal variation. When this is combined with the clear differences in response to storage under slow-growth conditions experienced not only between species but also between cultivars, it is clear that one cannot simply extrapolate from one fortunate example to all others. Thus, there is a pressing need for controlled experiments to test the genetic integrity of cultures stored in slow

growth, in comparison with controls maintained under normal-growth conditions, and ideally, with cryopreserved specimens, to gain an insight into the relative risks. An interesting model on which to conduct such an experiment would be *Musa*. Mass propagation in vitro is widely used for bananas and plantains, and a risk of somaclonal variation, even under optimal propagation conditions, is recognized. This is strongly linked to genotype. Studies of this phenomenon and parallel development of morphological, biochemical, and molecular methods for characterizing variants, have yielded a wealth of information on patterns of variation and potential markers to use in monitoring instability [19,198–202].

The risks of selection under slow-growth conditions should not be evaluated alone. Culture under any conditions carries risks. The risks of loss through human error are reduced by most storage measures. Risks caused by equipment failure are highly variable and, under some slow-growth conditions, might be considered to be greater than in normal growth. Thus, the equation to be drawn is not simple and must pragmatically take into account all of the pertaining circumstances, not least of all the risks of not applying the best available storage conditions.

B. Cryopreservation

Cryopreservation involves a series of stresses that may destabilize the plant material and lead to modifications in recovered cultures and regenerated plants. Therefore, it is necessary to verify the genetic stability of material recovered from cryopreserved samples before this technique is routinely used for the long-term conservation of plant germ plasm. Even though freezing protocols have been developed for many species, only a limited number of studies have considered this aspect. No modification at the phenotypic, biochemical, chromosomal, or molecular level that could be attributed with certainty to cryopreservation has yet been reported. This correlates with observations from other biological systems.

In cell suspensions, numerous examples are now available to illustrate that cryopreserved cells maintain their biosynthetic and morphogenic potential [203–205]. The only published exception concerns lavender *Lavandula vera* cell suspensions submitted to repeated freeze-thaw cycles: the number of colonies recovered from cryopreserved cells increased with the number of freeze-thaw cycles, suggesting that the selection of more freeze-tolerant cells was taking place [206]. However, no modifications were noted in the biosynthetic and regenerative capacities of cryopreserved cells, implying a change in population structure, rather than genetic change.

Plants regenerated from cryopreserved apices of strawberry and cassava were phenotypically normal [207,208]. No differences were noted in the vegetative and floral development of several hundred oil palm plants regenerated from control and cryopreserved somatic embryos [73]. Harding and Benson [209] noted that the ability of potato plants that were regenerated from cryopreserved apices was not impaired. However, following recovery of apices on certain media, plants failed to produce flowers in the first regeneration cycle. The authors suggest that this is more likely to be attributable to tissue culture than to cryopreservation as such.

Electrophoretic profiles of two enzymatic systems were comparable in plants regenerated from control and cryopreserved apices of sugarcane [210] and sweet orange (*Citrus sinensis* L., Osb.) somatic embryos [211]. With the latter material, there was no modification noted in the pattern of total soluble proteins. The ploidy level was not modified by cryopreservation in plants regenerated from oilseed rape somatic embryos [164] and sensitive dihaploids of potato [212]. Finally, restriction fragment length polymorphism (RFLP) patterns of plants regenerated from cryopreserved potato shoot-tips [213], embryogenic cell suspensions, and apices of sugarcane [210,214] were identical with those of unfrozen controls.

VI. CONCLUSIONS

A. Current Use of In Vitro Conservation Techniques

Classic in vitro slow-growth conservation techniques have been developed for a wide range of species, including temperate woody plants [215], fruit trees [18], and horticultural crops [216], as well as many tropical species [13,73,217]. A recent literature survey [71] indicated that shoot tips and node cuttings are the explants most frequently employed for slow-growth storage. For the tuber-producing species, medium-term storage of microtubers may represent an interesting solution, as shown [218] in experiments performed with potato. The slow-growth method most commonly employed is temperature reduction, the next most common is manipulation of the culture medium, then a combination of both parameters.

However, there are still only a few examples of in vitro slow-growth storage being used routinely as a complementary technique for the conservation of genetic resources of a given plant species. These notably include banana, potato, and cassava, which are conserved in regional and international germ plasm conservation centers such as INIBAP (now part of IPGRI), CIAT, CIP, and IITA.

Alternative medium-term conservation techniques are still at the experimental stage. Low-oxygen storage at room temperature may be interesting for tropical, cold-sensitive species because it allows growth reduction at the normal growth temperature. However, it still has to be tested with additional species and over longer storage periods.

Medium-term storage of desiccated (and possibly encapsulated) somatic embryos will facilitate the management of large-scale production of elite genotypes. For genetic resources conservation, encapsulated apices stored at low temperature may become the material of choice. However, further research is needed to increase the duration of storage.

Cryopreservation techniques have been developed for about 80 different plant species cultivated under various forms as cell suspensions, calluses, apices, somatic and zygotic embryos [16,18,19,205,219,220]. Most of this work has been performed in the framework of academic studies and has involved only one or a few genotypes. However, owing to the development in the last 3–4 years of new cryopreservation procedures for apices and embryos (encapsulation-desiccation, desiccation, pregrowth-desiccation, and vitrification), reports involving many genotypes or varieties are becoming more frequent. These new freezing procedures generally lead to satisfactory survival rates with a wide range of genotypes by using the same technique. The best example of large-scale experimentation is potato, for which a cryopreservation technique has already been successfully applied to more than 60 different varieties [221].

There is an increasing number of examples for which techniques can be considered operational. This is notably true with sugarcane, because cryopreservation procedures are now available for cell suspensions [222], embryogenic calluses [223], and apices [158,160].

Routine application of cryopreservation is still restricted almost exclusively to the conservation of cell lines in research laboratories [203]. The only example of routine application of cryopreservation to another type of material is oil palm. For this species, somatic embryos of 80 different clones are stored in liquid nitrogen, and frozen samples are thawed on request for plant production [185].

B. Future Needs and Prospects for In Vitro Conservation

When comparing the current status of in vitro conservation for plant genetic resources with the situation 20, or even 10, years ago, dramatic advances can be appreciated. Whereas in the 1970s it was a suggestion largely supported by extrapolation from the cryopreservation of

other biological systems, it is now a very realistic option for many species and culture systems. It has, through the development of slow-growth storage, revolutionized the medium-term genetic conservation of a substantial number of clonally propagated staple crops of the developing world. In vitro active gene banks of these crops can be maintained, independently of prevailing climatic conditions; thus, an important world collection of *Musa* is housed in Belgium where it is free of the risks that would be ever present if maintained in a *Musa*-growing country [65]. Similarly, collections can be safely duplicated to second sites and moved from location to location with ease.

A natural caution in the take-up of in vitro conservation has been observed over recent years. This was fully understandable at a time when the techniques were at a more experimental stage. However, there are now many instances where both slow growth and cryopreservation techniques could be more widely applied, to the benefit of both germ plasm management and research. Slow growth could be more widely used to provide safer alternatives to the field gene bank for species that are readily propagated in vitro by low-risk methods. Cryopreservation is not yet at as advanced stage of development as slow growth, particularly for organized cultures, despite the recent advances in technological development described here. Nevertheless, for cell and callus cultures, especially embryogenic systems, the effort to tailor published methods to specific materials would almost certainly pay off.

For organized shoot and embryo cultures, it must be recognized that there is still some way to go before routine methods can be applied without any such tailoring. However, it is time that the primary focus of experimentation for such materials moved from the research laboratory to the gene bank, or at least to research units within institutes that have a conservation mandate. Close liaison between in vitro conservation specialists and gene bank personnel will assure the development of method and conservation routines that meet the necessary criteria required in the gene bank context. This will also allow the know-how of the gene bank staff in relation to genetic stability and genetic characterization to be incorporated into in vitro conservation research and development.

Above all else, it is important that in vitro conservation be demystified to convey it as a realistic option that is not in the province of high biotechnology, but a very practical, convenient approach to consider alongside other conservation methods. An important step in that process will be development of an understanding for the flexibility of in vitro conservation protocols. A level of precision and exactitude that is unrealistic and, in fact, unnecessary will serve as a serious deterrent to the take-up of any technique.

The locations in which in vitro conservation could be most beneficial are developing country laboratories and gene banks in which facilities, although adequate, may not be practically adaptable to the many different conditions described in the literature. Thus, it is important to obtain a clear idea of the flexibility of storage protocols (i.e., the window within which effective conservation may be achieved). This is truly a situation in which "the best is the enemy of the good." Optimizing storage conditions to a fine degree of tuning for every genotype will be far less effective than developing more generalized procedures that have an adequate degree of flexibility that can accommodate slightly different culture conditions or a different pair of hands applying them.

Another crucial issue is the complementarity of in vitro conservation for other genetic conservation strategies [12,224,225]. In vitro conservation is not being presented as a replacement for conventional ex situ and in situ approaches. It is one more tool to place in the hands of the curator to cover the conservation requirements of the gene pool in question. In some circumstances it will be appropriate; in others it will not. Factors to take into account in making the selection will be effectiveness, security, cost, available facilities, and needs for access by users, including breeders. Sometimes the need will be to store clonal genotypes for

the short- to medium-term; sometimes, the need will be to store genes for the long term. Each situation needs to be matched by the best combination of conservation options, each option offering advantages and offsetting disadvantages of other methods. In this context, it is easy to see that in vitro conservation could be taken up much more readily than at present, its research and development, proceeding toward routine use with the safety net of other conservation methods that, at the same time, serve the purpose of experimental controls against which to evaluate its effectiveness.

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Asexual Cell Genetics

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1. INTRODUCTION

Asexual genetics sounds like a contradiction in itself, but today the biotechnological tools applied on single plant cells allow one to produce genetic variability asexually, to modify somatic genomes in a durable genetic manner, and to perform selection in such asexually combined genotypes. Thus, the central genetic prerequisites for crop improvement—production of variability and selection—are fulfilled (Fig. 1).

In this chapter, the use of these asexual genetic methods during in vitro culture of agronomically important plants will be outlined. In particular this is work with somatic and gametophytic cells as well as somatic cell fusion. These three tools offer to applied-breeding programs the following opportunities:

1. Somatic cells and protoplasts enable handling of extremely large numbers of individual genotypes in the very small space of a petri dish.
2. The chance to treat gametophytic, haploid cells the same as sporophytic ones, for which the simpler genomes of microspores allow the uncovering of recessive traits and additive characters and, after doubling, directly result in homozygous lines.
3. The sporophytic or gametophytic cells may serve as a tool for exchanging or taking up genetic information asexually by symmetric or asymmetric fusion, by vectors, or by direct DNA-transfer techniques.
4. Only through asexual, somatic genetics does it become possible to recombine and create new cytoplasm, allowing modification of the nuclear cytoplasmic interaction.
5. In vitro culture is not influenced by the natural environment, which makes it easier to measure slight quantitative genetic differences of polygenically inherited traits.

When trying to make use of a new technology one should always be aware that the application of these tools will be judged by the success of classic-breeding techniques. They have achieved tremendous improvements and also, in the future, will guarantee better resistance, higher

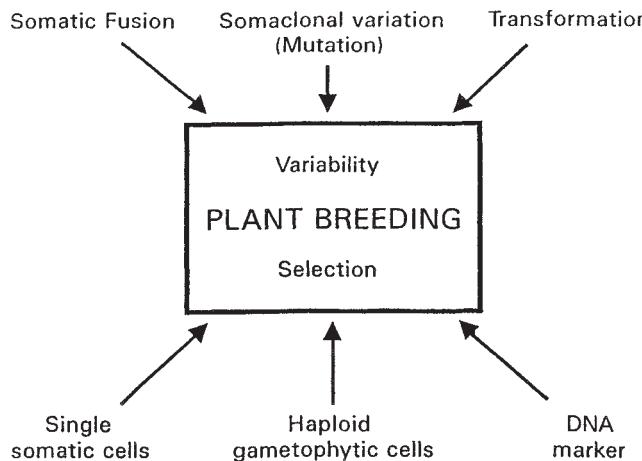


Fig. 1 Asexual tools using cell cultures in plant-breeding procedures.

quality, and more stable yields. As long as there is a chance of detecting new genetic resources, to collect, and to evaluate them, classic breeding is in principle without any limitation. Its most critical factor is the time needed until a new cultivar can be released. Particularly, from the standpoint of combining quantitative complex inherited traits, such as durable resistance against diseases, or quality characteristics, the population size and the time and accuracy of selection are very crucial factors. Consequently, those biotechnological procedures that have the most chance of succeeding are primarily those that are superior to classic combination-breeding strategies and accepted selection procedures.

II. NEW VARIABILITY IN SOMATIC CELL CULTURES

Plant breeders are normally not short of variability; thus, one prerequisite for successful selection—the availability of a gene pool expressing variability—is normally at hand. This gene pool is either a rather natural one conserved in wild varieties, primitive cultivars, or land races, or it is created by recombination of genotypes. Once, it was hoped that mutation research might broaden the variability in a predictable way, but today; we can conclude that success in using mutants is restricted to rather specific plant groups, such as the ornamentals, or to specific cases [1]. Having normally no restrictions in heterozygous plant populations, finding the optimal genotype in a population by selection is still the more critical process in breeding programs.

A. Types of Somatic Variation

Cell culture systems offer the possibility of growing higher plants in a manner similar to that for microorganisms *in vitro*. When regenerating single cells, it became obvious rather rapidly that the stress of a completely artificial environment, of the medium, and in particular, of the phytohormones increase the variability within a cell culture-derived plant population. In principle, each cell of such a population might be a different genetically defined individual. Cells of suspension cultures, protoplasts, and microspores, offer such single-cell systems from the sporophyte or the gametophyte. Haploid, gametophytic, cells even allow a direct uncovering

of recessive traits arising by, for examples mutations, and of additive characters because they are equivalent to the F_n generation. Furthermore, systems that begin with single cells can help circumvent problems of cross-breeding and chimerism which, in complex tissues, can hide a desired trait.

Depending on the type of somatic cell, *somaclonal* variation—originating from callus or suspensions—and *protoclonal* variation—originating from protoplasts—are distinguished. As a third phrase, the asexual variability expressed, in addition to the variability caused by sexual recombination in regenerants from microspores, is called *gametoclonal* variation.

1. Somaclonal Variation

Spontaneous alterations of the phenotype in regenerates from cell cultures have long been described. Particularly when seeking rapid propagation, this new variability was looked at as a negative feature of cell cultures; for example, those aiming for rapid propagation. This negative view was strengthened even more when, as the basis for the variability, chromosomal and genomic mutations were found [2]. The evolving new genotypes were preponderantly aneuploids which were of little use for work at the whole-plant level. In addition such variation is quite often unstable, or even a characteristic found at the cell level is not expressed in the functional plant. After a boom in reports on superior somaclones, only a few examples express the somatic characteristics sufficiently under field conditions. Consequently, this use of somaclonal variation has always been controversial. Either it was judged as a new, but not very useful mutagen [3], or it was viewed as an excellent technique for intracultivar improvement [4]. In the latter instances, it was anticipated that the genetic changes appearing in plants regenerated from dedifferentiated and redifferentiated cells during in vitro culture are stable, are expressed sexually, or are at least vegetatively transmitted. This approach is of particular value if the new character does not exist in the natural gene pools.

One of the explanations for the effective installation of useful cell lines in vitro, and the rare development of stable regenerates under field conditions is that these traits result from a physiological adaptation. Habituated, epigenetic changes may even hide the selection of cells with stable genetic mutations. Such epigenetic differences are often due to differences in DNA methylation; this could be confirmed by restriction fragment length polymorphism (RFLP) analysis using methylation-sensitive restriction endonucleases [5]. Similar methylation mechanisms are also under discussion for the partial instability observed in transformed plants, particularly in cereals transformed by microbombardment. But only the regeneration of fertile plants and the transmission of the phenotype to sexual progeny provides definitive proof for genetic changes and will be eventually useful in agriculture.

A severe drawback of the present strategies for in vitro selection is the limited knowledge of causal physiological or biochemical reactions responsible for the desired phenotype. Somaclonal variants cannot yet become a general breeding tool, because the diagnostic systems to identify their genetic base are too weak. Often, this lack of a profound knowledge of plant physiology has made working with somatic cells more of an art than a reproducible scientific tool. DNA diagnosis allows the immediate detection of alterations of the genome. Since 1985 when the polymerase chain reaction (PCR) was developed [6], hybridization of very small portions of plant material has become routine, and this technology can be applied to cell cultures and even single cells [7]. These techniques will help one to understand the nature of somaclonal variation, as reviewed by Peschke and Phillips [8].

The efficiency of increasing the amount of new variability in an existing cultivar increases with the application of selection pressure. Somaclonal variation might become superior to simple mutation breeding when selection is applied during the in vitro phase.

2. Protoclonal Variation

As in cell and callus culture, during the regeneration of protoplasts protoclonal variation often also appears. Because protoplasts are a single-cell system that can be handled in any species, they can be regenerated easier than cells from suspension cultures, Shepard [9] postulated the usefulness of protoplasts for creating new genotypes. He found tremendous variability in protoclones from the potato cultivar Russet Burbank. Up to 30% of the regenerated protoclones were claimed to be useful variants. However, only a few variants were genetically stable.

Landsmann and Uhrig [10] showed in potato that 2 of 12 clones, regenerated from the same protoplast donor clone, were variant when probed with one of a set of random potato DNA probes. Because the polymorphic DNA probe turned out to represent 25S-rDNA, the two variants can be considered to carry a mutation in RNA genes. By shortening the callus phase, this variability could be reduced, but not absolutely prevented.

In experiments with dihaploid potatoes ($2n=2x=24$), we have regenerated more than 3000 clones from protoplasts [11]. Only a few grossly aberrant types were found, most of which turned out to be aneuploids with no practical value. It can be concluded that the number of variant phenotypes depends on the ploidy level: In polyploids more mutants will survive than in diploids (or haploid microspores); at low ploidy levels unfavorable mutations cause lethality.

B. Use of the Asexual Variability from Cell Populations

A cultivar that performs better as food, feed, or industrial raw material is the central goal of breeding. Progress more easily and more quickly finding the superior plant within a segregating population means success. In classic plant-breeding programs the selection is carried out in the field and in plant populations of limited size. Especially in breeding for characteristics that do not follow qualitative inheritance—mostly monogenic—but show only quantitative inheritance—mostly polygenic—the selection is consequently uncertain and needs parallel trials and repetitions.

1. Selection for Disease Resistance

Because durable healthy plants are of increasing importance for not only economic but also for ecological considerations, breeding for disease resistance is becoming a central goal. Starting with the use of spontaneously arising somaclones, dual culture systems and alterations induced by selection pressure were screened for increased disease resistance.

With use of asexual cell populations, spontaneous new disease resistance among regenerated plants was first reported in sugarcane. Heinz et al. [12] found regenerates from calli that were resistant to the Fijivirus disease, and they later found resistance to *Helminthosporium sacchari*. A somaclonal variant of *Apium graveolens* resistant to *Fusarium*, was obtained from a susceptible cultivar [13]. These characteristics were heritable and could be incorporated into breeding lines.

Nearly 50 years ago Morel [14] realized the possible advantage of growing pathogens together with their hosts (*Vitis vinifera* and *Plasmopara viticola*) in vitro to detect new genes for pathogen resistance. However, the further development of such a dual culture technique has not been rapid. Recently, significant differences in growth of *Phytophthora cinnamomi* on resistant and susceptible pine callus lines were found [15]. Potato protoclones with significantly enhanced tuber resistance to *Erwinia* spp. have been found in nonselected populations of protoplast-derived regenerates [16]. Protoplast-derived callus was exposed to different inoculum concentrations of *Erwinia carotovora* subsp. *carotovora*. The reaction to the pathogen varied considerably within the callus populations, and callus lines with

improved resistance have been identified. But the number of regenerates was too small to field test for soft rot, or to make any evident correlation between in vitro and in vivo reaction [17].

As an improvement, toxins or culture filtrates produced by the pathogen were used for selection instead of the total pathogen. The assumption in this approach is that toxic metabolites of the pathogen play a role in pathogenesis and that they can be used to exert a selection pressure for cells that are resistant. Although it cannot be assumed a priori that plants regenerated from the resistant cells will be resistant to the pathogen, it has been applied to several plant-pathogen systems.

The first successful experiment in which a controlled selection for resistance to a pathogen was made with the responsible toxin alone was reported in the easily handled *Solanaceae* in 1972. The findings on the role of a toxin by Helgeson et al. [18] proved that tobacco calli resistant to *Phytophthora parasitica* can be obtained from resistant plants and that susceptible plants yield only susceptible calli. In *Solanum tuberosum*, the potato, protoplasts of dihaploid clones were regenerated in the presence of selective concentrations of extracts from *Fusarium sulfurium*, *F. coeruleum*, or *Phytophthora infestans* [19,20]. A significant higher number of protoclones proved to be unaffected by the toxin in secondary callus or in a leaf test, compared with regenerated protoclones that had developed without selection pressure. Although it has been proved that in vitro selection for fungal resistance works on the in vitro level, the correlation to subsequent field tests was not always reliable [21]. This may be due to not only the rather artificial in vitro screening, and the not too sensitive field tests that detect only differences greater than differences coming from average protoclonal variation, but also due to insignificant genetic changes. Tomato plants could also be regenerated from cotyledonary explants resistant to the culture filtrate of *Phytophthora infestans* [22].

For the first screening, it is surely important to use selective agents that are essentially correlated with the infection process such as, for example, the toxin sirodesmin PL produced by *Phoma lingam* in *Brassica napus*. Protoplasts isolated from resistant and susceptible varieties died when treated with the toxin in a concentration higher than 1 µM, whereas in cell aggregates significant differences between resistant and susceptible varieties were obtained. The same clear correlation between sensitivity to the toxin and susceptibility to the pathogen has been found in the intact plants [23]. Secondary embryoid lines of single-cell origin of *B. napus* differed in sensitivity to a selection medium containing partially purified *Alternaria brassicola* filtrate. Some regenerated plants were more resistant to the pathogen than seed-grown plants of the same cultivar, although there was no correlation between sensitivity to the selection medium and susceptibility to the pathogen [24].

In 1977, Gengenbach et al. [25] selected certain maize plants that were resistant to *Helminthosporium maydis* by using the T toxin. The genetic change in the resistant somaclones was located in the mitochondrial DNA, which lost, in a parallel fashion, the character male sterility. Thus, this result was of no practical importance. Calli of barley and wheat genotypes were also screened for their resistance to purified culture filtrate produced by the fungus *Helminthosporium sativum*. Plants have been regenerated from somaclones surviving the toxin treatment, and the first in vitro-derived generation gave significantly improved lines [26]. Similar results were obtained in wheat tissue culture in which a selection was carried out with syringomycin produced by the bacterium *Pseudomonas syringae* [27]. The correlation to subsequent field tests for *Helminthosporium* resistance was, however, unreliable [21]. Hunold et al. [28] regenerated barley plants after toxin-selection from *Drechslera teres*. The regenerated plants showed improvements in the resistance level, but so did the unselected control plants.

Severe problems with this in vitro selection system arise when hypersensitive reactions are screened for at the cell level. In this type of resistance, the cells in the resistant plant are killed so quickly that the pathogen cannot survive, multiply, or spread, thereby allowing all other tissue parts to survive. This type of cell-cell interaction demands an organized tissue. Additional examples dealing with in vitro selection for disease resistance are reviewed by Wenzel and Foroughi-Wehr [29].

2. Selection for Abiotic Stress

Selection for tolerance to abiotic stresses involves exposure of cells to the stress for which resistance is desired. Numerous results from selection in somatic cell cultures for abiotic stresses are discussed by Loh [30]. Here only a few studies are quoted as examples of how asexually new traits may be found in cell cultures.

a. *Temperature Tolerance.* Selection for freezing tolerance was carried out in somaclones derived from callus cultures of the winter wheat variety Norstar. Fewer and more freeze-tolerant somaclones were found, and it was stated that this variation was heritable [31]. Also in wheat [32] and in potato [33] tolerant somaclones were detected. Frost tolerance was coupled with an accumulation of proline in the leaves. This led to a decrease in the osmotic potential and an increase in the frost tolerance. From a cell suspension of dihaploid potato clones, somaclones were selected with a proline content up to 25 times higher than the wild type. In contrast to the resistance expressed in potato leaves, the tuber frost tolerance was not improved [33].

For high-temperature stress, in vitro selection is also a possible tool. Trolinder and Shang [34] succeeded in in vitro selection of heat-tolerant lines of cotton, and Wang and Nguyen [35] compared the reaction of wheat to heat treatment with the formation of heat-shock proteins and concluded that the selected heat-tolerant genotype synthesized greater amounts of heat-shock protein, with molecular weights of 16 and 17 kDa than did the heat-susceptible genotype.

b. *Salt Tolerance.* For selection of cells on media enriched in salts and a subsequent regeneration at least to calli has been frequently reported. [Table 1](#) summarizes some of the reports on crop plants.

Normal plants could rarely be regenerated, primarily because of the long callus phase, resulting in too many additional deleterious mutations. Even if plants could be regenerated from salt-tolerant callus of japonica or indica rice, often the proof of the genetic alteration was still missing. Vajrabhaya et al. [52] obtained two indica rice cultivars from callus of 450,000 embryos: one line showed a heritable salt-tolerant character in the progeny. In maize, plant regeneration was obtained from a long-term salt-tolerant cell culture, and the tolerance could be preserved in vitro for 3 months and also in the absence of NaCl; but the seed set of the regenerates was very poor. Among the regenerated salt-tolerant plants, four survived in soil and were selfed or crossed with each other; however, an increased salt tolerance in the offspring was not evident, although a higher vigor was observed in a few lines [39]. Plants were regenerated from a salt-tolerant cell line of *Linum usitatissimum*; the progenies of these plants were superior in both saline soil and normal soil. This indicates that the selected trait is a more general mechanism that might be due to an increase in total vigor of the regenerates [38]. Jain et al. [53] concluded from their experiments with *Brassica juncea* that different somaclones may have different salt-tolerance mechanisms, because they selected lines that reacted differently in their salt tolerance during vegetative and reproductive phases. In summary, for the selection of salt-tolerance, cells are not superior

Table 1 Examples for the Selection of Salt-Tolerant Plants in Somatic Cell Cultures

Plant species	NaCl	Na ₂ SO ₄	Resistant cells	Resistant regenerates	Ref.
Alfalfa	+		+	+	36
Citrus	+		+	+	37
Flax	+			+	38
Maize	+		+	+	39,40
Oats	+	+	+		41
Poncirus	+		+	+	42
Potato	+		+		43
Rapeseed		+	+		44
Rice	+		+	+	45,46
Sugar beet	+	+	+	+	47,48
Tomato	+		+	+	49
Wheat	+		+	+	50,51

to seedlings for selection because the problems of regeneration to normal plants are much easier after seedling selection.

c. *Metal Tolerance.* Similar in vitro selections have been performed for resistance to metals such as Cd, Zn, or Al. In a first report of this nature, Meredith in 1978 [54], used tomato cell cultures in selection experiments for resistance to aluminum. The selected clones remained resistant after 2–4 months of growth in the absence of the stress, but no plants were regenerated. Later, selection in cell suspensions supplemented with Al ions, either as Fe-EDTA or as Al₂(SO₄)₃, produced tolerant cell lines of *Daucus carota* [55], of *Nicotiana plumbaginifolia* [56], and of *Solanum tuberosum* [57]. In *D. carota*, young seedlings grown from the selected regenerated plant seeds also showed tolerance. Conner and Meredith [56] reported a 50% constancy in *N. plumbaginifolia*, and found a single dominant mutation causing the Al tolerance.

d. *Herbicide Tolerance.* Since the early work of Chaleff and Parsons [58] on a plicloram-resistant tobacco somaclone in 1978, numerous reports on tolerance to herbicides have appeared. Some interest developed in use of in vitro mutants for transferring mutant characters to crop plants, for example, the glyphosate resistance from petunia [59], or to tag the responsible gene in such mutants. For paraquat, enzymes convert the toxins to inactive products, whereas amplification of target genes confers resistance to glyphosate and l-phosphotricin. Suspension culture of *Brassica napus* cv. Jet Neuf was used for isolation of sulfonylurea herbicide resistance. The medium was supplemented with chlorsulfuron in a concentration of 5×10⁻⁸ M. Surviving cells were regenerated, and the resistant variants formed callus on the selection medium, indicating that the resistance was not lost during organogenesis [60]. Atrazine- and diuron-tolerant calli and plants were recovered from a protoplast culture of *Nicotiana plumbaginifolia* after addition of a plastome mutagen, N-ethyl-N-nitrosourea, to freshly isolated protoplasts. The surviving calli were regenerated into plants and sprayed with the herbicide. Plants of six and four potato clones were resistant to atrazine and diuron, respectively [61]. Ballie et al. [62] found improved tolerance to chlorsulfuron in barley. For a wide range of other herbicides in vitro cultures were used to detect herbicide-tolerant tissue. Usually regeneration of plants expressing some level of tolerance was possible.

The use of somatic cells for gene transformation and the subsequent selection steps are described in [Chapter 8](#).

III. ASEXUAL PRODUCTION OF HOMOZYGOUS LINES

As well as sporophytic cells, gametophytic cells can also be regenerated into functional plants. Such haploid cells already exist as egg cell or microspore as a single-cell system in nature. A microspore can be induced *in vitro* to asexually form a haploid plant. All the advantages of haploids were defined as soon as the first haploid plant was described in the 1920s [63], but it took 40 years before haploids gained broader scientific interest, and another 10 years until they were taken up in private breeding stations. Only today do breeders have access to sufficient numbers of haploids and doubled haploids (DH) from barley, wheat, potato, and rapeseed to run practical field experiments.

The main advantage of using haploids is the rapid and complete homozygosity of the offspring, making phenotype selection for quantitative, and particularly for qualitative, inherited characters much easier, and therefore breeding more efficient. In polyploids, such as *Medicago* and potato, haploidization is used to produce diploids, not only for breeding by classic techniques with such diploids, but also as a prerequisite for the successful application of somatic hybridization. In most tetraploid crops it has become possible by using this approach, to combine wild species with the cultivated forms or to combine complex genotypes.

From the performance of DH populations and the segregations in such populations data can be generated for the characterization of the parents of the anther donor hybrid. Table 2 gives an example showing that different numbers of alleles were present in lines with barley yellow mosaic virus resistance. Unadapted material was combined with German cultivars, and in the case of Bizen Wase 5, a strong overrepresentation of resistant DH lines was obtained. By such analytical screening of the parental type, breeding programs can be run in a much more efficient manner.

A rather recent application of DHs is their use in molecular genome mapping. In a population of DH lines, the identification of phenotypic markers is much more secure, for most intermediate expressions resulting from heterozygosity are excluded. In both the expression at the plant level as well as at the DNA marker level, a gene will segregate in a 1:1 ratio. Actually, it is very difficult to reproducibly map and correlate DNA polymorphisms when the bands segregate in a 3:1 or a 1:2:1 ratio. This is of particular importance when a polygenically coded characteristic is to be mapped in a QTL analysis. For barley, the unique combination of RFLP [65] or RAPD [66] and haploid cell techniques has facilitated tremendous progress and set the stage for a detailed characterization of this genome relative to both basic and applied research. By using DH lines, the likelihood of recovering a distinct homozygous genotype in a progeny is $1/2^n$, whereas it is $1/4^n$ in an F_2 progeny (n =number of

Table 2 Segregation of Barley DH Populations Extracted from F_1 Hybrids Between Susceptible German Cultivars and an Unadapted Resistant Parent for Barley Yellow Mosaic Virus Resistance

Resistant parent	Number of susceptible cultivars used	DH lines produced	Segregation Susceptibles:Resistant
Kobinkatangi	4	166	1.2:1
Cebada	4	210	1:1.2
Smooth	2	136	1.2:1
Chikurin	6	508	1:1.1
PI 329037	1	71	1:2.4
Kersho	4	749	1:1.7
Cl 9346	3	279	1:3
Bizen Wase	2	129	1:4
Bizen Wase 5	3	485	1:4.4

Source: Ref. 64.

Table 3 QTLs Mapped with Molecular Markers on Double Haploid Barley Lines

Trait	No. of loci	Variance ^a (%)	Ref.
Powdery mildew resistance	2	20	67
Kernel yield	6	58	68,69
Lodging	6	71	68,69
Plant height	10	72	68–70
Heading date	2	83	69,70
Ear emergence	11	67	68
Grain protein	6	56	68
Amylase activity	9	63	68
Diastatic power	9	67	68
Leaf spots	4	60	69
Resistance <i>Puccinia striiformis</i>	2	61	71
Winterhardiness	1	79	72

^aPortion of genotypic variance that is explained by the markers.

unlinked genes). Thus, to select a desired recombinant of five genes with a 95% probability, about 3000 F₂ plants and only 100 F₁-derived DH lines have to be tested [65]. Already a large number of quantitative trait loci (QTLs) have been mapped in barley for a series of traits with this approach (Table 3).

A. Androgenetic Haploid Induction

For many species, the most promising and also most successful method of inducing DH lines is microspore androgenesis. Parthenogenetic techniques are normally not asexual systems and thus are not part of this review. In spite of the numerous application of the androgenetic method, we know very little about the induction mechanisms responsible for transforming a microspore into a sporophyte. For a number of goals using androgenetic haploids or DH populations the absence of any selection is occurring during microspore regeneration. De Vicente and Tanksley [73] found, however, in tomato that the frequency of meiotic recombination differs between male and female gametes. A single F₁ plant was backcrossed to each of *Lycopersicon esculentum* and *L. pennelli*, as the male and the female parent. With 85 RFLP markers they constructed a map of two backcross populations. Because both recurrent parents were homozygous, recombination measured in each population reflects crossing-over rates leading to male and female gametes. Significantly less recombination was observed for male gametes. Mohler and Graner [74] found in experiments with barley using the same procedures as De Vicente and Tanksly [73] that from 53 DNA intervals analyzed, 28 were larger and the other 25 were smaller in the backcross population which was made with the barley genotype Igri as the female parent compared with the one with Igri as the male parent. They conclude that for barley no total difference in the recombination for female and male gamets exist. This does not mean, however, that during the subsequent regeneration steps independent mechanisms of selection become active. To some extent this is visible from areas with distorted segregations in the crosses with Igri for which the characteristic "regeneration capacity" might be responsible. This assumption is in accordance with the overall accumulation of alleles from the parent which better responds to in vitro culture.

1. Culture of Microspores Within the Anther

The easiest procedure for microspore culture is anther culture. More tricky and thus not so easy for routine procedures is the culture of microspores in isolation from the anther. In both

cases the genotype of the microspore donor plant is of utmost importance. Particularly during regeneration of microspores from monocots, numerous albino plants develop. This problem, particularly in many cereals, has been investigated for barley, for which it now became obvious by the use of organelle-specific DNA markers that, in particular, the chloroplast DNA is responsible [75]. Replication errors and recombination occurred, leading to deletions and rearrangements of the cpDNA, which caused albinism in the regenerated plants.

2. Culture of Isolated Microspores

Besides culture of the microspores within the anther, they may be cultured as shed-pollen culture or after mechanical isolation from the anther. For potato (Table 4), both techniques are compared. Here, isolated culture means that the anthers are cultured in liquid medium where they shed isolated microspores after about 3 weeks. These structures then grow in isolation from the anther tissue and develop into embryoids [76]. Kuhlmann and Foroughi-Wehr [77] were able to regenerate a high frequency of green barley plants in liquid Ficoll medium containing barley starch and maltose, but they obtained almost the same results in a maltosefree medium. The addition of maltose accelerates the microspore development more than starch, resulting in a higher regeneration frequency. The best results were gained by addition of both starch and maltose. A calculation of the price of such media for applied purposes gives a better chance to the cheaper starch medium [65].

The success of isolated microspore culture, measured in the numbers of green plant formation, is highly superior to anther culture for *Brassica napus* and for specific genotypes of potato (see Table 4). In most other cases the culture of isolated microspores is possible, but anther culture gives better results and is easier for routine haploid production. Nevertheless, for some high-responding genotypes and under circumstances other than only for maximal plantlet formation, isolated microspore cultures offer a more effective system of regenerating a random sample from the microspore population than does anther culture. This is particularly important when the desired trait is linked with low plasticity. In addition, these cultures are a good source for in vitro selection and gene transfer. The advantages of using microspores for selection purposes include the following:

1. A huge population of single haploid cells is available.
2. A lower level of somaclonal variation will result in fewer plants with undesired characteristics.
3. The selection can be carried out at the earliest imaginal breeding stage.
4. The expression of recessive traits and selected individuals will be homozygous after doubling.

Table 4 Comparison of Isolated Microspore Culture and Anther Culture in Potato

Genotype	Microspores ^a	(%)	Anthers	(%)
H3-703	120/7	6	120/1	1
H81.1022	24/80	333	24/0	0
H82.4200	48/2	4	48/3	6
H84.4004	120/7	6	144/0	0
H87.4100	24/9	38	24/7	29
H87.4103	112/160	143	144/129	90

^aThe number of anthers from which the microspores were isolated.

Source: Ref. 76.

3. Regeneration Capacity

A central parameter in increasing regeneration success in cell culture was the selection of clones with a high capacity to respond in cell culture and to transfer this ability to their progeny by sexual crosses. In potato [78–80], rape seed [81,82], rye [83,84], barley [85], wheat [86,87], and maize [88] high-responding genotypes were crossed with agronomically important varieties. The F₁ generation showed an intermediate reaction between both parents, making it feasible that a high response in anther culture is heritable [89]. In winter barley the highest responding genotype is still “Igri” [85]. For potato it was demonstrated that the combination of two heterozygous clones (probably also heterozygous for the characteristic regeneration capacity) resulted in a rather quantitative response, the combination of a probable homozygous responsive clone with a probable homozygous wild-type one without any tissue culture responsiveness resulted in a qualitative segregation [90]. These results were confirmed by Sonnino et al. [91], who combined a dihaploid clone with regeneration capacity with a root-knot nematode-resistant clone. Among 19 F₁ progenies tested a wide range of androgenetic capability was found. The authors conclude that the characteristic of regeneration capacity is controlled by more than one major gene. This is in contrast with the conclusion of Singsit and Veilleux [92]. They deduced from data from a backcross between a highly responsive and its unresponsive parent that the anther culture capacity may be under control of a single dominant gene. Meyer et al. [93] analyzed the regeneration capacity even further and claimed a few, perhaps only two, responsible major genes, one of which controls the induction of embryoid formation, the other one controls its frequency. Additionally, minor genes are involved. When using this procedure for anther culture improvement, Uhrig and Salamini [80] were able to obtain more than 100 embryoids per flower after a prebreeding phase for regeneration capacity. Although this genetic analysis was performed with dihaploid potato clones, the genetic basis is also valid for 4x anther donor clones.

In wheat, the good regeneration capacity of the variety “Centurk” could be transferred in reciprocal crosses to the F₁ progeny, whereas the cytoplasmic effects were negligible [86]. This is in contrast to other findings for which, in a diallele population produced from four Hungarian winter wheat cultivars, the additive genetic effects are more important than nonadditive effects in determining the inheritance of good-responding anthers [94]. The significance of reciprocal effects suggested that the variation observed was due to not only nuclear genetic effects, but also to cytoplasmic factors. Henry and de Buyser [95] stated that haploid production in wheat is controlled by at least three different and independently inherited traits: embryo induction, regeneration ability, and the ratio of green to albino plants. They suggested that regeneration ability could be linked to the 1BL-1RS translocation. The results were verified in other investigations [87,96,97]. In 1994, Henry and De Buyser [98] suggested that the involvement of the 1RS arm from rye translocated into the 1 BL wheat arm documents the effect of a “gametophytic gene.” Higgins and Mathias [99] analyzed the chromosomal effect of regeneration capacity from callus cultures and reported significantly increased morphogenesis of different substitution lines of 4B chromosome in Chinese Spring donor material.

Although progress has been made in developing highly androgenetic germ plasm, anther culture in maize remains highly genotype-dependent. Here, RFLP analysis was used to map the genes that condition high response to anther culture. Given that analysis, the high response of one genotype (139/39–05) is conditioned by two major genes that are epistatic and two minor genes. With this genetic description 57% of the variability among means of 98 families tested is explained [100,101].

4. Microspores as Targets in Transformation

Isolated microspores are an attractive explant for transformation when they can be easily regenerated. Particularly in monocots in which *Agrobacterium*-mediated transformation has limitations microspores are ideal. The best regeneration of cereal microspores has been achieved in barley; thus, first transformations with direct DNA transfer or transfer by electroporation and microbombardment have been started. Improved regeneration systems from isolated microspores of barley and numerous transformation strategies with particle bombardment have been developed [102,103]. For further details see [Chapter 8](#).

B. Haploid Cell Cultures Used for Selection

Ye et al. [104] combined anther culture and in vitro selection. They cultured anthers from a barley F₁ hybrid—a cross between a salt-tolerant and a normal sensitive cultivar—in a liquid medium containing up to 0.8% Na₂SO₄. No progeny from the F₁ microspore cultured in the high-salt medium was as susceptible as the susceptible parent. The results indicate that, in these experiments, sensitive microspores were eliminated. Lines were selected exhibiting elevated levels of tolerance of salt from recombination, rather than from gametoclonal variation.

For potato, such a selection system was applied using the crude toxin from *Phytophthora infestans* as a biochemical marker [105]. From seven dihaploid donor plants the microspore population was screened with different *Phytophthora* toxin concentrations. After the addition of toxin, three genotypes reacted with a general increase of the embryoid regeneration rate per anther. There existed neither a correlation with the *Phytophthora* resistance of the donor plants nor were surviving and regenerated androgenetic plants more resistant than the starting material.

Wheat microspore populations, descending from F₁ hybrids of varieties with different levels of resistances to *Fusarium*, were screened with a phytotoxin of *Fusarium* before and during regeneration. Two selection methods were compared: either embryos and calli were first initiated from anthers in toxin-free medium and then grown on medium with the toxin, or anthers were immediately cultured on regeneration medium containing the toxin. Microspores from donor hybrids that were produced from two susceptible cultivars were killed by lower toxin concentrations than microspores from hybrids of parents that were both somewhat resistant. Microspores descending from combinations resistant × susceptible showed an intermediate reaction (Table 5). The principle of this selection is not the formation of new variability through

Table 5 Estimation of the Toxin Concentration That Kills About 90% of Regenerating Microspores Compared with the Growth of Untreated Controls

Anther donors (F ₁ hybrids)	Numbers of anthers/ regenerants (− toxin)	Toxin concentration (ml/l)	Numbers of anthers/ regenerants (+ toxin)
Florida (5) ^a × Carisuper (3)	8250/5057	2.5	10300/722
Florida (5) × Falke (5)	9955/4496	2.5	9165/285
Florida (5) × Kraka (6)	8230/4850	2.5	9165/510
Florida (5) × Boxer (9)	9510/4290	2	8850/221
Carisuper (3) × Falke (5)	1175/245	2	7060/141
Carisuper (3) × Kraka (6)	6085/2192	2	7995/160
Carisuper (3) × Boxer (9)	3330/885	2	7010/140
Kraka (6) × Boxer (9)	1220/200	1	5490/109

^a1, very resistant; 9, very susceptible.

Source: Ref. 87.

mutation, but rather, the use of recombination. The selection should uncover a gene complex that should be present in the microspore population [87]. Also Sagi et al. [106] report on the successful isolation of *Fusarium*-resistant wheat lines; from a total of 200 unselected and 43 toxin-selected somaclones 17% and 20%, respectively, were superior in resistance to their donor varieties.

Segregated microspore populations of *Brassica* were screened for their tolerance to herbicides as imidazolinones or sulfonylurea [107,108]. Doubled haploid plants could be raised to maturity and the offspring could tolerate at least two times the field-recommended rates of the herbicide containing the selective agent [108].

C. Incorporation of Doubled Haploid Lines in Breeding Programs

Since Wark [109] in 1977 obtained improved, doubled haploid tobacco lines by anther culture, this technique has worked in practice in potato, rapeseed, and the cereals. In those crops for which the technical problems of haploid induction are solved, the question of their incorporation into breeding schemes is presently under investigation. For aims such as rapid incorporation of a monogenic trait or combination of monogenic traits, the time savings are interesting, but much more important are strategies that allow the combination of quantitatively inherited characteristics with a polygenic genetical basis. Here, the in vitro procedures have their highest potential, as they allow a very secure selection of polygenic traits in a very early generation. That an A_1 is equivalent to a F_n is probably the most far-reaching advantage of the ability to produce an isogenic line in one step, and in vitro production of these lines is the most recommended technique.

In potato, Wenzel and Uhrig [90] produced androgenetic doubled monohaploid clones possessing potato X, Y, and leaf role viruses (PVX, PVY, PLRV) and *Globodera pallida* resistance in the homozygous condition. These experiments include monogenically inherited traits (PVX/Y resistance) and polygenic ones (PLRV, *G. pallida* resistance), demonstrating that recombination works sufficiently. The technique is used in experimental programs, particularly for the establishment of strong field resistance to PLRV, *G. pallida*, and *Phytophthora*. In diploid crops the use of doubled haploids reaches the level of a variety much earlier; consequently, lines derived from microspores of barley have been released or are in official yield trials.

Snape and Simpson [110] discussed the genetic effects when starting from different filial generations. The question is: Can isogenic lines be extracted more economically from an F_1 generation, or should the haploid step be started from a preselected F_2 or F_3 ? It could be demonstrated that one haploid step followed by selection in the greenhouse and in the field during the first androgenetic (A_1) and two subsequent selfed generations (A_2 and A_3) is the most efficient procedure, if characteristics from related varieties are to be combined. Haploids can also increase the efficiency of the most complex-breeding problem: the transfer of quantitatively inherited traits from distant genotypes to cultivars. For such complex-breeding programs a combination of recurrent selection, which guarantees variability, and haploid selection, which guarantees secure selection, has been developed [111]. This breeding technique is called recurrent selection alternating with haploid steps. The degree of relation of the two parents determines the number of backcrosses needed. The necessary offspring size of DH population for quantitative characteristics depends on the degree of linkage and on the number of genes involved. The main advantage of using this method is to speed up the breeding process and to make selection of quantitatively inherited traits more effective and reliable. Because DNA hybridization techniques are now available that allow secure selection of

characteristics on a single-plant level [66,112], selection and combination can be done even more quickly.

IV. ASEXUAL PRODUCTION OF SOMATIC HYBRIDS

Breeding deals mainly with complex characteristics, the inheritance of which follows the laws of population genetics. Somatic hybridization presents a great opportunity to combine such quantitatively inherited traits without meiotic recombination. With classic methods, such combinations demand enormous populations and at the same time sensitive selection procedures. Consequently, the most attractive advantage of somatic fusion is that under applied circumstances the opportunity is offered to combine asexually two heterozygous nuclear genomes and to pool polygenic characteristics. In a vegetatively propagated crop, such as potato, the hybrids can be vegetatively propagated as clones, despite their highly heterozygous nature.

Very briefly the prerequisite for somatic combination (i.e., successful protoplast regeneration) should be mentioned. Leaves or axenically grown shoots are enzymatically macerated into single cells and protoplasts that are cultured in hypertonic liquid media. Recently, from a number of plants the regeneration of which was difficult, successful regeneration has been reported. Besides improvement in the monocots—rice [113] and ryegrass [114]—success has been obtained in other recalcitrant species: sunflower [115,116], soybean [117], faba beans [118], and pea [119]. For the sugar beet Hall et al. [120] developed a semiautomatic computer-controlled cell finder to identify those cells with the capacity to divide. Surprisingly, the most totipotent cells were traced back to single stomatal guard cells. Normal mesophyll cells, which constitute nearly 90% of the protoplast population were blocked in development before cytokinesis.

In protoplast culture again, a strong influence of the genotype on the regeneration frequency of protoplasts exists. For breeding programs, a much more genotype-independent response is required. This can be achieved in several genotypes by addition to the culture medium of substances that inhibit ethylene biosynthesis. Perl et al [121] reported that the addition of silver thiosulfate increased the protoplast yield of potato leaves. Möllers et al. [122] found that 1.5 mg/L silver thiosulfate led to an increase of potato leaf material harvested per culture vessel. Depending on genotype, the increase ranged from 1.2 to 2.3 times the yield obtained from normal medium. The addition of silver thiosulfate also promoted shoot regeneration from protoplast-derived potato calli.

Furthermore, for applied purposes it must be clarified why hybrids from the same parents are often genetically not identical. Even though complete genomes are added, there seems to be a strong influence from the cytoplasm, which segregates in different clones, thereby ending up in different nucleocytoplasmic combinations. Furthermore, the fusion technique has showed that different clones have different genetic strengths: it became clear that for somatic combination of potato clones the rules of specific and general combining ability may be applied.

A. Symmetric Somatic Fusion

Fusion can be induced chemically by the polycation polyethylene glycol (PEG) or by electrofusion. Although for *Brassica*, PEG is still a powerful tool, particularly in combination with cell sorting [123], in potato most groups turned to electrofusion. Even though in the potato efficient methods exist [124–128], for applied purposes, some bottlenecks must be overcome. Most important is the widening of the fusion procedure to many more genotypes. This could be achieved to some extent by optimizing the culture conditions [129,130].

In potato, for the incorporation of new resistance, wide hybridizations were favored, in particular *S. tuberosum* (+) *S. brevidens*. Austin et al. [124] and Gibson et al. [131] transferred resistance to PLRV and late blight from *S. brevidens* into *S. tuberosum* hybrids. They further demonstrated the presence of *Erwinia* and *Phytophthora* resistance in such hybrids and found enough fertility to produce sexual progenies [132]. In a similar approach, Austin et al. [133] could transfer resistance to the nematode *Meloidogyne* from *S. bulbocastanum* clones by fusion into *S. tuberosum*. Some of the somatic hybrids were even fertile and could be used in potato-breeding programs to incorporate a valuable pest resistance. The same group transferred PVY resistance from *S. etuberosum* by fusion with a dihaploid hybrid into a *S. tuberosum* [134,135]. Cardi et al. [136] transferred frost-tolerance from *S. commersonii* to *S. tuberosum*. The somatic hybrids showed an introgression of genes for frost tolerance. Also, from *S. circaeifolium*, resistance to *Phytophthora infestans* and *Globodera pallida* was brought into somatic potato hybrids [137]. Schilde-Rentschler (personal communication) transferred *Phytophthora* resistance from *S. pinnatisectum*, *S. circaeifolium*, and *S. bulbocastanum*, by asymmetric fusion procedures, into *S. tuberosum* clones.

Because the distance between the fusion parents in these combinations is rather large, applied use of the hybrids needs much further classic-breeding work. Parallel to classic breeding a quicker success is expected from fusion within the *S. tuberosum* genome. In such experiments Möllers et al. [138] could demonstrate high flower production, a pollen fertility of 60–90%, and a substantial yield increase by comparing the mean tuber yield of the parents with the tuber yield of the hybrids (Table 6). In general it could be demonstrated in these first experiments that somatic hybrids reached the yield of cultivars grown as controls. Besides the yield differences of different fusion combinations among clones from the same fusion parents yield variation also appeared. Möllers and Wenzel [130] fused dihaploids containing the *G. rostochiensis* resistance gene *Ro 1* with susceptible clones. In the somatic hybrids of such combinations all hybrids were scored as *Ro 1*-resistant. Cooper-Bland et al. [139] could transfer, in a similar approach, the resistance for *Globodera pallida* from dihaploid clones to tetraploid somatic hybrids. For sunflower [140,141] and ryegrass [142], first reports on successful somatic hybridization already exist.

Table 6 Mean Yield of Tetraploid Somatic Potato Hybrids and Their Dihaploid Parents

Hybrid	Midparent yield (g)	Average hybrid yield (g)	Yield increase in % of	
			MPV ^a	BPV ^a
Hy 1	234	566	142	105
Hy 2	291	672	131	72
Hy 3	266	468	76	70
Hy 4	332	576	73	48
Hy 5	289	578	100	50
Hy 6	348	722	108	85
Hy 9	156	518	232	127
Hy 10	487	875	80	49
Hy 12	272	698	177	94
Hy 13	381	725	119	113

^aMPV, midparent value; BPV, best-parent value.

Source: Ref. 138.

B. Asymmetric Somatic Fusion

In somatic fusion it will usually be necessary to reduce the ploidy level before or after protoplast fusion to the level desired in the crop. This problem may be overcome when one fusion partner, the donor type, is irradiated to partially or completely inactivate its nuclear genome. The recipient is normally not treated. During such asymmetric hybridization, chromosome elimination is intended. Wolf et al. [143] could observe a clear dose-response to irradiation when they followed the fate of molecular markers in a gamma-irradiated and subsequently fused donor genotype with untreated recipient protoplasts.

The difference between symmetric and asymmetric fusion is, however, not sharp. Even in symmetric fusions with both parents untreated, chromosome losses will result in asymmetric hybrids. For example, Valkonen et al. [144] in potato, combined both fusion approaches to bring PLRV, PVY, and PVX resistance from *S. brevidens* into hybrids with dihaploid *S. tuberosum* clones. The results obtained suggest that resistance to PLRV in *S. brevidens* is controlled by a gene or genes different from those controlling resistance to PVY and PVX, and the genes for resistance to PVY and PVX are linked in *S. brevidens*. Puite and Schaart [145] and Xu et al. [146] performed the same fusion combination *S. tuberosum* (+) *S. brevidens* and used chromosome-specific RFLP probes to investigate elimination of specific *S. brevidens* chromosomes in the hybrids. Of the donor genome of *S. brevidens* 20–60% was eliminated, but the donor genome of *S. tuberosum* had increased numbers of chromosomes.

Asymmetric intergeneric fusion began in 1978 with the fusion of potato and tomato by Melchers [147], and even today, tomato and potato are important families in symmetric [148–150] and asymmetric fusion experiments [151,152]. McCabe et al. [153] tagged chromosome 12 of a *Lycopersicon esculentum* × *L. pennellii* (+) potato hybrid with the T-DNA of *Agrobacterium*. After asymmetric fusion, all hybrid plants tested had more than the 4 × ploidy level and always contained the tagged chromosome 12. Derkx et al. [154] performed a series of asymmetric fusions between protoplasts of a cytoplasmic albino mutant of tomato and gamma-irradiated protoplasts of *L. hirsutum*, *S. commersonii*, *S. etuberosum*, and *S. nigrum*. The hybrids were analyzed for their morphology, nuclear DNA composition, cytoplasmic and (cp)DNA origin. The hybrids between the two *Lycopersicon* species contain the chloroplasts from *L. hirsutum* and the flower morphology from *L. esculentum*. Because no chromosomes of the irradiated parent were found, they are true cybrids. In all other fusion products nuclear DNA from both parents was detected. The cpDNA of all hybrids was of the donor, suggesting that chloroplast transfer by asymmetric fusion can overcome problems associated with the large phylogenetic distance between parental plants in intergeneric hybridization.

The plant family used most often in asymmetric fusions is the Brassicaceae [123,155]. Gerdemann-Knörck et al. [156] fused *Brassica napus* (+) *B. nigra* which was transgenic for hygromycin resistance. *B. nigra* was used as the donor and possessed genetic resistance to *Phoma lingam* and *Plasmodiophora brassicae*. By using hygromycin for selection, more than 300 calli were obtained, 10% of which produced plants that could be tested for pathogen resistance. For both pathogens 23% (*P. brassicae*) and 52% (*P. lingam*) resistant *B. napus* plants could be detected. Furthermore, the resistance against *Phoma lingam* could be transferred from different resistant *Brassica* species to *Brassica napus* by asymmetric fusion. The donor protoplasts were irradiated with X-rays and after fusion with the *Brassica napus* protoplasts, selection was applied on the hybrid cell cultures with the toxin, sirodesmin PL, produced by *Phoma lingam* [155]. The whole spectrum of fusions obtained is summarized in Figure 2.

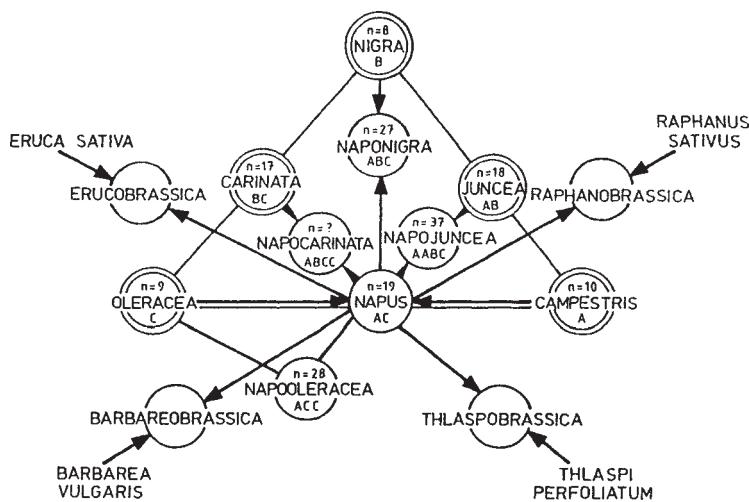


Fig. 2 An overview of different somatic hybridization experiments in the genus *Brassica* and related genera. The simple circles indicate newly produced somatic hybrids. (From Ref. 157.)

1. Transfer of Cytoplasmic Male Sterility

One important aim in asymmetric fusion is the transfer of cytoplasmic male sterility (CMS) by fusion from sexually incompatible species. This was achieved at first in *Brassica* species by Pelletier et al. [158] in 1988. Now the transfer of CMS in sugar beets has been reported [159]. From several sugar beet lines protoplasts were regenerated into plants after treatment with BAP or thidiazuron. The protoplasts could be transformed with the plasmid pGL2 for hygromycin resistance and developed into Calli, the transgenic nature of which could be proved by Southern-blotting. Results of asymmetric hybridization and cybridization to transfer CMS were started and the transfer CMS into some of the regenerated fusion products could be demonstrated using CMS-specific probes [159].

Other than in crop plants, the CMS characteristic is also important in vegetables. Horticulturally improved *Brassica* vegetable lines were obtained by fusion of *B. oleracea* protoplasts carrying the Ogura type of CMS with gamma-irradiated protoplast from fertile lines of *B. rapa* [160]. Through greenhouse and field screening, cold tolerant CMS lines with good female fertility and horticultural characteristics were selected.

2. Cybrids

The term *cybrids* describes protoplast fusion-derived cells and plants in which most or all nuclear genome of one (organelle-donor) fusion partner is eliminated, whereas this partner does contribute some or all the organelle (plastid or mitochondria) genomes [161]. Cybrids are consequently the extreme in asymmetric hybridization. The more we know of the genes located on the plastom and the chondriom, the more the importance of such cybrids with exchanged cytoplasms will increase.

C. Selection of Somatic Hybrids

One limitation for the application of somatic fusion is the difficulty in differentiating heterocaryotic fusion products from unfused and homocaryotically fused protoplasts. It is not practical to regenerate

all plants and to carry out the selection at the plant level. Besides a number of selection systems using complementation or partial lethality [162], hybrid vigor of fusion products may be a rather universal selection system. Such a system was used in potato for hybrid detection in 2x (+) 2x fusions [126,129,163]. After electrofusion 50% of the callus clones selected after 21 days were heterocaryotic fusions, but with increasing developmental period, the number of real hybrids selected, was reduced [164]. This selection is, however, rather pragmatic, as only combinations expressing a positive heterosis are found; surely there also exist combining abilities in somatic hybridization that lead to negative heterosis. Such combinations are overlooked. The isozyme analysis of both parents and the regenerating clones is a recommended method for proving the hybrid nature of the fusion products [129].

For a wider application of the fusion process, the selection techniques for the fusion products should be further improved. Experiments for potato are in progress to combining the fusion procedure for ploidy level detection with flow cytometry [165,166] and in Brassicaceae with a cell sorter [167]. With these techniques it becomes possible to combine a huge number of different *Brassica* species and additionally to widen the *Brassica* gene pool by incorporation of related genera (see Fig. 2) [168]. Direct DNA diagnoses allows the immediate detection of hybrids in those cases for which isoenzymes do not work. They further allow the use of undifferentiated calli, so that waiting for morphogenesis is not necessary. For DNA probing repetitive [169] as well as single-copy probes, RFLP, or RAPD [170,171] can be used. With such tests using repetitive DNA probes Schweizer et al. [169] found a possible correlation between a high amount of repetitive DNA and a good agronomic performance of a clone; in contrast clones with a lower level of repetitive sequences expressed good regeneration capacity. An increase in the amount of repetitive sequences might promote successful somatic hybridization.

D. Combining Ability in Somatic Fusion

Binding et al. [172] used mixed populations of protoplasts to study interactions between the protoplasts and their derivatives. His studies resulted in a first feeling for interactions between the different genome compartments during fusion. In potatoes clear differences exist in the usability of clones as fusion parents. Behaviors similar to those for specific and general combining ability in sexual hybridization programs were observed [173].

The assumption that ploidy level and vigor of hybrids alone are sufficient to select hybrids, as postulated by Debnath and Wenzel [163], could not be generalized. This still seems to be true for tissue culture-adapted potato clones. As soon as nonadapted clones and a wider range of genotypes was incorporated into the fusion combinations, only a few fusion products expressed extended vigor that could be used for selection. To identify the combining ability of protoplasts in somatic fusion, Frei [173] made fusions using up to five genotypes in a pooled fusion experiment. The identification of the clones present in the hybrids could be performed by RFLP or RAPD fingerprints. These experiments demonstrated that fusion success not only depends on the single genotypes and their regeneration ability, but there is considerable influence on the combination of genotypes. The data obtained during regeneration showed that several different features have to be considered when selecting a genotype for fusion experiments. Cell division activity, macrocalli formation, and regeneration ability only partially describe the problem. Especially the interaction between genotypes, their positive or negative influence on the development of the other genotypes in the mixture are important for a successful regeneration and the production of heterocaryotic hybrids of homocaryotic parental types. Along this line, in a fusion program, 40 different fusion combinations, with 10–12 genotypes, were performed [174]. A total of 240 macrocalli of the different fusion combinations were analyzed for their genotype with an RFLP probe. If in the reaction the phases of

microcallus formation, macrocallus growth, and plant regeneration were distinguished, four classes of genotypes could be identified.

1. A class of genotypes with good cell division activity, good macrocallus formation, and high regeneration ability that predominated among the first regenerates independently of fusion partner.
2. A class of genotypes with extraordinary good callus formation, but no regeneration ability.
3. Intermediate genotypes that gave good hybrid recoveries in some combinations, but failed in others.
4. Genotypes showing poor cell division activity and, in most combinations, also negatively influenced the other fusion partner.

Identification of such characters before fusion will enhance the success of somatic hybridization in applied programs.

E. Field Performance of Somatic Hybrids

Cell fusion is a technique to not only broaden the gene pool of a species, but also a fast procedure for the combination of traits within one gene pool. In potato [130,137–139,175,176] and in *Brassica* [177], first reports on the field performance of such intraspecific hybrids exist. In all cases, clones and lines could be detected that gave a yield comparable with classically produced material (see Table 6.).

The field evaluations have revealed, however, a new variability within the hybrids of a single fusion combination not only for potato [138], but also for *Medicago* hybrids [178]. In a detailed analysis of the level of resistance to *Phytophthora* it was found that although most of the hybrids showed an intermediate resistance, transgression to both sides also existed. The same was true for field resistance to PVX and PVY, indicative that after all fusion experiments the hybrids originating from the same two parents showed variability in quantitatively inherited traits. For the monogenetically inherited dominant hypersensitive reaction to PVX and PVY in potato it was demonstrated that after fusion of dihaploids with the gene *Rx* and other clones carrying the gene *Ry*, the expected combination of the resistances was observed. But in several hybrids either the PVY or both resistances are missing. The differences between hybrids originating from the same parents can be ascribed to several reasons, such as protoclonal variation, aneuploidy, the composition of the cytoplasm, and the interaction of different cytoplasms with the same hybrid nucleus.

F. Nuclear Cytoplasmic Interaction in Somatic Hybrids

Interspecific and intergeneric protoplast fusions were developed to introduce novel germ plasm from sexually noncompatible species to broaden the genetic base of the parents. Zubko et al. [179] and Gleba [180] described that as well as nuclear incompatibility, the phenomenon of nucleocytoplasmic incompatibility is also present. The described multifusion of potato [173] or wider plant groups [172] can help understand these phenomena. In all fusion events, however, a new type of organelle segregation and recombination takes place. Here, a new type of genome combinations is now developing, opening the opportunity to optimize not only the genetic composition of the nuclear genome, but also that of the cytoplasm and the interaction of both. Besides somatic fusion, transformation of organelles will also be a useful tool for further improvements along this line [181,182].

Molecular analysis of somatic hybrids, especially of their cytoplasmic composition, has been performed for the various organelles in *Brassica* [183]. In plants regenerated after fusion between *B. oleracea* var. *italica* (broccoli) containing the petaloid *B. nigra* type of CMS and

an atrazine-resistant biotype of *B. campestris* var. *oleifera* plants were regenerated that were CMS and atrazine-resistant up to the level of 25 µM. Molecular analysis showed that they contain chloroplasts from the atrazine-resistant *B. campestris* parent and mitochondria from the *B. nigra*-containing parent. No recombination or rearrangement of the mitochondrial genomes in the fusion products was detected. In potato, most of the analysis of the nuclear-organellar interaction refers to studies on cybrids, to avoid any influence originating from the hybrid nucleus [184].

1. Variability Caused by the Nucleus

To determine the reason for the new variability in the nuclear DNA genome, in hybrid regenerates the genomic DNA was hybridized after restriction with endonucleases with 24 cDNA probes, each of which was representative for one chromosome. Deviating banding patterns were identified for nearly all chromosomes. Between different fusion combinations the percentage of new variability was linked to the duration of the in vitro culture phase. It was not possible, however, to show any influence or direct correlation between this percentage and the phenotypic data obtained in field experiments [185].

2. The Role of the Plastids

Chloroplast segregation has been reported for somatic hybrids by Maliga and Menczel [186]. Chloroplasts normally segregated after a few cell cycles in several *Nicotiana* species [187,188], *Brassica* [157], and potato [131]. In potato and *Brassica*, however, different ratios of parental plastids were observed, perhaps owing to different organelle replication rates [157,168]. In potato, for which such segregations of hybrids were compared with the field data, no similarity was detectable between the type of plastome and yield characteristic; nor was there any obvious correlation patterns for the mitochondrial (mt)DNA [185].

3. Recombination of Mitochondria

For mitochondria Xu et al. [146,170] reported in potato about 75% recombination of the mtDNA. Such recombinations already occur during normal protoclinal variation without any fusion event [189]. Similar results are found in the genus *Brassica*, in which Temple et al.

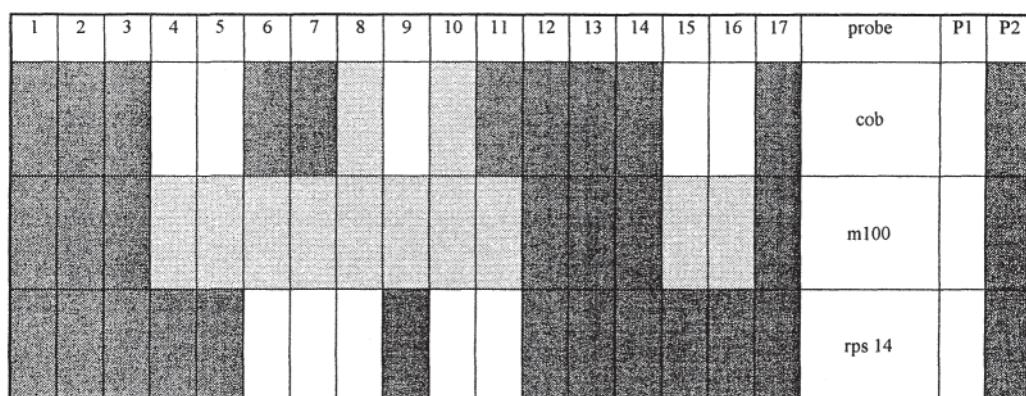


Fig. 3 Schematic summary of experiments for probing the mitochondrial genome of 17 somatic potato hybrids with three mtDNA markers; the parents P₁ and P₂ express for all probes polymorphism (here, either white or dark gray), whereas none of the hybrids contains only mitochondria of P₁, but numerous mixtures and recombined mitochondria (light gray). (From Ref. 191.)

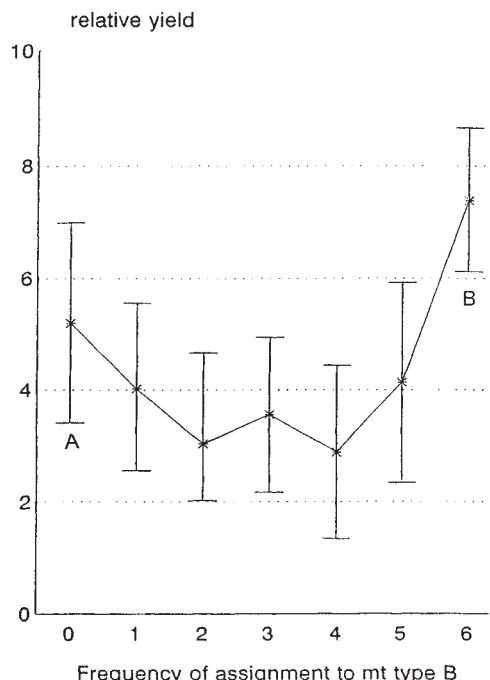


Fig. 4 Comparisons of the RFLP and yield data of one population of somatic potato hybrids A (+) B. Assignments to mainly mt-type of parent A or B and the yield of hybrids with mixed or recombined mitochondria. (From Ref. 185).

[190] located regeneration breakpoints in the mtDNA. Similar observations were reported by Walters and Earle [183], who found mitochondrial recombination in one-third of somatic *Brassica* hybrids.

For intraspecific potato hybrids, Figure 3 demonstrates the amount of mitochondrial recombination.

Besides a simple description of the fact of mitochondrial recombination, Lössl et al. [185] found a correlation between the mitochondrial composition and yield parameters of somatic potato hybrids (Fig. 4). Different levels of starch content and tuber yield could be ascribed to different mitochondrial genotypes. Especially for yield, mitochondria from one parent were superior to the mitochondria of the other parent. Most recombinations or mixtures of mitochondria resulted in a decrease in vigor. It is suggested that plant vigor measured by tuber yield correlates positively with the homogeneity of the mitochondrial genome. Because in sexual hybridizations only one, the female, plasmone is inherited by the next generation, the inferiority of mitochondrial recombination is logical. The evolution has selected for the best but uniform mitochondria. There are, however, chances that by this new type of mitochondrial recombination better mitochondria can also appear. If this can be demonstrated, protoplast fusion also offers for other crops a means for the construction of new mitochondrial genomes.

V. CONCLUSION

When discussing the use of asexual processes in genetics, there is no doubt that the in vitro procedures offer large gains for breeding. In vegetatively propagated crops, protoplast fusion

is probably one procedure that will pay, because asexual combination of polygenic traits is a challenge. In sexually propagated crops the symmetric fusion will be replaced by the asymmetric hybridization with the maximum of cybrid formation. Both approaches open up a new emphasis on cytoplasmic genetics for the formation of superior mitochondrial genomes.

In vitro procedures for DH line production have already shifted from research to application. In most cases, the microspore androgenesis has been improved so much that the numbers of green haploid plants necessary for applied work—normally about 100 per donor genotype—can be produced. This is particularly true for most cereals and for the Brassicaceae. Unfortunately, this statement can still not be generalized, as several major crops are very recalcitrant: these include nearly all the legumes and maize. The outbreeders maize and rye, for which a rapid production of isogenic lines for broadening the hybrid seed production would be very important, are not yet improved by this in vitro technique. The knowledge of which parameters have to be altered in which direction is still closer to an art than to a logical science. A basic knowledge of regeneration processes is lacking, but now molecular approaches for a better understanding of these are beginning to pay. Here, rapid progress is anticipated. Marker-aided selection will then also improve success in difficult species.

Such molecular selection steps may also help revive the use of somaclonal and protoclonal variation. When it becomes possible to screen for specific mutations that are responsible for a specific step in the biochemical pathway, the present need for good luck during selection can be turned to more reliability.

As a last conclusion it should be stressed that regardless of whether in vitro selection or whether single-cell combination is concerned, the use of asexual and somatic cell genetics must be accompanied by and combined with classical-breeding procedures, and any breeding product has to prove its quality finally under field conditions. Asexual genetics can only be successful when sexual breeding is continued with all possible sophistication.

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6

Genetic Engineering of Crop Plants

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I. INTRODUCTION

Crop improvement was, so far, based on selection of naturally occurring variation within a given species. Induced mutations increased the available genetic variation only moderately. Techniques that permitted rescue of offspring from interspecific crosses and that favored introgression of chromosome segments allowed the limited combination of characters between species. The technique of gene transfer to plants has changed the situation dramatically. In principle, the plant breeder can from now on use any given character, from any given organism, in any given species. And he can decide in which organ the novel character should be expressed, at which strength, and in response to which external trigger or endogenous signal. Gene transfer also offers the opportunity to inactivate endogenous genes. This is all true in theory. In reality, however, there are still numerous problems interfering with too easy a construction of novel miracle crop plants. Genes of agronomic importance are often not easy to identify and to isolate. Expression of the genes often depends more on the (random) integration into the host genome than on the appropriate expression signals. Gene transfer techniques that yield thousands of transgenic plants with laboratory model species might require an enormous investment in manpower and persistence when the production of just a few transgenic plants from a specific commercial variety is needed for some important crop species. However, in the short time between the recovery of the first transgenic “model plant” in 1983 [1] and now, it has been possible to develop the state-of-the-art of gene transfer to plants to a level at which many of the major crop plants are accessible to the technique and for which we have good reason to believe there is no basic biological principle that will prevent gene transfer to a specific crop plant.

Three gene-transfer techniques have been developed to such a stage of reproducibility and practical applicability that they can be recommended for future practical work. These are *Agrobacterium-mediated* gene transfer, *ballistic* gene transfer, and *direct* gene transfer to *protoplasts*. These techniques are described in some detail in the following chapter, and Table 1 summarizes which crop plants have been transformed with which of these techniques. There

Table 1 Transgenic Crop Plants Obtained by *Agrobacterium*-Mediated Transformation (A), Ballistic Methods (B), or Direct Gene Transfer to Protoplasts (P)

English name	Latin name	Method	Ref.
Alfalfa	<i>Medicago sativa</i>	A	111,112
Apple	<i>Malus pumila</i>	A	113,114
Apricot	<i>Prunus armeniaca</i>	A	115
Asparagus	<i>Asparagus officinalis</i>	A	116
Aspen	<i>Populus tremula</i>	A	117,118
Barley	<i>Hordeum vulgare</i>	B	119–121
Bean	<i>Phaseolus vulgaris</i>	B	122
Birdsfoot trefoil	<i>Lotus corniculatus</i>	A	123
Black currant	<i>Ribes nigrum</i>	A	124
Carnation	<i>Dianthus sp. caryophyllus</i>	A	125
Carizzo Citrange	<i>Citrus</i>	A	126
Carrot	<i>Daucus carota</i>	A	127
Cauliflower	<i>Brassica oleracea</i>	A	128
Celery	<i>Apium graveoleus</i>	A	129
Chickpea	<i>Cicer arietinum</i>	A	130
Chicory	<i>Cichorium endivia</i>	A	131
Chrysanthemum	<i>Chrysanthemum sp.</i>	A	132
Cotton	<i>Gossypium hirsutum</i>	A	133
		B	134,135
Corn	<i>Zea mays</i>	P	136
		B	55,76,137
Cranberry	<i>Vaccinium macrocarpon</i>	B	138
Cucumber	<i>Cucumis sativus</i>	A	139
Fescue, red	<i>Festuca rubra</i>	P	140
		B	141
Fescue, tall	<i>F. arundinacea</i>	P	142
		B	141
Flax	<i>Linum usitatissimum</i>	A	143,144
Gerbera	<i>Gerbera hybrida</i>	A	145
Grapevine	<i>Vitis spp.</i>	A	146,147
Kiwi fruit	<i>Actinidia chinensis</i>	A	148,149
Lettuce	<i>Lactuca sativa</i>	A	150
Muskmelon	<i>Cucumis melo</i>	A	151,152
Mustard	<i>Brassica juncea</i>	A	153
Oat	<i>Avena sativa</i>	B	154
Oilseed rape (canola)	<i>Brassica napus</i>	B	72
		A	128,155
Orchardgrass	<i>Dactylis glomerata</i>	P	156
Orchid	<i>Dendrobium sp.</i>	B	157
Papaya	<i>Carica papaya</i>	A	158
		B	159
Passionfruit	<i>Passiflora edulis</i>	A	160
Pea	<i>Pisum sativum</i>	A	161–163
Peanut	<i>Arachis Hypogaea</i>	A	164
		B	165
Pecan	<i>Carya ovata</i>	A	166
Penennial ryegrass	<i>Lolium perenne</i>		167
Pepino	<i>Solanum muricatum</i>	A	168
Petunia	<i>Petunia hybrida</i>	P	169
Plum	<i>Prunus domestica</i>	A	170,171

Table 1 Continued

English name	Latin name	Method	Ref.
Poplar	<i>Populus</i> sp.	A	172
		B	173,174
Potato	<i>Solanum tuberosum</i>	A	175,176
		P	177
Ramie	<i>Boehmeria nivea</i>	A	178
Rice	<i>Oryza sativa</i>	A	179
		P	180,181
Rye	<i>Secale cereale</i>	B	184
		B	185
Sorghum	<i>Sorghum bicolor</i>	A	186,187
Soybean	<i>Glycine max</i>	B	71
Strawberry	<i>Fragaria × Anannassa</i>	A	188,189
Stylo	<i>Stylosanthes humilis</i>	A	190
Subterranean clover	<i>Trifolium subterraneum</i>	A	191
Sugar beet	<i>Beta vulgaris</i>	A	192
Sugarcane	<i>Saccharum officinarum</i>	B	193
Sunflower	<i>Helianthus annuus</i>	A	194–196
		B	82
Sweetgum	<i>Liquidambar styraciflua</i>	A	197
Tamarillo	<i>Cyphomandra betacea</i>	A	198
Tomatillo	<i>Physalis ixocarpa</i>	A	199
Tomato	<i>Lycopersicon esculentum</i>	A	200,201
Trifoliate orange	<i>Poncirus trifoliata</i>	A	202
Triticale	<i>Hordeum × Triticum</i>	B	203
Tobacco	<i>Nicotiana tabacum</i>	A	1
		P	20
Turf grasses: creeping bentgrass; Redtop	<i>Agrostis alba</i> , <i>Agrostis palustris</i>	B	204
		P	205
Walnut	<i>Juglans regia</i>	A	206
Watermelon	<i>Citrullus vulgaris</i>	A	207,208
Wheat	<i>Triticum aestivum</i>	P	209
		B	210
White clover	<i>Trifolium repens</i>	A	68,211–214
		A	215,216
White mustard	<i>Sinapis alba</i>	A	217
White spruce	<i>Picea glauca</i>	B	218

are additional techniques that have yielded confirmed transgenic plants and that show some promise for future development, such as *microtargeting*, *microinjection*, *electroporation* into tissues, and *whiskers*. These techniques are described to a smaller extent and the literature citations will support further reading. There is a further group of techniques for which unequivocal evidence for the production of transgenic plants is still missing such as the pollen tube-pathway, and for which the reader is referred to a more detailed assessment [2,3].

The recovery of transgenic crop plants requires not only effective gene transfer techniques, but also depends on the appropriate use of signals for the regulation of transgene expression, and on vectors for transgene amplification and transfer. Transformation is normally a rare event; selectable marker genes or visible reporter genes, therefore, are cotransformed with the gene of agronomic

interest. This allows the identification of the transgenic clones among large populations of untransformed cells. Expression signals, vectors, marker and reporter genes that have so far been successfully used in crop plant transformation are discussed in the subsequent chapters.

An extensive list of references finally provides an overview of original publications on the subject, thereby giving easy access to detailed experimental protocols. A step-by-step laboratory manual, describing in detail all pertinent techniques, has been recently published [4].

II. GENE TRANSFER TECHNIQUES

A. Agrobacterium-Mediated Gene Transfer

The crown gall and hairy root diseases of dicotyledonous plants involved gene transfer to plant cells from bacterial virulence plasmids harbored by the gram-negative soil bacteria *Agrobacterium tumefaciens* and *A.rhizogenes*, respectively. These diseases have been the focus of considerable attention by plant pathologists, molecular biologists, and plant genetic engineers. Corresponding extensive information has been published in many comprehensive reviews during the last 15 years [5–16]. *A.tumefaciens* and *A.rhizogenes* possess the natural capacity to introduce segments of oncogenic DNA derived from their large, ca. 250-kb, plasmids, respectively called Ti (tumor-inducing) and Ri (root-inducing), into cells at plant wound sites. These plasmids bear nontransferred virulence (*vir*) genes, and the corresponding *vir* gene products promote the transfer to and integration into the nuclear genome in competent plant cells of another plasmid region, called T-region. The T-region is flanked by 25-bp imperfect direct repeats, called *border repeats*. The transferred DNA, called *T-DNA*, is integrated into the plant genome by illegitimate recombination. Genes encoded within the integrated T-DNA express novel enzymes, resulting in the synthesis of phytohormones and in the hydrolysis of phytohormone conjugates in transformed cells. These functional differences are responsible for the tumor-inducing and root-inducing properties of the Ti plasmid (pTi) and of the Ri plasmid (pRi), respectively. Besides these oncogenes, other T-DNA genes encode enzymes that catalyze, depending on the *Agrobacterium* strain, the production of different opines. The opines formed in transformed plant cells can be metabolized by the agrobacteria, but not by the plant cells and most other soil organisms. Thus, by the genetic modification of plant cells, the *agrobacterium* generates a favorable niche for its multiplication.

The expression of *A.tumefaciens* oncogenes is incompatible with the regeneration of whole plants, because the T-DNA-containing (transformed) plant cells are tumorous. The deletion of the tumor-inducing genes from the T-region of pTi does not interfere with the T-DNA transfer into plant cells, for only the border repeats are the necessary recognition signals for the transfer system. Thus, to obtain genetically transformed plants, using vectors derived from pTi, it is accordingly required to disarm the T-region. Plant cells transformed with such disarmed T-DNA behave similarly to untransformed cells in cell culture and subsequent plant regeneration protocols. Thus, by using virulent *A.tumefaciens* strains bearing disarmed pTi it is possible to regenerate from the transformed cells fertile transgenic plants of normal appearance (Fig. 1). In contrast, the Ri plasmids do not need to be disarmed to allow the regeneration of fertile plants from transformed roots. The T-DNA integrated in the genome of the transgenic plant will be transmitted to its progeny in a Mendelian manner. Given these natural DNA transfer systems, two main strategies are currently used to genetically transform plant cells and to obtain transgenic plants. The *binary vector* strategy is based on the separation of *vir* and T-regions on two compatible independent replicons. The second strategy involves intermediate vectors that do not replicate in *agrobacteria*, but will cointegrate with the resident pRi or disarmed pTi through recombination. Selectable

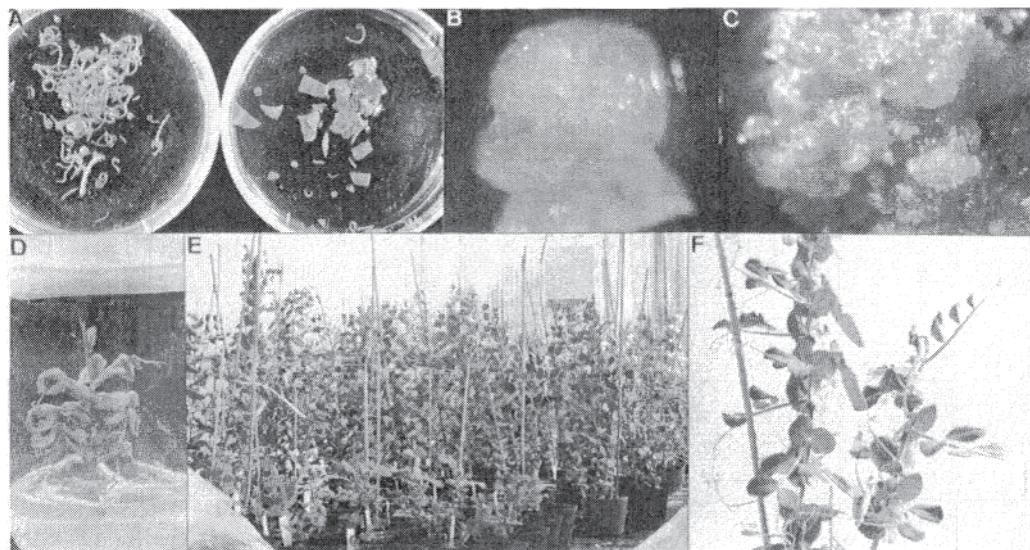


Fig. 1 *Agrobacterium*-mediated gene transfer and regeneration of transgenic plants in pea (*Pisum sativum*) [see Refs. 161; 162]: (A) Cocultivation of leaf and shoot explants of pea with *A.tumefaciens* strain bearing Ti-plasmid with a chimeric *hpt* gene as selectable marker; (B) Selection of transformed pea callus on hygromycin-containing proliferation medium; (C) induction of shoot formation from transformed pea callus; (D) in vitro growing transformed pea plant; (E) adult transgenic pea plants growing in soil under greenhouse conditions; (F) fertile transgenic pea plants obtained from *Agrobacterium*-mediated gene transfer.

marker genes, required to confer a selectable phenotype on the transformed plant cells, thereby allowing their enrichment (see [Section III](#)) and expression of the genes of interest, can thus be introduced into plants by using *Agrobacterium* strains either bearing intermediate or binary vector systems. If the *Agrobacterium* strain used contains more than one different T-region, these may be cotransferred to the recipient plant cells at relatively high frequencies. Furthermore, gene targeting based on the *Agrobacterium* vector system is possible (however, at very low frequencies) because the presence of DNA sequences homologous to the plant genome in the T-DNA may result in homologous recombination events between T-DNA and plant genomic DNA.

Agrobacterium-mediated DNA transfer has been reported for many different plant species; however, the efficiency of this T-DNA transfer may vary significantly, depending on the species, the plant genotype, and the explants used as targets for transformation. Further sources of experimental variation may be due to the *Agrobacterium* strain used, the physiological state of the donor plant, as well as the tissue culture procedures applied. As shown in [Table 1](#), the recovery of transgenic plants based on *Agrobacterium*-mediated DNA transfer has been described for many different crops.

B. Direct Gene Transfer to Protoplasts

For many years of genetic manipulation in plants, direct uptake of naked DNA by plant protoplasts has been the sole alternative to *Agrobacterium*-mediated gene transfer. *Protoplasts* are cells depleted of the cell wall by enzymatic digestion and can be isolated from callus material, cell suspension cultures, and a wide range of differentiated plant tissues. First experiments demonstrating direct gene transfer included the delivery of isolated plasmid DNA

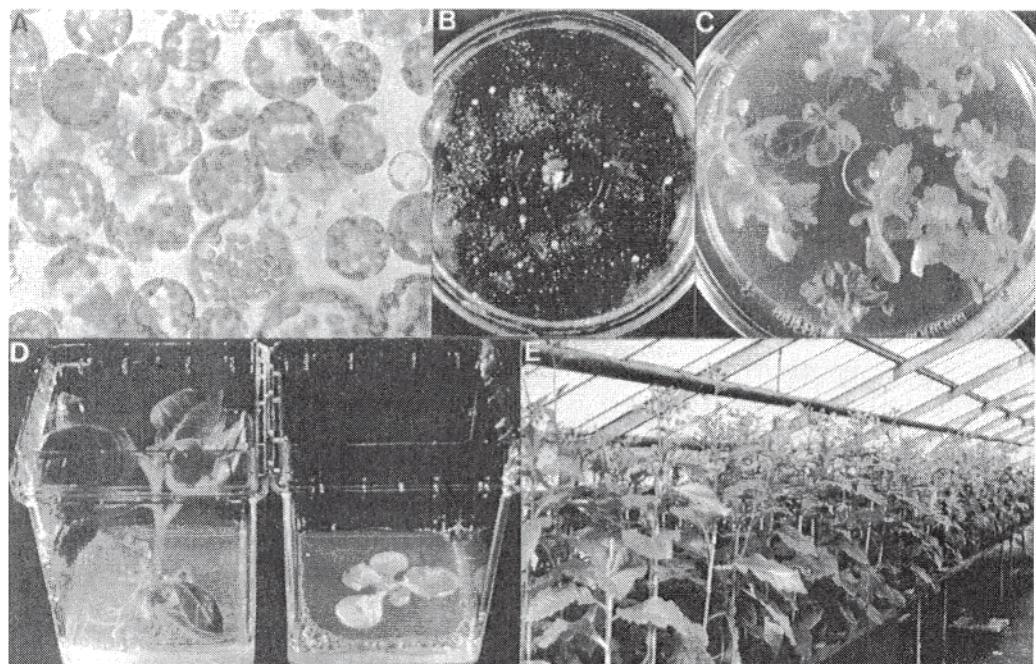


Fig. 2 Direct gene transfer to leaf protoplasts and regeneration of transgenic plants in tobacco (*Nicotiana tabacum*) [see Ref. 20]: (A) Freshly isolated leaf protoplasts of tobacco treated with polyethylene glycol and plasmid DNA bearing a chimeric *npt2* gene as selectable marker; (B) selection of kanamycin-resistant transformed colonies from protoplasts in bead-type culture; (C) shoot differentiation from tobacco calli obtained from transformed protoplasts; (D) rooting of transformed (left) and untransformed (right) shoot from protoplasts on selective medium; (E) fertile transgenic tobacco plants obtained from protoplasts.

to protoplasts of petunia and tobacco in the presence of poly-L-ornithine or polyethylene glycol (PEG) [17–20]. Coincubation of protoplasts with the nucleic acids and, for example, PEG leads to reversible permeabilization of the plasma membranes and allows the nucleic acids to enter the protoplasts. During the following years, protoplast transformation mediated by PEG [21] or electroporation [22–25] was substantially simplified, and the efficiency of the protocols was increased by several orders of magnitude [see Ref. 26 for details on the protocol]. No special equipment is necessary for PEG-mediated gene transfer; dozens of protoplast samples can be treated in a single experiment, and thousands of individual transgenic plants can be obtained in model systems such as tobacco (Fig. 2). In contrast to *Agrobacterium*-mediated gene transfer, there is almost no restriction for the manipulation of nucleic acids before transformation, and there are no host range limitations. These advantages allowed the development of various transient and integrative gene expression assays, which have become important tools to investigate the regulatory mechanisms of gene expression. Recently, electroporation conditions have been found that also deliver DNA molecules into plant cells that are still surrounded by parts of or intact walls [27–32] (see Sec. II.D.3. for a discussion of electroporation). Nucleic acids, especially RNA molecules, can also be delivered to protoplasts after encapsidation by liposome vesicles, followed by fusion of the vesicles with the plasma membrane or by endocytosis [33].

Among the most important parameters that affect the efficiency of PEG-mediated gene transfer to *Nicotiana* protoplasts are the concentration of magnesium or calcium ions in the incubation mixture, the presence of inert carrier DNA, and the molecular weight and concentration of PEG[21]. The physical configuration of nucleic acids also impinges on gene transfer efficiency: linearized double-stranded plasmid DNA molecules are more efficiently expressed and integrated into the genome than supercoiled forms [e.g., 21,34]. After delivery to protoplasts, single-stranded DNA molecules were efficiently used as templates for *in vivo* duplex formation, followed by genomic integration [35,36]. The mRNA molecules transferred to electroporated protoplasts of dicotyledonous and monocotyledonous species were efficiently translated [37]. Multiple copy integration of the foreign DNA and rearrangements of the original sequences are frequently observed [38–40]. As with other transformation techniques, integration of foreign DNA into the nuclear genome occurs primarily at random sites; frequencies of site-directed integration events obtained in tobacco and *Arabidopsis* mesophyll protoplasts ranged from 10^{-4} to 10^{-5} [41,42]. In contrast, PEG-mediated stable transformation of tobacco chloroplasts [43, 44] led to integration of the foreign DNA primarily at homologous sites within the plastome (see Sec. II.C.3. for organelle transformation).

The production of transgenic plants by direct gene transfer to protoplasts depends on protoplast-to-plant regeneration and on efficient selection systems for transgenic clones. Early gene transfer experiments focused on protoplasts of *Solanaceae* species, which are easily regenerable, and on the use of the bacterial gene for neomycin phosphotransferase (*npt2*), conferring antibiotic resistance to transformed clones. During the past few years, protoplast-to-plant regeneration was achieved for many other plant species, and transgenic plants of many important crops have been created (see Table 1). Natural resistance of many monocotyledonous species to the antibiotic kanamycin [45,46] made the development of other selection systems necessary. In addition to the *npt2* gene, the genes for hygromycin phosphotransferase (*hpt*) [47] (Fig. 3), phosphinothricin acetyltransferase (*pat*) [48], and a mutant dihydrofolate reductase, conferring resistance to methotrexate [49], have proved useful for the selection of stably transformed colonies in mono- and dicotyledonous species (see Sec. III.B. for more details on selectable marker genes).

C. Ballistic Gene Transfer

In many important crop species, the limiting factors for gene transfer are the host-range specificity of *Agrobacterium* or the protoplast-to-plant regeneration protocols [2]. Alternative methods require a system for the transport of DNA through cell walls. The currently most successful approach to overcome this mechanical barrier uses accelerated DNA-coated microprojectiles (Fig. 4). The projectiles are in a size range of about 1 μm and are accelerated to a velocity that is sufficient for penetration through the cell wall. Inside the cell, DNA molecules are released from the particles and eventually become integrated into the nuclear or organellar genome of the host cell [50]. The success of this approach in gene transfer to recalcitrant crop species is impressive. It has been reviewed by Christou [51] and is updated in Table 1. Ballistic methods have been used to deliver genes to cells of a wide variety of regenerable tissues, such as embryogenic cell suspensions or callus, scutella of immature embryos, shoot apical meristems, inflorescence tissues, or microspores, of an ever-increasing number of species. Reported stable transformation frequencies range from 0.1 to almost 10% per treated explant. Microprojectile-mediated gene transfer has good chances to become a general technique for nuclear and organellar transformation and, provided that reasonable regeneration frequencies of the target tissue and a tight screening or selection system are available, an essential tool for the generation of transgenic crops.

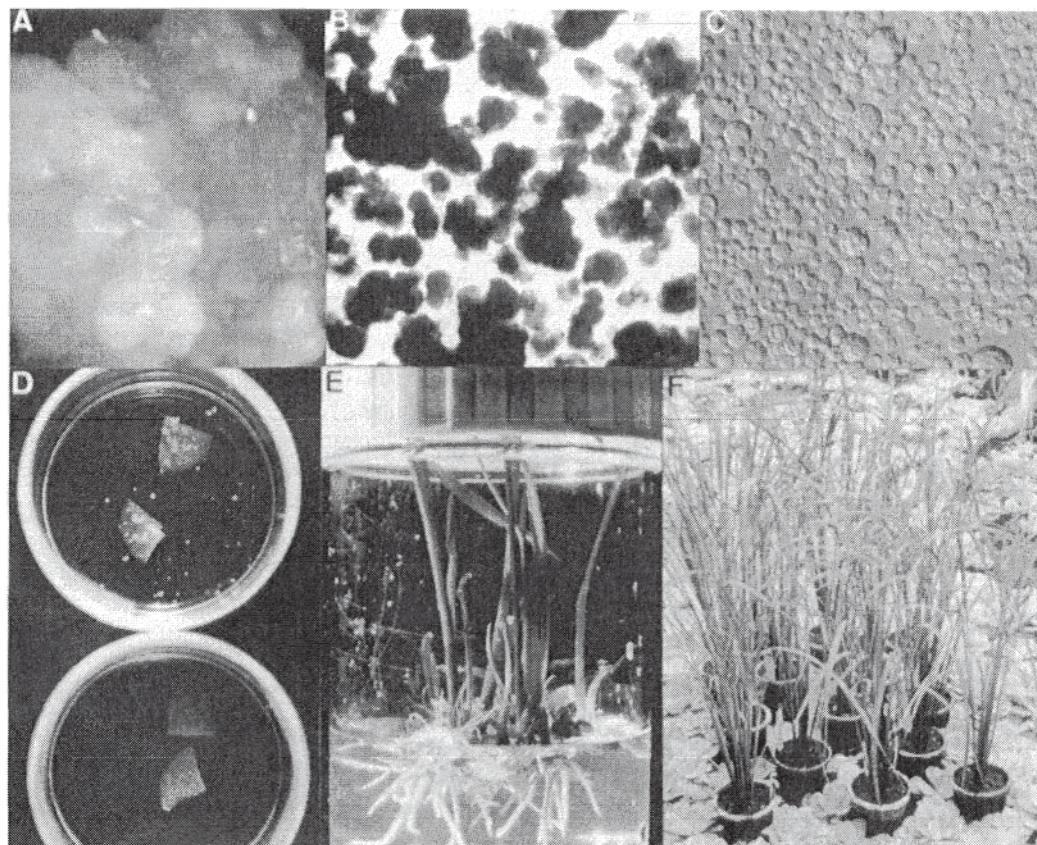


Fig. 3 Direct gene transfer to embryogenic cell suspension-derived protoplasts and regeneration of transgenic plants in rice (*Oryza sativa*) [see Refs. 78,180]: (A) Embryogenic rice callus used for initiation of suspension cultures; (B) established embryogenic cell suspension culture; (C) protoplasts isolated from embryogenic cell suspensions and treated with polyethylene glycol and plasmid DNA bearing a chimeric *hpt* gene as selectable marker; (D) selection in bead-type culture of rice protoplasts treated with (upper) and without (lower) plasmid DNA and polyethylene glycol; (E) *In vitro* growing plant regenerated from hygromycin-resistant callus obtained from transformed protoplasts; (F) fertile transgenic rice plants obtained from protoplasts.

1. Instruments

The first ballistic device used gun powder and a macroprojectile in a barrel for acceleration of microprojectiles on top of the macroprojectile. The latter was stopped at the end of the barrel, and the microprojectiles continued their travel toward the plant tissue [50,52]. This first instrument was suitable to generate transgenic plants [53–55]. In principle, the ballistic approach is independent of the technique used to accelerate the microprojectiles [56]. It became clear that gun powder releases oxidation products that interfere with the viability of the plant material [57]. Moreover, it was demonstrated that the use of macroprojectiles, which might be a drawback for control of the microprojectile velocity, could be dispensable [58]. Therefore, several modifications of the prototype have been described, and alternative constructions were developed to replace gun powder and to control various parameters of the acceleration system. A good overview over the most advanced constructions, together with citations of

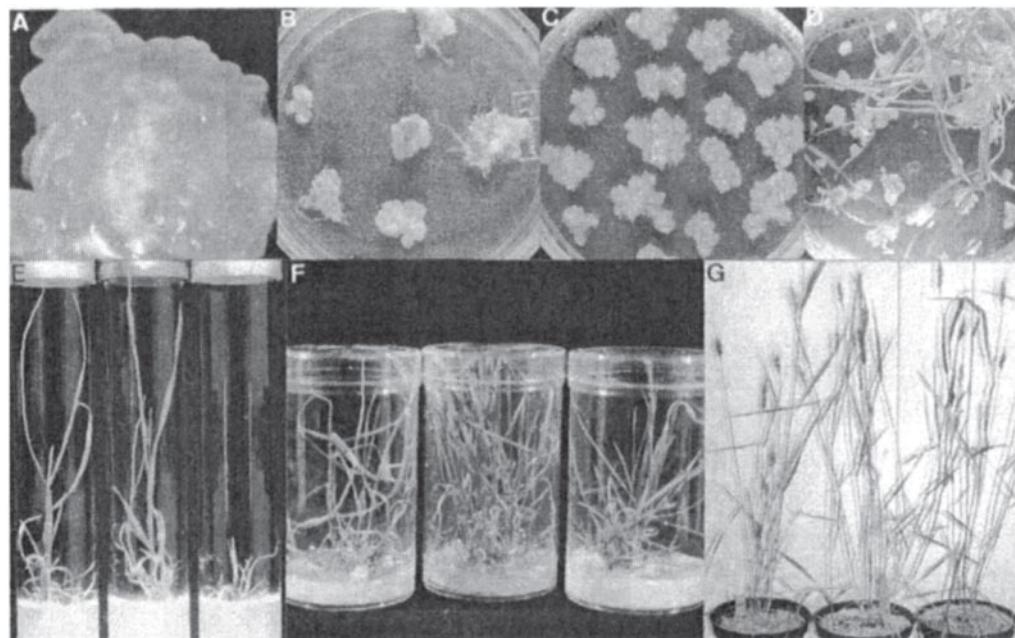


Fig. 4 Microprojectile bombardment of cultured immature zygotic embryos and regeneration of transgenic plants in wheat (*Triticum aestivum*) [see Ref. 74]: (A) Cultured immature zygotic embryo of wheat before microprojectile bombardment; (B) microprojectile bombarded precultured wheat embryos after proliferation and transfer to regeneration medium in absence of selective agent; (C) phosphinothricin-resistant regenerating callus obtained from precultured immature zygotic embryos bombarded with microprojectiles coated with plasmid DNA bearing a chimeric *bar* gene as selectable marker; (D) methotrexate-resistant plantlet regenerated from precultured immature zygotic embryos bombarded with microprojectiles coated with plasmid DNA bearing a chimeric *dhfr* gene as selectable marker; (E) Transformed (center) wheat plantlet obtained from microprojectile bombardment using a chimeric *bar* gene as selectable marker and untransformed controls (left and right) after transfer to phosphinothricin-containing rooting medium (left); (F) *in vitro* growing methotrexate-resistant wheat plantlets from microprojectile bombardment using a chimeric *dhfr* gene as selectable marker; (G) fertile transgenic wheat plants from microprojectile bombardment to precultured immature zygotic embryos.

older publications, has been reviewed [59]. It includes the modified original Biolistic device [60], the electric discharge gun (ACCELL) [61], the airgun device [62], the particle inflow gun (PIG) [63], and the microtargeting instrument for meristem bombardment [64]. Almost all modified systems use compressed gas to accelerate the projectiles and, in the latter two instruments, macroprojectiles are omitted and the microprojectiles are directly accelerated by a gas-pressure pulse.

2. Parameters

Important for the success of the ballistic gene transfer approach is the optimization of a variety of parameters. They range from merely physical bombardment conditions and the form of the offered DNA, to physiological tissue culture conditions during preculture and posttreatment of the target tissue, selection of transformants, and plant regeneration. In some instances, these parameters simply influence the efficiency, in others, they might be essential for the recovery of any transgenic plant. A comprehensive overview on the different physical

and biological parameters that can influence the results in microparticle treatments are given by Birch and Bower [65].

a. Physical Parameters. Penetration of the projectiles depends on their velocity, size, and shape. Because the orientation of the projectiles cannot be controlled in any of the published acceleration systems, round-shaped microparticles should be preferred. A measure for the impact of accelerated projectiles on the target cell wall is their impulse (mv , where m is the mass and v the velocity). Furthermore, their penetration depends on their kinetic energy ($v^2m/2$). These two formulas demonstrate that the size of the projectiles is very important for the control of microparticle penetration, because the diameter is included in the mass by the power of 3. The size of the projectiles proved to be important for DNA delivery into subepidermal cells of cereal meristems [66]. For most ballistic transformation systems yet published, it might even be an advantage to have a wide range of different penetration depths applied in one shot. This is of interest when the location of competent cells within a target tissue is unclear. The distribution of the projectiles on the target tissue should be adjusted to provide one projectile to every cell [67]. Although transient expression of marker genes can be observed, even if more than one projectile entered the cell, the viability of the cells might decrease rapidly with an increasing number of projectiles inside the cells. However, it is very difficult to study the effect of particle number per discrete cell after stable transformation (i.e., viability and division of the cell). Becker et al. [68] reduced the number of projectiles per shot and, by this, increased the number of transgenics. The material of the projectiles should be chemically inert and of high density. Gold particles of highly regular shape have been used successfully in transformation experiments, but tungsten particles are also widely used [69], in spite of their toxic oxidation products [57], their irregular shape, and their relatively low density owing to endogenous cavities [50]. Recently, direct shooting of plasmid-containing bacteria has been reported [70].

To deliver sufficient DNA together with the projectiles, the DNA in most ballistic systems is either tightly coupled to the particles by spermidine and Ca^{2+} [52], or precipitated to the beads [71]. DNA binding to the projectiles is a critical step, therefore, it is important to work with an established protocol. The addition of free, uncoupled DNA to the coupled DNA can enhance the transformation frequency [72]. At least in a system in which the DNA is not coupled to the projectiles, but freely dissolved in a watery particle suspension (microtargeting), linearized DNA is more effective in stable transformation than supercoiled DNA [58], as could be expected from earlier results with PEG-mediated gene transfer [23].

The viability of the cells that receive a projectile is in general low [69]. The explosion of the gun powder or the gas blast used to accelerate the projectiles is thought to create an acoustic shock wave that can damage the tissue. In fact, a steel grid ("baffle") inserted between the target tissue and the projectile source can significantly increase the survival of the bombarded cells [73], leading to improved transient gene expression, as well as to stable transformation [63]. In addition, a baffle grid might also reduce the number of projectile aggregates created by the DNA coating of the particles. A critical parameter is the size of the mesh (in the range of ca. 0.5 mm) and the distance of the baffle from the tissue [74]. Although a baffle can improve the viability of the hit cells, it cannot change the unfavorable relation between only a few stable transformation events and the huge number of transient-expressing cells. Actually, many of the transient-expressing cells might be lethally damaged [75].

b. Physiological Parameters. The turgor of the cells seems to be an important parameter affecting their survival after impact with the projectiles [76]. Incubation in a medium with high osmotic value for a short period reduces the turgor for optimum transient and stable transformation. Long-term incubation leads to adaptation and restoration of the original

turgor in the high osmoticum (C.Sautter, unpublished results). The optimum value must be evaluated and depends on the species and the tissue used. In wheat, for example, meristems need a higher osmotic value in the preincubation than do scutella of immature embryos [66,77]. The chemicals used to increase the osmotic pressure might interfere with the proliferation and regeneration of the explants. Although sucrose often causes no problem, maltose stimulates the growth in tissue culture of wheat and rice, whereas sucrose inhibits growth at higher concentrations [78]. Nonmetabolizable sugars, such as mannitol and sorbitol, might otherwise be preferable [77]. After microprojectile treatment, the explants should be kept for some additional time at a high osmotic value to avoid osmotic shock immediately after penetration of the projectiles. Once the cell that originally received a single projectile has divided, one daughter cell should be free of particles. This cell should be able to proliferate and regenerate as does any other cell of the tissue.

c. Intracellular Parameters. Integration of the foreign DNA into a chromosome as well as the transcriptional steps of transient gene expression are events taking place inside the nucleus. In ballistic gene delivery this means that the DNA (perhaps together with the projectile) has to penetrate the nuclear envelope. Yamashita et al. [79] observed that more than 90% of the transiently expressing cells in a tobacco cell culture contain a projectile in the nucleus. This indicates a relatively low chance for foreign DNA molecules to cross the nuclear envelope if they are transported to only the cytoplasm. Unfortunately, stable transformation has not been studied relative to the subcellular location of DNA delivery. Because the stable/transient ratio, in all ballistic systems, is in the range of 10^{-2} to 10^{-3} [65], or even worse, it cannot be ruled out that the impact of the projectile with the nucleus prevents its correct mitotic division. Thus, the relatively few cases of detectable stable transformations might be due to some rare events of cytosolic DNA delivery, followed by import of the DNA into the intact nucleus. Actually, in plant cells during mitosis, the nuclear envelope disappears for a short period and reestablishes afterward. This opens the possibility for large pieces of DNA to enter the nucleosol. This speculation is supported by a study of ida and co-workers [80], who observed significantly higher expression in synchronized tobacco cultures if the gene was ballistically delivered during the G₂ and the M phase, compared with the G₁ and S phase. This might indicate facilitated entrance of the foreign DNA into the nucleus during mitosis. Unfortunately, stable transformation was not investigated in this study.

The nuclear envelope as an assumed barrier for DNA can be overcome by *Agrobacterium*. The virulence genes code for at least two proteins that are involved in T-DNA transfer to the nucleus of the host cell and that contain at least one nuclear-targeting sequence [81]. Cocultivation of bombarded tobacco leaves or sunflower meristems with *agrobacteria* increased the number of sectors by at least 100 times when compared with conventional microprojectile-mediated DNA delivery [82]. Although the effect of *agrobacteria* on nuclear integrative transformation is unclear, it is evident that the nuclear import of the T-DNA is obviously no problem.

3. Organelle Transformation

Soon after the first publications of the ballistic system it was applied to the unicellular green alga *Chlamydomonas reinhardtii* to transform the chloroplast genome (plastome). Defective mutants of chlorophyll biosynthesis have been used as targets for restoration of the defect with the transgene. This enabled the selection for autotrophy [83]. In contrast with *Chlamydomonas*, which contains only one single plastid per cell, chloroplast transformation in higher plants is more difficult, because the cells of higher plants usually contain some 100 chloroplasts, each of them with hundreds of genome copies, of which usually only one is transformed. Therefore, a highly

proliferating tissue culture system is required to select for homoplasmy [84,85]. Foreign DNA is integrated into the chloroplast genome by homologous recombination [86, 87], whereas integration into nuclear chromosomes is usually by illegitimate recombination, regardless of which transformation technique is used. In the last reviews on this topic [88,89], chloroplast transformation was suggested to become useful for all higher plants.

Microballistic gene transfer to mitochondria was shown in yeast [90]. A suitable selection system that leads to homogeneous mitochondrial populations in transgenic cells needs to be developed before mitochondrion transformation will also become applicable to higher plants.

D. Developing Techniques that Might Be Useful in the Future

Several alternative approaches for gene transfer have been described that might offer the chance to transform highly regenerable tissues or to simplify the current methods. Although these methods have already been used to produce transgenic plants, so far they have been successful only in model plant systems, or they have been applied to only a very limited number of plant species, for which other transformation systems are already available. Nevertheless, we would like to discuss such methods here because they might become important in the future.

1. Microinjection

A microscopically small glass capillary with a diameter in the range of 1 to a few microns can be used to inject DNA solution into the cytoplasm or into the nucleus of living cells [91]. Hence, microinjection directs the DNA not only to a target cell, but also even into a desired cellular compartment. To provide the necessary resolution, this technique requires an inverted microscope, one or two micromanipulators, and a pressure injector for the dosage to small volumes. The technique can be applied to isolated cells as well as to cells *in situ* located in the tissue. Microinjection into germline cells is widely used in gene transfer to animal species and has been apparently very successful in the past [91–95]. In contrast with animal cells, plant cells usually exhibit an osmotic turgor and are surrounded by a cell wall. This presents a high mechanical barrier to microinjection, which was circumvented in the first trials by using protoplasts [96–100] or isolated nuclei [101]. Additionally, plant cells produce plenty of different sugars and glycoproteins that have a tendency to block the injection capillary after only few injections. Microinjection of isolated tobacco cells has been used for stable transformation [96,102]. It has been reported that meristems survive microinjection [103], and microinjection has produced transgenic sectors after injection into meristem cells [104]. In the area of gene transfer, microinjection has its potential probably in systems that provide highly efficient regeneration, such as egg cells, immature proembryos, or meristems. Despite the expensive instrumental equipment and the high technical skill required for operation of microinjection, it is theoretically an attractive approach.

2. Microtargeting

The microballistic targeting approach has been already mentioned in Section II.C in dealing with ballistic gene transfer. The system controls precisely the penetration and distribution of the microprojectiles. The instrument can direct microprojectiles to very small areas, requiring a stereomicroscope to aim at targets smaller than 0.15 mm in diameter. It has been particularly designed for targeting the projectiles to shoot apical meristems to transfer genes to this highly regenerable tissue. This approach has the potential for overcoming the genotype-dependent problems of tissue culture. Microtargeting was very efficiently used for stable tobacco

transformation: one of 1000 microtargeted cells in microcolonies gave rise to a transgenic plant [58]. Microtargeting to meristems produced transient results and partwise large sectors, but until now no transgenic progenies have been produced [66,67,77]. Microtargeting is also a tool for studying whatever might be the limiting steps for foreign gene integration in meristem cells.

3. Electroporation

Short-time high-voltage pulses are able to transiently perforate cellular membranes, thereby allowing the transfer of foreign DNA, without affecting the viability of the treated cells. Electroporation is usually performed in modified photometer cuvettes and requires a commercially available electrical supply with an additional capacitor. Electroporation and electrofusion are widely used methods for protoplasts (see Sec. II.B), but they can also be applied to cells *in situ* after a partial cell wall digestion e.g., in tissues of regeneration systems such as embryos [30] or after cutting the tissue to open a number of the cells [105]. Stable transformation has been achieved in maize [30] and rice [105]. Cowpea and wheat have been transiently transformed [32,106]. The study in wheat has also made apparent a drawback of electroporation: with this method, only the first cell layer is accessible for the foreign DNA. However, this cell layer is often not competent for regeneration, as in wheat scutella [74]. This might limit the applicability of electroporation of cells *in situ*, for electrophoresis of DNA through plant cell walls is questionable, although reported [107]. A possible escape might be the approach by Xu and Li [105], who electroporated with embryos cut into halves. Thus they reached deeper cell layers, although it should not be necessary in rice, because somatic embryogenesis arises from the outermost cell layer according to earlier reports [108].

4. Silicon Whiskers

Silicon carbide forms microscopically small needles (silicon whiskers) that can be used to perforate plant cell walls and plasmalemma of suspension cells or protoplasts. Technically, the methods is very simple: a mix of whiskers and plant cells in a small test tube is shaken. Thus, only Eppendorf tubes and a vortex is sufficient equipment. This opens the way for DNA diffuse into the cells [109]. Although, details of the mechanism by which silicon carbide whiskers work is unclear, stable transformation with maize has been recently published [110]. The overall efficiency of the whiskers approach is between one-fifth and one-tenth that of conventional microprojectile shooting, but its advantage would be its "low tech" character, dispensing with any sophisticated or expensive instrumentation. However, currently, only maize has been transformed and a highly regenerating system was used. It remains an open question whether the whiskers approach will be useful in organized tissue.

III. PLANT GENE EXPRESSION SIGNALS AND GENETIC MARKERS

A. Expression Signals

Foreign genes are introduced into plants to alter the properties of the recipient. This is achieved by either expressing a completely new gene or by altering the expression characteristics of an endogenous gene [examples are listed in Ref. 38, 219–223]. In both instances, a protein or RNA product has to be produced from the transformed DNA in sufficiently high amounts and at the proper time and place. A prerequisite for proper gene expression is the presence of *cis*-active signals within the transgene that's in association with cellular factors, cause

production of the RNA (*transcription*), RNA maturation (*splicing*, 3'-end formation), transport of the RNA to the cytoplasm, *translation* of the RNA into a protein, and modification and transport of the respective proteins. Here, we will give a brief review of signals involved in RNA and protein production that have been used to express foreign genes in plant cells and plants.

1. Constitutive Promoters

Transcription initiation in RNA polymerase II-dependent genes is largely controlled by sequences upstream from the transcribed region. These promoter sequences are the main determinants of quantity and temporal and spatial control of RNA production. A large number of plant genes have been isolated, and for many of these, promoters have been partially characterized. In general, all promoters consist of a mosaic of different sequence elements, the interaction of which with specific cellular factors results in the particular expression characteristics. For most of the early applications of plant genetic engineering, promoters have been used that cause expression in all or almost all tissues of a transgenic plant and that are independent of developmental or environmental stimuli.

The most widely used of these constitutive promoters is the 35S promoter of the pararetrovirus cauliflower mosaic virus (CaMV). A short DNA region of about 350 bp contains all signals required for activity: a 42-bp-long core promoter responsible for a low, basal activity and a variety of upstream sequence elements conferring particular tissue specificities and containing quantitative determinants. The interplay of these elements results in efficient expression in most, but not all, plant tissues [224,225]. The precise expression patterns vary among different transgenic plants. The CaMV 35S promoter has been modified by enhancer duplication, resulting in a two- to tenfold increased transcription [226]. Chimeric promoters combining elements of the 35S promoter and the agrobacterial *mas* promoter for strong expression in dicots (particularly roots [227]) or upstream or downstream elements from monocot promoters for use in monocots have also been constructed [228].

Other constitutive dicot promoters are the CaMV 19S promoter and the *nos*, *ocs*, and *mas* promoters derived from *Agrobacterium tumefaciens*. These promoters are about 50-fold weaker than the 35S promoter, and the activity of the agrobacterial promoters is influenced by hormones and wounding [229–231].

Agrobacterial promoters are mainly used in dicots, although activity for the *nos* and *mas* promoter in monocots has been described [232].

Useful future alternatives to the CaMV 35S promoter could be the corresponding promoter of figwort mosaic virus [233] or a strong constitutive dicot promoter from an *Arabidopsis thaliana* ubiquitin gene [234] that have been described as strong, constitutive dicot promoters, but are as yet not used frequently for purposes of genetic engineering.

For monocots, the CaMV 35S promoter is usable, although its activity is relatively low [235]. Five- to tenfold stronger promoters from rice *actin1* [236,237] or maize *ubiquitin1* [238] have been characterized and are used together with the first intron of the respective genes for monocot transformation [239; reviewed in Refs. 240,241]. Expression from the maize *ubiquitin1* promoter can be further increased by heat shock and other stresses in a tissue-independent manner [242,243]. The recently described promoter-first intron of the rice triosephosphate isomerase gene may constitute an additional useful constitutive expression signal for monocots [244].

The normally inducible maize alcohol dehydrogenase 1 gene (*adh1*) promoter has been modified to a constitutive promoter by insertion of transcription-regulating sequence motives [245].

Constitutive promoters are used for the expression of selectable marker genes and reporter genes during the establishment of transformation protocols, but they are also always useful when it is not necessary to restrict transgene expression to particular cells. Such applications are expression of antiviral, antifungal, or insecticidal proteins, or occasionally expression of antisense RNAs.

2. *Tissue-Specific and Inducible Promoters*

When transgene expression should be directed to certain tissues or time points during plant development, regulated promoters are required. A great number of tissue-specific genes have been characterized from many different plant species [246]. Again specificity and amplitude of gene expression is controlled by an interplay of *cis*- and *trans*-acting elements. Promoter elements conferring specificity for seeds, tubers, vegetative organs, and green tissues have been studied most extensively. Sequence elements controlling specificity are apparently quite conserved; therefore, such promoters can often be used in heterologous systems either as complete promoters [247–250] or as isolated elements fused to an unspecific core promoter [251]. However, even if specificity is conserved, the expression levels can vary drastically [252] and, in many cases, particularly with dicot elements in monocots or vice versa, regulation will not work properly in a heterologous system [247,249,253]. The use of a promoter from the recipient plant, therefore, may be preferable, although this may cause problems of gene silencing [254–256] (also see later discussion).

Despite these potential difficulties, a variety of such promoters have been used to specifically express transgenes in homologous or heterologous plants. Particular targets have been the storage tissues in seeds [257] (e.g., using the napin gene promoter of *Brassica napus* [258,259] or the 2S albumin gene promoter of *Arabidopsis thaliana* [260]) and tubers (promoter of a potato patatin gene [261–263]) and the vegetative organs (tapetum-specific promoter from tobacco [264,265]). For gene expression in monocot seeds, the promoters of a maize zein gene [266] and a rice glutelin gene [252] may prove useful.

Promoters that respond to environmental influences, particularly light and wounding or pathogens, but also to anaerobiosis and heat or cold stress, have also been studied extensively and with results similar to those found for tissue-specific promoters [267,268]. Pathogen-induced promoters can often be induced also by treatment with salicylic acid or other chemical substances; therefore, they are of particular interest because they allow the induction of gene activity independently from the developmental stage of the plant at any appropriate time [e.g., Ref. 269, and for review Ref. 270]. The *pr-1a* promoter from tobacco has been used for inducible expression of the insecticidal CrylA(b) protein of *Bacillus thuringiensis* [271]. So far most of these inducible promoters are derived from dicots, but it appears that at least some major aspects of the induction pathway is conserved between dicots and monocots and thus even inducible promoters can be used in widely heterologous systems (e.g., the promoter of a rice chitinase gene works in tobacco [272] and the potato *pin2* promoter was wound-inducible in rice [273]). Expression in rice from the *pin2* promoter can be enhanced without loss of the specificity by presence of the *act1* intron, suggesting that, in these cases, expression levels and specificity can also be modulated independently.

Inducible promoters, in principle, offer the possibility to express a transgene only in response to an external stimulus. However, natural-inducible promoters are often also under developmental control, which may result in unwanted expression of a transgene during plant growth [274–276]. Likewise, usually a whole group of promoters will respond to a given natural stimulus. Artificial, inducible promoters have been developed using bacterial

repressor-operator systems [277–279]. But, so far, promoter induction requires the removal of a repressing compound and, therefore, is not useful for applied purposes outside the laboratory.

3. Transcription Terminators and Polyadenylation Signals

Efficient RNA production involves not only transcription initiation, but also its termination. Generation of a functional RNA 3'-end requires the presence of a processing signal (called terminator or polyadenylation signal). Very few plant polyadenylation signals have been characterized to any extent. No species nor tissue specificity has yet been reported in plants. Model signals contain an AATAAA sequence 10–30 nts upstream from the polyadenylation site and a GT-rich region upstream or downstream [280–282]. The AATAAA sequence is not present in all signals and, even if present, may not be important for polyadenylation [283]. The most frequently used signals are derived from CaMV or the *nos* or *ocs* genes of the agrobacterial T-DNA and are used as about 200- to 500-bp-long DNA fragments. The effects of different polyadenylation signals on gene expression have rarely been studied, but up to 100-fold differences have been obtained [284,285]. Unfortunately, these comparative studies did not include the most widely used elements. It is assumed, that the differences are not due to different 3'-end-processing capacities of the elements, but to a contribution of the 3'-untranslated region which is at least partially derived from the termination signal.

4. Introns

Primary RNA transcripts usually contain introns that have to be removed by splicing. Production of mature mRNAs thus involves transcription, splicing, and 3'-end formation in a well-coordinated process, and omission of any one of the steps may interfere with correct expression patterns. Although intronless genes clearly are expressed, insertion of introns has improved expression efficiency in some systems. In maize cells, gene expression from the CaMV 35S promoter or the maize *adh1* promoter could be enhanced up to 100-fold when introns from maize *adh1*, *bronzel*, or *shrunken1* genes were inserted [286–288]. These introns were also effective in wheat [289,290] and rice [291]. Other monocot introns with stimulatory effect are derived from the rice *actin1* gene [236], and a maize ubiquitin gene [238]. The first exon and intron of the rice triosephosphate isomerase gene are even absolutely required for promoter activity in rice [244]. The positive effect on introns is, however, not universal. In monocots such as *Lolium* and sugarcane, the same introns have no or even a negative effect [292,293]. It is possible that introns in a heterologous context are spliced inefficiently [294] or that different splice sites are used than in their natural setting [295].

Although stimulatory effects were also obtained with the first intron of a castor bean catalase gene in rice [295], monocot introns in dicot plants are usually inhibitory [244,293,295] because splicing is inefficient [296–298]. Taken together, the usefulness of intron constructs has to be carefully tested in the target plant before their routine use in expression constructs.

5. Translation

The main determinant of the competitiveness of an mRNA for ribosomes seems to be the structure of the RNA 5'-end. Unstructured ends are thought to allow a more efficient binding of 43S ribosomal subunits to the capped RNA end and their subsequent migration to the start codon. Because the start codon is almost always approached by linear scanning, the absence of possible alternative translation initiation sites upstream from the protein-coding sequence is also important for efficient expression [for reviews see Refs 299–301]. Some 5'-untranslated regions (leaders) of plant viruses support translation particularly effectively. The best-studied

and most widely used example is the 68-nucleotide-long tobacco mosaic virus Ω leader sequence which enhances translation in protoplasts, plants, and *in vitro* systems [37,302,303]. The enhancing effect is variable and probably depends on the particular translation conditions. In dicots, the effect ranged from 4- to about 80-fold, in maize and rice only small effects have been observed. Other leader sequences causing a 4- to 20-fold enhancement of translation are derived from alfalfa mosaic virus [304], potato virus X [305,306], tobacco etch virus [307], CaMV [303], brome mosaic virus [37], satellite tobacco necrosis virus [308], and also from the maize *shrunken-1* gene [288].

Translation initiation efficiency is influenced by the sequence context surrounding the ATG start codon. A survey of plant gene sequences suggested an optimal context of AACAAATGGC [309,310]. The most important bases are located at positions -3 and +4 (with the A of the ATG as position +1). The positive effects of the A and G at -3 and +4 have been verified by mutation analysis in transgenic plants [311] and in protoplasts [312]. However, the importance of an optimal consensus seems to be variable (two- to eightfold) and, under natural conditions, differences in start codon usage may occur that are not explainable by context rules [313].

It has been speculated that in plants as in other eukaryotes, translation may also be influenced at the level of elongation (e.g., by the presence of clusters of codons for which only low levels of charged tRNAs are available). The codon usage differs slightly within the plant kingdom, but significant differences exist between plants and bacteria and other eukaryotes [314,315]. Extensive modification of a *Bacillus thuringiensis* toxin gene to comply with the plant codon usage resulted in up to 100-fold better expression [137,316,317]. However, in these cases, the contribution of translation to the increase is unclear because other elements possibly involved in aberrant RNA processing and RNA instability were also altered.

Little is known about the effects of 3'-untranslated regions on gene expression besides the positive effects on RNA stability and translation of the poly(A) tail that is present on at least the vast majority of mRNAs [300,318]. Unique RNA sequences derived from some plant viruses can have similar effects [318–320], but it is unclear whether these viral elements will also act in the context of a normally polyadenylated RNA. In other eukaryotes, the 3'-untranslated region frequently contains determinants of RNA stability [321]. Determinants of RNA stability in plants are poorly understood [reviewed on Refs 301,322]. Destabilizing sequences may be found anywhere on the RNA [323], but at least the destabilizing elements of plant SAUR genes also are located in the RNA 3'-region [324]. Because translatability may influence RNA stability [325], long untranslatable regions may provoke a more rapid RNA degradation.

6. Effects of Sequences Outside of the Expression Cassette

Even with thoughtful construction of expression cassettes, transgene expression, particularly in the progeny of a transgenic plant, is not always predictable [40,326]. Sequences outside of the expression cassette can have profound influences on transgene expression. For other eukaryotes, such sequences can be located in the bacterial plasmid that harbors the expression unit [e.g., see Ref. 327]. We found occasionally that plasmid sequences can have eukaryotic promoter activities and cause transcription through the expression unit in sense or antisense orientation. If such sequences are transferred to the transgenic plant, they can cause a reduction of gene expression. Transcriptional effects are also thought to be, at least partly, responsible for the widely different activities of an identical expression unit in independent transgenic plants or for the different activities of closely linked expression cassettes. Transcription from outside into the expression unit may interfere with the intended activity by antisense RNA-mediated mechanisms [328] or by promoter occlusion [329]. In principle such transcription can be avoided

by inclusion of additional polyadenylation signals, and such a signal cloned in anti-sense orientation downstream from a transgene has indeed reduced position effects in transgenic tobacco calli [330].

Scaffold attachment regions (SARs, also called matrix attached regions [MARs]) are DNA segments that seem to organize eukaryotic chromosomes into domains of different activity. The flanking of transgenes by such regions reduces position effects [331–334]. Transgene expression often is not related to the actual copy number of transgenes. This is explained by down-regulation or silencing of additional gene copies. Interestingly, SAR-flanked transgenes in some cases were expressed proportional to the copy number of transgene inserts [332,333]. A potential antisilencing effect of SARs has been inferred from this observation. SAR regions used so far typically are 500–1500 long and are AT-rich. They contain a number of conserved sequence elements and apparently are uniformly active in all organisms. However, data are still limited, and it remains to be seen whether all SARs are equivalent.

7. Optimized Transgene Expression Units

The basal expression characteristics depend on the respective promoter. Quantitative enhancement of expression independently of specificity is achieved by introduction of an efficiently spliced intron near the 5'-end of the transcribed region (particularly for cereal mono-cots) and the use of a polyadenylation signal from an efficiently expressed plant or plant virus gene. If the intended gene product is a protein, expression can be further promoted by an mRNA leader sequence (possibly derived from a plant virus) lacking secondary structure and potential translation initiation codons, a start codon in a good sequence context, and a coding sequence devoid of clusters of rare codons. If the measure of mutagenizing the coding region is taken to comply with the plant codon usage, at the same time, signals resembling polyadenylation signals or splice sites or known RNA instability signals can be removed.

To reduce position effects, transgenes could be flanked by SARs, which may also inhibit gene silencing. It is as yet unknown whether sequences similar to the SP1 sites in some mammalian genes [335,336] that inhibit methylation of adjacent genes also exist in plants and could be used to interfere with transgene methylation.

To allow for later removal of transgenes (e.g., the selectable marker gene), the respective parts of the expression cassette can be flanked by sequences that are targets for site-specific recombinases [reviewed in Refs. 337,338].

8. Expression Vectors

Most of the currently used expression vectors are simply a combination of strong promoters (mostly constitutive) with some polylinker sequences for simple cloning and a termination signal. A great number of such vectors for monocot and dicot transformation have been described [239,339]. Vectors for monocot transformation often also contain introns to boost expression [236,238,239,292].

Plasmids designed for *Agrobacterium*-mediated gene transfer require additional functions that allow maintenance in *Escherichia coli* and *Agrobacterium* (replicons and selectable markers) and transfer from *E. coli* and *Agrobacterium*, and T-DNA border fragments for the transfer to the plant. Integrative vectors are *E. coli* plasmids that, after transfer into *agrobacteria*, integrate into the T-DNA region of an endogenous Ti plasmid by homologous recombination. *Binary vectors* are plasmids that can be episomally propagated in both types of bacteria and, besides, replicons for both hosts contain the expression cassette between T-DNA border sequences. Transfer to plants is accomplished by the action of agrobacterial *vir* gene products on these border sequences in *trans*. Descriptions of such vectors with a variety

of additional features can be found: [340] (pMON series), [341] (pGV series), [342] (pGA series), [343] (pCG series with pRiHRI replicon), [339] (pBI series), [344] (pGPTV series), [345,346] (pCIT series), and [347].

B. Selectable Marker and Reporter Genes

1. Selectable Marker Genes

In plant transformation systems, the frequency of stable gene transfer is low. Only a fraction of the cells exposed to the foreign DNA integrate the DNA into the genome, thereby providing the basis for regeneration of transgenic plants. Therefore, systems are required that allow selection of such transformed clones. A selection system consists of a selective agent (i.e., a phytotoxic compound interfering with the plant cellular metabolism), and a selectable marker gene (i.e., a DNA sequence coding for a product that allows detoxification or evasion of the selective agent through enzymatic modification of the selective agent or expression of an altered target, respectively). Clones with integrated selectable marker genes can be detected through negative selection of untransformed cells or tissue *in vitro* or in whole plants *in vivo*. Cotransformation of nonselectable sequences linked or nonlinked with selectable marker genes allows recovery of tissue or plants that contain the nonselectable sequence.

A selection system for transformed plant cells must meet several requirements: The selectable marker gene should allow expression in a variety of plant tissues; the background metabolic activity or resistance should be minimal; and a clear change of phenotype should be visible. Over the past decade, several such systems have been developed (Table 2). They include the use of antibiotics, herbicides, substrate analogues, or high concentrations of other compounds. Several of them are widely used in plant transformation work.

a. *Neomycin Phosphotransferase*. The *npt2* or *neo* gene, also known as *aphA2* or *aph(3')II*, was isolated from transposon Tn5 from *E. coli* K12. *npt2* codes for neomycin phosphotransferase (NPTII) which detoxifies a range of aminoglycoside antibiotics, most importantly kanamycin (Km) and geneticin (G418) [368]. So far, *npt2* has been widely used in plant transformation work. A wide range of mono- and dicotyledonous plant species have successfully been transformed with *npt2*, among them many crop species. Transformation of plastids with *npt2*, followed by selection on kanamycin has been reported [369].

Kanamycin and geneticin are the selectable agents that are usually applied in combination with *npt2*. The choice of the selective agent is important according to the plant species to be transformed.

For *in vitro* selection of transformed cells and tissues of dicotyledonous species, kanamycin is used at concentrations of 25–100 mg/L. In flax, kanamycin provided better selection than geneticin [370]. However, in lettuce, kanamycin was insufficient, whereas geneticin was used successfully [150].

In several monocotyledonous species, problems with selection on kanamycin have been observed. *Lolium multiflorum*, *Triticum monococcum*, *Saccharum officinale*, or *T. aestivum* show a high degree of insensitivity to kanamycin, which limits its use in a selection system [45,46,371]. However, transgenic, fertile maize plants have been obtained after selection on 200 mg/L Km [30]. The application of geneticin allows a tighter selection. Transgenic wheat and sugarcane plants have been obtained after *in vitro* selection on geneticin [193,214].

In japonica and indica rice, Km seems to interfere with the regeneration of transformed cells to green plants [349,350]. In both cases, application of geneticin lead to successful regeneration of fertile plants. In barley, plants transformed with *npt2* were screened for NPTII activity, leading

Table 2 References on Selectable Marker Genes Used in Transformation of Crop Plants

Species	<i>npt2</i>	<i>hpt</i>	<i>bar</i>
Wheat	214	—	211
Barley	120	—	119
Maize	30	348	55
Rice (<i>japonica</i>)	349	180	182
Rice (<i>indica</i>)	350	181	351
Oat	—	—	154
Sorghum	—	—	185
Sugarcane	193	—	
Soybean	71,186	352	
Pea	353	162	163
Peanut	354	355	165
Alfalfa	356	112	112
Rapeseed	357	—	358
<i>Arabidopsis</i>	359	360	361
Cotton	133	134	
Sugar beet	192	—	362
Sunflower	195	—	
Potato	363	—	358
Tobacco	1	364	358
Tomato	201	—	358
Lettuce	150	—	
Carrot	365	—	
Cucumber	366	—	
Melon	151	—	
Apple	113	—	
Citrus	126	—	
Poplar	367	—	117

to recovery of transgenic offspring. However, selection systems for primary transformants with Km or G418 were impractical owing to increased albinism in barley plants [120].

NPTII activity in plant cell extracts is detectable by an enzymatic assay [372]. KM and [γ -³²P] ATP are used as substrates. Phosphorylated aminoglycosides are detected either by thin-layer chromatography or by less elaborate dot-blot analysis [373,374]. An immunological assay has also been developed [375].

The safety for human consumption of the NPTII protein has been assessed without finding any concerns [376].

b. Hygromycin Phosphotransferase. The *hpt* gene, also known as *hph* or *aph(3')IV*, has been isolated from *E. coli*. It codes for hygromycin phosphotransferase (HPT) [47]. This enzyme detoxifies the antibiotic hygromycin B (Hm). Several crop species have been transformed with *hpt*.

Most plant tissues show a higher sensitivity to hygromycin B than to kanamycin [232]. Hygromycin B allows selection in cereals such as maize and rice [180,181,348]. Selection in vitro is applied at concentrations ranging from 20 mg/L Hm for *A. thaliana* to 200 mg/L Hm for tall fescue [142,360].

The bacterial *hpt*-coding sequence has been modified for optimized expression in plant cells. Amino acid residues near the carboxy-terminus of the enzyme were deleted or substituted, which increased the level of resistance in tobacco protoplasts. The hydrophilic carboxy-terminal end itself, however, was preserved and may be essential for strong HPT activity [377].

HPT activity in plant cells is detectable by an enzymatic assay similar to the NPTII-assay [181]. HM and [γ - 32 P]ATP are used as substrates. Hygromycin B should be handled carefully because of its high human toxicity.

c. *Phosphinothricin Acetyltransferase*. The *bar* gene from *Streptomyces hygroscopicus* and the *pat* gene from *S. viridochromogenes* code for phosphinothricin acetyltransferase (PAT), which confers on plant tissue resistance to the herbicide compound phosphinothricin [48,378,379]. This compound is the herbicide substance most widely used today for the selection of transgenic plant tissue. As a glutamate analogue, it is an irreversible inhibitor of glutamine synthetase, a plant enzyme involved in ammonia assimilation [358]. Phosphinothricin derivatives with phytotoxic activity; for example, bialaphos (Herbiace, Meiji Seika, Japan), a tripeptide with a phosphinothricin residue produced by *S. hygroscopicus*, and Basta (Hoechst, Germany), a synthetic formulation of phosphinothricin (glufosinate ammonium) are nonselective herbicides used in agriculture and can both be used in selection schemes.

PAT can be used as a selectable marker in mono- and dicotyledonous plant species. In cereal transformation, its use is more widespread than the other selectable markers. Selection can be applied in the medium *in vitro* as well as by spraying whole plants either *in vitro* or in the field or green-house [380]. As Basta shows no systemic activity, local applications to plant organs (e.g., by brushing the herbicide onto leaves) is possible without lethal effect to the plant [362].

Selection schemes have been developed for various plant species and the herbicide compounds just mentioned. Concentrations for selection *in vitro* range from 0.5 mg/L PPT for tobacco protoplasts to 20 mg/L PPT for rice [351, 358]. In wheat, 5 mg/L PPT provided a tight selection [68]. Weeks *et al.* [212] used 1 mg/L bialaphos for selection *in vitro*. Becker *et al.* [68] found a combination of PPT *in vitro* and spraying of regenerants with Basta most effective in wheat.

PAT activity can be detected by an enzymatic assay where PPT and [14 C]acetyl-CoA is incubated with a cellular extract. An immunological assay has been developed [362].

d. *Dihydrofolate Reductase*. Dihydrofolate reductase (DHFR) is inhibited by the folic acid analogue methotrexate (MTX) [381]. Plant cells are very sensitive to MTX at low concentrations [382]. Genes coding for MTX-insensitive DHFR have been isolated; such *dhfr* genes can be transferred into plants to confer resistance against MTX.

A *dhfr* gene from *E. coli* plasmid R67, which is practically insensitive to MTX [383], is able to confer MTX-resistance to tobacco [49]. Transgenic, MTX-resistant tobacco plants were obtained after selection for transformed cells in liquid culture at 0.1 mg/L MTX, followed by selection for resistant regenerants in solid medium required 0.5 mg/L MTX. 0.1% active charcoal has to be added to the solid medium to prevent deleterious effects of the MTX selection [384]. Fertile transgenic wheat resistant to MTX has been obtained by using an *E. coli* *dhfr* gene [385].

An altered *dhfr* gene from mouse conferred MTX resistance to transgenic petunia at 1 μ M MTX [382]. The alteration, a change of one amino acid residue, is correlated with a 270-fold lower affinity of the enzyme for MTX [381,386]. Tobacco and *A. thaliana* were successfully transformed with the altered mouse *dhfr* [387]. In *A. thaliana*, transformation with *dhfr* followed by selection on MTX yielded lower background of nontransformed escapes than other selection systems. However, an influence of MTX on the efficiency of the root transformation method used seemed to be possible [388].

Because of the human toxicity of methotrexate, careful working procedures are recommended.

2. Reporter Genes

In contrast to selectable marker genes, reporter genes do not confer resistance to plant cells against a chemical agent normally inhibitory to plant development. Reporter genes code for products that can be detected directly, or they catalyze specific reactions the products of which are detectable. Reporter genes allow the investigation of regulatory, *cis*-acting sequences as well as *trans*-acting factors in transient and stable transformation experiments. There are a number of reporter genes presently available for which expression can be measured visually or biochemically.

a. *β-Glucuronidase*. The *gusA* or *uidA* gene encodes β-glucuronidase (GUS), a hydrolase that cleaves a variety of β-glucuronides. *gusA* was originally isolated from *E. coli* K12 [389,390]. The GUS reporter gene system meets several criteria required for use in plants, such as easy quantification, high sensitivity, sufficient specificity of the enzymatic reaction to minimize interference with normal cellular metabolism, possibility of histochemical localization of the reaction, and activity of the enzyme in translational fusions. Substrates for GUS are available for spectrometric, fluorometric, and histochemical assays. There is little or no detectable β-glucuronidase activity in almost any higher plant at pH levels used in the assay. Under more acidic conditions, however, intrinsic GUS activity can be observed, which is distinguishable from *gusA*-mediated GUS activity.

The spectrometric assay uses *p*-nitrophenyl-β-D-glucuronide as substrate and can be performed without sophisticated instruments. The more sensitive fluorometric assay uses 4-methylumbelliferyl glucuronide as the substrate, which is cleaved by GUS to yield the blue fluorescent compound 4-methylumbelliferon. Both assays are quantitative and can be performed only with cell extracts *in vitro*.

For localization of GUS activity in intact tissues and cells, a histochemical assay can be performed. The histochemical assay uses 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as the substrate. The colorless, water-soluble product of the enzymatic cleavage of X-Gluc must undergo an oxidative dimerization to yield an indigo blue precipitate. This second reaction of the assay can be enhanced by oxidation catalysts to prevent leaking of the intermediate product to neighboring cells. The highly colored dye can then be detected cell specifically [391]. However, the sensitivity is lower than with the fluorimetric assay [392], and preferential staining of vascular tissue in transgenic plants may be intrinsic to the reporter and detection system [393].

Unfortunately, none of the assays described is viable. A nondestructive assay has been developed that is based on excreted GUS in spent media of *in vitro* plant culture [394]. A fluorogenic substrate for viable GUS staining of plant cells, ImaGene Green has been proposed (Molecular Probes, Eugene OR), but data on the suitability of this substrate are still limited [395].

Assaying only parts of putatively transgenic plants (e.g., root or leaf pieces), may also allow recovery of transgenic offspring. In a cotton transformation experiment, leaves of putatively transformed plants were screened for GUS activity. Buds in the axils of transformed leaves were subcultured and yielded transgenic plants [135]. In peanut, shoot meristem cells were transformed with *gusA*. Developing shoots were screened for GUS activity, and transgenic plants could be recovered [165].

GUS has a half-life of about 50 h in mesophyll protoplasts. Therefore, quantitative results should be interpreted as integrals of transcription and translation, rather than rates [390].

b. *Luciferase*. Luciferase reporter gene systems use bioluminescence to detect gene expression. Bacterial and animal luciferases can be used [396,397]. Both luciferase activities can be detected

in plant cellular extracts as well as in intact, living plant tissue. An *in vivo*, noninvasive assay is therefore possible. The assay can be performed easily; however, a relatively expensive luminometry equipment is needed. The bacterial luciferase originates from *Vibrio harveyi*. It is a heterodimer protein complex, the monomers being encoded by the *luxA* and *luxB* genes [398,399]. Expression of both genes is required for luminescence. Gene fusions have been constructed to render the system more versatile in eukaryotic cells. *V. harveyi* luciferase is thermolabile. The protein dimer is inactivated above 35°C [400]; the fusion protein is even less stable [398, 399]. *V. harveyi* luciferase uses a flavin and an aldehyde of at least eight-carbon-chain length as substrates. The flavin FMNH₂ with the highest quantum yield, and decanal are used in a quantitative *in vitro* assay. Luciferase activity leads to light emission at a 490-nm wavelength, with rapidly decreasing intensity. If the substrates are added in excess, the duration of luminescence reflects the luciferase concentration in the extract. An *in vivo* assay in whole plants is possible with decanal added as substrate; however, the light emission from living transgenic tissue is rather low [401].

The firefly luciferase from the North American firefly *Photinus pyralis* is encoded by the *luc* gene. The firefly luciferase uses luciferin and ATP as substrates. The assay is extremely sensitive: 2000 luciferase molecules can be detected; the sensitivity is about 60 times higher than with bacterial luciferase. Firefly luciferase activity can be detected as a light flash, followed by a decreasing signal of 592-nm wavelength. Addition of coenzyme A stabilizes the signal to a plateau of 30s or more [402]. An *in vivo* assay can be performed by incubation of protoplasts in a substrate solution. Tissues or cells can be assayed by application of an aqueous solution of luciferin by spraying; however, the essay can lead to decreased cell viability, possibly owing to depletion of ATP in plant cells. Addition of ATP to the substrate is therefore recommended (M.Schnorf, personal communication). Unlike GUS, firefly luciferase activity is rather unstable *in vivo* and reflects mRNA abundance. As a result, real-time, quantitative measurement of gene expression in intact organisms is possible [403]. Transgenic tobacco plants have been obtained by assaying single cells, calli, and shoots expressing *luc* [404].

c. *Anthocyanine Pigments.* Anthocyanines are red or purple pigments that are accumulated in the cell vacuoles of various plant species [405]. Several genes that control anthocyanine biosynthesis at the transcriptional level have been identified. In maize, there are two families of genes, *R* and *C1*, that encode transcription factors [406]. Both of these factors have to be present in a cell to promote anthocyanine production. The genes can be used as visible reporter genes in plant genotypes able to express the structural genes needed for the anthocyanine biosynthesis pathway. The use of anthocyanines as markers has several advantages. The visualization is easy and nondestructive, and neither processing nor addition of any substrate is needed. The cells autonomous expression allows one to follow the development of clonal sectors in tissues.

Anthocyanine regulatory genes can be used as reporters for the efficiency of gene delivery into cells. The regulatory genes *Bperu* [407] and *C1* [408] from maize were used to monitor foreign gene expression in wheat after microprojectile bombardment of shoot apical cells [66] and electroporation of scutellum cells [32].

d. *Chloramphenicol Acetyltransferase.* The *cat* gene from *E. coli* [409] encodes chloramphenicol acetyltransferase (CAT), which modifies the antibiotic compound chloramphenicol. In eukaryotic cells, *cat* can be used as a reporter gene. The expression can be assayed with cellular extracts and [¹⁴C]chloramphenicol by thin-layer chromatography of the reaction products [410]. The assay is simple and very sensitive, and quantitative data can be obtained after transient transformation of plant cells. Background CAT-activity in plant cells is generally not detectable. In *Brassica* species, however, endogenous activity has been observed. This

activity is heat-sensitive and can be reduced to a neglectable level by a heat treatment of 68°C for 150 min. The *E. coli* CAT activity is not affected by this treatment [411].

The *cat* gene has been expressed in dicot and monocot protoplasts after electroporation and PEG-mediated gene transfer [22,412] as well as in tumor cells after *Agrobacterium*-mediated gene transfer to tobacco seedlings [413].

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Engineering of Crop Plants for Industrial Traits

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I. DEVELOPMENT OF INDUSTRIAL TRAITS

During mankind's history most requirements for human life were based on biomaterials: crops for human consumption, wood for constructions and burning, clothing, paints, lubricants, lamp oil, and so forth. This changed when industrialization began, and about 1850, the chemical industry started to develop with the availability of coal as a raw material. Since 1930, mineral oil became more and more important as a raw material for the chemical industry and, since about 1960, it is the primary resource for petrochemistry. Industrialization was dependent on large quantities of cheap energy, which is still being reflected. From a total of 3128 million metric tons of mineral oil produced in 1992, about 93% was used as energy supply and only approximately 7% was used as raw material in the chemical industry ([Table 1](#)).

In summary, the development during the last 150 years has led to the exploitation of these fossil resources. Nowadays public discussions have resulted in reconsideration about the use of biomaterials as feedstocks for the chemical industry. Arguments are that (1) biomaterials are renewable depending on sun power, (2) they are CO₂-neutral, (3) they contain complex molecules, and (4) some of them are already of high enough quality to be used directly as a raw material without further purification. But in economic terms only a limited number of compounds are available (i.e., sufficiently pure, of constant supply, and cheap). On the other hand, the chemical industry is capable of building highly complex molecules, starting from simple and inexpensive compounds such as ethylene. Nevertheless, the fossil resources are limited, and alternatives are required. Crop plants do provide a substantial potential as environmentally friendly bioreactors for the production of highly demanded products, such as proteins, carbohydrates, oils, and fats.

Two lines of industrial products are distinguished: (1) low-priced bulk commodities and (2) high-priced specialized products. Crops still suffer from limitations in the production of commodities, yet considerable amounts are already used in the relevant industry (see [Table 1](#)).

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Table 1 Estimated Industrial Demand of Resources (1988) Worldwide

Raw material	1000 metric tons
Mineral oil	200,000
Oil and fat	9,500
Cellulose	5,014
Starch	1,750
Sugar	800

Source: Modified from Ref. 1.

To reduce downstream processing costs, relatively pure compounds should be available, which are now found in only a few examples, such as high oleic acid sunflower oil or high ricinoleic acid castor oil, with a total of 80–90% of the respective fatty acid. Thus, the breeding goal for quality improvement according to the requirements of the chemical industry is the production of a desired compound of higher than 90% purity at a low cost. However, certain industrial applications require a defined ratio of compounds to obtain the desired properties for utilization (see Sec. IV.C.1). Further breeding goals include improved properties for a mechanical harvesting of fruits and production of proteins that fit nutritional and industrial demands.

II. ACHIEVEMENTS AND LIMITATIONS OF CLASSIC BREEDING METHODS

Crop yield depends on four factors: (1) the variety with its inherent genetic potential, (2) agricultural training of farmers, (3) soil, and (4) climate conditions. In Central Europe, at the end of the 18th century, wheat yielded about 0.7 metric tons per hectare (t/ha). In 1850, on average, 1.6 t/ha and in 1900 2.0–2.5 t/ha were harvested. These improvements resulted from new agricultural developments: fruit rotation, including nitrogen-fixing legumes, and organic and mineral fertilizers. The increase in yield to 6.0–7.0 t/ha wheat in 1992 was based on an improved agricultural training (fertilization, pest control, and harvest equipment), but it mostly depended on a substantial success in plant breeding.

The introduction of new methods considerably accelerated the progress in plant breeding. Mass selection, performed since 1850, was replaced by combination breeding after 1900, with the rediscovery of the Mendelian rules. Hybrid breeding, introduced in the 1930s and 1940s, and cell biological techniques introduced in the 1980s were milestones of new plant-breeding techniques. Molecular biology techniques, genetic engineering, and genomic analysis are currently being introduced into breeding programs. All these techniques contribute to today's breeding process, which is the generation and selection of a desired genotype:

1. To improve a crop, the natural genetic variability within a species is used by crossing techniques: Conventional breeding starts with an evaluation process, which means screening a population for a desired trait. Depending on the variability within the gene pool, this might result in a basic germ plasm, which is introduced into elite lines.
2. If a desired trait has not been identified in a breeding population, a mutagenesis program can be initiated and, for example, the progeny of ethylmethanesulfonate (EMS)-induced mutants (M_1 or following generations) have to be scored for the trait of interest. Traits that are affected by more than a single gene are rather unlikely to be identified.
3. Alternatively, variability of a genus or related species can be used in cell biology techniques, and a new trait may be introduced by wide crossings. Usually, this requires

intermediate cell culture steps and is restricted to related species. In the 1970s, protoplast fusions, resulting in cybrid plants, were developed to increase genetic variability. This technique can be applied between totally unrelated organisms. However, the less related the species involved, the smaller the chances of a successful regeneration of fertile cybrid plants. The weakness of this method is usually the quality of a screening system for cybrids.

On the contrary, in principle the total genetic pool existing in nature can be used by genetic engineering. Since 1983, chimeric genes can be transferred between species, providing a new tool for crop improvement. This has led to an enormously rapid development, which resulted in the first commercialized crop in 1994, Calgene's "flavr savr" tomato.

III. BIOTECHNOLOGICAL APPROACHES

The first transgenic plants with chimeric genes were raised more than a decade ago, and since then the field of plant genetic engineering has boomed. However, the strategies for improving plants rely on three basic concepts: (1) inhibition of gene expression (cosuppression, antisense, and ribozyme repression), (2) overexpression of endogenous genes, and (3) the expression of foreign genes. These strategies require genes or parts of them, specific regulatory elements, and transformation technologies. Independently from the strategy applied, the amount of one or more gene products is altered. This results in metabolic alterations, such as fruit maturation because of reduced enzyme activity, production of new peptides or proteins, and in terms of engineered metabolic pathways, in changes of the metabolite content in a plant tissue. To achieve such alterations the aforementioned tools and a detailed knowledge of the pathways are required. During the last few years our knowledge of individual pathways has substantially increased and has been used for their manipulation. This has been documented in several recent reviews for storage protein [2,3], carbohydrates [4–7], and lipids [8–12].

IV. GENETICALLY ENGINEERED TRAITS

A. Tomato Fruit Ripening

The industrialized agriculture requires not only vital and high-yielding crops, but these crops also need to be adapted to mechanical harvesting. Tomatoes, for instance, are harvested in the green stage (40% mature green and 60% immature green stage [13]) to handle the fruits mechanically and with sufficient shelf life. Consequently, the fruits cannot develop full flavor on the vine. Therefore, breeding was directed to select for rigid lines, which resist mechanical harvesting. Selection was performed in the United States with a special device for testing car bumpers. Interestingly, these bumpers must resist a crash with a speed of 13 km/h, whereas breeding resulted in tomatoes, that resist a crash of 35 km/h without being damaged [14].

However, to obtain fruits, that can be easily harvested mechanically and, moreover, that can fully mature on the vine, genetic engineering strategies have been followed. One strategy is to inhibit polygalacturonase (PG; EC 3.2.1.15), a cell wall-degrading enzyme that leads to fruit softening during ripening. Antisense expression under the control of the cauliflower mosaic virus (CaMV) 35S RNA promoter of a cDNA-encoding tomato PG, substantially reduced the PG activity, without interfering with the ripening process [15,16]. In homozygous lines, a reduction to less than 1% of normal tomato PG activity was detected, thereby preventing cell wall degradation and concomitant softening during normal ripening (17). This results in fruits with a better shelf life and improved processing quality.

Another strategy followed the use of chimeric genes to interfere with the hormonal balance during ripening by inhibition of ethylene biosynthesis. Ethylene formation proceeds from methionine through the intermediate 1-aminocyclopropane-1-carboxylate (ACC). This compound can be selectively degraded by ACC deaminase (EC 4.1.99.4). Thus, an ACC deaminase was isolated from a *Pseudomonas* strain, previously identified to grow on ACC as sole carbon source (18). Under the control of the CaMV 35S RNA promoter, the ACC deaminase gene was introduced into tomato, and it reduced ethylene synthesis up to 90%. These plants were phenotypically indistinguishable from controls, except for differences in the progression of ripening. Detached fruits ripen more slowly and show a reduced degree of softening and no overripening, as compared with normal tomatoes. After exogenous treatment with ethylene, maturation of transformed tomato fruits proceeds similarly to that of nontransformed controls. Thus, the inhibition of ethylene biosynthesis seems to be an alternative way to improve the processing behavior of tomatoes while allowing optimal flavor development on the vine.

B. Proteins

Until now, plant proteins were primarily used as nutrients occurring in complex mixtures together with lipids, carbohydrates, and other compounds. Nutritional properties of proteins are influenced by parameters such as amino acid composition, protein digestibility, or foaming and emulsifying ability. To increase nutritional properties, postharvest modifications consist of the addition of limiting amino acid, thermal denaturation, or limited proteolysis. An alternative to this might be modifications on the crop level by genetic engineering. However, such modifications are not likely to increase the price of the crop, whereas genetic engineering of peptides or proteins in plants eventually result in high-priced specialized products.

Thus, approaches to modify the protein content of crops can be classified as (1) modification of the amino acid composition for nutritional purpose, and (2) the production of high-value proteins (e.g., for pharmaceutical applications). Because up to now plant breeding has had only limited success in improving the nutritional quality of seed proteins, genetic engineering is expected to strongly support the breeders' efforts. However, difficulties may arise because seed storage proteins assume specific conformational structures, and modifications to these proteins must allow their proper deposition in the cell. On the other hand, new proteins might not accumulate (e.g., because of rapid degradation). Such concerns might be overcome by introducing in parallel stabilizing functions (e.g., involving the ubiquitin-dependent proteolytic pathways as reviewed recently [19]).

1. Improvement of Amino Acid Composition

The amino acid composition of storage proteins for nutritional purpose is frequently not optimal, lacking essential amino acids. For example, soybean protein lacks the sulfur-containing amino acids methionine and cysteine, and corn protein lacks lysine and tryptophan. To circumvent the problem of a suboptimal amino acid composition, genes encoding storage proteins with sufficient amounts of the desired amino acids can be introduced into a crop. Altenbach and co-workers [20] expressed a methionine-rich (18% Met) 2S albumin from Brazil nut under the control of a phaseolin gene promoter in transgenic tobacco. The seeds contained up to 8% 2S albumin in the total salt-extractable seed proteins and up to 30% more methionine compared with control plants. A similar experiment was performed by using a 2S albumin gene of *Arabidopsis thaliana*, which was engineered for methionine-rich sequences from Brazil nut 2S albumin [21]. Transgenic plants of *A. thaliana*, *Brassica napus*, and *Nicotiana tabacum* showed 1–2% engineered protein in the total salt-extractable seed proteins.

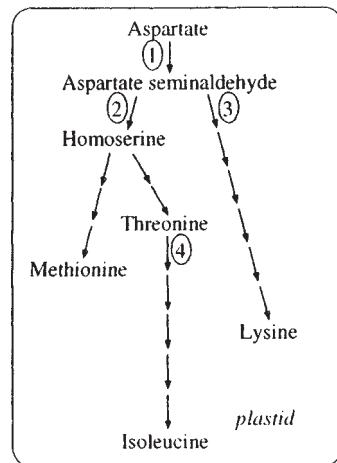


Fig. 1 Aspartate family pathway: sites of manipulation: 1, aspartate kinase; 2, homoserine dehydrogenase; 3, dihydronicotinate synthase; 4, threonine dehydratase.

An alternative strategy for the improvement of the amino acid composition is the attempt to directly interfere with the amino acid biosynthesis pathway. In plants as well as in bacteria, the biosynthesis of lysine, threonine, methionine, and isoleucine originates from aspartate by the aspartate family pathway (Fig. 1). The pathway is regulated by a few key enzymes that exist as isoforms showing different modes of feedback inhibition: (1) aspartate kinase (AK; EC 2.7.2.4) the major rate-limiting enzyme for threonine synthesis, which is feedback-inhibited by threonine and lysine; (2) dihydronicotinate synthase (DHPS; EC 4.2.1.52), the major rate-limiting enzyme for lysine synthesis, which is feedback-inhibited by lysine; (3) homoserine dehydrogenase (HSDH; EC 1.1.1.3), one isoform of which is feedback-inhibited by threonine; and (4) threonine dehydratase (TDHT; EC 4.2.1.16), which is feedback-regulated by isoleucine. In bacteria DHPS is less sensitive to feedback inhibition by lysine than the plant enzyme. Thus, a chimeric *Escherichia coli* DHPS gene under the control of the CaMV 35S RNA promoter was expressed in the cytoplasm and in the plastid of transgenic tobacco, respectively. As a result, an up to 15-fold higher level of free lysine was monitored, with expression in the chloroplast [22]. Similarly a mutant desensitized AK from *E. coli* was expressed in the chloroplasts of tobacco plants, resulting in an up to ninefold threonine-overproducing phenotype [23]. After crossing these two types of transgenic plants, levels of free lysine exceeded by far the level obtained by DHPS alone. However, concomitantly, a reduction in threonine, compared with plants expressing only AK, suggested a concerted regulation of AK and DHPS, which might involve also HSDH (24). In fact, it turned out that the regulation is still more complex: Although considerable accumulations of free lysine were detected in leaves of transgenic plants expressing *E. coli* AK and DHPS, in seeds the amounts of lysine declined to control levels. This might be due to the catabolism of free lysine in tobacco seeds [25]. In transgenic rapeseed the situation might be different, because a total increase in lysine was found in the seeds after transfer and expression of bacterial AK and DHPS genes [26].

2. High-Value Peptides and Proteins

In 1989, Vandekerckhove and co-workers first introduced a pharmaceutically relevant peptide, the pentapeptide leu-enkephalin, into rapeseed through an engineered 2S albumin gene of

Arabidopsis thaliana. The chimeric gene was constructed such that the codons for leuenkephalin replaced six codons of the 2S albumin gene sequence. After expression in transgenic rapeseed, leu-enkephalin could be released from purified chimeric 2S albumin at designed tryptic cleavage sites and removal of an extra lysine residue. These primary transformants could already yield 15–75 g of peptide per hectare [27]. The results of this concept are quite promising, but existing limitations and questions (see later discussion) need to be recognized and taken into consideration for further projects [28].

In addition to peptides, polypeptides have also been expressed in plants, such as the human serum albumin in transgenic potato and tobacco, providing a potential source for human serum albumin that is free of human pathogens [29]. Similarly, the trout growth hormone gene was introduced into transgenic plants and was expressed and correctly processed in leaves, but not in seeds [30]. A further example is the production of antibodies [31,32] and single-chain Fv antibodies [33,34] in transgenic plants. Such plant-based antibodies and their derivatives might be obtained in sufficient quantities for medical applications (e.g., for passive immunization and diagnostics, or for the development of novel concepts of plant tolerance against pathogens). Thus, a gene for a single-chain Fv antibody directed against artichoke mottled crinkle virus (AMCV) resulted in a reduction of virus infection following intracellular expression [34]. After secretion into the intercellular space, an antibody raised against tobacco mosaic virus (TMV) proved biologically active by reducing plant infection with TMV [35]. Similarly, experiments are in progress to engineer plants for resistance against *Erwinia* by expression of single-chain Fv antibodies directed against secreted pectolytic enzymes of *Erwinia carotovora* (T.Winkler, A.Schots, and K.Düring, unpublished data).

Although substantial progress in the production of proteins in transgenic plants has been made in the recent past, several parameters are not yet effectively controlled, including gene expression, protein folding, and protein processing, to guarantee the synthesis of sufficiently high amounts of a desired protein that is biologically functional.

C. Carbohydrates

Two major classes of carbohydrates are distinguished in plants: (1) high molecular weight glucans, such as cellulose, the major cell wall constituent, and starch, the primary storage carbohydrate in plants; and (2) low molecular weight compounds (sugars) such as glucose and fructose as intermediate metabolites, as well as sucrose, the primary transport metabolite, which exceptionally, also occurs as a storage compound in sugar beet or sugarcane. Cellulose, starch, and sucrose already contribute considerably (see Table 1) to important applications as industrial raw materials (Table 2).

1. Starch Synthesis and Sites of Manipulation

Starch consists of amylose, a polymer of 300–1200 $\alpha(1\rightarrow 4)$ -linked glucose relative molecular mass residues (50–150 kDa), and amylopectin, a branched polymer composed of 1500–12000 $\alpha(1\rightarrow 4)$ - and $\alpha(1\rightarrow 6)$ -linked glucose (300–2000 kDa). In amylopectin, branches occur, on average, at each 25th glucose residue. In the carbohydrate metabolism of plants, two types of starch can be distinguished: transitory starch and reserve starch. Transitory starch accumulates for a short time in chloroplasts before deposition as reserve starch in amyloplasts for long-term storage. Three enzymes in the biosynthesis of starch have been considered to catalyze key regulatory steps (Fig. 2): (1) ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27), (2) starch synthase (SSS and GBSS; EC 2.4.1.21), and (3) branching enzyme (BE; EC 2.4.1.18).

To modify natural types of starch Visser and Jacobsen describe various potential strategies, including overexpression or antisense repression of AGPase, SSS, GBSS, and BE [7]. Because

Table 2 Examples for Applications of Cellulose, Starch, and Sugar

Product	Application
Cellulose	Paper
	Textiles
	Chemically modified fibers
	Explosives (nitrocellulose)
	Cosmetics (toothpaste, creams)
Starch	Paper, cardboard
	Building material (plaster plates, mineral fiber plates)
	Adhesives
	Plastics (packing material, foils)
	Laundries (soaps, washing powder)
	Cosmetics (tooth paste, dry shampoo, face powder)
	Pharmaceuticals (tablet, antibiotics, vitamin C)
Sugar	Pharmaceuticals (amino acids, vitamin B ₁₂ , antibiotics, citric acid)
	Dyes, paints
	Adhesives
	Soaps, washing powder
	Plasticizer

the level of phosphate, particularly in starch from potato tubers, affects the viscosity of gelatinized starch, a modulation of the degree of starch phosphorylation occurring concurrently with de novo synthesis [36] is desirable. This is of particular interest for improvement of the properties of starch from corn, which is not naturally phosphorylated.

2. Starch Yield

Besides altering the quality of carbohydrates, quantitative improvements have been achieved. Stark and co-workers [37] introduced the *glgC16* gene from *E. coli*, encoding a mutated form

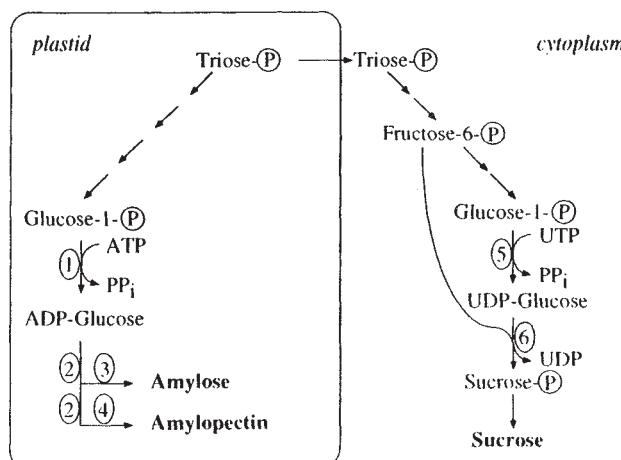


Fig. 2 Pathway of starch and sucrose metabolism; sites of manipulation: ATP, adenosine triphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; P, phosphate group; PP_i, inorganic pyrophosphate; 1, ADP-glucose pyrophosphorylase; 2, soluble starch synthase; 3, granulebound starch synthase; 4, branching enzyme; 5, UDP-glucose pyrophosphorylase; 6, sucrose-phosphate synthase.

of ADP-glucose pyrophosphorylase (AGPase) into potato tubers. AGPase catalyzes a key regulatory step in starch biosynthesis—the formation of ADP-glucose from glucose-1-phosphate and ATP. In *E. coli* the mutated gene resulted in an approximately 33% increase in glycogen accumulation compared with the wild-type AGPase owing to altered allosteric properties of the protein. In transgenic potato the *glgC16* gene fused to a transit peptide of the small subunit ribulose 1,5-bisphosphate carboxylase gene from *Arabidopsis* and controlled by a tuber-specific patatin promoter, resulted, on average, in 35% more starch than in controls and amounted to an increased starch content of nearly 60% in the best lines. In comparison with this, expression of the *E. coli* wild-type AGPase gene did not result in a significant increase in starch content in transgenic potato tubers [37]. Therefore, the altered allosteric properties make the mutated *glgC16* gene a valuable tool for the quantitative improvement of starch in plants and proves that AGPase is rate-limiting in starch biosynthesis because of its allosteric regulation, rather than the amount of enzyme.

On the other hand, a reduction of starch synthesis was achieved when AGPase antisense constructs were introduced into the potato genome [38]. The antisense construct of one of the AGPase subunits, under the control of the CaMV 35S RNA promoter, abolished starch formation in tubers. Concomitantly, an increasing amount of soluble sugars was detected: up to 30% sucrose and up to 8% glucose, based on dry weight. Simultaneously, more tubers were formed, but they were smaller and accompanied by an increase in fresh weight and a decrease in dry weight.

The increase in starch yield owing to overexpression of the *glgC16* AGPase and the formation of sugars owing to antisense inhibition of the resident AGPase demonstrated the key regulatory function of this enzyme in starch metabolism. Therefore, controlling the expression of this protein by genetic engineering revealed a major key for alterations of the starch biosynthetic pathway. This includes the replacement of starch biosynthesis by other metabolic pathways of interest (e.g., formation of cyclodextrins [39] or polyhydroxybutyrate [40]).

As already shown for lipids (see Sec. IV.D.), mutants affecting the quality of storage products might be useful to assemble novel or altered biosynthetic pathways. Mutants affecting the composition and content of reserve starch in higher plants have been reviewed [7]. Highamylose and high-amyllopectin types have been found in different plant species, but these mutants showed a significantly reduced yield. The introduction of the *E. coli* *glgC16* gene for AGPase, for instance, into these mutants might result in a restoration of the reduced yield.

3. High-Amylose or High-Amylopectin Starch

The use of starch depends on the structure of starch granules as well as on its composition of amylose, amylopectin, and minor compounds (lipid, protein, phosphate). It is the ratio of amylose to amylopectin and the distribution of low molecular weight glucans that affects starch granule properties such as gelatinization temperature, retrogradation, and viscosity. Owing to its physicochemical, nutritional, and textural properties starch is widely used in food industry. However, the capacity of water imbibition, of film formation, and the viscosity are typical characteristics of starch for industrial use. Traditionally in the nonfood sector, starch is used primarily in the manufacture of paper and cardboard, but also for chemicals, coatings, pharmaceuticals, adhesives, and textiles. To date, more than 600 products contain starch with its use still increasing [41]. Products based on starch fermentation, starch-based polymers, binding material, and specialized chemicals after chemical modification belong to the most promising extended applications for starch as raw material.

The requirements for the use of starch depend on the kind of industrial application. Good separations of starch from protein, lipids, and fibers are general prerequisites. Small grains

and minor variation in grain size are important as filling material in thermoplasts. Starch consisting of more than 90% amylose seems to be suitable for the production of films. Depending on the application, both amylose-rich starch and amylopectin-rich starch are required as binding materials. In summary, the industrial applications of starch depend on its particular chemical composition in a selected genotype of a certain plant species. Breeding programs for altered starch composition have frequently resulted in lines with substantially reduced yields, which are unable to compete on the market. Thus, there is an interest in exploring the possibilities of improving starch quality by genetic engineering.

The primary goals for manipulation of carbohydrate metabolism toward starch quality was to produce only one type of the starch constituents, amylose or amylopectin. The expression of an antisense gene for granule-bound starch synthase (GBSS), the key enzyme in amylose formation, under the control of the CaMV 35S RNA promoter in transgenic potato plants, resulted in potato tubers devoid of amylose [42]. This antisense construct gave rise to changes in starch composition both in tubers and in aerial parts of the plant. In potato tubers an inhibition of GBSS of 70–100% was measured. A partial reduction of GBSS resulted in a reduced amount of amylose, whereas total inhibition of GBSS resulted in tubers lacking amylose. Inhibition of amylose was also found with expression of a GBSS cDNA under the control of the potato GBSS gene promoter [43], which is highly active in tubers. Moreover, this promoter also resulted in complete inhibition of GBSS in transgenic potato plants [44] when used to control the antisense expression of a heterologous GBSS cDNA from cassava (*Manihot esculenta*), which shows nucleotides that are 74% identical with the potato GBSS gene.

However, despite the various possibilities of reducing the level of GBSS in potato by antisense repression, it is crucial that the introduced trait is stable in the field. This could be clearly demonstrated by comparison of four transgenic lines in a series of field experiments carried out in two successive years [43,45]. These lines were selected for different levels of reduced amylose content, depending on the antisense gene construct. The field trials revealed stable and total inhibition of GBSS gene expression in one of these lines. Additionally, these experiments showed that stable clones have to be selected from several independent transgenic lines. Probably owing to position effects of the integrated T-DNA, some lines turned out to be not totally stable, whereas others showed clear antisense phenotypes in the soil that were previously less pronounced in vitro. Moreover, the field experiments revealed no obvious effect on yield and dry matter content [43].

Another starch synthase, the *E. coli* glycogen synthase *glgA* (EC 2.4.1.21), was fused to a chimeric transit peptide of the small-subunit ribulose 1,5-bisphosphate carboxylase gene under the control of a tuber-specific patatin promoter [46]. This construct expressed in transgenic potato resulted in a rather low accumulation of mRNA and small amounts (0.007–0.028%) glycogen synthase protein. Nevertheless, clearcut alterations of the carbohydrate content could be monitored: a 60–80% increase in soluble sugar content, a 20–50% reduction in starch content, reduced starch phosphorylation and, most strikingly, an increase in amylopectin content and the number of its branches. AIC though probably a soluble starch synthase, in transgenic potatoes *E. coli* glycogen synthase obviously resulted in an increased degree of branching of amylopectin by an as yet unknown mechanism. Notwithstanding this, on transfer and expression of the *glgA* gene, a novel highly branched glucan was found, showing unique physicochemical properties [46].

A second target for engineering starch quality might be the engineering of BE expression. A cDNA of potato BE has been cloned by Kossmann and co-workers [47], which might be useful for an antisense approach. However, as yet no obvious effect of an antisense BE cDNA controlled by the CaMV 35S RNA promoter has been observed in transgenic potato (B. Müller-Röber, personal communication).

4. Fructans

Additional, however less common, storage carbohydrates are fructans, which occur predominantly as unbranched polyfructosylsucrose. For instance, inuline, a $\beta(2\text{-}1)$ -linked fructan of up to 35 fructosyl residues with one terminal glucose molecule, is found among others in dahlia or Jerusalem artichoke. Most fructans contain a terminal glucose, indicating that they are synthesized by addition of fructosyl moieties onto a sucrose molecule as primer. They are water-soluble and are deposited mainly as storage compounds in vacuoles. Fructans may also have other functions, because they are involved in mediation of cold tolerance (e.g., levans are found in leaves and stems of many cool-season grasses). Levans consist of $\beta(2\text{-}6)$ -linked fructosyl residues, with a degree of polymerization, ranging from a few to up to 300 fructose units, with a terminal glucose molecule. Fructans are also found in certain microorganism and may consist of more than 100,000 fructose units [48]. To produce fructans in transgenic plants, van der Meer and co-workers introduced two different microbial genes, the *sacB* gene from *Bacillus subtilis*, encoding levansucrase (EC 2.4.1.10), and the *ftf* gene from *Streptomyces mutans*, encoding a fructosyltransferase into potato. The two genes were expressed under the control of the CaMV 35S RNA promoter, using vacuolar targeting sequences. Transgenic potato plants contained fructans up to 30% of dry weight in leaves and up to 7% of dry weight in microtubers. The degree of polymerization exceeded 25,000 fructosyl residues [49]. Thus, the production of unusual carbohydrates in potatoes opens up a field for further diversification of carbohydrates in crops. As a sucrose-storing and high-yielding crop sugar beet might be an ideal target for genetic engineering of fructane production in plants.

5. Sugars

Sucrose, a disaccharide of $\alpha(1\text{-}2)$ -linked glucose and fructose, is the number 1 sweetener used in food industry. For nonfood applications sucrose became an important raw material ([Table 2](#)) owing to its high purity (important for fermentation and chemical syntheses) and its physical properties (e.g., hydrophilicity). The synthesis of sucrose is schematically outlined in [Figure 2](#). The key enzymes in sucrose synthesis are sucrose-phosphate synthase (SPS; EC 2.4.1.14) and sucrose phosphatase (EC 3.1.3.24). Worrel et al. [50] proved that SPS plays a crucial role in photosynthate partitioning by expressing a maize SPS cDNA in transgenic tomato plants. SPS expression under the control of the small-subunit ribulose 1,5-bisphosphate carboxylase promoter revealed an increase of sucrose up to 50% in leaf tissue, with a concomitant reduction in starch. In contrast, investigation of the potato uridine diphosphate-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) controlled by the CaMV 35S RNA promoter showed no substantial effect on carbohydrate metabolism in potato plants expressing only 4% of the wild-type UGPase activity [51].

D. Oils and Fats

Oils and fats are the most energy-rich constituents used in human consumption, as well as in animal feeding; most of the produced oils and fats are used for nutritional purposes. In 1991, for instance, the worldwide production totaled 81.5 million metric tons, 87% of which were used for food and feed, whereas 13% went into technical applications or oleochemical syntheses [52]. The primary constituents of oils and fats are the triacylglycerols (TAGs), which are composed of fatty acids and glycerol. Those TAGs that are liquid at room temperature are called oils, those solid under these conditions are named fats. Their physical properties and taste make them useful for the production of margerine, shortenings, salad oils, and such. In

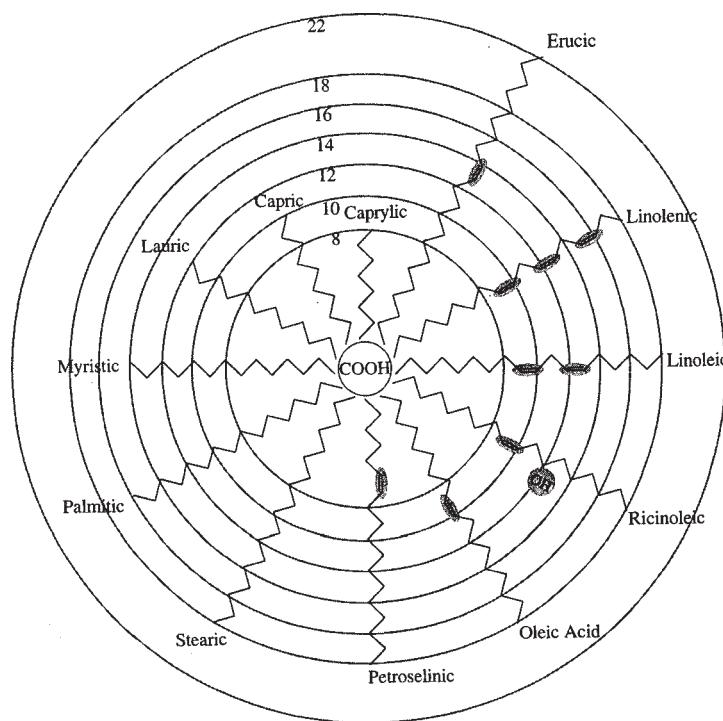


Fig. 3 Types of fatty acids in plant storage oil: Double bonds and functional groups; circles indicate chain length.

addition, the fatty acid composition of TAGs determine the range of applications of oils and fats for nutritional purposes, because certain lipids are considered to be cholesterogenic, whereas others are not. Furthermore, particular fatty acids open up a large field of technical applications for oleochemical syntheses. These nonfood applications depend on the fatty acids composition of TAGs and, in particular, on the availability of homogeneous fatty acyl residues. More than 210 different kinds of fatty acids are found in seeds and fruits of plants, but as yet, just a few are of importance for nonfood applications. Examples are lauric acid ($C_{12:0}$), which is widely used for production of detergents and surfactants; linoleic and linolenic acid ($\Delta 9, 12C_{18:2}, \Delta 9, 12, 15C_{18:3}$), used for paints, varnishes, and coatings; also ricinolic acid ($\Delta 9, 12OH-C_{18:1}$) for coatings, and lubricants; as well as erucic acid ($\Delta 13C_{22:1}$) as lubricant, antislip agent, for nylon formation and such. These examples describe the differences found in fatty acids (Fig. 3): (1) variation in chain length and (2) variation in position and number of double bonds and functional groups. There is one general breeding goal: a desired fatty acid of more than 90% purity. The typical crops for the production of new oils and fats are annual crops. Rapeseed is currently the most efficiently engineered oil crop, followed by soybean. Both crops provide not only the oil that is used, but also protein for animal feeding, which contributes largely to the price of the final product oil.

1. Fatty Acid Biosynthesis and Sites of Manipulation

Oleic acid is the major product of fatty acid biosynthesis in plants and is incorporated into storage TAGs during seed maturation. In rapeseed, for instance, the photosynthate, sucrose, is

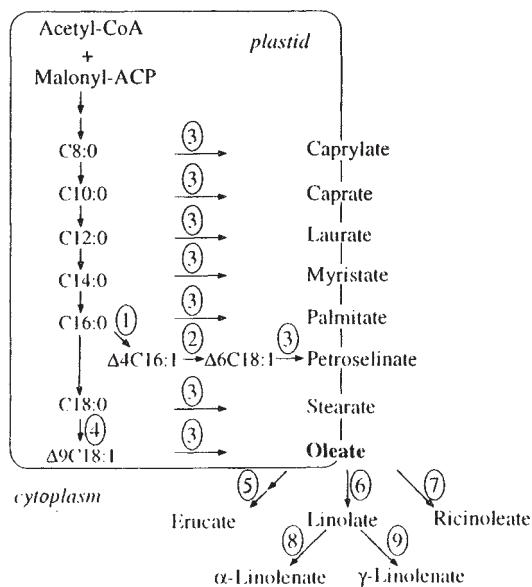


Fig. 4 Pathway of fatty acid metabolism; sites of manipulation: CoA, coenzyme A; 1, Δ4-palmitoyl[ACP] desaturase; 2, β-ketoacyl-[ACP] synthase; 3, acyl-[ACP] thioesterase; 4, Δ9-stearoyl-[ACP] desaturase; 5, oleoyl-CoA elongase; 6, Δ12-oleate desaturase; 7, Δ12-oleate hydroxylase; 8, Δ15-linolate desaturase; 9, Δ6-linolate desaturase.

transported from leaves and pericarp to the maturing seeds, where its activated breakdown product acetyl-CoA is available for the *de novo* fatty acid biosynthesis in plastids of embryos. The individual enzymes of fatty acid synthase (type II) consecutively polymerize C₂-units derived from acetyl-CoA to a growing acyl chain, which is bound to a small protein (acyl carrier protein; ACP). In a series of seven condensation cycles a C_{16:0} acyl thioester, palmitoyl[ACP] is produced, which requires among others the action of a condensing enzyme (β-ketoacyl-[ACP] synthase I [KAS II]; Fig. 4). Premature chain termination by thiolytic cleavage of acyl-[ACP] intermediates through the expression of heterologous acyl-[ACP] thioesterases can result in the formation of C_{8:0}–C_{14:0} short- to medium-chain fatty acids.

In coriander seeds palmitoyl-[ACP] is the branch-point for petroselinic acid production. However, in most lipid-accumulating plant tissues palmitoyl-[ACP] is elongated to C_{18:0} stearoyl-[ACP] by a specific KAS II. The subsequent first desaturation of stearoyl-[ACP] to oleoyl-[ACP] is catalyzed by a soluble Δ9 stearoyl-[ACP] desaturase (EC 1.14.99.6) in the plastids and, hence, constitutes the target for specific downregulation of this enzyme to produce oil with high contents of saturated fatty acids. The reverse, a specific up-regulation of stearoyl-[ACP] desaturase, can result in almost no stearic acid [53] (Table 3). Usually, oleic acid is the major fatty acid intermediate of this plastidial pathway, which is released by the action of an oleoyl-[ACP] thioesterase (EC 3.1.2.14) and while exported to the cytosol is activated to oleoyl-CoA by an acyl-CoA synthetase in the outer envelope. This is required for the stepwise acylation of glycerol-3-phosphate. The final products of this sequence of events, localized in the membranes of the endoplasmic reticulum (ER), are TAGs, which are deposited in oleosomes. The introduction of additional double bonds catalyzed by ER membrane-bound enzymes, interrupts the direct acylation sequence. With the help of separate enzyme systems, the acyl residues are transferred onto phospholipids, desaturated, and returned to complete TAG synthesis. One of the best

Table 3 Alteration in Fatty Acid Profiles of *Brassica napus* (mol%)

Fatty acids ^a	C _{8:0}	C _{12:0}	C _{10:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{22:1}
++ rapeseed					3	1	17	14	9	50
00 rapeseed (Canola)					4	2	62	22	10	0
<i>CITEg100</i>	1	3								
<i>CITEg200</i>					7	15				
<i>CtTE</i>	11	27								
<i>UcTE</i>				50						
<i>CtTE</i>						25				
<i>BcΔ9DES</i>							0.7			
<i>BcΔ9DESas</i>							40			
<i>BnΔ12DES(fad2)as</i>								83		
<i>BnΔ12DES(fad2)as</i> × IMC 129 (78%)								88		
<i>ScKAS</i> elong. × Canola									20	

^a++, conventional rapeseed containing erucic acid, 00, conventional rapeseed without erucic acid (canola); *CITEg100* and *CITEg200*, acyl-[ACP] thioesterase genes from *Cuphea lanceolata*; *UcTE*, acyl-[ACP] thioesterase gene from *Umbellularia californica*; *CtTE*, acyl-[ACP] thioesterase gene from *Carthamus tinctorius*; *BcΔ9DES*, Δ9 desaturase gene from *Brassica rapa (campestris)*; *BcΔ9DESas*, antisense Δ9 desaturase gene from *B. rapa*; *BnΔ12DES(fad2)as*, antisense Δ12 desaturase gene from *B. napus*; *BnΔ12DES(fad2)* as × IMC 129, *BnΔ12DES(fad2)as* plant crossed with the IMC129 mutant of rapeseed; *ScKAS*, β-ketoacyl-[ACP]synthase of the elongase from *Somondisia chinensis* in canola.

substrates for desaturation is phosphatidylcholine [54,55]. The relative activities of TAG synthesis and desaturation determine the amount of plastid-exported oleic acid to be deposited as trioleate in oleosomes [56]. Therefore, down-regulation of the microsomal Δ12-desaturase (EC 1.3.1.35) should prevent further desaturation of oleate residues to linolate and linolenate and, hence, result in a high oleic acid rapeseed.

In castor bean seeds the pathway for the introduction of a functional hydroxy group at the Δ12-position of oleate residues proceeds in the same manner as that for the introduction of additional double bonds. Efficient substitution of the down-regulated desaturation pathway by insertion of a gene coding for castor bean oleate Δ12-hydroxylase (EC 1.14.13.26) into high oleic acid rapeseed varieties is an obvious itinerary for high ricinoleic acid production. Yet a different metabolic pathway starting from oleoyl-CoA ($\Delta 9 C_{18:1}$) is the two-step elongation to erucoyl-CoA ($\Delta 13 C_{22:1}$), which is active in erucic acid rapeseed varieties and subject of genetic engineering. Modifications of the biochemical pathways for fatty acid biosynthesis and TAG assembly in and beyond the afore-highlighted and the following outlined projects have been greatly facilitated by the recent availability of numerous genes for these pathways [for review, see 57,58].

2. Saturated Fatty Acids

The first successful alteration of fatty acid composition in a storage oil was the increase in stearic acid content in rapeseed. In an antisense RNA approach Knutzon and co-workers [59] transferred a chimeric seed-specific gene encoding a Δ9-stearoyl-[ACP] desaturase from *Brassica rapa* into rapeseed. In seeds of these plants the amount of desaturase protein was reduced, resulting in a less efficient formation of $C_{18:1}$ and thus up to 40% stearate was measured (see Table 3). The oil might be used as cocoa butter substitute for margerine formulations and the production of confectionery fats.

3. Short- and Medium-Chain Fatty Acids

At the same time, redirection of fatty acid biosynthesis toward lauric acid production in a temperate oilseed crop was reported [60], which is considered a major breakthrough in the field. Current sources of laurate are from tropical crops, including coconut and palmkernel oils for industrial uses as major component in laundry detergent, shampoo, and other surfactants. For this use saturated fatty acids with a 12-carbon chain length, such as laurate, have ideal properties, because this chain length provides a balance of solubility in both aqueous and nonaqueous environments. The world demand for laurate is primarily satisfied by the aforementioned tropical oils. Alternative sources, such as domestication of *Cuphea* species, each harboring a distinct type of medium-chain fatty acids (C₈–C₁₄) in high individual quantities [61] or chemical oxidation of petroselinic acid (see Sec. IV.D.4) have been discussed. Yet a totally different approach to a novel and domestic laurate source was paced by Voelker and his colleagues. They attempted to engineer the production of lauric acid into canola, a crop of temperate climate requirements. Therefore, a prerequisite was to determine the mechanism by which some plants are able to produce high levels of medium-chain fatty acids. Seeds of the California bay tree (*Umbellularia californica*) storing up to 70% laurate in their TAGs provided a highly active and specific acyl-[ACP] thioesterase [62]. An isolated cDNA encoding this enzyme was introduced under the control of an early seed-specific napin promoter—terminator cassette into the canola background. The pivotal role played by this enzyme in prematurely hydrolyzing the growing ACP-bound acylchain, thereby controlling lauric acid production, was confirmed when transgenic *Arabidopsis* plants expressing this thioesterase accumulated up to 25% lauric acid in their seed oils [60]. More recently, field trials of the most advanced transgenic rapeseeds with approximately 45% C_{12:0} showed that appearance, seed oil yield, and other agronomic traits are not changed, despite the seeds producing a totally different kind of storage oil [63]. The high-laurate canola line contains nine integration events. In October 1994, the USDA Federal Register gave nonregulated status to this genetically engineered laurate canola; hence, a market-sustained cultivation with a limited acreage has already been commenced in southern Georgia (V.Knauf, personal communication). To increase laurate levels beyond the observed 50% levels, a coconut gene encoding a specific lysophosphatidic acid acyltransferase (LPAAT; EC 2.3.1.51) will be introduced into high-laurate-producing rapeseed lines [64]. Developments in this area have progressed very much and using various acyl-[ACP] thioesterase genes from *Cuphea hookeriana* (K.Dehesh, D.S.Knutzon, A.Jones, and T.A. Voelker, unpublished data), *Cuphea lanceolata* (N.Martini and R.Töpfer, unpublished data), and *Carthamus tinctorius* (A.Jones, H.M.Davies, and T.A.Voelker, unpublished data) they have already resulted in the novel formation of C_{8:0}, C_{10:0}, and C_{14:0}, as well as in an increase of C_{16:0} in transgenic rapeseed lines (see Table 3).

4. Petroselinic Acid

In coriander seeds, an isomer of oleic acid, petroselinic acid (Δ6C_{18:1}) is stored up to 80%. Petroselinic acid may be of commercial interest because it can be oxidized by ozone to form lauric acid, which is used in detergents, and adipic acid, a C_{6:0} dicarboxylic acid, which is used as monomer for nylon 6,6 formation. In addition, petroselinic acid may have useful properties for cosmetic and pharmaceutical applications [9]. For instance, this acid melts at 33°C in contrast with its more common isomer oleic acid (melting point: 12°C). Therefore, attempts are being made to develop coriander into a useful crop. Besides raising the yield of coriander oil, there is a need to decrease the susceptibility of coriander to various diseases. There are small breeding programs going on, which would be intensified if an increased market developed. In fact, there is no significant market for coriander as a crop, because there is as yet no

constant supply of good quality coriander oil at a low price level. As an alternative to exploit coriander seeds there are attempts to genetically engineer petroselinic acid in a high-yielding oil crop, such as rapeseed. Initial transformation of tobacco with a cDNA encoding an acyl-[ACP] desaturase involved in petroselinic acid formation in coriander resulted in a 5% petroselinic acid production of the total fatty acid synthesized in tobacco [65]. In parallel the biosynthetic pathway of petroselinic acid in coriander was elegantly elaborated by Ohlrogge and coworkers [12,66]. A specific desaturase ($\Delta 4$ -palmitoyl-[ACP] desaturase) introduces a *cis* double bond in the $\Delta 4$ -position of palmitoyl-[ACP], The 16-carbon *cis*- $\Delta 4$ C_{16:1} is elongated by a proposed specific condensing enzyme to form petroselinoyl-[ACP]. A specialized acyl-ACP thioesterase with high selectivity for petroselinoyl-[ACP] is responsible for the release of petroselinic acid [66]. This example demonstrates that besides a number of factors that may contribute to the low yield of petroselinic acid in transgenic tobacco, not always will the transfer of a single gene be sufficient to result in high-yielding products. In petroselinic acid production, at least three different enzymes appear to be involved.

5. High Oleic Acid Rapeseed

Classic breeding for high oleate (sunflower, rapeseed) and high linolenate oil seeds (linseed) demonstrated, that a reduced degree of desaturation of fatty acids had no effect on the amount of TAGs. In rapeseed, the increase of oleic acid from about 60 to approximately 80% (see the IMC129 mutant in Table 3) had no obvious harmful effects on the crop. This high oleic acid content is followed by a reduction in polyunsaturated fatty acids. Further increase does not seem possible because the desaturases that need to be inactivated are active in both storage and leaf tissues [67]. A biotechnological approach that specifically down-regulates a particular enzyme function in embryos is likely to reduce further the proportion of linoleic and linolenic acid and, thereby, increase the oleic acid content, without changing the oil yield. Transformation of rapeseed with a cDNA coding for a microsomal $\Delta 12$ -desaturase in antisense orientation should prevent the formation of linolate and, consequently, of linolenate. Thus, antisense repression of the $\Delta 12$ -desaturase in rapeseed resulted in an increase in oleic acid up to 83% (see Table 3). Crossing of this line with the aforementioned IMC129 mutant resulted in a rape-seed line of 88% oleic acid (see Table 3) in the TAG fraction [68]. This is a substantial increase of oleic acid in rapeseed and, because this is a currently developing area, it is expected that rapeseed lines will become available that produce more than 90% oleate. The supply with more oleate per hectare will drastically reduce the costs for downstream processing for industrial applications. This includes, for instance, ozone oxidation of oleic acid to produce azelaic acid, which is a C₉ straight-chain, saturated, dibasic acid used in the manufacture of polymers, fibers, films, and adhesives. In addition it can be used as plasticizer [69].

6. Ricinoleic Acid

In 1993, about 460,000 tons of castor oil were produced worldwide [70]. Brazil, India, the former USSR, and the United States are the main producers, whereas in the European Union only Italy, Spain, and France contribute on a limited scale. Castor oil consists of up to 90% ricinoleic acid ($\Delta 12$ -hydroxyoleic acid) highly enriched in triricinolein (68%), which is derived from the seeds of castor bean (*Ricinus communis*). The first pressing yields No. 1 oil (United States), with 2% free fatty acid suitable for medicinal application and virtually free of the three toxins, which remain in the residual meal: ricinine, a mildly toxic alkaloid; ricin, an extremely poisonous protein; and a heat-stable allergen, making up approximately 12% of the cake. In addition, castor oil is used to produce polymers, cosmetics, lubricants, plasticizers, coatings, and surfactants [71]. Besides the poisonous by-products for both humans and animals,

wide fluctuations in commodity prices ranging from 450 to 1200 dollars a ton of seeds during the last decade have been observed. These fluctuations are mainly due to the high susceptibility of the castorbean crop to climatic variations. As a consequence, these adverse conditions have led to a reluctance of companies to invest in research and development programs to expand further applications of castor oil [70]. There are two possible alternatives to obtain hydroxylated oils: (1) by chemical hydroxylation or (2) by in planta hydroxylation of high oleic acid rapeseed oil. For the latter, isolation of the hydroxylase gene from castorbean is in progress in several laboratories in the United Kingdom and the United States. Once the gene is available it will be inserted into a high-oleic acid-producing rapeseed variety to produce the desired high-ricinoleic acid seed oil in a crop that can be cultivated under temperate climatic conditions.

7. High-Erucic Acid Rapeseed

The long-chain monounsaturated 22-carbon atoms of erucic acid ($\Delta 13C_{22:1}$) give rise to several applications in lipochemistry: as additive to lubricants and solvents, as softener for textiles, or as its amide derivative used in polymer techniques. In particular, the cleavage of the double bond in erucic acid by oxidation results in the production of pelargonic acid with 9 carbon atoms and brassic acid, a dicarboxylic acid, with 13 carbon atoms. Besides the application of brassic acid as perfumery agent, it may well be the base for the development of a C_{13} chemistry. The lack of a sufficient supply of erucic acid prevents this development. Erucic acid might have a larger potential as a precursor to nylon 13,13, which is a high-temperature thermoplastic. For this, and many other nonfood market segments, raw materials from plants frequently compete economically with petrochemicals. If the yield in erucic acid from rapeseed was increased from its current level of about 50% to more than 90%, the costs could be reduced substantially, providing both economically and environmentally (CO_2 -neutral) reasonable alternative routes to use of petroleum as petrochemical source [12]. In 1992 high-erucic acid rapeseed (HEAR) was cultivated in Europe on about 5000 ha, a prognostic view estimates that 150,000 ha HEAR could be cultivated without facing the problem of overproduction [72]. The established worldwide market for erucic acid is currently estimated at over 100,000 tons a year of HEAR oil, and is expected to grow at a rate of 5% a year. This is mainly due to the increased demand of erucic acid for the production of erucamides, lubricants, surfactants, and cosmetics. The typical erucic acid content of most HEAR varieties is between 45 and 55%. Future classic-breeding efforts may allow increased proportions to as high as the theoretical 66%, whereas it is unlikely that even higher levels of erucic acid can be achieved applying traditional sources of genetic variation. Rapeseed appears to exclude specifically erucic acid from the *sn*-2 position of the glycerol backbone in triacylglycerol formation, unlike for example, seeds of *Limnanthes* species, which provide the appropriate catalytic microsomal 1-acylglycerol-3-phosphate acyltransferase or lysophosphatidic acid acyltransferase (LPAAT), allowing trierucin assembly in these species [73]. To genetically engineer this desirable trait into existing HEAR varieties the LPAAT protein in rapeseed has to be replaced. Therefore, cDNAs from *Limnanthes douglasii* encoding an erucoyl-CoA-specific LPAAT have been isolated [74]. It is proposed that overexpression of an erucoyl-CoA-specific LPAAT cDNA and concomitant down-regulation-inhibition of the inherent LPAAT gene by an antisense RNA approach in rapeseed might produce oil containing trierucin. To supply sufficient amounts of erucoyl-CoA to drive the yield of the desired product to up to 90%, there might well be the need to add genes encoding catalytic microsomal fatty acid elongation functions, such as $C_{18:1}$ -CoA/ $C_{20:1}$ -CoA synthase. A recent report on the successful transfer of a jojoba elongase gene in a canola background, resulting in 20% erucic acid (see Table 3), demonstrates that this particular gene function is now also at hand [75].

E. Polyhydroxybutyrate

In all organisms the precursor of acyl lipid formation is acetyl-CoA, whereas in some bacteria, such as *Alcaligenes eutrophus*, the acetyl-CoA pool is also shunted for the production of the commercially valuable, aliphatic polyester with thermoplastic properties, polyhydroxybutyrate (PHB), rather than TAGs. The pathways for PHB synthesis from acetyl-CoA involves three consecutive catalytic steps: β -ketothiolase, acetoacetyl-CoA reductase, and PHB synthase. In turn, a wide variety of microorganisms can hydrolyze PHB and other polyhydroxyalkanoate (PHA) polyesters, which serve as carbon source. Therefore, these polymers have attracted interest as a potential source of renewable biodegradable thermoplastics. PHB production by means of bacterial fermentation results in significantly higher input than the cost of starch or oil production from crop plants. Initial transformation of the acetoacetyl-CoA reductase and PHB synthase genes from *A. eutrophus* in *Arabidopsis* was designed to explore the feasibility of PHB synthesis in plants. The cytosolic expression of these genes in *Arabidopsis* resulted in the accumulation of small quantities of PHB granules, with properties similar to the bacterial PHB, throughout the cells in leaves of transgenic plants. Unfortunately, plants were severely stunted in growth. This may be due to the depletion of the cytoplasmic acetyl-CoA pool by diverting acetoacetyl-CoA from mevalonate synthesis to PHB formation [76]. A potential strategy to circumvent this problem, is to direct the gene products to the plastids, for two obvious reasons: (1) plastids are the site of fatty acid biosynthesis for membrane and storage lipids; therefore, the flux through acetyl-CoA is relatively high; and (2) the high level of starch accumulation in this organelle is apparently not interfering with its function (i.e., plastids can cope with the deposition of insoluble storage compounds). Therefore, all three PHB biosynthetic genes (including that coding for β -ketothiolase) were each transferred into *Arabidopsis* under the control of a constitutive promoter and a pea chloroplast-targeting sequence to ensure expression of the gene products and PHB accumulation only in the plastids. Following sexual crosses, plants were obtained expressing all three genes and accumulating PHB up to 14% of the dry weight as 0.2- 0.7- μm granules within plastids, without showing any obvious effect on growth or fertility of the transgenic plants [40]. In parallel, efforts have been made to introduce the trait for PHB accumulation into rapeseed under the control of seed-specific promoter elements containing plastid-targeting sequences. The envisaged aim is to obtain PHB deposition in rapeseed, where up to half of the seed oil is substituted by PHB. These transgenic seeds should be able to germinate while they also contain economically useful quantities of PHB [11,77].

V. CONCLUSIONS

The strategies for genetic engineering developed during the last decade are well established and have proved successful. Major breakthroughs for the engineering of metabolic pathways have been achieved. Now the fine-tuning and product development is needed as well as a success of the new crops and products on the market. In certain cases, the plant material used for transformation needs to be preselected for a desired trait, and genetic engineering might help overcome key steps of a given pathway limiting optimal plant product formation. If we consider industrial traits, it is still difficult to predict which degree of alteration an engineered plant can tolerate relative to protein stability and function, changing metabolites, and introduction of new pathways, respectively. For the future, more insight into the biochemistry as well as the regulation and interaction of pathways will contribute to a foreseeable improvement of plant products for industrial purpose.

Present limits of engineering pathways will be overcome by a major contribution of gene technology itself. The generation of mutants by transformation will provide a tool for in vivo analysis of pathways leading to a far better understanding of a particular pathway and, in addition, of the interaction of pathways. The study of partitioning of photosynthate will enable us to substantially improve yield characteristics for more productive plants. The present major bottleneck for this seems to be our limited understanding of the complexity of metabolic pathways, which will progressively be overcome, enabling us to design desired alterations and products in crops.

However, the perspectives of gene technology that are currently being inserted into the concert of existing methods in plant breeding, should not result in a further limitation of the number of crop species cultivated. I contrast, it should lead to the introduction of new crops into the market, in particular for nonfood purpose. These plants need to be improved for the plant product itself, as well as for other agricultural traits. Genetic engineering seems to be the only technique that is expected to result in plants for the production of renewable resources in sufficient quality and at low price. However, it is anticipated that the demand of 200 million metric tons of mineral oil (see Table 1) cannot simply be substituted by natural products. A concert of several different activities is required.

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Production of Secondary Metabolites

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I. INTRODUCTION

Concerns for health and well-being have made plants collected for drugs, colors, spices, and fragrances a focal point of curiosity since the dawn of history. The arduous route from there to analysis and production of plant drugs is marked by medieval herbals, by the discovery of morphine in opium (Serturner, 1783–1841), and synthesis of acetylsalicylic acid (Aspirin/BAYER-Leverkusen) by H.Dreser in 1893. Biotechnology-assisted production of drugs from plants is rooted in physiological plant anatomy (Haberlandt, 1854–1945), in vitro culture of plant cells [White, 1939; Gautheret, 1939], phytochemistry (Mothes, 1900–1983), the design of bioreactors [Tulecke and Nickell, 1959], and plant transformation [Horsch et al., 1985]. Today, recognition of ecologic implications mandates that research and development (R&D) personnel better understand the synthesis and function of plant metabolites and guarantee supply while guarding natural resources. The recent paclitaxel (Taxol) supply crisis [Cragg et al., 1993] may underscore academic and public demand.

Primary metabolites in plants (i.e., proteins, oils, and starches) maintain essential physiological processes. Secondary metabolites, such as alkaloids, terpenoids, flavonoids, and a host of glycosides, mediate the relation between plants and their environment. Specifically, they constitute a chemical response to pollinators and distributors, to competitors and herbivores, and to pathogens and stress. Manipulation of secondary metabolites, therefore, not only may change the quality and quantity of products, but also may significantly change plant interaction with the environment. Low-alkaloid lupine mutants, for example, are said to have been widely appreciated by rabbits. Modern zero-glucosinolate rapeseed plants may or may not affect flea beetle populations.

The 20,000 plus organic compounds listed as secondary metabolites show an enormous diversity and, thus, allowed chemical description to complement conventional classification of plants by morphological features. They subsequently became subject of studies to understand

*Retired.

their biosynthesis and function. Although during the 1950s they were regarded as end products of deluxe metabolism, ecorelations were recognized in the 1960s. More recently, biotechnologies have become indispensable in efforts to experimentally widen our knowledge of the ontogeny, metabolism, function, and production of secondary metabolites.

As commodities, secondary metabolites can be subject to supply problems owing to adverse climatic conditions, pests, and political instability in cropping areas, and some are prone to misuse, and to ecological concerns. Wild cropping of medicinal botanicals has come under restrictions if not outright closure in countries worldwide. Already, a startling number of plants known for their medicinal value are threatened in their habitat; for example *Lithospermum erythrorhizon* [Tabata, personal communication], *Hydrastis canadensis* [Constabel, personal observation], *Cephaelis ipecacuanha* [Teshima et al., 1992], *Rauwolfia serpentina* [Sharma and Chandel, 1992], *Podophyllum peltatum* [Oostdam et al., 1993], or *Artemisia genipi* [Botany Congress Berlin, 1987].

Research and development for the production of secondary metabolites have been directed primarily at cell technologies. Arguments in support of plant cell culture stress the advantages of year-round availability of plant material, process isolation, and magnification-accentuation of chemical reactions under growth control. Zenk's [1991] contribution entitled "Chasing the enzymes of secondary metabolism: plant cell cultures as a pot of gold" aptly illustrates this concept. Second, *in vitro* culture of plant cells may result tomorrow in improved yields and cost/benefit ratios superior to those of plantations [Verpoorte et al., 1993]. Recombinant DNA technologies are supplementing cell technologies at an ever-accelerating pace [Nessler, 1994; Hashimoto and Yamada, 1994].

Early history of metabolite production with plant cells cultured *in vitro* is not crystalline science, but rather accidental. Prof. Reinhard in Tuebingen, Germany, may have been one of the first to discover secondary metabolites *in vitro* by the fragrance emanating from *Ruta graveolens* callus stored in his laboratory. In 1956, at a Christmas party at Prof. Gautheret's in Paris, this author (FC) obtained proof of flavor production by carrot (*Daucus carota*) with a cup of soup prepared from a two-pound lump of callus. Subsequent microscopic inspection of leftovers did not reveal pockets of essential oil, but islands of tracheary elements, in bundles, and lignified (phloroglucinol HCl). The following chapter will present R&D of secondary metabolite production and submit some concepts for new work at the bench.

This chapter on the production of secondary metabolites will not include a list of all compounds referred to in relevant literature. Rather, it will focus on concepts of how to alter and possibly enhance the formation of metabolites in plant material, on methods to increase yields, and present a few case studies to allow learning by analogy.

II. CONCEPTS

The production of secondary metabolites in plants can be understood as the result of plant development, which includes differentiation in metabolism as well as in morphogenesis [Constabel, 1988]. Or, the production of secondary metabolites is the expression of a plant genome under developmental control. On the other hand, secondary metabolites accidentally appear as a result of stress and disease. Such metabolites may be synthesized *de novo*, (i.e., by gene activation) and are referred to as *phytoalexins*. Other metabolites produced in response to stress and disease result from stimulation of enzymes active in regular metabolism and are referred to as *phytoanticipins* [van Etten et al., 1994]. Both concepts invite genetic manipulation to better understand the biology of secondary metabolites in plants and to increase yields.

A. Secondary Metabolites

1. Differentiation Products

The occurrence of secondary metabolites in tissues (calli) grown in vitro was unexpected, as long as the prevailing concept equaled a callus to a meristem (i.e., indefinite growth by cell division was the goal of cell culture at the time) [Gautheret, 1939; White, 1939]. Heterogeneity of cultured cells, however, was soon recognized and given thorough description [Gautheret, 1959]. Hence, pigmentation, flavor, and fragrance or tannin production was interpreted as the result of cell differentiation as observed in planta [Constabel, 1969]. Conversely, the lack of alkaloids in poppy or terpenoids in carrot cultures was readily explained with lack of laticifers or resin canals, respectively. A more recent observation—failure to demonstrate vindoline and vinblastine in cell cultures of periwinkle as found in seedlings—was convincingly interpreted similarly (i.e., with developmental regulation) [DeLuca et al., 1986, 1988]. Also, the recurrence of vindoline and vinblastine in cell cultures that had undergone shoot formation [Miura et al., 1988] underscores the point. And finally, the search for menthol in mint (*Mentha piperita*) cell cultures succeeded as soon as shoot cultures were obtained, a strong confirmation of developmental regulation of secondary metabolite production [Bricout and Paupardin, 1975; Spencer et al., 1993].

Today, the concept of secondary metabolite products as differentiation products is supported by the following four arguments: Secondary metabolites occur in vitro

1. During the stationary phase of plant cell cultures
2. Under hormonal control
3. During maturation of cells to tracheids, giant cells, laticifers, or idioblasts
4. During organ development (i.e., growth of roots and shoots)

Methodology designed to enhance synthesis and increase yields of secondary metabolites in cell cultures, therefore, must integrate the foregoing arguments in strategies for experimentation and application.

a. *Stationary Phase.* Kinetics of growth and secondary metabolite synthesis and accumulation have long and repeatedly been shown to co-occur with the stationary phase of cell cultures. Indole alkaloid accumulation as a function of growth in *Catharanthus roseus* cell suspensions has become a standard in tissue culture literature [Zenk et al., 1977]. Profitable management of cell cultures for industrial production by first boosting growth, then metabolite accumulation [Fujita et al., 1981], adds corroborating evidence. Still, not all experimental results are in accordance with this concept.

For one, in pokeweed (*Phytolacca americana*) cultures, betacyanins have been observed to be synthesized and accumulated in dividing cells over the period of accelerated growth. Actually, pigment accumulation was reduced when cell division was curtailed by treatment with inhibitors of DNA synthesis or antimicrotubule drugs [Hirano and Komamine, 1994]. This observation contrasts with anthocyanins in grape (*Vitis vinifera*) cell cultures, which appeared during the stationary phase, exclusively [Hirose et al., 1990]. Moreover, betacyanins of red beet (*Beta vulgaris*) or of different *Phytolacca americana* cell cultures temporarily changed from red to pale whenever over many years a callus had been subcultured onto fresh medium and went through the phase of accelerated growth [Constabel and Nassif-Makki, 1971; Constabel, unpublished data]. Discrepancies may stem from differences not perceived or recognized as critical at the time, such as specificity of source material, accidental selection, or shift in media composition.

b. *Hormonal Control.* Hormonal control of metabolite formation and accumulation has been well established for many systems. As a rule, deceleration of growth because of deletion or reduction of auxins in culture media prompts the appearance of products such as pigments or alkaloids [Constabel et al., 1971; Kutchan et al., 1983; Siah and Doran, 1991].

Cytokinins, on the other hand, have been demonstrated to stimulate production, as shown for nicotine in cell cultures of *Nicotiana tabacum* [Tabata et al., 1971]; for indole alkaloids in cultures of *Catharanthus roseus* [Decendit et al., 1992]; or for anthocyanins [Seitz and Hinderer, 1988], catechins, proanthocyanidins, and lignins [Zaprometov, 1988]. For *Thalictrum minus* the specific nature of cytokinin-driven increases in yield has been identified as stimulation of a specific O-methyltransferase activity. This enzyme catalyzes the first methylation of norcoclaurine to yield claurine, a principal intermediate in the biosynthesis of protoberberine alkaloids [Hara et al., 1993, 1994].

c. *Cell Maturation.* In *Papaver bracteatum* cell cultures, deprivation of hormones led to organogenesis (differentiation) and subsequently to an elevation of the morphinan alkaloid concentration. Concurrent microscopic analysis clearly revealed a correlation between the appearance of thebaine and laticifer-like cells [Kutchan et al., 1983]. Supplementary experimentation with seedlings confirmed that the presence of laticifers was necessary for the accumulation of morphinan alkaloids. Interestingly, it was not a precondition for the accumulation of related benzophenanthridine alkaloids (sanguinarine), or their mutual precursor, dopamine [Rusch et al., 1985]. Recently, cultures of *P. somniferum* derived from cv. 14-14, a Tasmanian breed, growing on hormone-free medium led to the formation and accumulation of morphine and codeine at levels of 0.3 and 0.25% dry weight (DW), respectively [Siah and Doran, 1991]. Here, alkaloid production was not accompanied by cell, tissue, and organ differentiation, excepting the formation of giant cells, and so a much disputed observation reported earlier may have found confirmation [Tam et al., 1980].

The relation between cell morphology and metabolite synthesis and accumulation again came to the fore when cells of protoplast-derived *Catharanthus roseus* clones were investigated [Kim et al., 1994]. In each clone, most of the cells maintained only one of two shapes, spherical or cylindrical. The cell aspect ratios (length/width) for most clones was stable for more than 2 years of subculture. Remarkably, the production of indole alkaloids ajmalicine and catharanthine was significantly greater when the cell aspect ratio was more than 2.8.

The degree of differentiation of a given tissue certainly makes for differences in the spectrum of products. The appearance of triterpenoids as a function of the degree of tissue differentiation is not surprising. The correlation of accumulation of laticifer-specific triterpenes in cell cultures of *Asclepias syriaca* [Biesboer, 1983] and *Taraxacum officinale* [Akashi et al., 1994] and the occurrence of proper laticifer cells in tissues and regenerants demonstrate the point. Regenerants (somaclonal variants) without latex, as observed once with *Papaver somniferum* cultures [Constabel, unpublished data] would have been the ultimate control material for such demonstration.

Maturation and, implicitly, specialization of cells and product formation, may be achieved by technology that allows cells to assemble in a mat, a pseudotissue (i.e., immobilization on surfaces of a matrix) [Kurz et al., 1990]. Cell-to-cell contact and nutrient gradients in cell layers may indeed convey physiological attributes of maturation; or mature cells within a given population could more effectively communicate maturation to their neighbors. Lately, this matter has become slightly less mysterious than before, because of the demonstration of a substance that effects pigmentation. Cocultivation of target cells (*Chenopodium album*) and nurse plants (*Wolffia arrhiza*) resulted in diffusion of an unknown substance causing differentiation and subsequent betalain production [Rudat and Ehwald, 1994]. The challenge

will be to analyze the chemical nature of the inducing agent—methyl jasmonate may be a candidate—and relegate the phenomenon not to a maturation process, but to a stress response.

Immobilization of cells cultured *in vitro* still might not be a maturation condition sufficient enough for product formation. For example, immobilized placental tissues of chili pepper (*Capsicum annuum*) exhibited a substantially higher capacity for capsaicin production than immobilized parenchyma cells. Only the placenta cells showed activity of the rate-limiting enzyme in capsaicin synthesis (i.e., caffeic acid-O-methyltransferase and capsaicinoid synthase) [Ochoa-Alejo and Gomez-Peralta, 1993].

d. Organ Development. The competency of *in vitro* cultures for metabolite synthesis and accumulation, once roots or shoots are formed, has been established rather solidly. Variants to this observation have been reported, however: For example, microspore-derived embryos of rapeseed (*Brassica napus*) cultured *in vitro* fail to accumulate detectable levels of glucosinolates until they are placed in a germination medium that supports shoot and root formation. The concomitant induction of glucosinolate accumulation, however, is initially restricted to indole derivatives, whereas the glucosinolate pattern becomes similar to that of zygotic seedlings, only later. Specifically, in microspore-derived emblings, tryptophan is incorporated into indole glucosinolates, methionine is not incorporated into alkenyl glucosinolates [McClellan et al., 1993].

The ultimate in morphological differentiation for accumulation of secondary metabolites is the formation of trichomes in shoot cultures. Their structure can be rather complex because they provide effective compartmentalization of products that are generally toxic. In mint, many if not all of the enzymes involved in terpenoid synthesis and catabolism occur in the cells of the glandular trichomes [Gershenson et al., 1989] and are capable of de novo biosynthesis when fed with precursors and cofactors [McCaskill, 1992]. Development of (transgenic) shoots *in vitro* is followed by differentiation of hairs that accumulate menthol at levels comparable with those in field-grown control plants [Spencer et al., 1993]. Gland hairs on leaves of tobacco (*Nicotiana tabacum*) produce diterpenes. Shoot cultures exhibited an inverse relation between the concentration of cytokinin in the medium and the density of trichomes (i.e., large numbers of trichomes were found only on leaves and stems of shoots grown in the presence of very low cytokinin levels [Miedzybrodzka and Yoeman, 1992]). Again, hair density, now effected by abscisic acid added to the medium, significantly affected monoterpene content in shoot cultures of *Lavandula latifolia* [Calvo and Sanchez-Gras, 1993]. Notably, *in vitro* studies using tobacco shoots had become desirable for an additional and particular reason (i.e., protection of diterpenes in hairs from degradation by microorganisms, as may occur with field-grown material [Miedzybrodzka and Yoeman, 1993]).

The most definite answer to metabolite production by organized tissues and organs is presented by hairy roots and shoots. These materials are known to grow profusely while retaining histological integrity. For example, poppy plants (*Papaver* spp.) readily respond to *in vitro* culture and may synthesize and accumulate benzophenanthridines such as sanguinarine. Recently, cocultivation of *P. somniferum* hypocotyl cells with *Agrobacterium rhizogenes* gave rise to transformed cell cultures and, subsequently, to adventitious shoots. These accumulated tenfold higher levels of morphinan alkaloids, specifically of thebaine and codeine, than controls. Conversion of these zero-morphine shoots to codeine plants is intriguing [Yoshimatsu and Shimomura, 1992].

A caveat to employing hairy roots and shoots concerns the quality of essential oils. The oil distilled from hairy roots and roots of field-grown *Artemisia absinthium* differed significantly in the bouquet of constituent monoterpenes. The observation was explained by changes in the spectrum of terpenes in essential oil, as occur in the course of root (and plant) development.

By comparison, hairy roots were judged to be the chemically younger material [Kennedy et al., 1993].

2. Stress Products

Secondary metabolite production has been viewed as the result of differentiation, exclusively, until the concept of chemical defense gained ground [Swain, 1977], and elicitors were employed for metabolite production in vitro [Wolters and Eilert, 1983]. In a wider context, the occurrence of some metabolites was seen and interpreted as a response to stress. The following are stress factors influencing the production of secondary metabolites in plant cell, tissue, and organ cultures:

1. Exposure to osmotica
2. Exposure to UV light
3. Exposure to elicitors

a. *Osmotic Stress.* The relation between osmotic stress caused by high levels of sucrose in nutrient media and production of secondary metabolites has been known for some time [Knobloch and Berlin, 1980], and corroborative observations have been added ever since. Interestingly, products now include not only anthocyanins [Do and Cormier, 1990] and alkaloids [Godoy-Hernandez and Loyola-Vargas, 1991], but also terpenoids. For example, increasing the carbohydrate concentration to 6% (3% sucrose plus 3% mannitol) and adding 25 µM abscisic acid significantly increased the accumulation of monoterpenes in shoot cultures of *Lavandula latifolia*, primarily of cineole [Calvo and Sanchez-Gras, 1993].

b. *Ultraviolet Radiation.* The response of plants in high altitudes to UV irradiation by anthocyanin formation is a common observation. Pigmentation in cell cultures of parsley (*Petroselinum hortense*) on treatment with UV light [Kreuzaler and Hahlbrock, 1973] has not only improved conditions for metabolite production, but also has significantly affected plant molecular biology in our understanding of the induction, signal transduction, and gene expression in the biosynthesis of plant metabolites [Hinderer and Seitz, 1988]. Although recognized as a major tool in experimental biology, UV irradiation led to the characterization of two isoforms of chalcone synthase in cell cultures of carrot, for instance [Gleitz and Seitz, 1989]. UV light has not been applied for production of secondary metabolites in vitro as much as had been expected.

c. *Biotic and Abiotic Stress.* Cell cultures have been exposed to very different kinds of stress, biotic and abiotic. Most radical was the use of fungal homogenates and extracts in nutrient media as elicitors of alkaloid synthesis and accumulation in plants and plant cell cultures [Eilert, 1987]. When applied to poppy (*P. somniferum*) cell cultures, not morphinan as expected, but sanguinarine production was the result [Eilert et al., 1985]. Shortly thereafter in periwinkle (*Catharanthus roseus*) cell cultures, fungal homogenates led to the formation of catharanthine [Eilert et al., 1986]. The list of materials successfully treated with fungal elicitors for production of secondary metabolites has grown rapidly and continues to do so. Investigations of elicitor-induced production of benzophenanthridine alkaloids in *Sanguinaria canadensis* cell cultures were extended to include the effect of pretreatment with a specific calcium chelatant and a calcium channel inhibitor. As a result, elicitor-induced alkaloid accumulation was seen as mediated by calcium and possibly calmodulin and/or protein kinase C [Mahady and Beecher, 1994].

Product formation through fungal elicitors was stimulated by heavy-metal ions such vanadyl ions [Smith et al., 1987]. Thus, rather different stress agents led to the same products, patterned

according to species. This observation called for activity of a common mechanism for receptor site binding, signal transduction, and gene activation, and jasmonic acid has been identified as signal transducer in elicitor-induced plant tissue, including plant cell cultures [Farmer and Ryan, 1990; Gundlach et al., 1992]. Jasmonic acid and its methyl ester accumulate rapidly and transiently after treatment of plant cell suspension cultures with an elicitor. Also, cell cultures of various plant species could be elicited to produce secondary metabolites by supplementing nutrient media with methyl jasmonate [Gundlach et al., 1992]. Notably, the occurrence of secondary metabolites resulting from elicitation is not a function of cell maturation or differentiation. On the other hand, highest yields will be achieved when elicitors are being introduced to *in vitro* systems at a time when carbohydrates are exhausted and when cells are in the deceleration mode of growth [Park et al., 1992].

3. Genetic Engineering Products

The manipulation of metabolism in plants through DNA technologies for enhanced production of secondary metabolites is in part curiosity-driven. Finally, this technology allows introduction of experimentation into biochemistry and phytochemistry, where previously the *modus operandi* was description. In part, recombinant DNA technologies are also pulled by the resource and processing industries owing to prospects of marketable and profitable botanicals. Here, the concept is that nature did not maximize product formation in plants, that value may be added through genetic engineering.

Generally, the strategy is aimed at enabling transgenic plants to synthesize new and novel products by

1. Completion of partial pathways
2. Amplification of regular pathways
3. Blockage of competing pathways
4. Interdiction of regular pathways
5. Revising metabolic regulation
6. Minimizing response cascades [see also Bailey, 1991; Nessler, 1994]

Caveat: The improvement of secondary metabolite production in crops by plant breeding has a long history and is being displayed daily in any vegetable market and all around the world. Also, inasmuch as mutants have been employed, they now continue to play a major role, in combination with genetic engineering. A case in point is a lignin variant mutant of *Arabidopsis thaliana*. This material is particularly valuable, because it has a small and well-analyzed genome and would allow one to answer specific questions on regulatory mechanisms of product (lignin) formation [Chapple et al., 1992].

a. *Insertion of Heterologous Genes.* Gene insertion for synthesis of enzymes that will upgrade natural products has been accomplished. Specifically, a hydroxylase gene from *Hyoscyamus niger* was introduced into hyoscyamine-rich *Atropa belladonna*, with the result that transgenic material now produced the more valuable scopolamine [Yun et al., 1992]. Interestingly, one single copy of the transgene was sufficient to create an all-scopolamine-type shoot. In the future, conventional breeding efforts for improved botanicals may well be complemented by including metabolically engineered plants, as demonstrated here.

b. *Amplification of Pathways.* Such amplification would "simply" require activation of ratelimiting enzymes by insertion of homo- or heterologous genes, thereby removing "bottlenecks." Apart from pigment synthesis in petunia [Meyer et al., 1987], the isolation, characterization, and heterologous (sense-) expression of the tryptophan decarboxylase

[DeLuca et al., 1989] and strictosidine synthase genes for monoterpene indole alkaloid synthesis in *Apocynaceae* [Kutchan et al., 1988; Kutchan, 1993] and of the berberine bridge enzyme in *Papaveraceae* [Dittrich and Kutchan, 1991], which catalyzes the conversion of (*S*)-reticuline to (*S*)-scoulerine in the pathway to benzophenanthridine alkaloids (sanguinarine), are significant recent steps toward the goal of genetically improving the quality and quantity of secondary metabolites. Yields, however, are not yet overwhelming. Attempts to enhance the production of nicotine in root cultures of *Nicotiana rustica* by overexpressing a heterologous ornithine decarboxylase gene amounted to a twofold increase of putrescine and, consequently, a twofold increase in nicotine [Hamill et al., 1990]. In short, performance of processes can be enhanced by genetic modification of cells and plants. However, more studies will be required to demonstrate the feasibility of metabolic-engineering methods to increase yields or rates of production.

c. *Competitive Pathway Blockade.* Glucosinolates accumulate in the leaves and seeds of *Cruciferae* and so in rapeseed plants. They give an unpleasant taste to meal and cause thyroid disorders in livestock. The strategy to reduce glucosinolates in rapeseed crops would be to insert genes encoding enzymes that compete without harm for a common precursor, here a sulfur compound (i.e., flavonol-3-sulfate). So far, expression in host material of a sulfotransferase gene has been low [Varin et al., 1992]. An alternative suggested strategy would be to produce rapeseed with tryptophan decarboxylase transgenes to direct tryptophan away from the synthesis of indole glucosinolates and to increasing the tryptamine pool. And, indeed, transgenic rapeseed plants responded with higher tryptamine and dramatically lower glucosinolate levels, only 3% of that found in nontransformed controls [Chavadej et al., 1994].

d. *Use of Antisense Genes.* Experimentation based on the insertion of antisense genes of rate-limiting enzymes of the phenylpropanoid pathway (i.e., phenylalanine ammonia-lyase and chalcone synthase) led to curtailment of transcription processes and inhibition of flower pigmentation in transgenic petunia and tobacco [van der Krol et al., 1988]. The result has had a strong influence on attempts to manipulate metabolite reduction in botanicals, as demanded by industry: lignin in wood and glucosinolates in rapeseed meal.

Reduction of the amount of lignin in cell walls of trees could assist the pulp and paper industry, and its reduction in herbs could benefit the forage industry. The omnipresence of lignin in higher plants has not made it any easier to elucidate its analysis, synthesis, polymerization, and deposition. The identification of rate-limiting enzymes in lignin synthesis has been slow, three enzyme systems have now been isolated and characterized. For one, an O-methyltransferase antisense gene from aspen has been expressed in tobacco. The expected suppression of precursor (i.e., ferulic and sinapic acid synthesis) did materialize and led to lignin reduction [Dwivedi et al., 1994; Dixon et al., 1994]. Second, antisense technology was applied to suppress, again in tobacco, the activity of two enzymes that catalyze the final steps of cinnamic acid derivatives to cinnamyl, feruyl, and syringyl alcohol (i.e., cinnamyl-CoA-reductase and cinnamyl alcohol dehydrogenase; CAD). As a result, CAD activity was reduced; however, lignin was not so much reduced in quantity as it was altered in quality. The new lignin was judged to be more accessible to chemical extraction, however, and thus may well affect the pulp industry. Interestingly, staining sections of tobacco with phloroglucinol.HCl made possible a quick first differentiation of transformed and control material [Halpin et al., 1994]. Lignin polymerization is catalyzed by peroxidases. However, transgenic tobacco plants with suppressed levels of this enzyme did not show changes in the amount of lignin in tissues [Kolattukudy et al., 1992].

e. Metabolic Expression Revision. Naturally, attention has been paid first to genes that encode enzymes which catalyze steps along the biosynthetic pathway of secondary metabolites. In analogy to studies of flower pigmentation in petunia, such experimentation will have to be followed by studies of regulatory genes that control the flux of compounds along a pathway for expression, at specific sites, for specific patterns. For anthocyanin synthesis in petunia flowers the dihydroflavonol 4-reductase gene has been characterized as such [Hufts et al., 1994]. It will be interesting to see to what extent organized and unorganized tissue respond to changes designed for control of product synthesis and accumulation, by regulatory genes.

A most promising step has been accomplished with identification and characterization of genes that encode the major latex proteins in poppy and, thus, will permit us to target these cell components for genetic manipulations much more specifically than before [Nessler, 1994].

f. Minimization of Response Cascades. The cascade of events following insertion of a heterologous gene may bring surprises rather than the expected outcome. It will be important to minimize unwanted response cascades [Bailey, 1991]. Transgenic plants with lower than normal phenylalanine ammonia lyase activity did show corresponding lignin reduction. However, they also exhibited several unusual and aberrant phenotypes, including stunted growth, altered leaf shape, reduced pollen viability, and altered flower morphology [Elkind et al., 1990]. Overexpression of genes encoding enzymes of anthocyanin synthesis in petunia did not always result in increased pigmentation. On the contrary, suppression has been observed. Such results demonstrate only the complexity of gene expression. The basic cause may be gene-gene interaction, interference of RNA strands with the transcription process, and methylation [van der Krol et al., 1990].

One handicap lies in the employment of constructs that are chimeric (i.e., the gene of interest has been aligned with a precursor derived from a heterologous donor, for example, a virus, as CaMV S35). For better specificity and expression, a homogenomic promoter sequence should be used. Significant progress has been made when the gene for a major latex protein of poppy was analyzed and characterized [Nessler and von der Haar, 1990]. The promoter sequence contained may well be suited to direct gene activation in laticifers for increase or interdiction of morphinan production, without a concomitant cascade of undesirable phenotypes. Besides, it can be expected that a zero-morphin poppy would benefit agriculture in the future as did the Bronowski, zero-glucosinolate rapeseed variety in the past [Nessler, 1994].

B. Preservation and Multiplication of Plant Resources

High-yielding cell lines, selected from wild or transgenic populations, comprise unique germ plasms that require preservation, best accomplished by cryotechnology using liquid nitrogen. Cryopreservation is well developed, as documented by the long list of cell lines already covered [Kartha and Engelmann, 1994]. Equally important is the cryopreservation of germ plasm of wild medicinal and industrial plants classified as rare and threatened by extinction, a task for gene banks around the world.

If we consider the production of secondary metabolites, the uniqueness of each species and, therefore, the loss to nature and to human benefit may lie not so much in the loss of an individual secondary metabolite, but rather, in the specific bouquet of all metabolites presented by one species. *Artemisia genipi* of the European alps, source of vermouth flavor, is a case in point, complicated by the occurrence of subspecies [Gautheret, personal communication]. Modern concepts to remedy the situation comprise germ plasm preservation by cryotechnology as mentioned and, more importantly, micropropagation of remaining stock for rehabilitation,

for domestication, and for establishment of plantations [Fay, 1992]. Socioeconomic considerations may dictate that, in future, only failure to domesticate certain germ plasm may permit initiation of *in vitro* culture systems.

Cryopreservation and micropropagation regrettably fail to draw adequate attention in biotechnology laboratories. On the other hand, the demand for user-friendly technology at the front line cannot be overstated. For cryopreservation, the introduction of encapsulation-dehydration technology may lessen the dependency on liquid nitrogen [Dereuddre et al., 1991]. For micropropagation, automation has been advanced and, conversely, the investment of labor has been reduced. At the front end of securing medicinal and industrial plant gene resources, however, more appropriate would be a one-stop technology embracing testing, explantation, and culture of organogenic and embryogenic tissue of a given species. Of particular interest would be a method that would allow one to determine the morphogenic capacity of a given tissue by molecular "fingerprinting."

III. METHODOLOGY

Concepts of biotechnology for enhanced production of secondary metabolites in plants and plant cell cultures need to be complemented by methods that are applicable in the laboratory. Because directed manipulation of product synthesis and accumulation in cells has only recently begun, (i.e., with the introduction of recombinant DNA technologies), assistance basically is to be found in empirical data. Therefore, progress can be expected from adjusting the approach of a problem to cases that have been proved successful. As a first step an electronic literature search is highly recommended to display the state of the art on any given topic. Various publications have covered the production of secondary metabolites in cell cultures and transgenic plants emphasizing methodology [Fontanel and Tabata, 1987; Constabel and Vasil, 1987; Robins and Rhodes, 1988; Lindsay, 1991; Bajaj, 1988, 1989, 1993; Ganapathi and Kargi, 1990; Stafford, 1991; Brodelius and Pedersen, 1993; Constabel and Tyler, 1994; Endress, 1994], Just as important are the proceedings of conferences on the topic of metabolite production [Kurz, 1989; WTC Florence, 1994; IAPMB Amsterdam, 1994; Ellis et al., 1994].

Methods for the enhancement of secondary metabolite production, excluding scale-up [see [Chap. 12](#)] and recombinant DNA technologies [see [Chaps 10 and 13](#)] include the following:

1. Selection of source material
2. Selection of superior cell lines
3. Optimization of culture conditions
4. Elicitation and stress
5. Genetic transformation
6. Cryopreservation
7. (Micro)propagation

A. Selection of Source Material

The initiation and maintenance of callus and cell cultures is the subject of several laboratory manuals [Wetter and Constabel, 1982; Seitz et al., 1985; Dixon, 1985; Lindsay, 1991]. Ancient *Cycadopsida* [Chavez et al., 1992] or quasi-aquatic plants [Brisson et al., 1988], rare specimens from far-away islands [Douglas, 1987], and modern transgenics [Nessler, 1994], all have been covered by modern cell culture technology. Species limitation, therefore, is no longer a problem when searching for source material to produce secondary metabolites *in vitro*.

Plants known to be rich in the product under investigation would naturally be considered the best starting material. Accordingly, tubers of red beet (*Beta vulgaris*) would be considered superior to cacti or pokeweed (*Phytolacca americana*) for pigment production. Experience shows, however, that the latter may result in cell cultures as high-yielding and stable as the former [Hirose et al., 1990]. *Vitis vinifera* was preferred as donor material for production of anthocyanin in cell cultures [Cormier et al., 1990], but may be outperformed by species little known for pigment production [Crouch et al., 1993; Sakamoto et al., 1994]. In short, following traditional advice is proper, but may not be the last word; thorough literature search and experimentation is. And today, consideration must also be given to legal implications of access and availability.

Given a specific natural product, explants should be taken from as many source species as indicated by the literature and available to the operator. For paclitaxel (Taxol), five species were taken into culture, and the callus of *Taxus cuspidata* was identified as the most suitable for further experimentation [Wickremesinhe and Artega, 1994]. For monoterpenoid indole alkaloid production, multiple species from at least five families were indicated [Kutchan, 1993]. *Catharanthus roseus* or *Rauwolfia serpentina* have won out owing to ready response to in vitro culture conditions, prior knowledge of phytochemistry, and the lure of valuable medicinals. Tobacco (*Nicotiana tabacum*) appears to have become the material of choice for heterologous gene expression ever since the demonstration of transgenic specimens [Horsch et al., 1985]. Here, the lack of precursors may not allow reaction products to be seen owing to de novo synthesis of enzymes [McKnight et al., 1991]. Still, transgenic tobacco plants expressed a specific hydroxylase-produced scopolamine when fed with the precursor hyoscyamine [Yun et al., 1993].

A plant offers a variety of tissues for in vitro culture. Leaf sections or internode and petiole segments would be easiest to handle; shoot tip meristem, seedling, or embryo explants may be the most productive. For the terpenoids of mint, textbooks will dictate a preference for aerial parts; for nicotine, it will be roots.

B. Selection of Superior Cell Lines

High-yielding cell lines are generally, first, a result of a screening and selection process. For obvious reasons this process is more easily accomplished with visible products (e.g., pigments), and flavors such as vanilla. Extraction and chemical analysis are the traditional procedures. Radioimmunoassays and enzyme-linked immunosorbent assay (ELISA) tests are being applied whenever possible [Kemp and Morgan, 1987; Stafford, 1991]. Nondestructive analyses by nuclear magnetic resonance (NMR) may soon become the method of choice.

Single-cell selection, aided by protoplast technology, has proved the variability in the response of plant material to in vitro culture (i.e., formation of specific alkaloid spectra or anthocyanins) [Constabel et al., 1981; Zubko et al., 1993], but it may not be the key to high-performance cell lines. Cell aggregate selection, on the other hand, has succeeded ever since its introduction [Yamamoto and Mizuguchi, 1982; Ozeki and Komamine, 1985]. A shining example are the green, yellow, orange, red, and purple lines obtained with one callus line of red beet [Girod and Zryd, 1987, 1991]. Superior cell lines have been obtained by mutant selection as well [Widholm, 1987]. A *Catharanthus* line resistant to 5-methyltryptophan (0.05 mmol/L 5-MT) has been widely exploited [Fujita et al., 1990].

Generally, the methods applied to screening have remained chemical analysis of plant material by chromatography and, more recently, by NMR [van Calsteren et al., 1991], over a substantial time. Such procedures have included hairy root cultures, as demonstrated for

Datura stramonium, for which the screening process began with 500 samples and lasted over 5 years, resulting in stable production of scopolamine that was two orders of magnitude higher than from source plants [Maldonado-Mendoza et al., 1993]. Also, shoot cultures have been established from axillary buds (11 strains) and seeds (1 strain) of *Digitalis lanata*, and after 1 year of monitoring the quality and quantity of cardenolides, significant differences have been recorded [Stuhlemmer et al., 1993]. Because industrial application will benefit from in vitro cultures that can be grown continuously (i.e., uninterrupted by subcultures), product release from cells has also become a goal of selection. Reselection of hairy root clones of *Duboisia leichhardtii* yielded a release of 75% of the 2.6 mg of scopolamine released by a 50-mL culture over a period of 4 weeks [Muranaka et al., 1993]. And finally, although strictosidine synthase cDNA from *Rauvolfia serpentina* has been positively hybridized with 35 Rubiales [Kutchan, 1993], such or similar experimentation in future may be extended to “fingerprint” plants and callus for performance in product formation in vitro.

C. Optimization of Culture Conditions

1. Light

Physical parameters of culturing plant cells are relatively easy to test and to adjust for optimum product formation. Light has been a significant factor in some instances. In red beet (*Beta vulgaris*) as in *Portulaca sp.* cv. “Jewel,” light was the controlling factor for betalain formation, or pigmentation disappeared with transfer of cultures into the dark [Girod and Zryd, 1987; Kishima et al., 1991]. Light has also induced the production of anthocyanin in *Daucus carota* [Takeda, 1988; Gleitz and Seitz, 1989] or *Centaurea cyanus* [Kasegawa et al., 1991] cell cultures. Consideration may have to be given to specific light requirements. In vinblastine production in shoot cultures of *Catharanthus roseus*, synthesis was stimulated by visible light that contained near ultraviolet light at lambda=270 nm [Hirata et al., 1993].

Light will be required for growth of shoot cultures and for production of metabolites in leaves, such as essential oils in glands and hairs or idioblasts. More complex is the interrelation of metabolite synthesis and accumulation with chloroplasts. Thus, in *Catharanthus roseus*, alkaloids such as vindoline, will appear with illumination of plantlets, not in illuminated cell cultures. Phytochrome is implicated in some biosynthetic steps [Aerts et al., 1992]. Or, the site of cardenolide biosynthesis in *Digitalis lanata* is the mesophyll cell. The synthesis is described as not being dependent on intact chloroplasts. However, shoot cultures furnished better results when kept in the light. *Digitalis* shoots maintained in this manner produced cardenolides over years. Products were identified as digitoxose-type cardenolides of “mature” plant parts, as opposed to those found in cell cultures (i.e., “early” or fucose-type cardenolides) [Stuhlemmer et al., 1993].

Light will lead to greening of callus and cell cultures and may, thus, hinder or interfere with the detection of desirable products, as has been experienced with paclitaxel formation in *Taxus* spp. explants [Wickremesinhe and Artega, 1994]. Also, cell suspensions of *Lithospermum erythrorhizon* transferred from growth to production medium will completely fail to synthesize shikonin when irradiated with either white or blue light. Light strongly inhibits the activity of *p*-hydroxybenzoic acid geranyltransferase, a key enzyme in shikonin synthesis [Heide et al., 1989].

Synthesis is also inhibited by addition of lumiflavin, a photodegradation product of flavin mononucleotide (FMN), or light inhibits shikonin production by decomposing the cofactor FMN to lumiflavin [Tabata et al., 1993].

2. Nutrient and Production Media

Growth of cells and tissues cultured in vitro and production of secondary metabolites demand the specification of nutritional requirements for each species, even with different genotypes of one species. The general procedure is to permute the components of the nutrient media (i.e., mineral salts, vitamins, organic supplements and, in particular, carbohydrates and hormones). A general approach begins with testing growth and product formation of tissues in the presence of the different common media [White, 1943; Murashige and Skoog, 1962; Gamborg et al., 1968; Schenk and Hildebrandt, 1972]. An expedient approach would be to optimize conditions by directing cells toward the production of biomass by exposure to a growth medium, followed by production of secondary metabolites by substituting the growth medium with a production medium, as introduced for alkaloids (serpentine and ajmalicine) [Zenk et al., 1977] and for naphthoquinones (shikonin) [Fujita et al., 1981].

For production media, a decrease in the level of auxin and nitrate and an increase in the level of ammonium and carbohydrates are generally recommended. Numerous efforts to optimize individual components of production media have been rewarded by high yields and have been incorporated into scale-up procedures (see [Chap. 12](#)).

A recent example for optimization has been presented for *Rauwolfia serpentina*, known to accumulate appreciable amounts of raucaffricine. Here, the highest yields (1.6 g/L alkaloid) were achieved by fortifying the alkaloid production medium [Zenk et al., 1977] with 2.5 g/L $Mg_2SO_4 \cdot 7H_2O$ and 100 g/L sucrose [Schubel et al., 1989]. For anthocyanin production of *Vitis vinifera* cell cultures, studies led to an increase of sucrose to 123 mM and a decrease of the nitrate level to 6.25 mM. An increase of the ammonium level from 2 to 6–8 mM made for a shift from peonidin-3-glucoside to peonidin-3-coumaryl-glucoside [Do and Cormier, 1991].

Today, the term *two-phase culture* applied to cultures exposed to growth medium first and production medium thereafter has been widened to include the supplementation of (growth) media with specific compounds, meant to trigger product formation (i.e., elicitors and adsorbents) 4 days after subculture [e.g., Byun et al., 1990].

Determining the quality and quantity of *hormone supplements* to nutrient media has played a major role in efforts to increase product synthesis and accumulation in cell cultures. As a rule, the concentration of auxins has been reduced to a minimum or eliminated altogether, and cytokinins have been adjusted to levels of 0.1–1 mg/L. Still, in *Papaver somniferum* cell cultures, only complete deprivation of all hormones led to unusual and sizable yields of codeine and morphine, 3 and 2.5 mg/g DW, respectively [Siah and Doran, 1991]. In *Thalictrum minus* cell cultures, adding 6-benzyladenine resulted in rapid conversion of precursor, l-tyrosine, into berberine, which was then released into the medium [Hara et al., 1993].

The effect of gibberellic (GA3) and abscisic acids (ABA) has generally been judged ambivalent. For example, the production of shikonin in cell cultures of *Lithospermum erythrorhizon* [Yoshikawa et al., 1986] or of anthocyanins and flavonoids in those of carrot [Hinderer and Seitz, 1988] was inhibited by addition of GA3, in *Coptis japonica* and *Stephania cepharantha* cell cultures; however, GA3 did enhance the formation of alkaloids (berberine). Here, it appeared to activate primary metabolic steps leading to tyrosine biosynthesis, itself the precursor of these alkaloids [Hara et al., 1994]. Moreover, production of essential oils (aromatic alcohols and esters) in flowers of *Hyacinthus orientalis* regenerated in vitro could be realized only by supplementing nutrient media with 1 mg/L GA3-Nb, in vitro and field-grown flowers that substantially differed in the quality and quantity of essential oil constituents [Hosokawa and Fukunaga, 1995].

Increase in the yield of alkaloids by addition of abscisic acid has been reported for *Catharanthus roseus* cell cultures. (+)-ABA media supplementation (8.33 mg/L) in a 30-L

fermenter on day 5 after subculture resulted in a catharanthine yield of 27 mg/L with line JOH on day 12 [Smith et al. 1987].

The feeding of *precursors* is common practice for biochemists when elucidating biosynthetic pathways. Supplementation of nutrient media with substantial amounts of precursors is aimed at increasing yields of metabolites. Such a procedure must satisfy two concerns: the concentration of the precursor must be tolerated by the cells under investigation and, also, the precursor must be catalyzed to the desired end product and not be diverted to the synthesis of metabolites on branch pathways. Three examples from many show (1) the feeding of shikimic acid and of phenylalanine (50 mg/L) to cell cultures of *Cephaelis ipecacuanha* resulting in increased production of cephaelin. Notably, both precursors failed to influence emetine production [Veeresham et al., 1994]. (2) Feeding the earliest precursor of terpenoids (i.e., mevalonic acid) to cell cultures of *Catharanthus roseus* did not increase the production of monoterpene indole alkaloids, feeding secologanin, loganin, or loganic acid did [Moreno et al., 1993]. (3) Feeding tryptamine to hairy root cultures of *Peganum harmala* led to substantial increases in the content of serotonin, a tryptamine derivative, but not of harmane alkaloids, as may have been desired [Berlin et al., 1993].

The feeding of secondary metabolites to cells cultured *in vitro* raises the question of tolerance or the mechanism of protection from compounds that are potentially toxic. Recently, ajmaline was fed to cell cultures of *R. serpentina*. The cells did tolerate 1 g/L of added alkaloid. The process led, however, to several previously unknown compounds, for example, raumaclines [Endress et al., 1993]. Instant extra- and intracellular catalysis may be the response by cells and the answer to the foregoing question. Extracellular enzyme activity in plant cell cultures has been known for some time [Constabel, 1960, 1965].

Optimization should also be achieved by *decreasing catabolism* of desirable products. Concepts of how to achieve this remain uncharted. Observations would indicate, that alkaloids fed to *R. serpentina* or accumulated in *C. roseus* cell cultures may well be subject to substantial turnover [Endress et al., 1993; Dagnino et al., 1993].

The addition to nutrient media of as yet *undefined supplements* may underscore the degree of empiricism that governs some tissue culture experimentation. In the 1950s, coconut water was one such supplement. Its application paved the way, however, to the demonstration of plant biotechnology's greatest achievement, plant regeneration from single somatic cells. Induction of betacyanin formation in *Chenopodium album* cell cultures by cocultivation with duckweed *Wolffia arrhiza* [Rudat and Ehwald, 1994] demands analysis of the differentiation or elicitation factors involved here.

3. Elicitation

For practical purposes here, the term *elicitation* is used in its widest definition; that is, as induction and stimulation of synthesis of secondary metabolites by stress agents, regardless of whether metabolites are phytoalexins or phytoanticipins, as discussed recently [Van Etten et al., 1994]. Of interest, today, are the identification and characterization of more powerful elicitors for further improvement of product formation including manipulation of signals and transduction mechanisms [Scheel, 1990]. Elicitation parameters to be optimized to improve the production of secondary metabolites in plant cell cultures are the selection of cell line and type of elicitor, the concentration of elicitor, the timing of elicitation, and the general culture conditions during elicitation ([Table 1](#)) [Eilert, 1987].

The use of jasmonate, recently characterized as signal transducer of elicitor-induced plant response, as an agent to initiate production of secondary metabolites, should be given prime consideration and may outdate previous elicitors. Such a claim is substantiated by

Table 1 Elicitor Induced Accumulation of Secondary Metabolites in In Vitro Culture Systems

Elicitor	Plant material	Products	Refs.
Oligogalacturonides	<i>Lithospermum erythrorhizon</i>	Dihydroechinofurane	Tani et al., 1993
<i>Botrytis spp.</i>			
<i>Trichoderma viride</i>			
<i>Rhodotorula rubra</i>	<i>Tripterygium wilfordii</i>	Oleanane triterpenes	Kutney et al., 1993
<i>Sclerotinia sclerotiorum</i>			
Arachidonic acid	<i>Capsicum annuum</i>	Capsidiol	Hoshino et al., 1994
<i>Phytophthora cinnamomi</i>	<i>Hyoscyamus albus</i>	Lubimin	Miguel and Barroso, 1994
Polysaccharides	<i>Morinda citrifolia</i>	Anthraquinones	Doernenburg and Knorr, 1994

experimentation showing alkaloid synthesis in cell cultures of 36 species subsequent to media supplementation with 5 µL of an ethanolic solution of either jasmonic acid or methyl jasmonate per 1 mL of cell suspension [Gundlach et al., 1992].

4. Immobilization

Immobilization is meant to aggregate cells in a tissue-like manner, but well exposed to the medium for continuous culture and metabolite production in vitro [Brodelius, 1985] (Table 2). Most methods recommend the entrapment of cells in a polymeric matrix, be it alginate, agarose, carrageenan, or polyacrylamide gels, hollow fibers, or reticulate polyurethane found in membranes or stainless steel screens. Further development will depend on methods to force release of products and recovery by adsorption to resins. Usage appears to have decreased over the last few years.

D. Genetic Transformation

Inasmuch as roots are the site of product biosynthesis, the development of hairy root systems readily fulfilled expectations for enhanced in vitro production of such compounds Table 3. A few caveats concern the spectrum of products targeted. Hairy root cultures of *Hyoscyamus albus*, for example, strongly surpassed regular roots in the content of total alkaloids; however, they lacked a few minor tropan alkaloids, and more importantly, showed themselves as poor performers of scopolamine synthesis [Doerk-Schmitz et al., 1994]. Also, induction of hairy roots on *Trigonella foenum-graecum* as with other plants demands to first test *Agrobacterium rhizogenes* strains for virulence, antibiotics for elimination of bacteria during subsequent culture, and adjustments of the nutrient medium to be employed [Rodriguez-Mendiola et al., 1991] (Table 4).

Table 2 Immobilized Cell Systems for Production of Secondary Metabolites

Species	Matrix	Product	Ref.
<i>Coleus blumei</i>	Luffa	Rosmarinic acid	Park and Martinez, 1994
<i>Datura innoxia</i>	Calcium-alginate	Tropane alkaloids	Gontier et al., 1994

Table 3 Secondary Metabolite Production by Hairy Roots Cultured In Vitro

Species	<i>A. rhizogenes</i>	Metabolite	Ref.
<i>Artemisia absinthium</i> ^a	LBA 9402	Neryl isovalerate	Kennedy et al., 1993
<i>Salvia miltorrhiza</i>		Diterpenoids	Hu and Alfermann, 1993
<i>Sesamum indicum</i>		Naphthoquinones	Ogasawara et al., 1993
<i>Linum flavum</i> ^b		5-Methoxypodophyllotoxin	Oostdam et al., 1993
<i>Peganum harmala</i> ^c		Serotonin	Berlin et al., 1993
<i>Panax ginseng</i>		Saponins	Asada et al., 1993
<i>Biotransformed Datura stramonium</i>	TR-105,	Hyoscyamine	Maldonado-Mendoza et al., 1993
<i>Duboisia myoporoides</i> ^d	ATCC 15834	Scopolamine	
<i>Catharanthus roseus</i> ^e	HRI	Scopolamine	Yukimune et al., 1993
<i>Hyoscyamus albus</i>	ATCC 15834	Catharanthine	Jung et al., 1994
	R 1601	Tropanes	Doerk-Schmitz et al., 1994

^aMedium after Parr et al. (1988); yield, 47% of volatile oil.

^bMedium after Gamborg et al. (1968) half strength; yield, 1–3.5% DW.

^cMedium after Murashige and Skoog (1962) with 20 mg/L tryptamine; yield, 6.25% DW.

^dMedium after Nitsch and Nitsch (1969) with 10 µM IBA; top, yield after eight selections; yield, 3.2% DW.

^eMedium after Schenk and Hildebrandt (1972) with improved inorganic salt composition, yield, 60 mg/L.

Shoots transformed by cocultivation of stem segments with *Agrobacterium rhizogenes* have become the prime source material for production of essential oils.

Basic methodology of genetic manipulation of metabolism for directed alteration of plant products may be derived from the preceding chapter (see Chap. 8). Following is a listing of experimentation applied to secondary metabolism (**Table 5**).

E. Product Release and Adsorption

Cell cultures arriving from a phase of growth at the stationary phase will mature and sometimes show signs of cell deterioration and lysis. As a consequence, secondary metabolites will appear in the nutrient medium. Such occurrence may be exploited for downstream processing [Mathur et al., 1994]. A more controlled release of products from cells would be preferred. Hairy root clones of *Duboisia leichhardtii* releasing scopolamine have been obtained by selection. Although total scopolamine produced was 2.6 mg/50 mL culture, approximately 75% was released into the medium. Curiously enough, hyoscyamine was not released, it may have been completely transformed to scopolamine [Muranaka et al., 1993].

Release of berberine from cultured cells of *Thalictrum minus* into the medium was temperature-dependent and was suppressed by such inhibitors of membrane-bound ATPase as vanadate and diethylstilbestrol. These results indicate that berberine is secreted through an

Table 4 Secondary Metabolite Production by Transformed Shoots Cultured In Vitro

Species	Transforming Agent	Product	Ref.
<i>Mentha piperita</i> ^a	Expression cassette pFIH10	Monoterpenes	Spencer et al., 1993
<i>Mentha citrata</i> ^b	<i>Agrobacterium tumefaciens</i> strain T37		

^aMedium after Murashige and Skoog (1962) or Gamborg et al. (1968) without hormones; yield, 0.8 mg/flask (15 g FW).

^bMedium as above; yield, 2 mg/flask (11 g FW).

Table 5 Expression of Transgenes in Plants and In Vitro Systems for Improved Production of Secondary Metabolites

Species	Material	Transgene	Product	Ref.
<i>Peganum harmala</i>	Roots Cell suspension	Tryptophan decarboxylase	Serotonin	Berlin et al., 1993
<i>Nicotiana tabacum</i>	Hairy roots	Lysine decarboxylase	Cadaverine	Fecker et al., 1993
<i>Atropa belladonna</i>	Roots	Hyoscyamine hydroxylase	Anabasine; scopolamine	Hashimoto et al., 1993
<i>N. tabacum</i>	Plant	Peroxidase	Lignin	Lagrimini et al., 1993
<i>N. tabacum</i>	Plant	Cinnamyl dehydrogenase	Lignin	Halpin et al., 1994
<i>N. tabacum</i>	Plant	O-Methyl transferase	Lignin	Dwivedi et al., 1994; Ni et al., 1994.

energyrequiring process located in the plasma membrane of berberine-producing *T. minus* cells, an active transport system [Yamamoto et al., 1987]. The engineering of active release of products by cells may be the major challenge for future genetic manipulation of in vitro systems.

The release of metabolites, again, triggers the question of whether these compounds would not be toxic to cells generating them or whether they may be resorbed. Uptake has been investigated and led to the observation, that products indigenous for a given species will indeed be taken up from the medium and stored in vacuoles [Deus-Neumann and Zenk, 1986], but not indiscriminately. For example, in *Coptis japonica* protoberberine alkaloids, such as berberine, coptisine, jatrorrhizine, and columbamine, were taken up from the medium and stored in vacuoles; magnoflorine and papaverine, isoquinoline alkaloids, were also taken up, but at a much lower rate [Sato et al., 1993].

Forced release of secondary metabolites from cells and the harvesting of these products from the medium by adsorption would be a biological scoop and, at the same time, facilitate continuous culture processes that may reduce costs of production and purification and allow one to economize immobilized systems as well as hairy root and shoot cultures. The release of desirable products from vacuoles without inferring damage to the cells remains the actual challenge to be met. Strategies could include a reversal of the ion-trap mechanism that governs the accumulation of some products, such as indole alkaloids in vacuoles of *C. roseus*, by acidification of the culture medium [Bouyssou et al., 1987; Jardin et al., 1991].

Passive diffusion, unmitigated or stimulated by elicitation or enhanced by permeabilization with dimethyl sulfoxide (DMSO) or other agents, and subsequent extracellular accumulation by adsorption of metabolites has been demonstrated (Table 6). Since the inception of the idea [Beiderbeck, 1982], several solid and liquid adsorbents of secondary metabolites have been introduced into nutrient media, thus adding an extraction phase to a growth phase (i.e., establishing a two-phase culture) [Beiderbeck and Knoop, 1987]. Such a procedure would increase product yields by adding an additional source (i.e., the culture medium), but more importantly, by suppressing end-product inhibition, by suppressing enzymatic or nonenzymatic metabolism of extracellular products, and by capturing volatile products. It requires adsorbents that are nontoxic, do not interact with the nutrient media, bind in as specific a manner as possible to desirable metabolites, and release metabolites during subsequent extraction processes. Moreover, the introduction of adsorbents benefits continuous culture of plant cells and organs and, thus, immobilized cell culture systems.

A combination of agents that permeabilize cells, such as DMSO [Brodelius, 1988], and of adsorbents [Maisch et al., 1986] may enhance both release and the productivity of cell

Table 6 Adsorbents for Accumulation of Secondary Metabolites in the Medium of Cell Cultures In Vitro

Species	Material	Adsorbent	Product	Ref.
<i>Vitis vinifera</i>	Cell suspension	Amberlite IR- 1 20	Anthocyanins	Cormier et al., 1992
<i>Eschscholtzia californica</i>	Cell suspension	Dimethyl siloxane	Benzophen anthridines	Byun et al., 1990
<i>Lithospermum erythrorhizon</i>	Hairy roots ^a	XAD-4; XAD-7	Shikonins	Shimomura et al., 1991
<i>Coleus blumei</i>	Immobilized cells	DMSO	Rosmarinic acid	Park and Martinez, 1994
<i>Papaver somniferum</i>	Immobilized cells	XAD-4; XAD-7	Sanguinarine	Kurz et al., 1990

^aAbout 85–90% of shikonin produced was trapped in the resin, whereas only 1–5% was found in the medium and 10–15% in the hairy roots. XAD-4 and XAD-7 adsorbed less shikonin, and lower productivity of the hairy roots was observed. Maximum production of shikonin was with XAD-2 (i.e. twice control).

suspension by scavenging even trace amounts of chemicals from the medium, thereby avoiding any kind of feedback inhibition that may hinder product formation. Chemical permeabilization with, for instance, menthol, and adsorbents, such Amberlite IR-120, affected the release of up to 70–80% of vascular anthocyanins [Cormier et al., 1992].

F. Cryopreservation

Technology for successful cryopreservation of cells, tissues, and organs of medicinal plants has been described and reviewed [Kartha, 1985; Kartha and Engelmann, 1994]. It basically involves the viable freezing of cells and tissues, their subsequent storage at ultralow temperature, preferably at that of liquid nitrogen (-196°C), and their eventual retrieval in a genetically unaltered condition. Over the years, development of cryopreservation technology revolved around examining various freezing methods, such as slow or two-stage freezing, rapid freezing, droplet freezing, and vitrification (Table 7). With high-yielding cell lines, chemical analyses will always have to confirm the success of cryopreservation. Procedures are best derived from manuals [Kartha, 1987].

G. (Micro)propagation

Domestication and establishment of plantations with cuttings or with plants regenerated from in vitro grown cells, tissues, and organs have become the goal when rare and high-yielding genotypes are to be maintained. The simpler method will be given preference over the more sophisticated one. Plant material that qualifies for (micro)propagation are elite plants, wild mutants or transgenics, and rare plants threatened by extinction. For a rare, high-vindolineyielding flowerless variant of *C. roseus* selected in the greenhouse, propagation by cuttings became the

Table 7 Recent Applications of Cryopreservation to Selected Cell Lines

Species	Material	Ref.
<i>Tabernaemontanum divaricata</i>	Cells	Schrijnemakers et al., 1990
<i>Catharanthus roseus</i>	Cells	Schrijnemakers et al., 1990
<i>Papaver somniferum</i>	Cells	Friesen et al., 1991

Table 8 Examples of Recent Micropropagation for Preservation of Wild Germ Plasm of Medicinal Plants

Species	Technology	Ref.
<i>Adhatoda beddomei</i>	Shoot proliferation from axillary meristem	Sudha and Seenii, 1994
<i>Panax ginseng</i>	Somatic embryogenesis	Asaka et al., 1994
<i>Stevia rebaudiana</i>	Multiple shoot mass propagation	Akita et al., 1994
<i>Turnera diffusa</i>	Organogenesis from leaves	Alcaraz-Melendez et al., 1994
<i>Woodfordia fruticosa</i>	Mass propagation from shoot segment explants	Krishnan and Seenii, 1994
<i>Iris pallida</i>	Embryogenesis and cloning	Jehan et al., 1994
<i>Iris germanica</i>	With a variety of explant tissues	
<i>Leucojum aestivum</i>	Mass propagation from bulbs	Stanilova et al., 1994

method of choice [Balsevich and Bishop, 1989]. Repeated plantations for vindoline production proved the benefit of such procedure [Balsevich, unpublished data] (Table 8).

The procedure to follow has been amply and repeatedly described in manuals [George and Sherrington, 1984; Lindsay, 1991], books [Pierik, 1987; Debergh and Zimmerman, 1991], and reviews [Rao, 1987; Krikorian, 1994]. In general, cuttings will be given preference over regenerants grown in vitro from seeds; or embryos and seedling tissue, over shoot, root, and rhizome segments, to achieve success in a short period and without intervening callus formation. All measures should be taken to avoid callusing and, thereby, the possibility of somaclonal variation. The latter would be of interest only in cases of extremely limited plant material, as for example, with *Sophora toromiro* (Leguminosae) of the Easter Islands, extinct since 1962. Automation of micropropagation procedures have been introduced by several (commercial) laboratories. Because micropropagation remains an art, examples describing the micropropagation of material similar to that under investigation are invaluable.

IV. CASE STUDIES

A. Monoterpenes: Menthol

Menthol (3; Fig. 1) is the main constituent (up to 50% plus) of the essential oil of *Mentha piperita*, a triple hybrid plant, multiplied by cuttings. It is derived from pulegone (1) via menthone (2). Functionally, menthol and other terpenoids of the essential oil of mint are phytoanticipins [van Etten et al., 1994], a chemical defense mechanism of the plant against insect pests [Charlwood and Charlwood, 1991]. Worldwide demand for mint oil as over-the-counter medication and food additive is met by plantations in many countries, particularly in Europe and North America. The challenge is to develop technology that will stimulate essential oil accumulation in cells and organs cultured in vitro for molecular biological studies and will increase the menthol constituent of essential oil in mint.

Undifferentiated, finely-dispersed cell suspension cultures derived from a wide variety of aromatic plants typically accumulate only insignificant amounts of monoterpenoids [Charlwood, 1993]. Thus, the production of essential oils by *Mentha* spp. cultures has been very low (0.1% FW) whereas intact plants yield oil at 0.1–5% FW. The lack of accumulation of terpenoids in undifferentiated cells of mint may not necessarily be due to lack of expression of genes encoding enzymes in terpenoid biosynthesis, rather these enzymes may be expressed only in differentiated cells and tissues, (i.e. trichomes). Also, essential oils may be subject to catabolism, potentially at a greater rate than synthesis. Finally, the toxicity of oil may become limiting if it exceeds 0.1% FW [Charlwood, 1993].

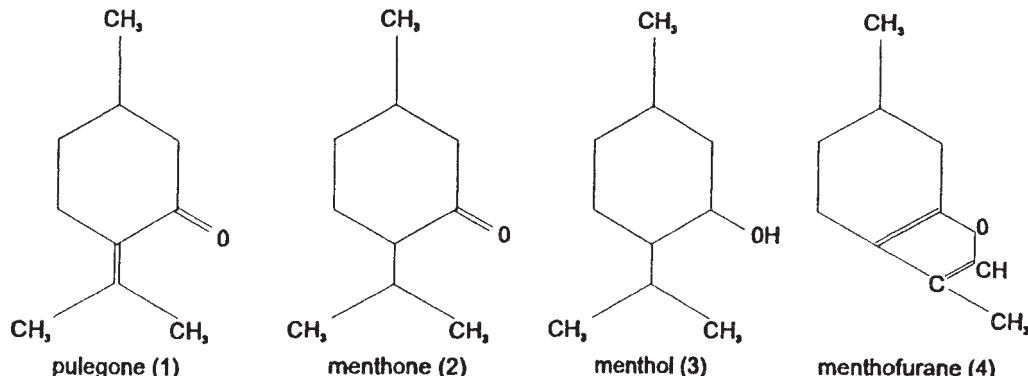


Fig. 1 Structure of pulegone (1), menthone (2), menthol (3), menthofurane (4).

Early investigations revealed clearly that callus cultures with shoot buds accumulate sizable amounts of essential oil of a quality different from the source plants. Instead of a preponderance of menthol as in source plants the oil of these bud-studded calli contained mainly pulegone (1) and menthofurane (4; see Fig. 1) [Bricout and Paupardin, 1975]. Addition of 0.01 mg/L colchicine increased the density of glands, thus the amount of oil, not the quality of the oil [Bricout and Paupardin, 1978]. High pulegone pools and traces only of menthol in in vitro cultured material would indicate a rate-limiting step downstream. The case is more complex, however, because *Mentha* cell cultures reduce *d*-pulegone to *d*-isomenthone and *l*-menthone to *d*-neomenthol [Rodov et al., 1982], not *l*-menthol. These results indicate only, biotransformation capability and stereospecificity relative to precursors and products. Besides, cell suspension cultures will glycosylate *l*-menthol added to the medium to *l*-menthyl glucoside [Berger and Drawert, 1988; Werrman and Knorr, 1993]. In *M. canadensis* cultures the conversion was more enantioselective and stereospecific than in *M. piperita* cells.

With the production of transgenic shoots, using disarmed strains of *Agrobacterium tumefaciens*, mint oil resembled that of parental material (i.e., menthol was the major component), but menthone levels were lower, and menthofuran levels were higher than in parent plants [Spencer et al., 1993] (Table 9).

[For a listing of all in vitro cultures employed in the production of monoterpenes see Charlwood, 1963].

Table 9 In Vitro Culture of *Mentha* × *piperita* for Production of Menthol

Material	Medium	Products	Ref.
Callus w. buds	MS ^a	Pulegone, menthofurane	Bricout and Paupardin, 1975
Callus w. buds	MS ^b	Pulegone, menthofurane	Bricout and Paupardin, 1978
Cell suspension		Monoterpenoids	Rodov et al., 1982
Cell suspension	B5 ^c	Monoterpenoids	Cormier and Do, 1988
Transformed shoots	B5, MS ^d	Menthol Menthone, menthofurane	Spencer et al., 1993

^aMedium after Murashige and Skoog (1962) with 1 mg/L each of IAA and BA.

^bMedium as for a with 0.01 mg/L colchicine.

^cMedium after Gamborg et al. (1968).

^dMedium as for a or c without any hormones.

B. Diterpenes: Paclitaxel (Taxol)

(Taxol; paclitaxel 1) and taxanes (2–5; Fig. 2) are complex oxygenated diterpenes, initially isolated from the stem bark of Pacific yew (*Taxus brevifolia* Nutt.) by Wani et al. [1971]. More recently, these compounds have also been demonstrated to occur in needles of various *Taxus* spp. [Witherup et al., 1990] and in the mycelium of *Taxomyces andreanae*, an endophytic fungus of Pacific yew [Stierle et al., 1993]. Little is known about the biosynthesis of paclitaxel, and the possibility of engineering its production remains frustratingly remote. Examination of gold-labeled thin sections has revealed binding of an antipaclitaxel antibody primarily to vacuolar inclusions in parenchyma cells associated with the phloem. Comparisons suggest that the inclusion bodies are tannin bodies. Tannins may trap paclitaxel and are the site of its sequestration [Ellis et al., 1993]. Treatment of plant, animal, and HeLa cells with low concentrations of paclitaxel results in the appearance of new microtubules and in the polymerization of existing microtubules [Mole'-Bajer and Bajer, 1983]. Importantly, this compound blocks the cell cycle at the G₂/M phase through microtubule polymerization and, thus, is a potent inhibitor of cell replication [Horwitz et al., 1986].

Paclitaxel has been effective in the treatment of refractive ovarian cancer, metastatic breast cancer, malignant melanoma, and other solid tumors [Slichenmyer and von Hoff, 1991]. The worldwide demand for paclitaxel as a drug will quickly exceed 100 kg/yr. At present, the commercial source is the bark of *T. brevifolia*, a tree limited to the North American Pacific Northwest. The bark required to yield 1 kg of paclitaxel is about 7000 kg [Cragg et al., 1993]. This situation has mandated the development of alternative sources of this substance.

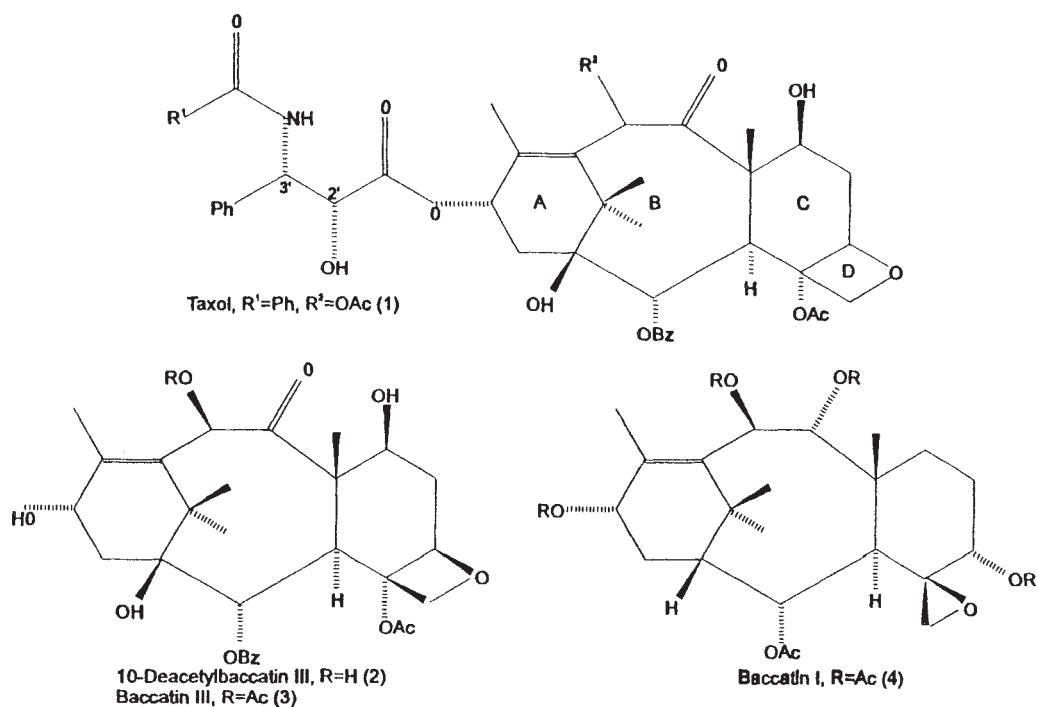


Fig. 2 Structure of paclitaxel (1) and taxanes (i.e., 10-deacetyl [10-D] baccatin III, 2; baccatin III, 3, baccatin I, 4).

Total synthesis of paclitaxel has been accomplished rather recently [Holton et al., 1994a,b]. It is unlikely, however, that total synthesis will ever contribute significantly to its supply, because the process is rather uneconomical [Kingston, 1994]. Production by the semisynthetic route from precursor taxanes will, however, increasingly replace the bark of *T. brevifolia*. Taxanes are readily obtainable from needles harvested from *Taxus×media cv. Hicksii* grown in plantations [Cragg et al., 1993].

The demand for paclitaxel has prompted several biotechnology laboratories to develop alternative methods for its production: (1) plant propagation and (2) cell and DNA technologies. Consequently, in vitro culture conditions have been established that allow embryo germination and subsequent conversion into seedlings in various *Taxus* spp., among them *Taxus×media cv. Hicksii*, in less than 3 months using a nutrient medium after Gupta and Durzan [Flores et al., 1993]. The result compares favorably with natural long dormancy periods observed with seeds grown in the field. The development of in vitro microppropagation methods, as has been accomplished for several coniferous species, remains to be shown. Such methods may become imperative, should cloning of elite specimens or production of transformed trees be required.

The establishment of callus and cell suspension cultures of *Taxus* spp. has been achieved, once browning and deterioration of explanted tissue had been overcome by supplementing nutrient media with ascorbic acid, activated charcoal, or polyvinylpyrrolidone. Cultured cells respond well to media after Gamborg et al. [1968] and Murashige and Skoog [1962] when supplemented with 2,4-D and kinetin (Table 10). The production of paclitaxel by some cell lines cultured in vitro has equaled, if not surpassed that of *T. brevifolia* bark. Remarkable is the enhancement of production by media supplements of phenylalanine [Fett-Neto et al., 1993].

Results in Table 10 are significant inasmuch as they show paclitaxel synthesis and accumulation in cells cultured in vitro. One may, thus, assume that paclitaxel occurs in parenchyma cells, likely in older cells and, according to one observation, in cells that tend to be rich in phenolics, for they easily turn brown over time [Fett-Neto et al., 1992]. Stimulation of paclitaxel production

Table 10 Production of Paclitaxel by *Taxus* spp. Trees and Cells Cultured In Vitro

Species	Material	Medium	% DW	Ref.
<i>T. brevifolia</i>	Bark	—	0.015	Vidensek et al., 1990
	Roots	—	0.0004	
	Needles	—	0.015	
	Needles		0.006	Witherup et al., 1990
<i>T. brevifolia</i>	Cell suspension	B5 ^a	—	Gibson et al., 1993
	Gall ^b cells	WP ^b	Trace	Han et al., 1994
<i>T. × media</i>	Callus	B5 ^c	0.001	Wickremesinhe and Artega, 1993
	Cell suspension	B5 ^d	Trace	Wickremesinhe and Artega, 1994
<i>T. cuspidata</i>	Callus	B5 ^e	0.02	Fett-Neto et al., 1992
	Immobilized cells	B5 ^e	0.012	Fett-Neto et al., 1992
	Callus	B5 ^f	0.04	Fett-Neto et al., 1993
<i>T. andreanae</i>	Mycelium		50 ng/L	Stierle et al., 1993

^aMedium after Gamborg et al (1968) with 1 mg/L 2,4-D and 0.2% casamino acids.

^b*Agrobacterium tumefaciens* gall-derived cells. Woody plant medium after Lloyd and McCown (1980) without hormones.

^cHabituuated callus. Medium after Gamborg et al. (1968) with 2×B5 vitamins, without hormones.

^dMedium as for c with 1 mg 2,4-D and 1 mg/L kinetin plus 20 g/L sucrose +2.5 g/L glucose +2.5 g/L fructose.

^eMedium after Gamborg et al. (1968) with 1 mg/L 2,4-D and 1 mg/L kinetin, plus 1.5% polyvinylpyrrolidone.

^fMedium after Gamborg et al. (1968) as for e plus 0.1 mg/L phenylalanine.

may well be achieved by permutation of concentrations of various media components, by application of two-phase systems, or by application of biotic and abiotic stress agents (elicitors). Phyton Catalytic (New York) is said to have increased paclitaxel yields to 1–3 mg/L of supernatant using elicitors. The idea of a cocultivation, as a mixture or separated by a membrane, of *Taxus* cells and fungus (*Taxomyces andreanae*) is intriguing.

For the bioengineering of paclitaxel production, prospects of success are slim, at the moment. For one, its biosynthetic pathway is undefined. The likely precursor is geranylgeranylpyrophosphate. Isolation and partial purification of an enzyme that catalyzes cyclization of this precursor has been reported [see Cragg et al., 1993]. Second, transgenic conifers have been demonstrated [Ellis et al., 1993; Bommieni et al., 1993]. Still, a workable concept on how to genetically manipulate paclitaxel synthesis and accumulation has not been devised. ESGA genetics (California) may succeed in using a by-way by employing hairy root cultures for improved production. As it stands, 1 kg paclitaxel would require the harvest of 500–1000 bioreactors of 500 L each filled with a heavy suspension of *Taxus* cells.

C. Isoquinoline Alkaloids: Sanguinarine

Sanguinarine (1; and Fig. 3) and related compounds, such as dihydrosanguinarine, chelerythrine (2), or chelirubine, are benzophenanthridine alkaloids derived from tyrosine. Together with morphinan and berberine alkaloids they share reticuline as common precursor. The biosynthetic pathway leading to benzophenanthridines has been substantially elucidated [Dittrich and Kutchan, 1991]. These alkaloids occur in Papaveraceae, sanguinarine being a major alkaloid in *Sanguinaria canadensis*, bloodroot [Preininger, 1986]. In plants, sanguinarine is thought to function as a phytoalexin, because it has proved to be an antibiotic, effective against fungi and bacteria. The site of sanguinarine accumulation is parenchyma organelles pelleting at 1000×g [Kutchan et al., 1986].

Since sanguinarine was described as an antiplaque agent [Southard et al., 1984], it has become a component of dental health care products, with a potentially wide market [Vipont, 1988]. Accordingly, pressure on natural sources of sanguinarine continues to rise. Domestication of *S.*

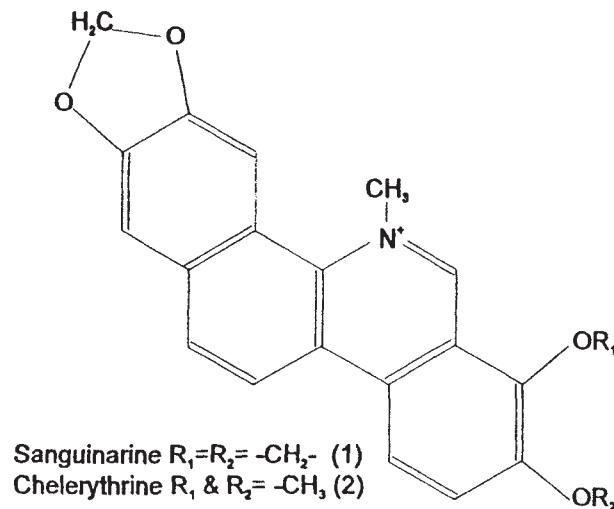


Fig. 3 Structure of sanguinarine, 1; chelerythrine, 2.

Table 11 Production of Sanguinarine by Cells of Various *Papaveraceae* When Cultured In Vitro

Species	Material	Medium	% DW	Ref.
<i>S. canadensis</i>	Rhizome	—	2.7	Bennet et al., 1984
<i>P. somniferum</i>	Root	—	Trace	Preininger, 1986
<i>S. canadensis</i>	Cell suspension	B5 ^a	1.3	Rho et al., 1992
	Immobilized cells	B5 ^a	1.0	Rho et al., 1992
	Cell suspension	MS ^b	0.02	Cline et al., 1993
	Cell suspension	B5 ^c	3.6	Mahady and Beecher, 1994
	Cell suspension	B5 ^d	0.22	Mahady and Beecher, 1995
<i>Chelidonium majus</i>	Cell suspension	B5 ^e	8.8	Tome and Colombo, 1992
<i>E. californica</i>	Cell suspension	B5 ^f	Trace	Berlin et al., 1983
	Cell suspension	B5 ^g	3.5	Byun et al., 1990
<i>P. bracteatum</i>	Cell suspension	B5 ^h	0.5	Lecky et al., 1992
<i>P. somniferum</i>	Cell suspension	B5 ⁱ	2.9	Eilert et al., 1985
	Cell suspension	B5 ^j	13	Park et al., 1992
	Cell suspension	MS ^j	3.9	Williams et al., 1992
	Transformed cells	B5 ^k	4.8	Williams and Ellis, 1993

^aMedium after Gamborg et al. (1968) with 1 mg/L 2,4-D, 0.1 mg A kinetin, plus nitrate×1/2.

^bMedium after Murashige and Skoog (1962) without hormones, plus *Verticillium dahliae* conidial suspension.

^cMedium after Gamborg et al. (1968) with *Penicillium expansum* homogenate; product includes chelerythrine (total = 362 µg/100 mg cells).

^dMedium after Gamborg et al. (1968) with 100 µM quercetin, product includes chelerythrine.

^eMedium after Gamborg et al. (1968), without elicitors.

^fMedium after Gamborg et al. (1968) with 5×10⁻⁶ M 2, 4-D, benzophenanthridine as dihydrosanguinarine.

^gMedium after Gamborg et al. (1968) with 5 µM 2, 4-D and 0.5 µM kinetin, second phase with a supplement of elicitor (yeast extract) and silicone fluid.

^hMedium after Murashige and Skoog (1962) with copper×2 or 4; production 2.5–0.5% DW dihydrosanguinarine.

ⁱMedium after Gamborg et al. (1968) with 1 mg/L 2, 4-D and *Botrytis* homogenate.

^jMedium after Murashige and Skoog (1962) plus polymeric resins (XAD-7) during production phase.

^kMedium after Gamborg et al. (1968) without hormones; production=400 µg FW* 0.63–6.31 % DW of rhizome.

canadensis has been met with limited success. *Macleaya cordata* plantations are said to furnish most of the material for extraction and processing, so far. The search for alternative sources of sanguinarine has led to vigorous biotechnological research and development.

Cell cultures of various *Papaveraceae* have successfully been established in several laboratories. Although sanguinarine production by such cultures has been observed to reach levels of 0.1% DW, concentrations have been increased very significantly by a variety of culture procedures, foremost elicitation (Table 11). Notably, the quality of both the cell line and the elicitor had to be optimized for highest response. *Verticillium* elicitor, for example, led to high levels of sanguinarine production in *P. bracteatum*, whereas this elicitor did not influence sanguinarine production in *S. canadensis* [Cline et al., 1993]. *Papaver somniferum* cells produced sanguinarine up to 2.9% DW after treatment of cells with *Botrytis* spp. elicitor [Eilert et al., 1985], whereas several years later, selected cell lines showed production levels of 13% DW [Park et al., 1992].

Sanguinarine accumulates in cells as well as in the medium [Kutchan et al., 1983; Eilert et al., 1985; Cline and Coscia, 1988; Tome and Colombo, 1992; Williams and Ellis, 1993]. If controlled by the cells, the release of sanguinarine into the medium would be an important feature to be subjected to future biotechnological research and development. Benzophenanthridine adsorption and subsequent extraction from dimethyl siloxane in cell

cultures of *Eschscholtzia californica* elicited with yeast has been very substantial (3.5% DW) [Byun et al., 1990].

Of the 17 enzymes implicated in the synthesis of sanguinarine from two molecules of tyrosine, 9 require molecular oxygen. Dihydrophenanthridine oxidase is an oxygen-dependent enzyme that catalyzes the conversion of dihydrosanguinarine to sanguinarine. The activity of this oxidase from *S. canadensis* cell cultures is elevated two- to threefold under elicitation conditions in the presence or absence of hormones [Cline et al., 1993]. The biosynthesis of sanguinarine does not appear to have become subject to genetic engineering; transgenic expression of the berberine bridge enzyme in Papaveraceae has not yet been reported.

Addition of silicone fluid to culture media had a surprising effect on product accumulation and harvesting. With cultures derived from *Eschscholtzia californica*, dimethyl siloxane has greatly increased the production of sanguinarine, is selective for anthridine alkaloids, allows release of bound alkaloids by simple subsequent treatment, is stable during sterilization of media by autoclaving, does not change the media composition, and is easily separated from the cells and the medium [Byun et al., 1990]. Finally, polymeric resins, Amberlite XAD-4 and XAD-7, have been employed in stimulation of sanguinarine production, and enhancement of harvest and extraction procedures. Yields of more than 4% DW were achieved [Byun et al., 1990; Kurz et al., 1990; Williams et al., 1992].

High-yielding cell lines of *P. somniferum* have successfully been stored by cryopreservation [Friesen et al., 1991].

D. Indole Alkaloids: Vinblastine

Vinblastine (3; Fig. 4), a dimeric compound composed of catharanthine (1) and vindoline (2), two monoterpene indole alkaloids, is found in *Catharanthus roseus* (L.) G.Don (*Apocynaceae*), Madagascar periwinkle. The biosynthetic pathway has been elucidated to the extent that initial steps from tryptophan and mevalonic acid to strictosidine and catharanthine [see Kutchan, 1993], and from tabersonine to vindoline [DeLuca et al., 1986; DeCarolis and DeLuca, 1993] have been well analyzed and described. Natural dimerization to vinblastine includes 3'4'-anhydrovinblastine. Chemical dimerization has been accomplished by way of the Polonovsky reaction [see Kutney, 1990].

In plants, vinblastine occurs at very low concentrations (5 g/2 tons FW). Owing to its cytotoxic effect, it may well function as a phytoalexin, a chemical defense agent. Vinblastine may occur in laticifers and idioblasts of *Catharanthus*, it accumulates in parenchyma throughout the plant. Of the two monomers, catharanthine has the highest concentrations in youngest, fully expanded leaves, the vindoline concentration was highest in leaves 5 and 7 from the apex [Balsevich and Bishop, 1989].

Ever since 1958 when vinblastine and its analogue vincristine were recognized as anticancer agents and established as important drugs in the chemotherapeutic treatment of Hodgkin's disease and acute leukemia [see Svoboda, 1975], its supply has been of great concern. Plantations of *C. roseus* in various parts of the world could match the demand, were it not for the extreme cost associated with extraction and purification. Alternative sources of higher value (i.e., rich in vinblastine and unaccompanied by a host of related alkaloids) are the goals targeted by R&D. Not surprisingly, the development of cell cultures of *C. roseus* began as soon as in vitro culture had been established as a method in plant physiology, with callus, crown-gall, and habituated tissue cultures [Carew, 1975]. More recently, these efforts have been complemented by DNA technologies.

Despite many years of effort and remarkable success in increasing the production of catharanthine in cell suspensions by treatment with biotic and abiotic elicitors (Table 12),

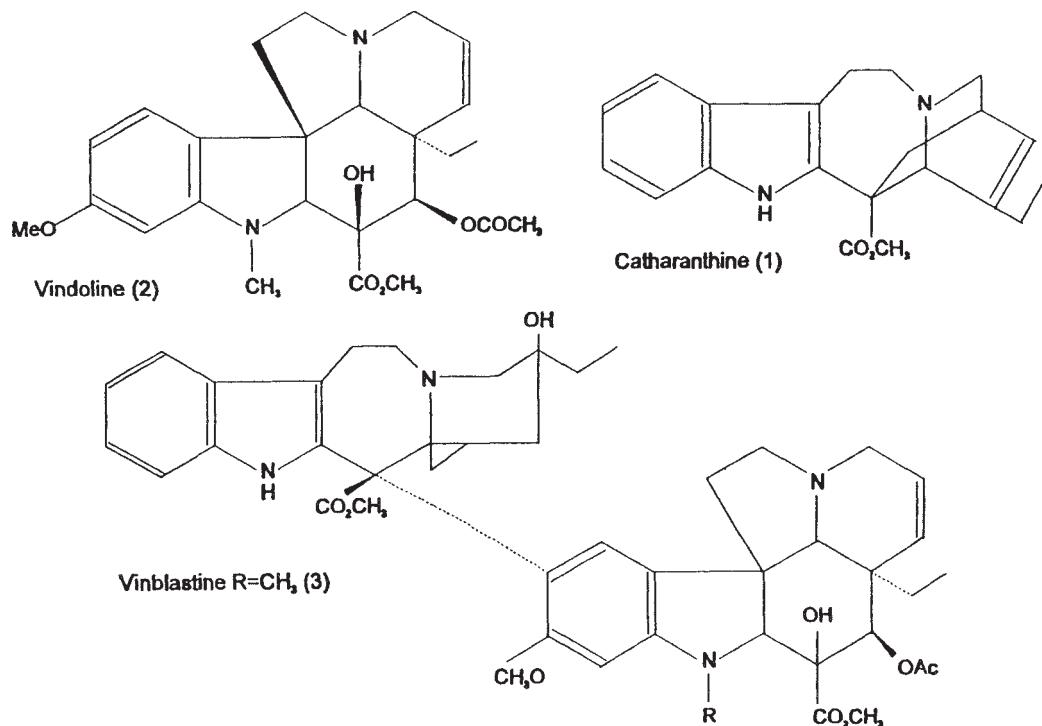


Fig. 4 Structure of catharanthine, 1; vindoline, 2; vinblastine 3.

vinblastine has eluded all laboratories. The problem is a lack of vindoline synthesis and accumulation in cell suspension cultures [DeLuca et al., 1986, 1988]. Still, vindoline recurs in shoots regenerated from callus [Constabel et al., 1982] and accumulates in multiple shoot cultures [Hirata et al., 1987, 1993; Miura et al., 1988]. This observation underpinned the notion that vindoline synthesis in material cultured *in vitro* requires prior differentiation of cells. Incidentally, differentiation of material as expressed in shoot cultures also stimulated the formation of vinblastine. Also, vindoline and catharanthine production in shoot cultures was influenced by phytohormone levels in the medium [Hirata et al., 1987] and treatment with near-ultraviolet light [Hirata et al., 1991, 1993]. Evidence for vindoline synthesis as a function of differentiation may be deduced from seedling analyses that show that enzymes involved in the transformation of tabersonine to vindoline are localized in the chloroplast, the endoplasmic reticulum, a putative alkaloid synthesizing vesicle, and cytoplasm, and are phytochrome-dependent, respectively [Aerts and DeLuca, 1992; DeLuca et al., 1993]. It remains to be seen whether photoautotrophic cells of *C. roseus* would produce sizable amounts of vinblastine. So far, photoautotrophic cell cultures have been incapable of synthesizing vindoline [Tyler et al., 1986].

Recently, vindoline-producing cell cultures of *C. roseus* have been presented [Mahady et al. personal communication]. These cultures had been treated with *A. tumefaciens* and characterized as habituated (i.e., growing in medium [Gamborg et al., 1968] without hormones under constant fluorescent light of 2500 lux). Hairy root cultures generated by employing *A. rhizogenes* have shown remarkable levels of catharanthine [Jung et al., 1994] and traces of vinblastine [Parr et al., 1988].

Table 12 Production of Catharanthine, Vindoline, and Vinblastine in Plant Tissues Derived from *Catharanthus roseus* and Cultured In Vitro

Material	Medium	% DW	Ref.
Catharanthine			
Leaf 5	—	0.2	Balsevich and Bishop, 1989
Leaf 3	—	0.3	Balsevich and Bishop, 1989
Cell suspension	B5 ^a	0.001	Eilert et al., 1986
Cell suspension	MS ^b	0.15	Smith et al., 1988
Hairy roots	SH ^c	0.3	Jung et al., 1994
Cell suspension	B5 ^d	6.8	Fujita et al., 1990
Vindoline			
Leaf 5	—	0.3	Balsevich and Bishop, 1989
Leaf 3	—	0.4	Balsevich and Bishop, 1989
Hairy roots	B5 ^e	Traces	Parr et al., 1988
Shoot cultures	MS ^f	0.0015	Miura et al., 1988
Regenerated shoots	—	0.01	Hirata et al., 1993
Vinblastine/anhydrovinblastine			
Leaf 5	—	0.3	Balsevich and Bishop, 1989
Leaf 9	—	0.4	Balsevich and Bishop, 1989
Hairy roots	B5 ^e	Traces	Parr et al., 1988
Shoot cultures	MS ^f	0.0015	Miura et al., 1988
Regenerated shoots	—	0.01	Mirata et al., 1993

^aMedium after Gamborg et al. (1968) with *Pythium aphanidermatum* homogenate.

^bMedium after Murashige and Skoog (1962) with 0.1 mg/L NAA, 0.1 mgA kinetin, plus 50 mg/L vanadylsulfate (selected cell line JOH).

^cMedium after Schenck and Hildebrandt (1972), two-phase culture with inorganic salts×1/3 in second phase.

^dMedium after Gamborg et al. (1968) using a high-yielding cell line at high inoculation density.

^eMedium after Gamborg et al. (1968) without hormones.

^fMedium after Murashige and Skoog (1962) with 1 mg/L benzyladenine.

Enhanced production of catharanthine and vindoline in planta and in vitro is expected to be effected by genetic manipulation of their biosynthesis [Kutchan, 1993] or by genetically directed changes of the cytoenvironment of alkaloid synthesis and accumulation. Early work has shown that elicitors would induce tryptophan decarboxylase and strictosidine activities in cell cultures of *C. roseus* [Eilert et al., 1987]. Since then, initial steps have been completed with the isolation and characterization of tryptophan decarboxylase cDNA [DeLuca et al., 1989] and the isolation and heterologous expression of cDNA and genes of strictosidine synthase from *R. serpentina* [Kutchan et al., 1988] and from *C. roseus* [McKnight et al., 1990].

Finally, given sufficient yields of catharanthine from cell cultures and vindoline from shoot cultures or plants, vinblastine can be obtained through dimerization catalyzed by cell-free extracts of cells cultured in vitro, by enzymes of such cultures [Kutney et al., 1990], and by horseradish peroxidase [Goodbody et al., 1988] with 3'4'-anhydrovinblastine as an intermediate. Following a procedure by Vućović [see Fujita et al., 1990], a chemical reaction involving Fe³⁺ as a catalyst may result in coupling of the two precursors to anhydrovinblastine at a yield of 90% and in a second step in the formation of vinblastine at a yield of 50% [Fujita et al., 1990]. The latter would appear to be the most promising technology for vinblastine production, to date.

Cryopreservation of *C. roseus* cells has been successfully demonstrated [Karthä, 1987].

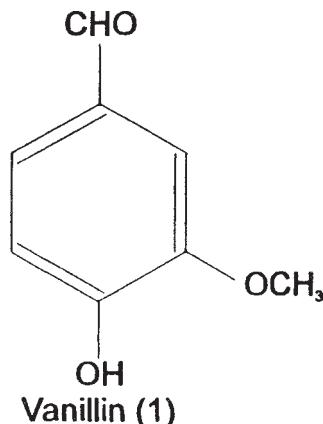


Fig. 5 Structure of 4-hydroxy-3-methoxybenzaldehyde, 1.

E. Phenylpropanoids: Vanillin

Vanillin (1; Fig. 5), a phenylpropanoid, is widely used for flavoring and in the perfume industry. It is produced through fermentation of unripe fruits ("beans") of *Vanilla planifolia* (Orchidaceae). Vanilla extracts contain vanillin as a major component (2–3%), the flavor, moreover, results from the composition of a host of many accompanying compounds, such as *p*-hydroxybenzaldehyde (2), 4-hydroxybenzaldehyde (2), and vanillic acid (3). Global production of natural vanilla rests on a dangerously narrow genetic base [Smith et al., 1992]. Plantations in many parts of the tropics are all derived from a single cutting introduced to Reunion from the Jardin des Plantes in Paris in 1827. Tissue culture technology is being used to conserve *Vanilla* germ plasm collected in tropical rainforests of Central and South America.

Vanilla is a vine orchid, that is difficult to grow; fruit set depends on hand-pollinating skills. Hence, there is a need for alternative production methods. As early as 1874, Tiemann devised a process of oxidizing coniferine of young conifer bark to vanillin. Biotransformation of eugenol to vanillin, practiced since 1891, has recently been improved by using microbial catalysts (BASF). The biology of vanillin production does not yet appear to have been touched. The surge in demand for natural vanillin may change this situation and has driven R&D technology to increase production. Little breeding has gone into *Vanilla*. However, because vanillin occurs not only in beans, but in several parts of the *Vanilla* plant, tissue culture has been embarked on to produce vanillin in vitro [Konowicz and Janick, 1984], and results are beginning to be published (Table 13).

The pathway has not been fully characterized [Funk and Brodelius, 1990]. Still, ferulic acid was found to be the best precursor for metabolism to vanillin.

Vanilla callus [Romognoli and Knorr, 1988] and cells selected for high production and secretion ability have been obtained [Knuth and Sahai, 1989]. And recently, a novel process

Table 13 Production of Vanillin in *Vanilla planifolia* Cells and Shoots Cultured In Vitro

Material	Medium	% DW	Ref.
Beans	—	0.002	Westcott et al., 1994
Transfected aerial roots	B5	0.7	Westcott et al., 1994

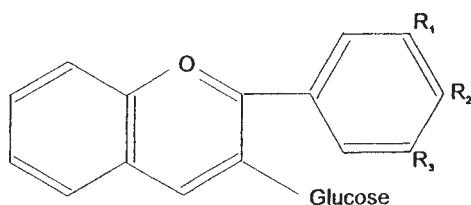
was demonstrated based on conversion of ferulic acid to vanillin using axenic aerial roots of *Vanilla* cultured in hormone-free medium [after Murashige and Skoog, 1962] as the biocatalyst. The use of charcoal as an adsorbent for the vanillin formed was critical to prevent product inhibition. The aerial root material could be reused several times [Westcott et al., 1994].

F. Flavonoids: Anthocyanins

Anthocyanins (1–3; Fig. 6) are widespread metabolites among seed plants, they are most conspicuous as pigments in flowers and fruits. They have a high potential as markers in experimental biology and as coloring in the market place. Their production at acceptable cost/benefit ratios, however, remains difficult to achieve. Still, as artificial colorings are being phased out, pressure on R&D to introduce natural nontoxic food colorings at competitive prices is mounting. Because anthocyanins generally occur as a mixture of glucosides, it would be important to reduce the number of glucosides for simplified processing of material. The ideal state of cells producing one anthocyanin at 100% purity has not yet been achieved.

Because anthocyanins have been discovered to readily accumulate in vacuoles of cells cultured *in vitro* which, in turn, have been derived from various source plants [Seitz and Hinderer, 1988], expectations were that increasing yields would only be a matter of selecting superior cell strains and optimizing culture conditions. A first wrinkle in such concept was caused by the observation that traditional source plants of anthocyanins, such as red grapes, may not render the most productive cell cultures, and may be outperformed by cell cultures of plants not known for exceptional anthocyanin accumulation, such as *Aralia cordata* [Sakamoto et al., 1994] or *Oxalis reclinata* [Crouch et al., 1993].

Culture conditions tested for improving anthocyanin synthesis and accumulation are the composition of the medium, including hormones, pH, and light. Remarkably, cell biomass and anthocyanin content in carrot (*Daucus carota*) cultures was highest on media containing a combination of galactose and glucose [Nagarajan et al., 1989, cited in Zwayyed et al., 1991]. With a different carrot culture growth was best on media with fructose, but anthocyanin production was best on glucose [Zwayyed et al., 1991]. An equimolar mixture of both carbohydrates, however, led to a lower performance in both categories. It is speculated that glucose might give a higher level of an intermediate required for anthocyanin synthesis than fructose. Stress clearly affects anthocyanin formation in plants; *in vitro* cultured material was exposed to UV irradiation [Gleitz and Seitz, 1989] with supplementation of media with carbohydrate, up to 8 and 10% (Table 14).



Pelargonidine-3-glucoside	$R_2=OH$	(1)
Cyanidine-3-glucoside	$R_2+R_3=OH$	(2)
Delphinididine-3-glucoside	$R_1+R_2+R_3=OH$	(3)

Fig. 6 Structure of pelargonidine-3-glucoside, 1; cyanidine-3-glucoside, 2; delphinididine-3-glucoside, 3.

Table 14 Production of Anthocyanin by Cells Cultured In Vitro

Species	Material	Medium	% DW	Ref.
<i>Aralia cordata</i>	Cell suspension	B5 ^a	cyanidin 3-xylosylglucoside 10.3	Sakamoto et al., 1994
<i>Vitis vinifera</i>	Cell suspension	B5 ^b		Do and Cormier, 1991
<i>Oxalis reclinata</i>	Cell suspension	MS ^c		Crouch et al., 1993

^aMedium after Gamborg et al. (1968) with 1 mg/L.

^bMedium after Gamborg et al. (1968) with 1 mg/L NAA, 0.1 mg/L kinetin, and 15 mM total N.

^cMedium after Murashige and Skoog (1962) with 5 mgA NAA and 0.5 mg/L kinetin.

With phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) identified as key enzymes in anthocyanin biosynthesis and with genes encoding both these enzymes available as cassette, genetic manipulation of cells for improved quality and yield of production has been applied to petunia plants and inaugurated molecular horticulture. PAL and CHS transgenes have not yet been expressed in cells cultured in vitro for production of anthocyanins.

Cryopreservation of anthocyanin cells was successfully accomplished by Seitz as early as 1985 [see Kartha, 1987].

V. OUTLOOK

Research and development of biotechnologies for the production of secondary metabolites has received a major impetus by the very positive response of cells and tissues, roots, and shoots cultured in vitro. Levels of production in selected cases have been very substantial, many times the level of the source material (Table 15). But interest in such tables has peaked. For one, they have been given too much emphasis in the past, they have not changed much recently, and finally, they may not be indicative of a better understanding the biology of product synthesis and accumulation. That the challenge to increase production levels and diversity in the spectrum of products is still very much alive, however, is in part a response to perceived public need.

When applied to furthering our understanding of plants and of the environment, R&D to advance biotechnologies for the production of secondary metabolites deserves full, not "secondary" attention and support. Specifically, insights into the function of vacuoles, catabolic processes, and the response of plants to environmental factors may be beneficial for further increasing the capacity of cells for product accumulation.

Table 15 Yield Records of a Few Secondary Metabolites from Plant Cells Cultured In Vitro

Species	Product	Culture/		Ref.
		% DW	Plant	
<i>Coptis japonica</i>	Berberine	8	3	Sato and Yamada, 1984
<i>Coleus blumei</i>	Rosmarinic acid	15	5	Razzaque and Ellis, 1977
<i>Morinda citrifolia</i>	Anthraquinones	10	8	Zenk et al., 1975
<i>Lithospermum erythrorhizon</i>	Shikonin	20	14	Fujita et al., 1981
<i>Datura stramonium</i>	Tropane alkaloids	1.2	15	Maldonado and Loyola, 1993
<i>Thalictrum minus</i>	Berberine	4	1,000	Nakagawa et al., 1984
<i>Papaver somniferum</i>	Sanguinarine	13	1,000	Park et al., 1992

Attention and efforts are now being shifted from enhanced production of secondary metabolites in *in vitro* systems to genetic manipulation of production in plants, accompanied by expectations of directing secondary metabolism toward desirable compounds. Besides, and more importantly, the problems of pattern formation, of cell and tissue interaction, and of the correlation between differentiation or stress and metabolite synthesis come into focus. Also, the occurrence of certain compounds in vastly different parts of the plant kingdom may be demystified through DNA techniques. In all, biology will come out ahead.

Much of the support for R&D has been considered an investment by industry in furthering human well-being. Disillusion, however, has spread recently owing to cost/benefit ratios in *in vitro* systems. Hurdles, it would appear, have grown in height over time, rather than diminished: merchandising in *in vitro*-grown products is hindered by costs of bioreactor operations, conservatism in adjusting regulations governing plant products, and the social condition of (un)employment in developing countries.

The outlook, thus, would see a return from the bioreactor to the field, now with nursery stock, more diverse and much improved by biotechnology-assisted breeding, for a time. And it is hoped that medicinal and industrial plants threatened in existence, today, can be included and planted, tomorrow.

ACKNOWLEDGMENT

This chapter is National Research Council of Canada publication no. 38467.

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Large-Scale Plant Tissue Culture

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I. INTRODUCTION: PRODUCTIVITY

Plant tissue culture was originally developed as a research tool to study the physiology and biochemistry of plants, without the complication of the whole-plant structure. The development can be traced to the early 1990s, but it has been only the last 30 years that techniques have developed sufficiently to encourage industrial interest. The first commercial process to be developed was micropropagation, which is now a worldwide industry producing millions of plants per annum.

Plants have long been used to supply colors, flavors, fragrances, and pharmaceuticals. The plant-derived pharmaceuticals have been under fierce competition from the microbial-based antibiotics, which proved easy to isolate and develop. Microorganisms were easy to collect from the soil, grow, and produced a dazzling array of antibiotics. This reduced the importance of the plant-derived pharmaceuticals for some time. However, more recently an ethnobotanical approach has been developed to study plants showing medicinal value, and the isolation of new pharmaceuticals. Initially, drugs such as digoxin and digitoxin, used for the treatment cardiac irregularity, were discovered because the plant foxglove (*Digitalis*) had been used in early days as an herbal remedy. Thus, plants still remain a major source of pharmaceuticals [1], and the recent development of the drug paclitaxel (Taxol) shows that plants can still produce valuable drugs. Chemical synthesis is often an alternative supply for many pharmaceuticals, but most of the plant-derived pharmaceutical compounds are complex in structure, and cannot be chemically synthesized at a competitive price. Thus, morphine and codeine are isolated from the latex of seed pods of the poppy *Papaver somniferum*, and quinine is extracted from the bark of the tree *Cinchona ledgeriana*.

Most of the plants used for the isolation of colors, flavors, and pharmaceuticals are grown in plantations. Plantation-grown plants are subject to climatic changes, diseases, and pests. All these can affect the yield of plant or product. Often the plants are grown in a country that, because of its climate, can be far removed from where the compounds are extracted the used,

which can lead to supply problems owing to the distances involved and the seasonal nature of many crops. The problems of supply of plant products, particularly with the pharmaceuticals, prompted the belief that plant tissue culture may be an alternative supply to the plantation-grown crops or chemical synthesis [2–4]. Plant cell cultures grown on a large scale have the advantage of their independence from environmental factors, producing a consistent supply of known quality, and having a high degree of flexibility. Any commercial production of phytochemicals by plant cell culture will require a medium-to large-scale process (100–100,000 L), which will inevitably involve growing cell suspension cultures in bioreactors. The possibility of using plant cell culture for the supply of phytochemicals was also prompted by the observation that many of the cultures were capable of producing compounds typical of the source plant. There have been many reviews on the potential of plant cell cultures [2–10], but often these have not fully appreciated the lack of knowledge of the biosynthetic pathways involved, and the difficulties of growing plant cells on the large scale. The first commercial plant cell product, shikonin, was marketed by Mitsui in 1983 [11], but no other process has as yet reached commercialization, although it has been suggested that berberine may be the next. Whole cells of *Panax ginseng* have been produced since 1988 for addition to tonic drinks, wines, soups, and such, in Japan. The shikonin produced by plant cell culture has been marketed only in Japan as a pharmaceutical. In the United States it was used as a dye to prepare a lipstick.

When introducing a new technology, the product should be of high value and low volume, and several examples have been cited in various reviews [2–7]. The other feature that is required is a high yield of the particular product. Plant cell cultures can give very high yields of some compounds. However, these high yields are generally for products of limited or no value and, although considerable effort has been expended in trying to obtain high yields of the commercial products, this has been to no avail. Examples of high yields and valuable products are shown in Table 1. Success in this area awaits further information on the pathways, controls, and accumulation sites of these phytochemicals.

The other feature that is required if a plant cell culture is to be commercialized is the growth of the cells in large volumes in bioreactors. It is the growth of plant cells in bioreactors that is the subject of this chapter.

One of the most important factors in the development of any process is its productivity in terms of gram product per liter of culture per day. Productivity is made up of three components:

Table 1 Potential Commercial Products and Yields from Plant Cell Cultures

Product	Plant species	Application	Yield (%)	Price (\$/kg)	Ref.
Rosmarinic acid	<i>Coleus blumei</i>	Nil	21–36	Nil	40
Anthraquinones	<i>Morinda citrifolia</i>	Nil	18	Nil	68
Shikonin	<i>Lithospermum erythrorhizon</i>	Antibacterial (Japan)	12.4	4500	69
Berberine	<i>Thalictrum minus</i>	Drug (Japan)	10.6	3250	70
Anthocyanins	<i>Perilla frutescens</i>	Nil	8.9	n/a	71
Diosgenin	<i>Dioscorea deltoidea</i>	Steroid	3.8	1000	72
Morphine	<i>Papaver somniferum</i>	Drug	0.025	340,000	4
Sanguinarine	<i>P. somniferum</i>	Antibiotic	2.5	4800	73
Paclitaxel (Taxol)	<i>Taxus brevifolia</i>	Anticancer	0.06	0.6M	74
Vincristine	<i>Catharanthus roseus</i>	Antileukaemic	0	20M	11

Table 2 Productivities of Plant Cell Cultures

Cell line Product	Biomass (g/L)	Time (d)	Yield (%)	Yield (g/L)	Productivity (g/L/d)	Ref.
<i>Coleus blumei</i> Rosmarinic acid	25.7	6	21.4	5.5	0.91	40
<i>Lithospermum erythrorhizon</i> Shikonin	—	23	12.4	3.67	0.0057	60
<i>Coptis japonica</i> Berberine	14	14	7.5	1.04	0.53	61
<i>Perilla frutescens</i> Anthocyanins	13.5	10	8.9	1.2	0.12	62
<i>Dioscorea spp.</i> Diosgenin	11.3	16	3.8	0.43	0.028	63
<i>Papaver somniferum</i> Sanguinarine	12.1	9	2.5	0.025	~0.0025	64
<i>Papaver somniferum</i> Morphine	16	21	—	0.375	0.0178	4
<i>Taxus brevifolia</i> Paclitaxel	10	20	0.06	0.006	0.0003	65
<i>Penicillium chrysogenum</i> Penicillin	—	7	—	10–15	1.4–2.1	63

the yield of product, the process time, and the final biomass level. The productivity of several plant cell cultures has reached values approaching those of the microorganisms used in antibiotic production (Table 2). However, those cultures exhibiting high productivity produce compounds of little value, as can be seen if Table 2 is compared with Table 1. It can be seen from Table 2 that the low productivities are due to low yields, long growth periods, and limited biomass, but probably the main problem is the low yield.

Most of the phytochemicals of industrial importance are what are known as secondary products. These compounds are not essential for the growth of the whole plant, but often confer some advantage, such as being toxic to insects. The compounds are often produced by special cells, and the product stored in the vacuole or in other specialized cells. In culture, secondary products are generally produced after growth has ceased, and often require a change in cultural conditions to initiate their synthesis. This observation has led to the development of a two-staged strategy for secondary product accumulation. In the first stage, growth is encouraged, and in the second stage, culture conditions or the medium are changed, which often stops growth and initiates secondary product accumulation. The best example of this form of two-staged process is the Mitsui production of shikonin using a culture of *Lithospermum erythrorhizon* [11]. In this process the cells are grown for 9 days in MG-5 medium in a 200-L bioreactor, the medium is removed from the white cells, and replaced with M-9 medium. The cells are incubated for another 14 days in a second 750-L bioreactor where the accumulation of shikonin is induced and the cells turn red (Fig. 1).

Considerable effort has been made to increase the yield of secondary product, including changing the medium composition, in particular the growth regulators; the culture conditions, such as light and temperature; and adding elicitors or removing the product. All these variations have been successful to some degree in certain cases, but no universal method has evolved. Accordingly, the productivity of those compounds of possible commercial interest has remained low.

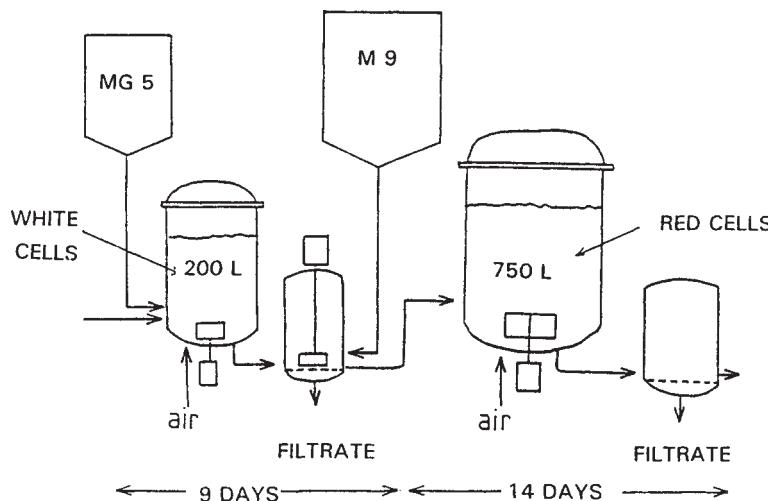


Fig. 1 The Mitsui process for the production of shikonin from cells of *Lithospermum erythrorhizon*: The process has two stages; in the first, the cells grow in MG-5 medium, and in the second, the medium is replaced with M-9 and shikonin accumulation takes place in a second bioreactor. (From Ref. 11.)

II. PRODUCTION OF SECONDARY PRODUCTS

A. Suspension Cultures

The first large-scale cultivation of plant cell suspensions was reported in 1959, for which bioreactors constructed from carboys and roller bottles were used [12]. The early types and designs have been well described in a review [13]. However, despite being considered similar to microorganisms, plant cell suspensions have numerous unique characteristics that have an influence on their mass cultivation. Some of the important differences between microbial cells, plant cell suspensions, and root, shoot, and embryo cultures are shown in Table 3. The large size and rigid cell wall indicate that the cells could be sensitive to shear and, in particular, the larger organized structures. A plant cell suspension contains mainly aggregates of sizes up to 2 mm in diameter, which in fine plant cell suspensions have mean diameters of about 300 nm. These aggregates settle out more rapidly than individual cells and, therefore, good mixing is required, but not so vigorous that it breaks up the aggregates and damages the cells. In addition, the cell aggregates make sample removal difficult and homogenous samples improbable. These problems are more acute with organized cultures. Plant cell suspensions require high inoculation densities owing to medium

Table 3 The Differences Between Plant Cell Suspensions, Organized Cultures, and Microbial Cells

Characteristic	Microbial	Plant cell suspension	Organized cultures
Size	2–10 μm	10–200 μm	200 μm <
Individual cells	Often	No, often aggregates	Cells in structures
Inoculation density	Low	High 5–10%	High 5–10% Low hairy roots
Growth rate ^a	Rapid td 1–2 h	Slow td 2–5 d	Slow td 2–8 d
Aeration	High	Low	Low
Shear sensitivity	No	Partial	Yes

^atd doubling time in hours or days.

conditioning, and often the requirement for cell-to-cell contact. This is close to 5–10%, which is considerably larger than that required for microbial cultures. The consequence of this characteristic is the need for larger inoculation vessels and lower scale-up steps when preparing large cultures. The growth rate of plant cell suspensions is much slower than that for microorganisms, and it is measured in days, rather than hours. This means that the bioreactor cultures may need to be run for up to 3 weeks, with the associated problems of maintaining sterility. Also, few bioreactor runs can be carried out per year, which means an increase in the number of bioreactors required to produce a given biomass. The one real advantage is that, as a consequence of their slower growth rate, plant cell suspensions require a lower rate of oxygen supply. The supply of oxygen to microbial cultures is often the rate-limiting step for growth so that the lower oxygen requirement means that lower K_{la} values for the bioreactors can be tolerated.

Plant cell suspensions were initially thought to be shear-sensitive, but recent data suggest that they may less sensitive. The organized culture, on the other hand, should be shear-sensitive, although no data have yet been presented. These differences between plant cell suspensions and microbial cultures also apply to the organized cultures.

Many of these problems were similar to those faced in mammalian cell culture. This resulted in the great variety of bioreactor designs used for mammalian cell cultures. These designs have to compete with the modified conventional bioreactors, and this is often possible because the volumes involved are small and the product expensive. However, if the final scale is to be thousands of liters, the design has to scale up, and competition with existing technology becomes more difficult. It is in this area that plant cell cultures have to function.

One feature of suspension cultures is that occasionally the product is released into the medium where it can accumulate or be broken down. The provision of an accumulation site, such as the resin XAD-7, lipophilic Miglyol, or charcoal, has shown that in some cases the culture has been producing secondary products, but these have been rapidly lost [14, 15]. The provision of an accumulation site may also encourage further export and synthesis, as high levels within the cells may be inhibitory.

B. Immobilized Cultures

The failure to obtain high yields of secondary products with suspension cultures has prompted the investigation of alternatives to the suspension cultures normally employed, one of which was cell immobilization. The immobilization of biocatalysts has received considerable attention over the last decade, and reviews on the immobilization of plant cells have been published [16,17].

There are many possible advantages of cell immobilization. The cells can be easily recovered and can be used over an extended period. The cells are easily separated from the medium and product and, thus, a continuous process is possible. The cells also show increased stability. Immobilization induces secondary product accumulation [18,19]. There has been some evidence that secondary product accumulation is encouraged by slower growth and cell-to-cell contact [20,21], which would be the conditions favored by cell immobilization. Immobilization also protects those cells sensitive to shear.

The two main methods of immobilizing cells are entrapment and binding (Fig. 2). Entrapment is the primary method used for cells, and this includes plant cells. Entrapment can take three main forms: in-formed or preformed polymers, behind semipermeable membranes, and in microcapsules. The most commonly used polymer to entrap plant cells is calcium alginate. Alginate is extracted from seaweed and consists of long chains of guluronic and mannuronic acids. These long chains can form gels by making cross-links between the guluronic acid

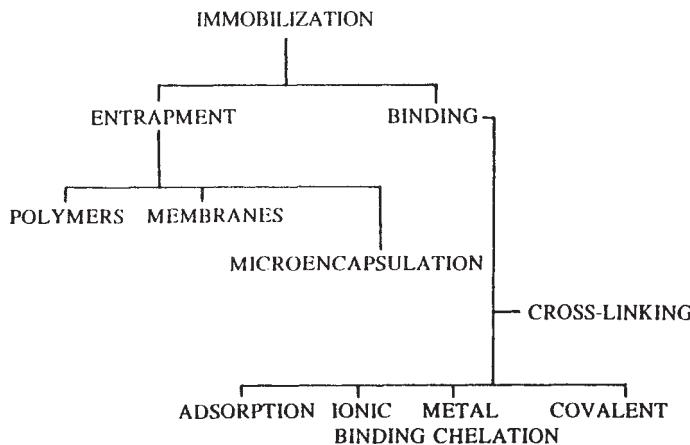


Fig. 2 The various methods which can be used to immobilize plant cells.

components with ions such as calcium. Gelation can occur at room temperature and, by removing the calcium, the gels can be dissolved. This gives very mild conditions for polymerization that are ideal for plant cells. Other polymers that can be used are agar, agarose, carageenan, gelatin, and polyacrylamide. These all form gels at temperatures higher than room temperature, and for the polyacrylamides chemical polymerization is needed. All these conditions may reduce cell viability and have not been widely used.

Preformed polymers, such as foams, have also been successful in immobilizing plant cells; polyurethane foams, one of the most widely used, can be used in various shapes and sizes. Entrapment is a passive process, but it is very effective in removing all cells from a suspension in a few days. Another gentle method of entrapment is the use of semipermeable membranes that can be incorporated into various bioreactor designs, including hollow fiber, flat plate, spirals, tubular, and multmembrane.

Of the other methods shown in Figure 2, only adsorption of various materials to form biofilms has shown promise. The materials used to adsorb plant cells have included fiberglass, polystyrene, plastics, ceramics, polyesters, and metals [22,23].

The disadvantages of using immobilized plant cells are the possible loss of activity on immobilization, and the extra handling, which adds to the costs and poses problems of contamination. The physiology of the cells may or may not be influenced by immobilization in a positive way. This may be a consequence of the increased cell-to-cell contact or the gradients that develop within the immobilized structure. Secondary products are often accumulated in the cell's vacuole or, if extracellular, bound to the cell wall. To function in a continuous manner any immobilized system will require the release of the product into the medium, and although this sometimes occurs [24], it has been difficult to ensure in all cases. Excretion of secondary products varies from one species to another, but various methods have been used to try and release the products, including temperature, electroporation, changes in medium, chemical treatments such as dimethyl sulfoxide (DMSO), and ultrasound. Although these methods do release the product, they can also affect the viability of the immobilized cells, as release is often by cell lysis. There is as yet no universal technique, but the mild processes, such as changing the medium composition and mild heat treatment, offer promise.

Table 4 Examples of Secondary Products Accumulated by Root and Shoot Cultures

Culture	Product	Ref.
Root Cultures		
<i>Atropa belladonna</i>	Tropane alkaloids	75
<i>Catharanthus roseus</i>	Ajmalicine	76
<i>Papaver bracteatum</i>	Thebane	77
<i>Papaver somniferum</i>	Codeine	78
<i>Senecio spp</i>	Pyrrolizidine alkaloids	79
Shoot cultures		
<i>Atropa belladonna</i>	Tropane alkaloids	80
<i>Rauwolfia serpentina</i>	Ajmalicine	81
<i>Digitalis purpurea</i>	Digitoxin	82
<i>Pelargonium fragrans</i>	Pinene	83
<i>Cinchona ledgeriana</i>	Quinine	84
<i>Centranthus macrosiphon</i>	Valpotriates	85
<i>Linum flavum</i>	Coniferin	86
<i>Coleus forskohii</i>	Forskolin	65

C. Organized Cultures

The lack of secondary product accumulation by some suspension cultures has prompted the investigation of organized cultures, such as roots and shoots, as alternative sources, because both roots and shoots have been cultured for some time [25,26]. In undifferentiated cells the relevant pathways are not expressed because some of the pathways appear to be under strict developmental control. There is also the diversion of substrate away from secondary product formation, loss of mechanisms for product removal, no storage site, and catabolism of the product in the dedifferentiated cells. Therefore, organized structures, such as roots and shoots, have been cultured to obtain a high yield of secondary product (Table 4).

More recently, transformed roots (hairy roots) have also been shown to accumulate secondary products [27]. Plants are induced to form roots at the point of infection by *Agrobacterium rhizogenes*, the causative agent of "hairy root" disease. The infection process is complex, but leads to the insertion of DNA from the R1 plasmid in the bacterium into the plant genome. The expression of the genes coding for auxin synthesis results in root formation at the site of infection. Transformed roots are characterized by a high degree of genetic stability and retain their biosynthetic stability for long periods. The rate of growth is high, compares well with plant cell suspensions, and is generally faster than normal roots [22] in a medium lacking growth regulators. Secondary product accumulation reflects the plant and exhibits the kinetics found for suspension cultures (Table 5). The products vary in the extent to which they are released from the roots.

Table 5 Examples of Phytochemicals Produced by Hairy Roots

Compound	Culture	Ref.
Shikonin	<i>Lithospermum erythrorhizon</i>	67
Quinine	<i>Cinchona ledgeriana</i>	87
Nicotine	<i>Nicotiana tabacum</i>	88
Ajmalicine	<i>Catharanthus roseus</i>	89
Hyoscyamine	<i>Datura stramonium</i>	90
Saponin	<i>Panax ginseng</i>	91

These alternatives require bioreactor designs and processes different from the suspension cultures when grown in large volumes. I should like to deal with each in turn, showing the different approaches that have been taken.

III. LARGE-SCALE CULTIVATION OF PLANT CELLS

A. Stirred-Tank Bioreactors

The scale-up of a process involves the growth of plant cells in a bioreactor, and the standard design of bioreactors used in industrial processes and laboratories is the stirred-tank. In this design mechanical agitation both mixes and improves the air supply by producing turbulent high-shear conditions (Fig. 3). The early work on the scale-up of plant cell suspensions did not fully appreciate the differences between plant cell suspensions and microbial cells, but despite this, stirred-tank bioreactors, using low impeller speeds (>100 rpm), were successfully used for

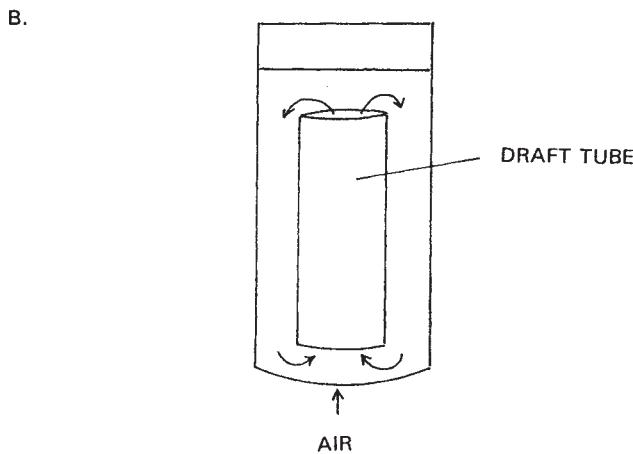
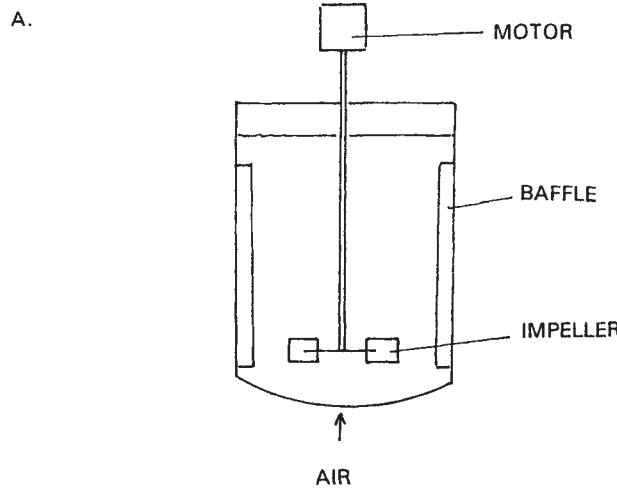


Fig. 3 The two main types of bioreactor design: (A) the stirred-tank; (B) the airlift.

Table 6 Bioreactor Designs Used to Cultivate Plant Cell Suspensions

Bioreactor Design	Volume (L)	Cell Line	Date
Stirred-tank	30	<i>Nicotiana tabacum</i>	1972
	130,600	<i>Nicotiana tabacum</i>	1972
	10,30,300	<i>Glycine max</i>	1974
	15,65	<i>Nicotiana tabacum</i>	1975
	15,30,230,	<i>Nicotiana tabacum</i>	1977
	15,500	<i>Nicotiana tabacum</i>	1977
	10	<i>Morinda citrifolia</i>	1977
	7.5	<i>Catharanthus roseus</i>	1981
	20,000	<i>Nicotiana tabacum</i>	1982
	500,750	<i>Lithospermum erythrorhizon</i>	1983
	30	<i>Panax ginseng</i>	1984
	32	<i>Coleus blumei</i>	1985
	14	<i>Catharanthus roseus</i>	1986
	70,750,800	<i>Catharanthus roseus</i> <i>Nicotiana tabacum</i> <i>Solanum demissum</i>	1986
	20,000	<i>Panax ginseng</i>	1986
	5,000	<i>Catharanthus roseus</i>	1986
	75,000	<i>Echinacea purpurea</i> <i>Rauwolfia serpentina</i>	1990
Airlift	10	<i>Morinda citrifolia</i>	1977
	20,30	<i>Catharanthus roseus</i>	1977
	10,100	<i>Catharanthus roseus</i>	1981
	20	<i>Trispterium spp.</i>	1981
	200	<i>Digitalis lanata</i>	1983
	5,10	<i>Catharanthus roseus</i>	1984
	20	<i>Berberis wilsonae</i>	1985
	80	<i>Catharanthus roseus</i>	1987
	80	<i>Helianthus annuus</i>	1989
	2.5–1,000	<i>Lithospermum erythrorhizon</i>	1983
Taylor-Couette	2	<i>Nicotiana tabacum</i>	1992
	2.5	<i>Beta vulgaris</i>	1987
Stirred membrane	21	<i>Thalictrum rugosum</i>	1988

Source: Ref. 104.

plant cell suspensions [29]. Much of this work was carried out with tobacco cell suspensions in Japan and, in 1977, a vessel of 20,000 L was used to grow tobacco cells continuously [30]. Quite a number of different designs have been used, but the two main bioreactor designs that have been used are the stirred-tank and the airlift (see Fig. 3). Table 6 lists some of the bioreactor designs used to cultivate plant cell suspension over the years, along with an estimate on the volume of culture. The largest cultures, up to 75,000 L, have been grown in stirred-tank bioreactors [31]. This probably reflects the availability of large stirred-tank reactors, rather than a preference for these reactors. The only process for the production of secondary products to be commercialized, Mitsui's production of shikonin from cultures of *L.*

erythrorhizon, used two stirred-tank bioreactors in a two-staged process (see Fig. 1). The cells are grown for 9 days in growth medium in a 200-L bioreactor, the medium removed and replaced with M-9 medium, which stimulates shikonin accumulation, and run into a second larger, 750-L bioreactor. After 14 days the cells are harvested and the shikonin extracted giving the yields shown in Table 2.

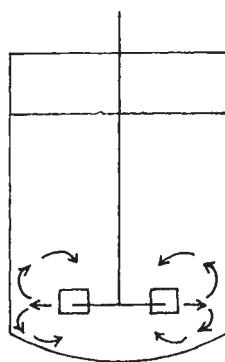
B. Impeller Designs

Although stirred-tank bioreactors could be used to cultivate plant cells, they were by no means successful with all cell suspensions. These problems were ascribed to the shear sensitivity of plant cell suspension [32–34].

These observations combined to suggest that plant cells in suspension were sensitive to shear and that any bioreactor used to cultivate plant cells should be able to provide low-shear conditions. Shear in stirred-tank bioreactors is mainly caused by the impeller, with the highest shear at the impeller tip. Therefore, research was directed toward two main areas of bioreactor design. The first was to modify the stirred-tank design to give good mixing, while still maintaining low shear. This largely involved the modification of the impeller to give it low-shear characteristics while retaining good mixing. Mixing is important, for plant cell suspensions contain aggregates, which sediment rapidly.

Stirred-tank bioreactors are normally operated with a Rushton turbine (Fig. 4), which is designed to give a high-shear region at the impeller tip to give good bubble breakup and improve oxygen transfer in the bioreactor. The Rushton impeller produces a radial flow within the bioreactor that gives good mixing and a high-power input into the reactor. In contrast, impeller designs, such as the paddle or propeller, give an axial flow which, although it still produces high shear at the impeller tip, has a reduced overall shear. This is due to the distribution of power input into a bioreactor between liquid flow and shear. The Rushton impeller is designed to give high shear for good bubble breakup, rather than high flow rates, whereas impellers giving axial flow have a reduced shear and a higher liquid flow. Hence, axial flow impellers were tried for the cultivation of plant cell suspensions. Furuya et al. [35] compared three impeller designs: Rushton turbine, anchor, and an angled disk turbine for the growth of *Panax ginseng* in a 30-L bioreactor. The best results were obtained with the angled turbine, which gave axial flow. A similar result was obtained when *Catharanthus roseus* cultures were grown in a 12-L bioreactor with either a Rushton turbine or inclined (30° and 60°) impellers [36]. The inclined impellers gave better growth and cell yield. In a similar study, large flat-bladed or sail impellers made of plastic were tried with cultures of *Nicotinia tabacum* [37] with some success (Fig. 5). Another impeller design, the cell-lift impeller, originally designed for animal cells, has been compared with flat-bladed and marine impellers with cultures of *Glycine max* and *Pinus elliotti* [38]. The cell-lift impeller is designed to lift cells and medium up the central tube and release them through three arms. This gives good mixing, but as the rotation is slow and the tubes wide, the shear is low. There was little difference in biomass yield, but the cell-lift impeller gave improved viability and an increased aggregate size. A hybrid system using the cell-lift impeller combined with a sparger at the base of the central tube has been used successfully for high-density (31 g/L) cultures of *Thalictrum rugosum* [39]. Other possible impeller designs with good mixing characteristics are the anchor and helical designs (see Fig. 5). The helical design was best for the culture of *Coleus blumei* at cell densities of 25 g/L compared with conventional or anchor impellers [40]. A double-helical impeller has been used to cultivate a high-density culture of *C. roseus* in a 11-L bioreactor [41], and a low-shear impeller, the INTERMIG, has been used for various cultures grown in a 75,000-L bioreactor [31]. It is clear from these results that a reduction in shear and an

A.



B.

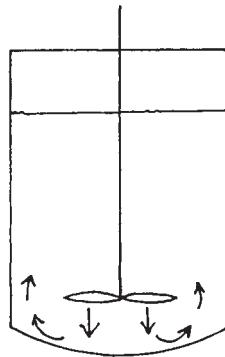


Fig. 4 The flow pattern from two impeller designs: (A) Rushton turbine; (B) propeller.

increase in flow from that found with the Rushton impeller will allow growth in a stirred-tank bioreactor, even at high cell densities.

C. Airlift Bioreactors

The second approach to the growth of plant cell suspensions was to develop bioreactor designs that were alternative to the stirred-tank. In the late 1970s the airlift bioreactor was developed in particular for the production of single-cell protein. The design relies on a stream of air sparged into the base of the bioreactor to both aerate and mix the culture (see Fig. 3). The bioreactor is divided into two unequal sections, which are linked. Sparging of air into one section causes a reduction in density and an upflow of liquid, this brings in liquid from the other section, and hence, a flow is established. The advantage of such a design was that it had no moving parts, had a simple construction, gave good mixing, had a large surface area, a high K_{la} , was inexpensive to run, and because there was no impeller, there were no areas of high shear. The low-shear characteristics of the airlift bioreactor perhaps were not fully appreciated by those working on single-cell protein, but it was ideal for those wishing to grow shear-sensitive cells, such as animal cells. The first reports of the use of airlift bioreactors for the growth of plant cells was in 1977 when *Morinda citrifolia* and *C. roseus* were grown in airlift bioreactors [42, 43]. A study was reported [42] in which several bioreactor designs were compared for growth and product formation in cultures of *M. citrifolia*. Although there were few differences between the growth,

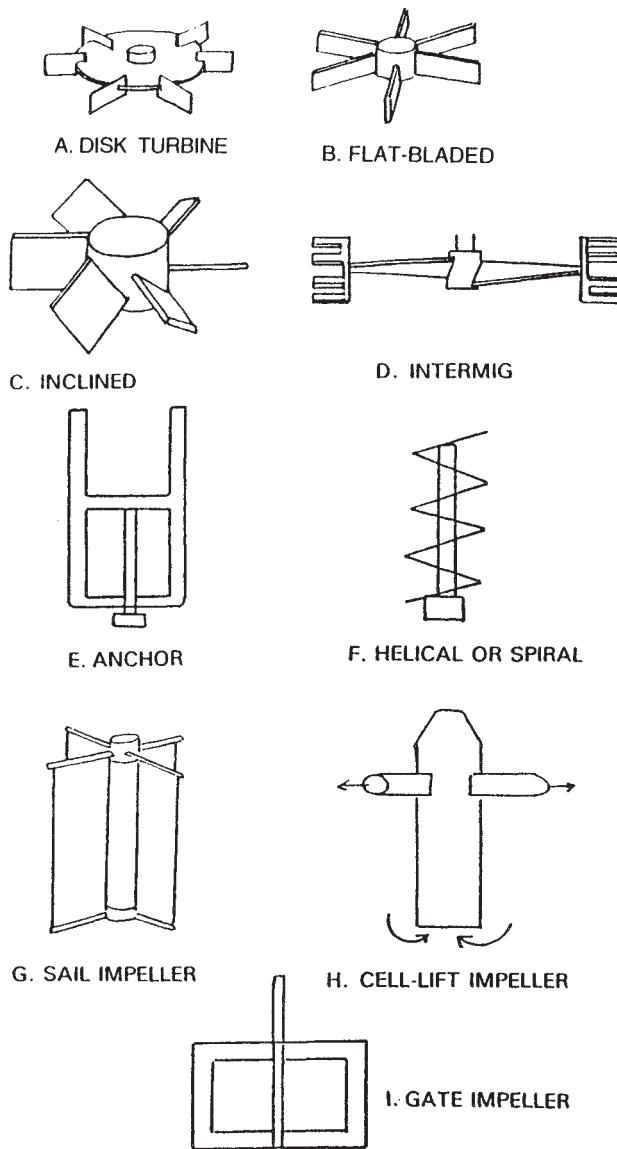


Fig. 5 Various impeller designs: (A) disk turbine; (B) flat-bladed; (C) inclined; (D) INTERMIG; (E) anchor; (F) helical or spiral; (G) sail impeller; (H) cell-lift impeller; (I) gate impeller. (From Ref. 96.)

anthraquinone accumulation was best in the airlift bioreactor. This encouraged the use of the airlift bioreactor for plant cells. At the same time Zenk et al. [43] showed that the airlift bioreactor was the only one that would support the growth of *C. roseus*. As can be seen from Table 6, airlift bioreactors have been successful in growing a wide range of plant cell cultures and at various volumes, up to 300 L.

D. Alternative Bioreactor Designs

Although the airlift and stirred-tank bioreactors have been the main designs used to cultivate plant cells, other designs have been developed. Here again, the object has been to provide good

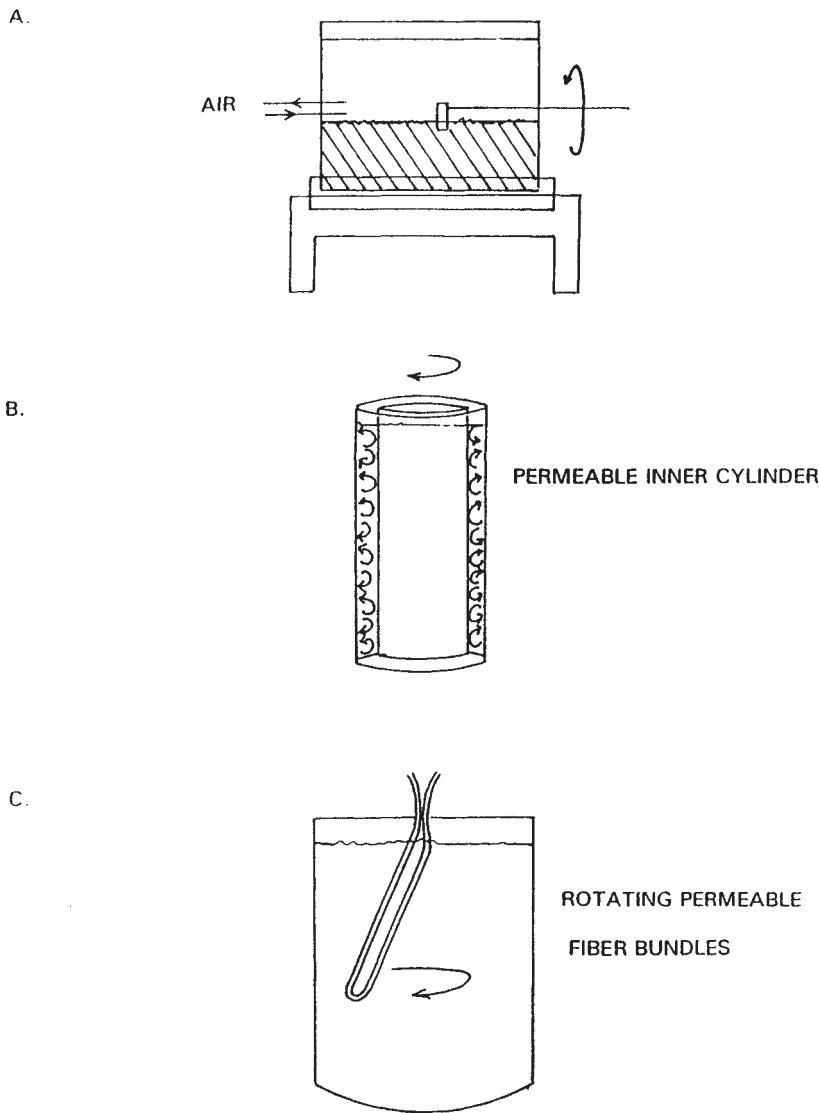


Fig. 6 Alternative bioreactor designs to the stirred-tank: (A) rotating drum; (B) Taylor-Couette annular vortex bioreactor; (C) membrane-stirred bioreactor. [From Refs.: (A) 44; (B) 45; (C) 46.]

mixing and air supply, while keeping the shear as low as possible. The rotating drum bioreactor was first reported in 1983 [44] when it was used to cultivate cultures of *C. roseus* and *L. erythrorhizon* (Fig. 6). Bioreactors of up to 1000 L have been constructed. The slowly rotating drum mixes the culture, while the high surface area provides sufficient oxygen in a low-shear environment.

Other designs have been produced in which the oxygen is supplied without having to sparge air into the bioreactor. In one design, low-shear mixing was provided by a rotating outer drum with a stationary inner drum. This produces vortices or Taylor-Couette flow, which provides the low-shear mixing, while the oxygen is supplied by an air supply to the semipermeable inner drum [45]. At present this has only been used to grow *Beta vulgaris*; hence, comparison with

Table 7 Effect of Shear Stress on the Viability of Plant Cells

Cell line	Effect
Short-term exposure (5 h)	
<i>Catharanthus roseus</i>	Tolerant
<i>Datura stramonium</i>	Sensitive
<i>Helianthus annuus</i>	Tolerant
<i>Vitis vinifera</i>	Tolerant
<i>Solanum tuberosum</i>	Tolerant
<i>Nicotiana tabacum</i>	Sensitive
<i>Picrasma quassoides</i>	Tolerant
<i>Cinchona robusta</i>	Tolerant
<i>Tabernaemontana divaricata</i>	Tolerant
Long-term exposure (growth)	
<i>Catharanthus roseus</i>	Tolerant
<i>Nicotiana tabacum</i>	Tolerant
<i>Cinchona robusta</i>	Sensitive
<i>Tabernaemontana divaricata</i>	Tolerant

Source: Ref. 92.

other designs is difficult. A similar approach has been adopted in the membrane-stirred bioreactor [46], which was developed for the cultivation of animal cells [47]. In this system, oxygen transfer is achieved using a basket or coil of silicone tubing that is permeable to oxygen placed within the bioreactor. The culture is mixed by a magnetic stirrer or by rotating the basket of tubing (see Fig. 6). This system has been used to culture *T. rugosum*, in a 21-L bioreactor, to high cell densities of up to 50 g/L, while maintaining a dissolved oxygen level of 30%. A recent development has continued to use gas-permeable plastic, but here, the plastic is formed into bags that have been used for the cultivation of plant cell suspensions [48]. Mixing appears not to be required, and these make a very simple cultivation system.

E. Shear Sensitivity

Plant cells in suspension are large (40–200 µm), have a rigid cell wall, and a large vacuole and, because of this, have been considered sensitive to shear stress [49]. Some of the difficulties in growing plant cells in stirred bioreactors has been attributed to their sensitivity to shear [34]. Recent studies, however, have shown that plant cells are not as sensitive to shear as was at first thought [50–53], both in the short-term and long-term. The results of these experiments are shown in Table 7, in which it can be seen that plant cells can withstand quite high shear levels over long periods. Although this is probably not true for all cell lines, it does alter our perception of what may be possible in bioreactor operation with many cell lines. The higher-shear tolerance may allow more vigorous mixing which, in turn, may mean that high-biomass levels can be obtained in bioreactors. The bioreactors used will also need less modification, and novel designs may not be required for many cell lines.

F. Comparison of Bioreactor Designs

The first comparison of bioreactor design was by Ulbrich et al. [40], who used cultures of *C. blumei*, to show that at biomass levels of 24 g/L a stirred-tank fitted with a spiral impeller was better than one fitted with an anchor impeller or an airlift bioreactor. The rotary drum bioreactor has been compared with an airlift design, using *N. tabacum* cultures [54], and gives higher growth rates. With suspension cultures of *Fragaria x ananassa* cv Brighton, airlift, stirred-tank,

Table 8 Growth of Strawberry Cell Suspensions in Various Bioreactors

Bioreactor type	Volume (mL)	Growth rate (d ⁻¹)	Yield
Shake flask	250	0.12	0.39
Airlift	700	0.14	0.44
Stirred jar	7000	Nil	—
Roller bottle	1850	0.15	0.41

Source: Ref. 55.

and roller-bottle bioreactors were compared [55]. Again, the roller-bottle was the better design for growth (Table 8). At this stage with the limited amount of data available, it is difficult to suggest which bioreactor design is the most suitable for plant cell suspensions.

G. Growth Rates

One of the components of the productivity of plant cell suspension is their growth rate. Most growth rates quoted are those obtained with shake flasks, rather than in bioreactors, in which the conditions are somewhat different. However, there is little reduction in growth rate in bioreactors, even in large volumes.

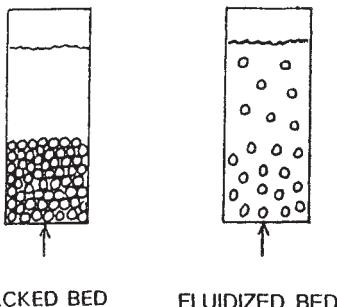
Unfortunately, there is less information on the effect of scale-up on the accumulation of secondary products. With a culture of *C. roseus*, serpentine accumulation was reduced when it was grown in bioreactors [56], whereas the scale-up of another line of *C. roseus* showed little loss of ajmalicine accumulation [57]. It is clear that conditions in a bioreactor will be different from that of a shake flask, and these differences will be in the areas of shear and gaseous conditions. Early data indicated that the gaseous conditions, in particular those of carbon dioxide and oxygen, affect both growth and product accumulation [58]. The shake flask is a partially closed system in which gas exchange is slow and as the culture grows, oxygen is depleted (13%), and carbon dioxide builds up to levels of close to 10% [59]. In contrast, the bioreactor is supplied with a constant flow of air, which can keep the dissolved oxygen level high. Recently, it has been shown that the dissolved oxygen level can have a profound effect on ajmalicine accumulation in *C. roseus*. Low levels of oxygen of 15% inhibited ajmalicine accumulation, whereas levels above 50% stimulated accumulation. Although this has been investigated only for *C. roseus*, high dissolved oxygen levels may be required for the accumulation of other secondary products.

Another feature of the growth of plant cells in bioreactors is an increase in biomass levels to increase productivity. Normal biomass levels are close to 5–15 g/L dry weight for initial

Table 9 High Biomass Levels in Bioreactors

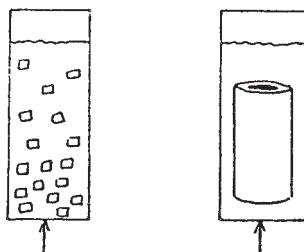
Culture	Biomass (g/L)	Format	Ref.
<i>Catharanthus roseus</i>	27.6	Batch	41
<i>Coleus blumei</i>	25.7	Fed-batch	40
<i>Coptis japonica</i>	55.0	Fed-batch	62
<i>Berberis wilsonae</i>	40.0	Fed-batch	93
<i>Catharanthus roseus</i>	23.4	Fed-batch	94
<i>Coptis japonica</i>	70.0	Perfusion	69
<i>Thalictrum rugosum</i>	27.6	Perfusion	39

A.



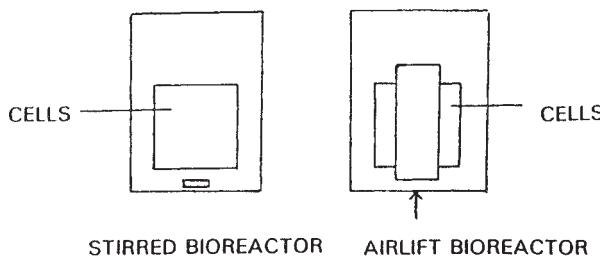
PACKED BED FLUIDIZED BED

B.



FLUIDIZED CUBES POLYURETHANE

C.



STIRRED BIOREACTOR AIRLIFT BIOREACTOR

D.

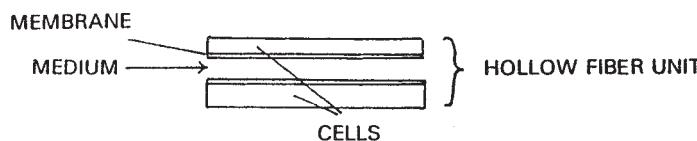


Table 10 Types of Bioreactor Used for Immobilized Plant Cells

Bioreactor type	Cell line
Gel-entrapped cells	
Stirred-tank	<i>Digitalis lanata</i>
Airlift	<i>D. lanata</i>
Fluidized-bed	<i>Dioscorea deltoidea</i>
	<i>Catharanthus roseus</i>
Packed-bed	<i>Daucus carota</i>
	<i>D. carota</i>
	<i>Catharanthus roseus</i>
Polyurethane-entrapped cells	
Circulating-bed	<i>Capsicum frutescens</i>
Fixed-bed	<i>C. frutescens</i>
	<i>Catharanthus roseus</i>
Membrane entrapment	
Tubular or hollow fiber	<i>Glycine max</i>
Flat-bed	<i>G. max</i>

Source: Ref. 95.

sugar concentrations of 1–3%. The maximum possible, assuming a dry weight of between 80–90%, would be 100–200 g/L dry weight. However, some liquid is required to dissolve the oxygen and other nutrients, and the cells are not spherical, so that a practical limit has been set at 35–60 g/L dry weight [61,62]. The biomass levels obtained with various cultures is given in Table 9. High biomass is not without its problems of high viscosity, reducing the mass transfer of oxygen and mixing.

H. Bioreactors for Immobilized Cells

The immobilization of plant cells has proved fine on a small scale, but on scale-up, conventional bioreactors are not generally suitable. The design and operation of any bioreactor will depend on the method and form of immobilization used. If alginate beads are formed, these can be used in stirred-tank bioreactors, airlift, packed bed, and fluidized bed (Fig. 7). Examples of these can be seen in Table 10.

Surface immobilization is versatile, because the immobilization material can be wound or formed into a shape able to fit into the normal bioreactor. One of the most extensive studies has been carried out with *C. roseus* immobilized on various materials fitted into both airlift and stirred-tank bioreactors [63].

Entrapment behind membranes requires the development of different bioreactors, such as the use of hollow fiber units and flat-bed systems (see Fig. 7).

I. Bioreactors for Organized Cultures

Organized structures, such as roots, shoots, and embryos, do present a very different problem from suspension cultures when it comes to growing them in bioreactors, as can be seen in

Fig. 7 Bioreactors for immobilized plant cells: (A) alginate-entrapped cells in bead form used in packed or fluidized beds; (B) polyurethane-entrapped cells used as cubes or as a sheet to replace the normal draft tube in an airlift bioreactor; (C) cell adsorbed to a surface that is incorporated into either an airlift or stirred tank bioreactor; (D) cells entrapped behind semipermeable membranes used in a flat-bed bioreactor or entrapped in a hollow fiber unit. (From Ref. 96.)

Table 11 Bioreactors Used for Organized Plant Cultures

Bioreactor		Culture	Form
Stirred-tank	(41)	<i>Daucus carota</i>	Embryo
Stirred-tank	(21)	<i>Medicago sativa</i>	Embryo
Stirred-tank	(101)	<i>Atropa belladonna</i>	Roots
Airlift	(21)	<i>Medicago sativa</i>	Embryo
Airlift	(51)	<i>Digitalis lanata</i>	Embryo
Airlift	(square 21)	<i>Artemisia annua</i>	Plantlets
Bubble column		<i>Gladiolus</i>	Minicorms
Bubble column	(11)	<i>Nephrolepis exaltata</i>	Shoots
Spin-filter	(11)	<i>Medicago sativa</i>	Embryo
Spin-filter	(11)	<i>Daucus carota</i>	Embryo
Spin-filter		<i>Panax ginseng</i>	Embryo
Bubble-free	(21)	<i>Euphorbia pulcherrima</i>	Embryo
Mist		<i>Musa, Cordyline, Nephrolepis</i>	Shoots

Source: Ref. 96.

Table 3. The structures will be large and although their shear sensitivity has not been determined, they are likely to be very shear-sensitive. Thus, of the bioreactor designs used to cultivate organized structures, all have been operated to ensure low-shear conditions. Most of the designs given in Table 11 were used to produce shoots or embryos as part of a mass propagation process and not for the accumulation of secondary products. Two exceptions are the use of adventitious root culture of *Atropa belladonna* to accumulate tropane alkaloids [64], and root cultures of *Coleus forskohlii*, which accumulate forskolin [65].

J. Bioreactors for Hairy Roots

Hairy roots once established can be grown in simple medium, from low inocula, and at growth rates similar to those of suspension cultures. The roots are characterized by a high degree of

Table 12 Bioreactors Used to Grow Hairy Roots

Bioreactor	Volume	Culture	Ref.
Stirred-tank	1.0	<i>Atropa belladonna</i>	97
	1.0	<i>Calystegia sepium</i>	97
	0.3	<i>Armoracia rusticana</i>	98
	1.0	<i>Atropa belladonna</i>	97
Stirred-tank with impeller isolated	1.0	<i>C. sepium</i>	97
	12.0	<i>Datura stramonium</i>	The impeller separated by a mesh from the roots 90
Bubble column	1.0	<i>Duboisia leichhardtii</i>	99
	1.0	<i>Catharanthus roseus</i>	89
	2.5	<i>Atropa belladonna</i>	100
Airlift	9.0	<i>T. foenum-graceum</i>	Draft tube 103
	9.0	<i>T. foenum-graceum</i>	Nylon mesh replacing draft tube 103
	0.3	<i>Armoracia rusticana</i>	Polyurethane foam insert 98
Trickle bed	2.0	<i>Hyoscyamus muticus</i>	27
Nutrient mist	1.4	<i>Beta vulgaris</i>	101
Rotating drum	1.0	<i>Daucus carota</i>	102

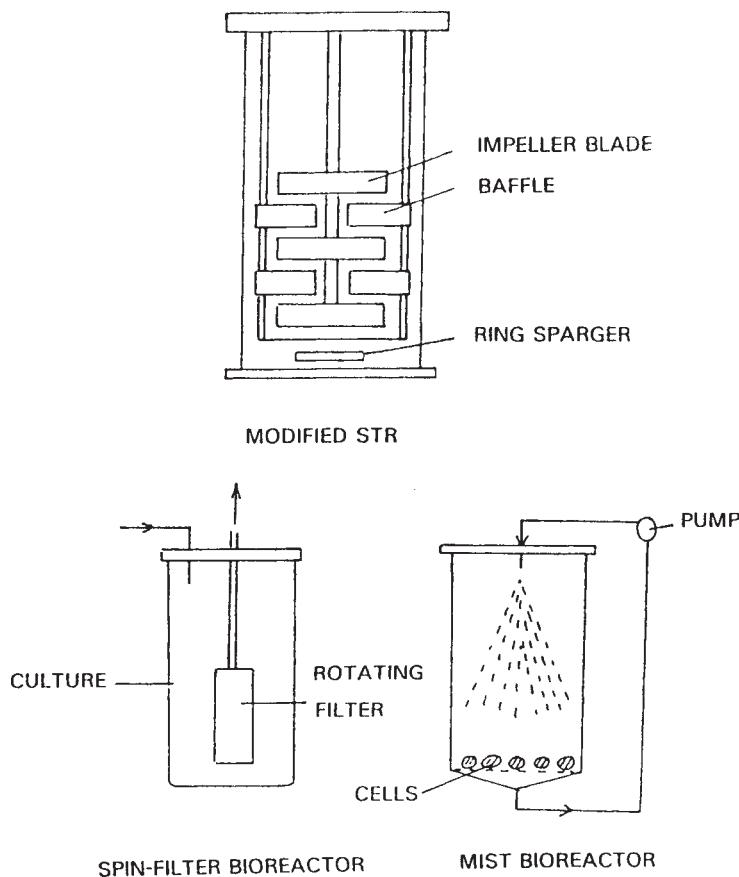


Fig. 8 Three examples of bioreactors used to grow organized plant tissues. The modified stirred-tank has large impellers and baffles and is agitated at a very low speed of 10 rpm. In the spin-filter bioreactor, the rotating filter mixes the culture and allows spent medium to be removed and fresh added. In the mist bioreactor the culture remains stationary and medium is sprayed onto it. Once the medium has passed through the culture it is recycled. (From Ref. 96.)

branching, which on scale-up leads to an interlocked network that affects medium circulation and mass transfer. Mechanical agitation causes breakup of the roots and callus formation. The network makes inoculation difficult and sampling impossible. These properties have encouraged the investigation of novel types of bioreactor design. Whatever design is used, the roots need to be fixed on some form of support, because the root mass will become lodged in some part of the bioreactor and this will restrict flow. Also, to maximize the biomass, an even distribution of the roots is needed within the bioreactor. Once the roots are fixed in place, the main problem is the supply of oxygen, and three methods have been used for this (Table 12). The first is the normal sparging of air into the base of the bioreactor, either in a stirred-tank or airlift. The second method is to aerate the medium in a second vessel and pump it past the roots. The third method is to spray the medium over the roots in a mist bioreactor. Examples of all three methods are given in Table 12 and Figure 8.

For the production of secondary products using hairy roots, some form of product release would be preferable to the harvesting of the whole biomass in a batch process. Polymeric

adsorbents have been connected to bioreactors to remove any product released. In this way shikonin and nicotine have been collected, and production has been increased [66,67].

IV. CONCLUSION

It is clear that whatever the form of tissue that needs to be grown in a bioreactor a design exists that will fulfill all the requirements, similar to animal cell culture. However, unlike animal cell-derived products, plant secondary products are required on a larger scale and, as a consequence, a more standard bioreactor design is probably the most economical to scale up. The shear tolerance of plant cell suspensions may be of great value, for this should allow the use of the standard stirred-tank bioreactor without many modifications. The one feature remaining to be solved is the yield of secondary product that is needed to achieve high productivity. Despite considerable efforts, the yields of valuable products remain low, which reflects the lack of knowledge of the pathways and their controls. This is under study; for example, the pathways for berberine and hyoscyamine have been elucidated and, if this can be used to increase yields, suspension cultures may be the best system to use, and there would be no need for the more specialized bioreactors.

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Production of Foreign Compounds in Transgenic Plants

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I. INTRODUCTION

Expression of bacterial genes, mammalian genes, or other plant genes is becoming routine for several plant species. Most transgenic plants are generated for basic research purposes, such as the evaluation of tissue-specific expression of genes, processing and secretion signals, and enhancer elements. A growing effort is being made to use transgenic plants for broader objectives. One of the most successful approaches has been to engineer resistance against insects, viruses, and other pathogens, as well as herbicides. Numerous examples of resistance mediated by expression of bacterial or viral genes are available (see [Chap. 11–13](#)).

Crop improvement by engineering for specific traits can also be achieved through production of enzymes and metabolites encoded by foreign genes. Thus, the amino acid composition of seed proteins has been altered and, for example, transgenic canola, expressing a Brazil nut albumin, contains up to 33% more methionine in its seed protein [1]. The alkaloid composition of some medicinal plants has been improved. The production of scopolamine, a medicinally important anticholinergic drug is highly increased in transgenic *Atropa belladonna* plants expressing hyoscyamine 6 β -hydroxylase from *Hyoscyamus niger*, which catalyzes the conversion of the precursor hyoscyamine to scopolamine [2]. The shelf life of fruits and vegetables can be extended. Tomato expressing a bacterial ACC deaminase gene reduces ethylene synthesis, and the mature fruits remain firm for at least 6 weeks longer than the nontransgenic controls [3]. Flavor can also be enhanced, transgenic lettuce and tomato have been obtained that express monellin, a sweet protein isolated from some African berries that elicits a flavor approximately 100,000 times sweeter than sugar on a molar basis [4]. The freezing properties of harvested produce can be improved and, in tomato plants expressing an antifreeze protein from the polar fish, winter flounder, ice recrystallization is inhibited [5], making accumulation of the protein cold-specific [6]. The toxicity of some vegetables can be reduced. More than 70% of human cadmium intake is contributed by vegetable products.

Shoots of transgenic tobacco expressing the mouse metallothioneine I gene contain about 24% lower cadmium concentration than nontransgenic plants, because the pollutant metal is sequestered in the roots as cadmium-metallothioneine complexes [7].

Production of valuable foreign compounds by genetically engineered plants has a strong potential and may become a suitable alternative for microbial expression systems when specific processing is required, or when large amounts of protein need to be produced, for the production of plant biomass is inexpensive. A few examples have already appeared in the literature, and many more are expected in the future years.

II. BIOACTIVE PEPTIDES

Biologically active peptides are chemically synthesized or obtained by extraction from biological materials or by expression as fusion proteins in prokaryotic or eukaryotic cells [8]. Leu-enkephalin, a pentapeptide (YGGFL) with opiate activity [9,10], has been produced in *Arabidopsis* and oilseed rape as part of chimeric plant seed storage proteins [11,12].

The 2S albumins, or napin-like proteins, are formed by two subunits linked by disulfide bridges [13] and are synthesized as prepropeptides, being co- and posttranslationally processed [14] and stored in the protein bodies. They are abundant proteins and may represent from 20 to over 60% of total seed protein in various species. Their purification is simplified by their solubility in low salt solutions and by their small size, thereby allowing purification by salt fractionation or dialysis. The 2S albumins from different species are structurally very similar, but contain a region between the sixth and seventh cysteine residues that is highly variable [15]. Vandekerckhove et al. [11] inserted sequences encoding the neuropeptide leu-enkephalin into the 2S1 gene from *Arabidopsis thaliana*, the variable region was substituted by the enkephalin peptide flanked by lysine residues to allow its cleavage from the remainder of the 2S albumin using a trypsin digestion. A remaining lysine on the COOH-terminus was removed using carboxypeptidase B. The small size of the protein simplifies the purification by high-performance liquid chromatography (HPLC) of the inserted peptide after excision, because a less complex mixture of fragments is obtained. The chimeric gene was expressed under its own 2S1 promoter and, consequently, the modified protein was expressed essentially only in seeds. Up to 200 nmol of peptide were recovered per gram of *Arabidopsis* seed, which represents a high-expression level, indicating that the stability of the engineered protein is similar to that of the endogenous albumins.

The small size of enkephalin probably produced a minimal disruption of the protein structure and allowed a stable, correctly processed hybrid protein. The feasibility of producing larger peptides in this manner remains to be determined. Seeds present the advantage of stable storage of proteins for long periods, but some plant seeds may contain contaminants that could cause purification problems. Longer peptides may contain unwanted cleavage sites, in this case, or for peptides that require special modifications, part of the peptide could be chemically synthesized and then linked to the plant-produced fragment.

III. HUMAN PROTEINS

Human serum albumin (HSA) has been expressed in transgenic potato [16]. It is a protein of broad clinical use and, consequently, of a high commercial value. In humans, it is synthesized as preproalbumin, released from the endoplasmic reticulum after removal of the prepeptide [17] and proteolytically processed by a serine protease that cleaves the six residues of the propeptide [18]. The expression of HSA has been reported in various microbial systems [19,20] but, unlike other smaller human proteins such as insulin or interferons, it has been difficult to obtain good levels of secreted HSA.

Similarly to HSA, secreted plant proteins contain NH₂-terminal signal peptides that are removed from the preprotein by a specific protease. Sijmons et al., in their constructs, used an enhanced cauliflower mosaic virus (CaMV) 35S promoter, followed by a synthetic leader sequence derived from alfalfa mosaic virus RNA4 [21], and to ensure protein secretion, they used either the preprosequence of HSA or the signal sequence from the extracellular tobacco protein PR-S. They obtained secretion of HSA with both types of signal sequences. The NH₂-terminal amino acid sequence analysis of purified HSA produced in transgenic potato plants showed that the processing of the protein depended on the type of signal sequence. Partial processing of the precursor and secretion of proHSA was obtained when the HSA signal sequence preceded the protein. Correctly processed HSA was secreted when a fusion of HSA and the plant signal peptide was used, indicating normal cleavage of the presequence. The two signal peptides were capable of targeting HSA extracellularly, but the serine protease necessary to remove the prosequence might not be present, because this kind of enzyme is rare in plants [22].

The HSA accumulated up to 0.02% of the total protein. This amount could probably be increased in tubers by using a tuber-specific promoter [23]. HSA added to ground untransformed potatoes did not show any degradation during the industrial process of starch extraction [16], as it was the protein obtained in the waste liquid fraction of the process. This observation suggests that transgenic starch tubers could well be used as an inexpensive source for protein production, provided that the levels of HSA could be increased.

Human interferons (INFs) are small defense proteins related to resistance to viral infection. They are high-value proteins and have been cloned and produced as recombinant proteins in bacterial and cultured animal cell systems. Interferon-like factors have been detected in virus-infected plants [24], and some have been isolated by their affinity for antibodies against human interferon beta, although they do not show structural homology to the interferons [25]. They have been identified as pathogenesis-related proteins [26]. Occasionally, human interferons have transiently inhibited viral infection in plants [27,28]. These facts indicate that plants could be an appropriate system to express and produce interferons. Interferon- α_D has been expressed in turnip using CaMV as expression vector [29]. The open-reading frame II of CaMV was replaced with the sequence of human interferon- α_D . Inoculation of turnip resulted in the production of biologically active protein in infected plants. Interferon was localized in the CaMV inclusion bodies, and the level of expression was extremely high. Activity measurements indicated a yield of 2 μg of interferon- α_D per gram of tissue fresh weight.

Human interferon β has been expressed in tobacco transgenic plants [30]. The interferon- β sequence was placed behind the CaMV 35S promoter. Interferon- β was detected by immunoblot analysis and extracts from positive plants inhibited the cytopathic effect of vesicular stomatitis virus in human Wish cells. The protein was also purified by immunoaffinity before the bioassay to confirm that the active component was interferon- β . About 17 μg of the protein were obtained from 100 g of fresh leaf material, which makes the system an inexpensive alternative to the mammalian tissue culture systems that are currently being employed to produce interferon- β . Although the glycosylation pattern is somewhat different in plants from that in mammals, the plant-produced interferon was active in a human system. Provided that these differences have no adverse immunological effect, transgenic plants producing interferon- β could be used as an inexpensive system to produce the protein.

IV. ENZYMES

Enzymes are used, soluble or immobilized, in a wide variety of industries. They are isolated from the organisms that produce them or they are obtained as recombinant proteins. Occasionally,

whole organisms or crude preparations are used, when relatively low purities are required. Transgenic plants may constitute an appropriate system to produce industrial enzymes, because the purification costs may be lowered owing to the absence of bacterial contaminants. A couple of examples of transgenic plant-produced enzymes are available.

Hoekema's group have expressed *Bacillus licheniformis* α -amylase in transgenic tobacco [31]. The enzyme α -amylase hydrolyzes α -1,4-glycosidic linkages in the starch components amylose and amylopectin [32], and it is used in the starch-processing industry, and also in others, such as the brewing, baking, wine and juice, and detergent industries. *Bacillus licheniformis* α -amylase is the most commonly used in starch liquefaction because of its heat stability and wide pH range of activity [33]. To express the enzyme in tobacco they used constructs containing the α -amylase sequence preceded by an enhanced CaMV 35S promoter and either the enzyme's signal peptide or the sequence encoding the signal peptide of the tobacco PR-S protein. Both signals functioned equally well in secretion of the α -amylase to the extracellular space and were cleaved correctly on secretion. The molecular mass of the enzyme produced in tobacco was 64 kDa; this value is higher than that of the *B. licheniformis* enzyme, which is only 55.2 kDa. This difference could be attributed to glycosylation in plants, as the protein's primary structure presents six potential asparagine-linked glycosylation sites [33]. Deglycosylation experiments with *endo*-N-acetylglucosaminidase H and trifluoromethanesulfonic acid showed that the incorporated carbohydrates were of the complex type. Complete deglycosylation reduced the molecular mass to 55 kDa.

Despite differences in glycosylation, the enzyme exhibited biological activity and had similar thermostability. It accumulated up to 0.3% of total soluble protein, with an expression level of 0.2% in seeds. To test the correct characteristics of the enzyme, it was applied in starch liquefaction. Milled transgenic tobacco seeds were added to corn and potato starches. HPLC analysis of the hydrolysis products showed no differences between the bacterial and the plant-produced α -amylases. The enzyme did not need any further purification.

Transgenic plants expressing α -amylase were phenotypically indistinguishable from nontransgenic tobacco plants, and leaves contained similar amounts of starch. This suggests that the α -amylase was located extracellularly and, consequently, it had no access to the intracellular starch. The production of transgenic potatoes expressing α -amylase is an interesting possibility. No degradation of the starch should occur until homogenization of the tubers, as enzyme and substrate are in separate locations. The α -amylase levels obtained in tobacco would be sufficient to hydrolyze the starch present in the tuber, and no addition of exogenous α -amylase should be needed.

Also Hoekema's laboratory has expressed *Aspergillus niger* phytase in transgenic tobacco [34]. Phytase catalyzes the conversion of phytate (myo-inositolhexaphosphate) into inorganic phosphate and myo-inositol [35]. Phytate is the main storage form of phosphorus in many plant seeds used as animal feed, but it is a poor nutrient for monogastric animals and, consequently, inorganic phosphate has to be added to the animal diet. The addition of phytase to feed constitutes an alternative; it optimizes phosphorus utilization and reduces excretion of phosphate by poultry and pigs [36,37]. A phytase from *A. niger* has been developed that functions well in the acidic conditions found in the gastrointestinal tract of these animals.

Tobacco was transformed with constructs containing the *A. niger* phytase-coding sequence, preceded by an enhanced CaMV 35S promoter and the sequence encoding the signal peptide of the tobacco PR-S protein. Seeds of transgenic plants were assayed for phytase activity and showed accumulation of the enzyme to 1% of soluble protein. The plant-produced enzyme had a molecular mass of 67 kDa, which is smaller than that of the *aspergillus* enzyme. The difference was due to different glycosylation, as the protein presents

a total of ten potential asparagine-linked sites, and the *aspergillus* enzyme is heavily glycosylated [38]. Deglycosylation of the protein resulted in a molecular mass of 60 kDa for enzyme from both sources.

Transgenic phytase seeds were tested *in vivo* by milling and subsequent addition to the basal diet of broilers. Diets supplemented with nontransgenic seeds, diets with and without added inorganic phosphate, and diets with added commercial phytase were used as controls. Diets supplemented with transgenic seeds resulted in significantly higher growth rates than those with control seeds or no addition, and were comparable with those with added inorganic phosphate or added phytase.

The plants expressing phytase were indistinguishable from controls, and germination of the seeds was not affected by the presence of the enzyme. The high expression levels indicated stable accumulation in seeds, and activity was not affected after 1 year of storage at room temperature of the milled seeds. Tobacco seeds can be applied in animal diets, but the enzyme could well be expressed in seeds from crops used in animal feed, thereby improving their nutritional value.

V. VACCINES

The expense of immunization programs in developing countries becomes prohibitive when large populations need to be treated. Plants can represent a less expensive production system here, being most appropriate for oral vaccines that could be expressed in edible plant tissues. Hepatitis B surface antigen (HBsAg), which is used as a vaccine against hepatitis B, has been successfully expressed in transgenic tobacco plants [39].

Hepatitis B virus infection is a widespread viral infection of humans that causes hepatitis and hepatocellular carcinoma [40]. The serum of infected individuals contains 22-nm, noninfectious viral particles, composed of elements of the viral envelope, including the HBsAg [41]. HBsAg for use in vaccines was purified from the serum of infected individuals until a recombinant form was expressed in yeast [42]. Intramuscular injection of HBsAg results in effective immunization and protection from viral infection in healthy individuals [43].

Tobacco was transformed with constructs containing the sequence encoding for the HBsAg preceded by an enhanced CaMV 35S promoter and the 5'-untranslated leader of tobacco etch virus [44]. The presence in transgenic leaf extracts of material that reacts specifically with monoclonal antibody against HBsAg was tested, and levels ranging up to 66 ng/mg of soluble protein were found. HBsAg was purified by immunoaffinity chromatography; negative staining and transmission electron microscopy revealed the presence of particles the average diameter of which was 22 nm, similar to the particles observed in human serum. This indicated that the plant-produced HBsAg is properly processed and retains the capacity for self-association in the highly immunogenic particle form.

The maximal levels of HBsAg obtained were approximately 0.01% of the soluble leaf protein, which is insufficient for the efficient use of plants as a production system for HBsAg. However, if these levels could be increased, the inexpensive plant-produced protein could be used in vaccination against hepatitis B.

VI. INDUSTRIAL PRODUCTS

Plants can also be engineered to produce foreign metabolites by introducing genes coding for the appropriate enzymes that convert compounds that are normally present in the plant into products of higher commercial value.

Cyclodextrins are cyclic oligosaccharides of six (α), seven (β) or eight (γ) α -1,4-linked glucopyranose molecules [45]. They have an apolar cavity and are capable of forming inclusion complexes with hydrophobic substances, thus providing new properties to the complexed molecule, such as improved stability or higher water solubility. They are used in the pharmaceutical industry as delivery systems and in the food industry for flavor and odor enhancement and for removal of undesired compounds, such as caffeine [46]. Cyclodextrins are prepared in vitro by the action of the bacterial enzyme cyclodextrin glycosyltransferase (CGT) on prehydrolyzed starch [47]. The gene encoding cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* has been cloned [48] and has been expressed in transgenic potato [49]. A tuber-specific promoter from a class I patatin gene [23] was used to restrict expression of the enzyme to the tuber, which stores large amounts of starch, the CGTs substrate. The protein was targeted to the amyloplast by using the transit peptide of the small subunit of ribulose bisphosphate carboxylase, which directs targeting to the chloroplast [50], an organelle that is developmentally related to the amyloplast.

Northern blot analysis of the transformants showed transcription of the chimeric gene, but no CGT was detected in tubers by immunoblot analysis. Tubers were analyzed for the presence of cyclodextrins, which can be purified by affinity chromatography and separated by thin-layer chromatography. Expression levels of 2–20 μg α -cyclodextrin 2–5 μg β -cyclodextrin per gram of fresh weight were obtained. The approximate ratio of α/β cyclodextrin is similar to that obtained in vitro with *K. pneumoniae* enzyme [51]. The presence of cyclodextrins indicated that the enzyme was correctly targeted to the amyloplast, for starch synthesis occurs only in this plastid [52].

A potato tuber contains approximately 14% starch, of which 0.001–0.01% was converted to cyclodextrins. For the commercial production of cyclodextrins, this value should be increased to 1–10%. This might be possible by using stronger promoters and more efficient targeting signals.

Plastics are probably the most used materials in industry nowadays. Their use creating environmental problems, because most of them need extremely long periods to biodegrade. Therefore, growing efforts are being made to obtain inexpensive biodegradable plastics.

Poly-D(-)-3-hydroxybutyrate (PHB) is a high molecular weight aliphatic polyester that is accumulated by many species of bacteria as storage material [53] and is a biodegradable thermoplastic. However, the cost of PHB produced by bacterial fermentation is rather high. In the bacterium *Alcaligenes eutrophus*, PHB is derived from acetyl-coenzyme (Co)A by a sequence of three enzymatic reactions [54], catalyzed by three different enzymes, 3-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB), and PHB synthase (phbC). The genes encoding the three enzymes have been cloned, and expression in *Escherichia coli* leads to PHB production. 3-Ketothiolase is also found in the cytoplasm of higher plants, involved in the synthesis of mevalonate, and genes encoding phbB and phbC have been introduced into *Arabidopsis thaliana* [55]. Plants were transformed with constructs containing the coding sequences of either phbB or phbC, preceded by the CaMV 35S promoter. Leaf extracts from transgenic plants that had the phbB exhibited acetoacetyl-CoA reductase activity, whereas transgenic plants containing phbC had no detectable PHB synthase activity, although proper integration and transcription of the gene were observed. The same lack of activity was observed when the enzyme was expressed alone in *E. coli* [56].

Homozygous transgenic plants expressing phbB and phbC were cross-pollinated. The presence of PHB was observed in the hybrid plants by gas chromatography and mass spectroscopy analyses. The highest amount of PHB accumulated in leaves was approximately 100 $\mu\text{g/g}$ of fresh weight, and it formed electron-lucent granules, the size and appearance of

which were similar to the granules found in bacteria. PHB was expected to appear in the cytoplasm, but granules were also observed in the nucleus and the vacuole.

Plants expressing *phbB* and *phbC* genes were smaller and produced fewer seeds than nontransgenic plants. This phenotype could be the result of the diversion of acetyl-CoA or acetoacetyl-CoA away from their essential biochemical pathways. Regulation of tissue specificity, time of expression, and cellular localization of the enzymes might avoid these phenotypical effects.

VII. ANTIBODIES

Of the variety of bioactive compounds expressed in transgenic plants, antibodies may offer the widest range of possible applications. An antibody, taken out of the context of an animal immune system, is simply a complex protein capable of binding, with high affinity, a single antigen. That antigen can be anything from another protein to a synthetic organic molecule. Applications that have been envisioned in the plant and that depend solely on the binding affinity of the antibody include pathogen resistance (viral, fungal, and insect), and modulation of metabolic pathways to produce new developmental or nutritional characteristics [57]. Production of antibodies by plant cells offers a variety of new possibilities for basic research in plant biology as well as for large-scale production of antibodies for use as therapeutic, diagnostic, or affinity reagents. The unparalleled capacity and flexibility of agricultural production suggests that antibodies derived from plants may be significantly less expensive than antibodies from any other source. Moreover, antibodies in plants may become useful reagents for isolating and processing environmental contaminants or industrial by-products.

Antibodies produced in plants possess all of the characteristics and are virtually indistinguishable from antibodies produced by hybridoma cells. Recently, it has been found that antibodies that possess catalytic capabilities can be isolated from mouse hybridoma cells [58]. This opens the possibility of introducing new catalytic capabilities into plants or for production of catalytic antibodies useful in industrial processes.

The high capacity and flexibility of agricultural production offers a number of advantages for obtaining antibodies. Genetically stable seed stocks of antibody-producing plants can be isolated and stored indefinitely at low cost; the seed stock can be converted into a harvest of any quantity of antibody within one growing season. Although tobacco has been used as the principal research tool to initiate the study of antibodies in plants, there may be more appropriate plants for production. A variety of common crop plants can be used as the production host. Acreages of perennial forage crops could be generated by clonal propagation or from seed and harvested numerous times in a growing season. The choice of species may depend on the quantity and nature of contaminants encountered during purification. Some candidates are alfalfa, soybean, tomato, and potato. Because large-scale production of antibodies is not yet commonplace, appropriate techniques for purification of hundreds or thousands of grams have yet to be perfected.

As in other eukaryotic cells, secretion of proteins from plant cells is accomplished by an endomembrane system. Recognition and initial processing of proteins destined for secretion occurs in the endoplasmic reticulum and subsequent processing and vesicularization is a function of the Golgi apparatus [59]. We know little about the fidelity with which secreted proteins from evolutionarily distant organisms can be recognized and processed by a plant host endomembrane system. Although signal sequences for secretion generally possess common features, the amino acid sequences are not highly conserved [60]. Data from heterologous *in vitro* translation of mRNAs have suggested that although there are similarities between animal and plant translocating systems, they are not truly homologous [61–63]. In addition, once association of

a heterologous protein with endomembrane has occurred, there are a variety of processing alternatives that could yield a protein different from the one synthesized in its native host [64]. For example, for immunoglobulins, an unusual glycosylation pattern of an antibody produced in yeast resulted in a structure that did not bind complement [65].

Subsequent to endomembrane association, proteins in plants can be subject to proteolytic processing, assembly with other subunits, glycosylation [66], as well as other possible modifications [64]. Little is known about the ability of plant cells to perform these functions on gene products derived from other organisms because relatively few transgenic plants have been regenerated that express, in abundance, proteins from animals or microorganisms. For that reason, we have chosen to evaluate some of the characteristics of murine antibodies produced by plant cells that could result from association with the plant endomembrane system.

Production of the functional antibody was completely dependent on the presence of the mouse signal sequence at the NH₂-terminus of the immunoglobulin. In the absence of the signal, immunoglobulin accumulation was very poor and heavy chain-light chain complexes were not detected. Dramatic increases in immunoglobulin accumulation were observed when signal sequences were included as well as when the individual chains were coexpressed in the same plant. These results suggested that the mouse signal sequences affected endomembrane localization, assembly, and possibly secretion of the antibody.

To optimize the secretion of immunoglobulins from plant cells, we designed an expression strategy to exploit secretion pathways known for other eukaryotes. We have replaced the native mouse leader sequence with a yeast signal peptide and prosequence from the α -mating factor peptide oligomer. In other eukaryotes, many peptide hormones are derived from precursors containing long prosequences, terminating in Lys-Arg residues that are removed before secretion [67]. A specific protease resident in the endoplasmic reticulum is responsible for cleavage [68]. Our results suggest that a similar protease, which selectively recognizes prosequences terminating in Lys-Arg pairs, is present as an integral membrane protein of tobacco leaf cells.

VIII. CONCLUDING REMARKS

A wide variety of foreign products can be obtained from transgenic plants, not only proteins, but also all kinds of modified compounds through the expression of the appropriate enzymes or even catalytic antibodies. The production of plant biomass is inexpensive, the plant cell machinery can provide specific processing unavailable from microbial systems, the isolated products are free of bacterial contaminants, the plant seeds can be used as a low-cost storage, and ultimately plants use sunlight as their source of energy. These facts make us believe that the production of foreign compounds in plants is becoming a competitive alternative system to traditional methods.

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Biocontrol of Bacteria and Phytopathogenic Fungi

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I. INTRODUCTION

Despite the many achievements of modern agriculture, certain cultural practices have actually enhanced the destructive potential of diseases. These practices include use of genetically similar crop plants in continuous monoculture, use of plant cultivars susceptible to pathogens, and use of nitrogenous fertilizers at concentrations that enhance disease susceptibility. Plant disease control, therefore, has now become heavily dependent on fungicides to combat the wide variety of fungal diseases that threaten agricultural crops [De Waard et al., 1993]. Recently, Ritter [1990] reported that over 70 pesticides, including soil fumigants, have been detected in groundwater in 38 states in the United States. Thirty-two of these pesticide detections were attributed to point sources or misuse. A landmark study published by the U.S. Environment Protection Agency (EPA) indicates that, in the United State alone, 3000–6000 cancer cases are induced annually by pesticide residues on foods, and another 50–100 by exposure to pesticides during application. This type of findings have made the governments of many countries increasingly aware of the drawbacks of many chemical pesticides, in terms of their effect on the environment, as well as on the growers and the consumers of agricultural products. Studies aimed at replacing pesticides with environmentally safer methods are currently being conducted at many research centers. The heightened scientific interest in biological control of plant pathogens is partly a response to growing public concerns over chemical pesticides. In this context, it is important to mention that few areas of research within plant pathology have attracted more interest during the past 28 years than has the use of introduced microorganisms for biological control of plant pathogens [Cook, 1993]. This follows an over 40-year period, starting in the mid 1920s, when biological control of plant diseases moved from the discovery of suppression in response to organic materials added to the soil, to attempts at biological control with single cultures of microorganisms added to soil, to disappointment and the emergence of an attitude that plant pathology has made little progress in biological control [Baker, 1987; Cook, 1991].

Biological control is a potent means of reducing the damage caused by plant pathogens. Commercialized systems for the biological control of plant diseases are few. Although intensive activity is currently being geared toward the introduction of an increasing number of biocontrol agents into the market [Elad and Chet, 1995], the performance of a biocontrol agent cannot be expected to equal that of an excellent fungicide; although some biocontrol agents have been reported to be as effective as fungicide control [Mukerji and Garg, 1988]. Usually, results so far are intermediate and inconsistent. Nevertheless, a moderately effective, but consistent agent, seems to be sufficient to establish nonchemical control of plant disease or to reduce the level of chemical residues in agricultural products.

However, there is an equally great or greater need for biological control of pathogens that presently go uncontrolled or only partially controlled. No practical or economical chemical control was replaced by *Agrobacterium radiobacter* strain K-84 [Kerr, 1980] for biological control of crown gall, nor would a chemical pesticide necessarily be replaced by ice-minus bacteria for control of the ice-nucleation active bacteria [Wilson and Lindow, 1993] responsible for the frost damage on potatoes. Biological control should and can be justified on its own merits, without giving it importance at the expense of chemical controls.

Potential agents for biocontrol activity are rhizosphere-competent fungi and bacteria which, in addition to their antagonistic activity, are capable of inducing growth responses by either controlling minor pathogens or by producing growth-stimulating factors [Harman et al., 1989].

Before biocontrol can become an important component of plant disease management, it must be effective, reliable, consistent, and economical. To meet these criteria, superior strains, together with delivery systems that enhance biocontrol activity, must be developed [Harman et al., 1989]. Existing biological control attributes can be enhanced by improving existing, known biocontrol agents, with genetic manipulation. Genetic manipulations of biocontrol agents not only can enhance their activity, but also can expand their spectrum.

The growing interest in biocontrol with microorganisms is also a response to the new tools of biotechnology. Plants and microorganisms can now be manipulated to deliver the same mechanism of biological control, as has been done for the production of the delta endotoxin encoding gene transferred from *Bacillus thuringiensis* to plants to control insect pests [Vaeck et al., 1987]. We can now think of microorganisms with inhibitory activity against plant pathogens as potential sources of genes for diseases resistance.

The successful control by biological means in the phylloplane that have been reported in the literature involve mainly rusts, powdery mildews, and diseases caused by the following genera of pathogens: *Alternaria*, *Epicoccum*, *Sclerotinia*, *Septoria*, *Drechslera*, *Venturia*, *Plasmopara*, *Erwinia*, and *Pseudomonas*. Good soil biocontrol systems have been reported for species of *Fusarium*, *Sclerotium*, *Sclerotinia*, *Phythium*, and *Rhizoctonia*. The following biocontrol agents have already been registered: *Agrobacterium radiobacter* against crown gall (USA, Australia, NZ); *Bacillus subtilis* for growth enhancement (USA); *Pseudomonas fluorescens* against bacterial blotch (Australia); *Pseudomonas fluorescens* for seedling diseases (USA); *Peniophora gigantea* against *Fomes annosus* (UK); *Pythium oligandrum* against *Phythium* spp. (USSR); *Trichoderma viride* against timber pathogens (Europe); *Trichoderma* spp. for root diseases (USSR); *Fusarium oxysporum* against *Fusarium oxysporum* (Japan); *Trichoderma harzianum* against root diseases (USA); *Gliocladium virens* for seedling diseases (USA); *Trichoderma harzianum/polysporum* against wood decay (USA) [Elad and Chet, 1995].

Biocontrol agents may employ several modes of action; therefore, it is important to know the proportion and timing of each mode of action that may occur. Information of this type can be obtained from in vitro studies or by using plants grown under gnotobiotic conditions during which the potential activity of biocontrol agents can be assessed. However, such studies do not provide information on their mode of action in vivo, particularly within plants for

which separation of plant response or antagonistic activity is not always possible or in soil where direct observation and chemical analysis are difficult. These limitations must be kept in mind when extrapolating results obtained in the laboratory to the natural environment. Furthermore, apart from the antagonistic activity of the applied agent, effective biocontrol involves the ability to survive in the habitat in which it is applied. Unfortunately, insufficient research efforts have been directed toward the selection of characteristics that enhance survival of the biological control agents. However, several techniques developed by microbial ecologist and the fermentation industry are now available to select for survival and to manipulate beneficial microorganisms under given environmental conditions, including temperature, osmotic pressure, radiant flux, and pH. Moreover, proper formulation of the biocontrol product can provide a preparation with long shelf life, the ability to withstand adverse conditions, and even with the necessary ingredients to induce its specific activity [Elad and Chet, 1995].

II. MECHANISMS OF BIOLOGICAL CONTROL OF PLANT DISEASES

A. Induced Resistance and Cross-Protection

Induced resistance is a plant response to challenge by microorganisms or abiotic agents such that following the inducing challenge *de novo* resistance to pathogens is shown in normally susceptible plants [de Wit, 1985]. Induced resistance can be localized, when it can be detected only in the area immediately adjacent to the inducing factor, or systemic, when resistance occurs subsequently at sites throughout the plant. Both localized and systemically induced resistance are nonspecific and can act against a whole range of pathogens, but whereas localized resistance occurs in many plant species, systemic resistance is limited to some plants. Cross-protection differs from induced resistance in that, following inoculation with avirulent strains of pathogens or other microorganisms, both inducing microorganisms and challenge pathogens occur on or within the protected tissue [de Wit, 1985].

During localized resistance, the plant reacts to the environmental stimulus by the activation of a variety of defense mechanisms that culminate in various biochemical and physical changes, including phytoalexin production and alterations to plant cell walls, such as increased production of suberin, hydroxyproline-rich glycoproteins, and lignification [Hammerschmidt et al., 1984], and correlations between resistance and lignin formation, peroxidase activity, and protease inhibitors have been found [Dean and Kuc, 1987; Roby et al., 1987]. In systemically protected tobacco or cucumber, increases in newly formed pathogenesis-related (PR) proteins have also been recorded, and these may be chitinase-, glucanase-, or osmotin-like [Fritig et al., 1987; Gianinazzi et al., 1980; Metraux et al., 1988].

The most commonly reported examples of cross-protection involving fungi are probably those used against vascular wilts. Inoculation with nonpathogenic strains or weakly virulent strains of pathogenic *formae speciales* of *Fusarium* and *Verticillium* species, or with other fungi or bacteria, all have shown different levels of cross-protection [Hillocks, 1986; Matta and Garibaldi, 1977; Ogawa and Komada, 1985; Sneh et al., 1985].

B. Hypovirulence

Hypovirulence is a term used to describe reduced virulence found in some strains of pathogens. This phenomenon was first observed in *Cryphonectria (Endothia) parasitica* (chestnut blight fungus) on European *Castanea sativa* in Italy, where naturally occurring hypovirulent strains were able to reduce the effect of virulent ones [Grente and Sauret, 1969b]. These slower-growing

hypovirulent strains contain a single cytoplasmic element of double-stranded RNA (dsRNA), similar to that found in mycoviruses, that was transmitted by anastomosis (fusion of the hyphae of two strains) in compatible strains through natural virulent populations of *C. parasitica* [Grente and Sauret, 1969a; Van Alfen et al., 1975]. In more recent studies, it was demonstrated that a full-length cDNA copy of the hypovirulence-associated virus (HAV) conferred the hypovirulence phenotype when introduced into virulent strains by DNA-mediated transformation [Choi and Nuss, 1992]. Hypovirulent strains of *C. parasitica* have been used as biocontrol agents of chestnut blight [Anagnostakis, 1982]. This may be considered a specialized form of cross-protection that is limited to the control of only established compatible strains [Van Alfen and Hansen, 1984].

Hypovirulence has also been reported in many other pathogens, including *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici* and *Ophiostoma ulmi*, but the transmissible elements responsible for hypovirulence or reduced vigor of the fungi are subject to debate and may be due to dsRNAs, plasmids, or viruses [Koltin et al., 1987; Rogers et al., 1986].

C. Competition

Competition occurs between microorganisms when space or nutrients (*i.e.*, carbon, nitrogen, and iron) are limiting, and its role in the biocontrol of plant pathogens has been studied for many years, with special emphasis on bacterial biocontrol agents [Weller, 1988]. Implicit in this definition is the understanding that combative interactions, such as antibiotic production or mycoparasitism, or the occurrence of induced resistance in the host are not included, even though these mechanisms may form an important part of the overall processes occurring in the interaction. In the rhizosphere competition for space as well as nutrients is of major importance. Thus, an important attribute of a successful rhizosphere biocontrol agent would be the ability to remain at high population density on the root surface, providing protection of the whole root for the duration of its life.

Mycorrhizal fungi can also be considered to act as a sophisticated form of competition or cross-protection, decreasing the incidence of root disease. With ectomycorrhizas, antibiosis against the pathogen, physical protection by the mantle, competition with the pathogen for nutrients coming from the roots, stimulation of antagonistic microflora associated with the mantle, and induction of host plant resistance, all have been suggested as possible mechanisms involved in the protection of roots [Chakravarty and Unestam, 1987; Marx, 1972]. Similarly, plants with endomycorrhizal associations can be more resistant to pathogens than nonmycorrhizal plants of similar size and developmental stage [Dehne, 1982]. Occasionally, the effect may be partially systemic [Rosendahl, 1985].

D. Antibiosis

The production of antibiotics by actinomycetes, bacteria, and fungi is very simply demonstrated *in vivo*. Numerous agar plate tests have been developed to detect volatile and nonvolatile antibiotic production by putative biocontrol agents and to quantify their effects on pathogens [Whipps, 1987]. In general, however, the role of antibiotic production in biological control *in vitro* remains unproved.

Secondary metabolite production is influenced by cultural conditions and, although many microorganisms produce antibiotics in culture, there is little evidence that antibiotics are produced in natural environments, except after input of organic materials. Even so, it is possible that detection techniques are insensitive, that antibiotics are rapidly degraded, or that they are bound to the substrate, such as clay particles in soil, preventing detection [Howell and Stipanovic, 1980; Papavizas and Lumsden, 1980; Williams and Vickers, 1986].

Species of *Gliocadium* and *Trichoderma* are well-known biological control agents that

produce a range of antibiotics that are active against pathogens *in vitro* [Claydon et al., 1987; Dennis and Webster, 1971a, b; Ghisalberti and Sivasithamparam, 1991] and, consequently, antibiotic production has commonly been suggested as a mode of action for these fungi.

Within bacterial biocontrol agents several species of the genus *Pseudomonas* produce antibiotics involved in their ability to control plant pathogens [Fravel, 1988].

E. Mycoparasitism

Mycoparasitism occurs when one fungus exists in intimate association with another from which it derives some or all its nutrients while conferring no benefit in return. Biotrophic mycoparasites have a persistent contact with or occupation of living cells, whereas necrotrophic mycoparasites kill the host cells, often in advance of contact and penetration [Lewis et al., 1989]. Mycoparasitism is a commonly observed phenomenon *in vitro* and *in vivo*, and its mode of action and its involvement in biological disease control has been reviewed [Baker, 1987; Chet, 1993; Handelsman and Parke, 1989]. There are several examples of this phenomenon. Tribe [1957] described the direct attack of sclerotia of *Sclerotinia trifoliarum* by *Coniothyrium mintans*. In a similar way, the mycoparasite *Sporidesmium sclerotivorum* traps the sclerotia of *Sclerotinia minor* [Ayers and Adams, 1979]; Lifshitz and collaborators [1984] found a new variety of *Pythium nunn* capable of lysing germinating sporangia of *Pythium ultimum* in soil. An example of a different aspect of parasitism is observed in *Anquillospora pseudolongissima*, which attacks the mycorrhizae *Glomus deserticola* [Paulitz and Baker, 1987]. However, most of the published studies on mycoparasitism refer to *Trichoderma* spp. because they attack a great variety of phytopathogenic fungi responsible for the most important diseases suffered by crops of major economic importance worldwide.

F. Biocontrol of Airborne Diseases

Many naturally occurring microorganisms have been used to control diseases on the aerial surfaces of plants [Andrews, 1992; Blakeman and Fokkema, 1982]. The more common bacterial species that have been used for the control of diseases in the phyllosphere include *Pseudomonas syringae*, *P. fluorescens*, *P. cepacia*, *Erwinia herbicola*, and *Bacillus subtilis*. Fungal genera that have been used for the control of airborne diseases include *Trichoderma*, *Ampelomyces*, and the yeasts *Tilletiopsis* and *Sporobolomyces*. The mechanisms of action proposed for these biocontrol agents, include competition for sites or nutrients, antibiosis, and hyperparasitism.

Several phytopathogenic bacteria exhibit an epiphytic phase before invasion, during which time they are susceptible to competition from other microorganisms. Although preemptive competitive exclusion of phytopathogenic bacteria in the phyllosphere can be achieved using naturally occurring strains, avirulent mutants of the pathogen, in which deleterious phenotypic traits have been removed, may be more effective because they occupy the same niche as the parental strain. Phytopathogenic bacteria possess several genes that encode phenotypes that allow them to parasitize plants and overcome defense responses elicited by the plant [Panopoulos and Peet, 1985]. In addition, phytopathogenic bacteria possess pathogenicity genes such as *hrp* [Willis et al., 1991]. Isogenic, avirulent mutants can be produced by insertional inactivation of genes involved in pathogenicity. A nonpathogenic strain of *P. syringae* pv. *tomato*, produced by *Tn5* insertional mutagenesis, prevented growth of pathogenic strains in the tomato phyllosphere, presumably by preemptive competitive exclusion [Cooksey, 1988]. Nonpathogenic mutants of *Erwinia amylovora*, produced by transposon mutagenesis, have also been used in the biological control of fire blight [Norelli et al., 1990].

Antibiosis has been proposed as the mechanism of control of several bacterial [Vanneste et

al., 1992] and fungal [Levy et al., 1992] diseases in the phyllosphere. Molecular biology techniques could be used to enhance the efficacy of biocontrol agents that use antibiosis as a mode of action. The transcriptional regulation of genes conferring antibiotic production could be altered by replacing its promoter region by one known to direct high levels of transcription. It may also be possible to transfer the genes required for antibiotic production from a poor-colonizing organism to one that colonizes more aggressively.

Biocontrol agents must normally achieve a high population in the phyllosphere to control other strains, but colonization by the agent may be reduced by competition with the indigenous microflora. Application of a bactericide to which most members of the microflora are sensitive, but to which the control agent is tolerant, can maximize colonization by the biocontrol agent. Integration of chemical pesticides and biocontrol agents has been reported with *Trichoderma* spp. [Elad et al., 1993; Gullino and Garibaldi, 1988; Sivan and Chet, 1993] and *P. syringae* pv. *tomato* [Cooksey, 1988]. Biocontrol agents tolerant to specific pesticides could be constructed using molecular techniques. Resistance to the fungicide benomyl is conferred by a single amino acid substitution in one of the β -tubulins of *Trichoderma viride*, the corresponding gene has been cloned and proved to work in other *Trichoderma* species [Goldman et al., 1993], thereby producing a biological control agent that could be applied simultaneously or in alternation with the fungicide. Molecular techniques may eventually be used to transfer several beneficial traits, such as the production of one or more antibiotics and pesticide tolerance, to an aggressive phyllosphere colonizer.

G. Biocontrol of Soilborne Diseases

Chemical control of soilborne plant diseases is frequently ineffective because of the physical and chemical heterogeneity of the soil, which may prevent effective concentrations of the chemical from reaching the pathogen. Biological control agents colonize the rhizosphere, the site requiring protection, and leave no toxic residues, as opposed to chemicals.

Microorganisms have been used extensively for the biological control of soilborne plant diseases as well as for promoting plant growth [Bakker et al., 1991; Thomashow and Weller, 1990; Weller, 1988]. *Fluorescent pseudomonads* are the most frequently used bacteria for biological control and plant growth promotion, but *Bacillus* and *Streptomyces* species have also been commonly used. *Trichoderma*, *Gliocadium*, and *Coniothyrium* species are the most frequently used fungal biocontrol agents. Perhaps the most successful biocontrol agent of a soilborne pathogen is *Agrobacterium radiobacter* strain K84, used against crown gall disease caused by *A. tumefaciens*. Biological control with *A. radiobacter* is mediated primarily by the bacteriocin agrocin 84 synthesis, which is directed by genes carried by the plasmid pAgK84. This plasmid also carries the genes needed for resistance to agrocin 84 and has conjugal transfer capacity. Consequently, pAgK84 may be transferred to *A. tumefaciens*, which would then be resistant to agrocin 84. To prevent this resistance, a transfer-deficient mutant of strain K84 was constructed. *A. radiobacter* strain K1026 is identical with the parental strain, except that the agrocin-producing plasmid, pAgK1026, has had the transfer region deleted [Jones et al., 1988].

Competition as a mechanism of biological control has been exploited with soilborne plant pathogens as with pathogens on the phylloplane. Naturally occurring, nonpathogenic strains of *Fusarium oxysporum* have been used to control wilt diseases caused by pathogenic *Fusarium* spp. [Alabouvette and Couteaudier, 1992]. Molecular techniques have been used to remove various deleterious traits of soilborne phytopathogenic bacteria to construct a competitive antagonist of the pathogen. Random *Tn5* insertions into the genome of *Pseudomonas solanacearum* [Trigalet and Demery, 1986; Trigalet and Trigalet-Demery, 1990] or insertion

of an interposon into the *hrp* cluster [Frey et al., 1993] produced avirulent mutants. The avirulent mutants exhibited various levels of invasiveness of tomato plants and provided protection against bacterial wilt disease caused by the pathogen [Frey et al., 1993]. The phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora* secretes various extracellular enzymes, including pectinases, cellulases, and proteases. Pectinases are known to be a major pathogenicity determinant in soft rot disease of potato. *E. carotovora* subsp. *carotovora* mutants defective in the production of pectate lyase [Roberts et al., 1986] have been used in the biocontrol of this disease [Stromber et al., 1990].

Molecular techniques have also facilitated the introduction of beneficial traits into rhizosphere-competent organisms to produce potential biocontrol agents. Chitin and β -(1,3)-glucan are the two major structural components of many plant pathogenic fungi, except by *Oomycetes*, which contain cellulose in their cell wall and no appreciable levels of chitin. Biological control of some soilborne fungal diseases has been correlated with chitinase production [Buxton et al., 1965], bacteria producing chitinases or glucanases exhibit antagonism in vitro against fungi [Fridlander et al., 1993; Gay et al., 1992], inhibition of fungal growth by plant chitinases and dissolution of fungal cell walls by a streptomycete chitinase and β -(1,3)-glucanase have been demonstrated [Schlumbaum et al., 1986; Skujins et al., 1965]. The importance of chitinase activity was further demonstrated by the loss of biocontrol efficacy in *Serratia marcescens* mutants in which the *chiA* gene had been inactivated [Jones et al., 1986]. Other organisms from which chitinase-encoding genes have been isolated include: rice [Zhu and Lamb, 1991], tobacco [Payne et al., 1990], cucumber [Metraux et al., 1988], potato [Gaynor and Unkenholz, 1989], *Bacillus circulans* [Joshi et al., 1988], *Streptomyces* [Robbins et al., 1988], and *Cellvibrio* [Wynne and Pemberton, 1986]. A recombinant *Escherichia coli* expressing the *chiA* gene from *S. marcescens* was effective in reducing disease incidence caused by *Sclerotium rolfsii* and *Rhizoctonia solani* [Oppenheim and Chet, 1992; Shapira et al., 1989]. In other studies, chitinase genes from *S. marcescens* have been expressed in *Pseudomonas* spp. and the plant symbiont *Rhizobium meliloti*. The modified *Pseudomonas* strain controlled the pathogens *F. oxysporum* f. sp. *redolens* and *Gauemannomyces graminis* var. *tritici* [Sundheim, 1990, 1992]. The antifungal activity of the transgenic *Rhizobium* during symbiosis on alfalfa roots was verified by lysis of *R. solani* hyphal tips treated with cell-free nodule extracts [Sitrit et al., 1993]. A β -(1,3)-glucanase-producing strain of *Pseudomonas cepacia* significantly decreases the incidence of diseases caused by *R. solani*, *S. rolfsii*, and *P. ultimum*. The biocontrol ability of this *Pseudomonas* strain was correlated with the induction of the β -(1,3)-glucanase by different fungal cell walls in synthetic medium.

Various extracellular antibiotics produced by *Pseudomonas* spp. are involved in the biocontrol ability of soilborne plant pathogens [Fravel, 1988], including phenazine-1-carboxilic acid (PCA) [Thomashow and Weller, 1988; Thomashow et al., 1990], oomycin A [Gutierrez et al., 1986; Howie and Suslow, 1991], pyoluteorin (PLT) [Haas et al., 1991; Keel et al., 1992], and 2,4-diacetyl-phloroglucinol (PHL) [Haas et al., 1991; Keel et al., 1992]. In systems in which antibiosis plays a primary role, molecular techniques can be used to enhance biocontrol efficacy by increasing levels of antibiotic synthesis, either by increasing the copy number of the biosynthetic genes or by modifying the regulatory signals that control their expression. This is particularly interesting for bacteria in which the biosynthetic genes are arranged either in operons or clusters, or when only one enzyme is missing, to achieve the synthesis of the antibiotic. For example, increased production of PLT and PHL and superior control of *Pythium ultimum* damping-off of cucumber was achieved by increasing the number of antibiotic biosynthesis genes in *P. fluorescens* strain CHAO [Haas et al., 1991; Maurhofer et al., 1992]. Constitutive synthesis of oomycin A in *P. fluorescens* strain HV37a was achieved by insertion

of a strong promoter from *E. coli* upstream of the *afuE* locus. The recombinant *P. fluorescens* strain containing the *tac-afuE* construct produced significantly higher levels of oomycin than the parental strain and provided greater control of *P. ultimum* infection [Guttersen, 1990; Guttersen et al., 1990]. Alternatively, biosynthetic genes can be introduced into a strain deficient in antibiotic production, or into one that produces a different antibiotic, to increase the spectrum of activity. Cloned PCA biosynthetic genes were transferred from *P. fluorescens* 2-79, which exhibits poor rhizosphere competence, into *P. putida* and *P. fluorescens* strains that exhibit superior rhizosphere competence. The recombinant strains, which synthesized PCA in vitro, are potentially superior biocontrol agents because of their ability to colonize the rhizosphere [Bull et al., 1991]. In a similar study, a cloned genomic fragment from *Pseudomonas* F113 was transferred into various *Pseudomonas* strains, one of which was subsequently able to produce PHL and inhibit *P. ultimum* damping-off of sugar beet [Fenton et al., 1992]. The spectrum of activity through expression of an additional antibiotic increased when genes conferring PHL synthesis were mobilized from *P. aureofaciens* into *P. fluorescens* 2-79, which normally produces only PCA. This procedure increased activity against *G. graminis* var. *tritici*, *P. ultimum*, and *R. solani* [Vincent et al., 1991].

III. THE TRICHODERMA SYSTEM

Trichoderma spp. act against a range of economically important aerial and soilborne plant pathogens. They have been used in the field and greenhouse against silver leaf (*Chondrostereum purpureum*), on plum, peach, and nectarine; Dutch elm disease (*Ophiostoma ulmi*) on elm; honey fungus (*Armillaria mellea*) on a range of tree species; and against rots on a wide range of crops, caused by *Fusarium*, *Rhizoctonia*, and *Pythium*, and sclerotium-forming pathogens such as *Sclerotinia* and *Sclerotium* [Chet, 1987; Dubos, 1987; Papavizas, 1985]. In many experiments, showing successful biological control, the antagonistic *Trichoderma* was a mycoparasite [Boosalis, 1964; Chet and Elad, 1982; Elad et al., 1983].

A. Mechanism of Action

From recent work, it appears that mycoparasitism is a complex process, including several successive steps. The first detectable interaction shows that the hyphae of the mycoparasite grows directly toward its host [Chet et al., 1981]. This phenomenon appears as a chemotropic growth of *Trichoderma* in response to some stimuli in the host's hyphae or toward a gradient of chemicals produced by the host [Chet and Elad, 1983]. Chemotactic responses in host-parasite relationship have been found in other systems, such as in lytic bacteria [Chet et al., 1971], nematode-trapping fungi [Jansson and Norbring-Hertz, 1979], and plant pathogenic bacteria [Ashby et al., 1987].

When the mycoparasite reaches the host, its hyphae often coil around it or are attached to it by forming hook-like structures (Fig. 1). In this respect, production of appressoria at the tips of short branches has been described for *T. hamatum* and *T. harzianum*. The interaction of *Trichoderma* with its host is specific [Chet and Baker, 1981; Elad et al., 1980; Sivan et al., 1984]. The possible role of agglutinins in the recognition process determining the fungal specificity has been recently examined. Indeed, recognition between *T. harzianum* and two of its major hosts, *R. solani* and *S.rolfsii*, was controlled by two different lectins present on the host hyphae. *R. solani* carries a lectin that binds to galactose and fucose residues on the *Trichoderma* cell walls [Elad et al., 1983a]. This lectin agglutinates conidia of a mycoparasitic strain of *T. harzianum*, but did not agglutinate two nonparasitic strains [Barak et al., 1985]. This agglutinin may play a role in prey recognition by the predator. Moreover, because it does

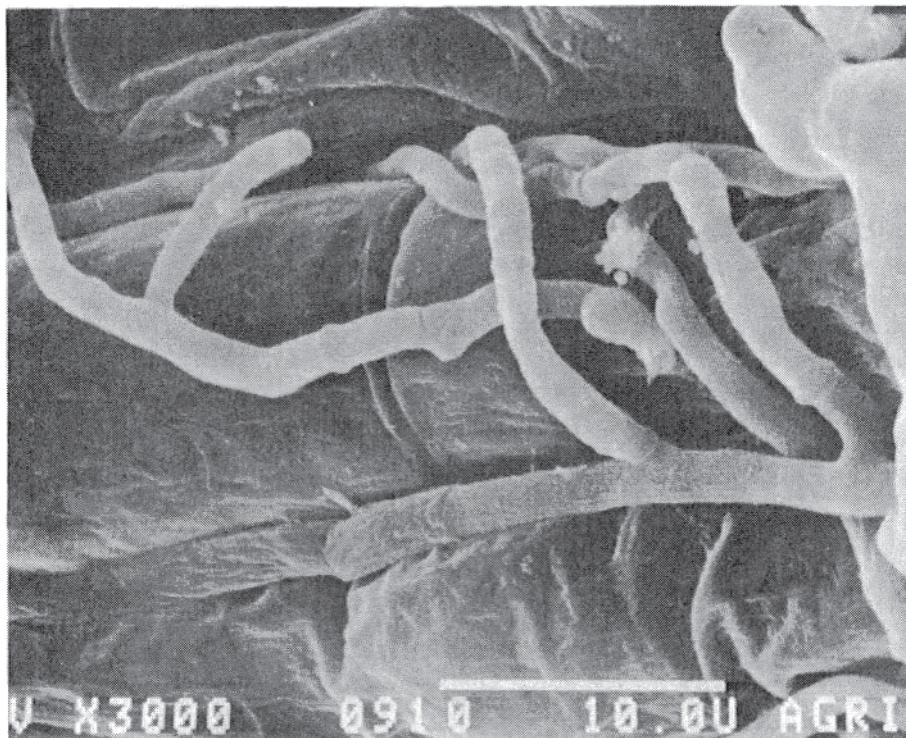


Fig. 1 *Trichoderma* coils around the pathogen.

not distinguish among biological variants of the pathogen, it enables the *Trichoderma* species to attack different *R. solani* isolates [Elad et al., 1983a]. The activity of a second lectin isolated from *S. rolfsii* was inhibited by d-glucose or d-mannose residues, apparently present on the cell walls of *T. harzianum*. This lectin has been isolated and purified from the culture filtrate of this plant pathogen [Barak and Chet, 1990]. Inbar and Chet [1992; 1994] were able to mimic the fungus-fungus interaction *in vitro* using nylon fibers coated with either concanavalin A or the purified *S. rolfsii* lectin. As previously shown *in vivo* for the fungal hyphae [Elad et al., 1983 b,c; Harman et al., 1980; Henis and Chet, 1975; Wells et al., 1972], during the interaction *Trichoderma* recognized and attached to the coated fibers, coiling around them and forming other mycoparasitism-related structures, such as appresorium-like bodies and hyphal loops (Fig. 2) [Inbar and Chet, 1992; Inbar and Chet, 1994].

Following these interactions, the mycoparasite sometimes penetrates the host mycelium (Fig. 3), apparently by partially degrading its cell wall [Elad et al., 1983b,c]. Microscopic observations [Benhamou and Chet, 1993; Hadar et al., 1979a,b; Liu and Baker, 1980; Weindling, 1932] led to the suggestion that *Trichoderma* spp. produced and secreted mycolytic enzymes responsible for the partial degradation of the host's cell wall. Results supporting this hypothesis have shown that indeed *Trichoderma* produces extracellularly β -(1,3)-glucanases, chitinases, lipases, and proteases when grown on cell walls of *R. solani* [Chet and Baker, 1981; Chet et al., 1979; Elad et al., 1982; Geremia et al., 1991; Hadar et al., 1979a]. The production of all cell wall-degrading enzymes secreted by *Trichoderma* has been studied during its growth in different carbon sources. Maximal β -(1,3)-and β -(1,6)-glucanase-specific activities were detected in media supplemented with either pustulan [β -(1,6)-glucan], nigeran [α -(1,3)-

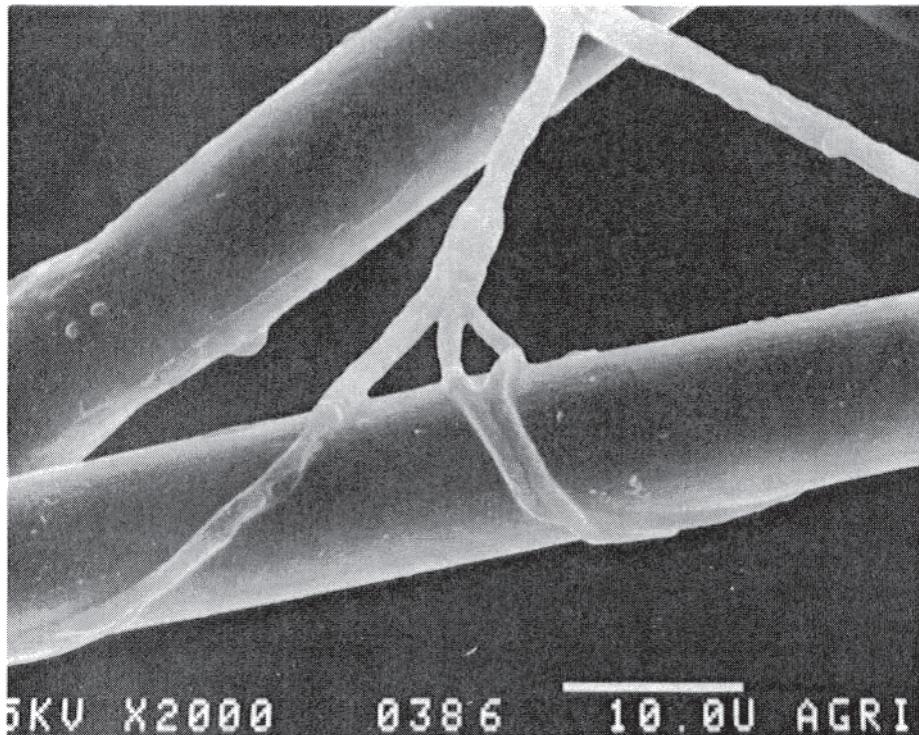


Fig. 2 Biomimics of the *Trichoderma*-host interaction. *Trichoderma* coils around lectin-coated nylon fibers.

glucan alternating with α -(1,4)-glucan] or *Botrytis cinerea* purified cell walls [De la Cruz et al., 1993; Geremia et al., 1991]. In contrast with the results of Geremia and coworkers [1991] where β -(1,3)-glucanase activity was not observed in media supplemented with chitin, De la Cruz and collaborators [1993] obtained high levels of specific activity of this enzyme in medium containing chitin as sole carbon source. These experiments, however, are not equivalent because two different *T. harzianum* strains were used and chitin was pretreated in a different way.

In 1993, Geremia and coworkers, reported the isolation of a 31-kDa basic protease that is secreted by *T. harzianum* during simulated mycoparasitism, an interesting observation was that chitin also appeared to strongly induce proteinase activity. The corresponding gene (*prb1*) was cloned and characterized [Geremia et al., 1993]. That was the first report of cloning of a mycoparasitism-related gene. Recently, Flores et al. [1996] showed that the gene is induced during fungus-fungus interaction and used it to generate transgenic *Trichoderma* strains carrying multiple copies of *prb1*. The resulting strains produced up to 20 times more protease, and one of them reduced the disease incidence caused by *R. solani* on cotton plants to only 6%, whereas the disease incidence for the nontransformed strain was 30% [Flores et al., 1996]. Although the results obtained with the protease-overproducing strains were clear, the use of such strains may be even more useful in the control of *F. oxysporum* for which it has been suggested that proteins in its cell wall may make them more resistant to degradation by the extracellular enzymes of *T. harzianum* [Sivan and Chet, 1989a].

The purification and characterization of three chitinases from *T. harzianum* has been reported

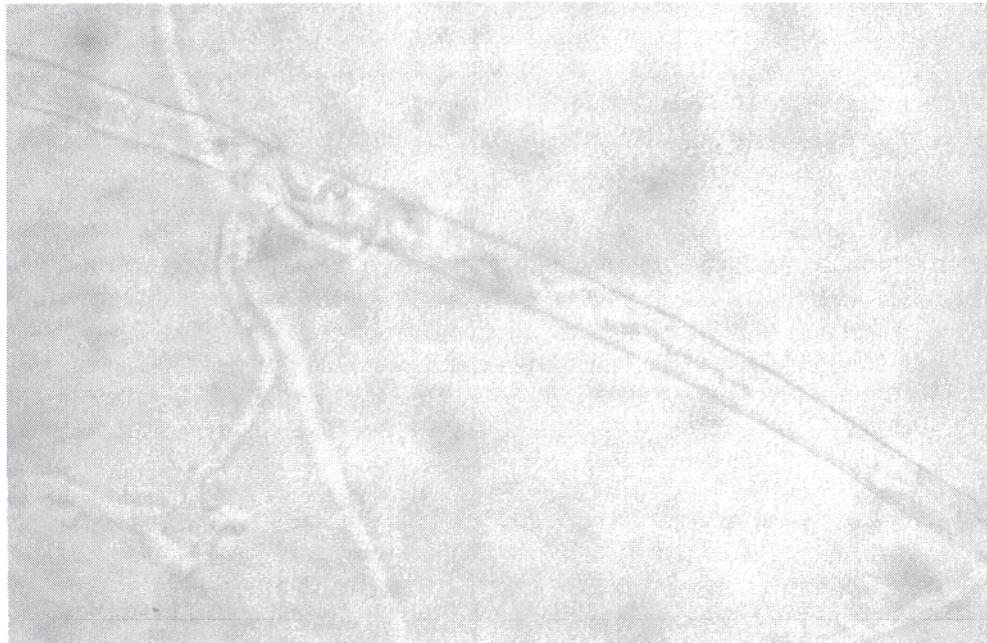


Fig. 3 *Trichoderma* penetrates the hyphae of its host *Rhizoctonia solani*.

[De la Cruz et al., 1992]. These authors reported the isozymes to be 37, 33, and 42 kDa, respectively. Only the purified 42-kDa chitinase hydrolyzed *Botrytis cinerea*-purified cell walls in vitro, but this effect was heightened in the presence of either of the other two isoenzymes [De la Cruz et al., 1992]. However, the chitinolytic system of *T. harzianum* was recently found to be more complex [Haran et al., 1995], consisting of six distinct enzymes. The system is apparently composed of two β -(1,4)-N-acetylglucosaminidases of 102 and 73 kDa, respectively, and four, endochitinases of 52, 42, 33, and 31 kDa, respectively. All the chitinolytic enzymes were induced and secreted during growth of *Trichoderma* on chitin as the sole carbon source. Only the 102-kDa β -(1,4)-N-acetylglucosaminidase was expressed intracellularly, at a low constitutive level, when *Trichoderma* was grown on glucose.

The complexity and diversity of the chitinolytic system of *T. harzianum* involves the complementary modes of action of six enzymes, all of which might be required for maximum efficiency against a broad spectrum of chitin-containing plant pathogenic fungi. Probably the most interesting individual enzyme of the system is the 42-kDa endochitinase because of its ability to hydrolyze *Botrytis cinerea* cell walls in vitro. Since the report of the purification of this enzyme, the corresponding gene has been cloned [Carsolio et al., 1994; Hayes et al., 1994]. Expression of the gene (*ech-42*) encoding Ech42 is strongly induced during fungus-fungus interaction. Its expression is apparently repressed by glucose and may be affected by other environmental factors, such as light, nutritional stress, and may even be developmentally regulated [Carsolio et al., 1994]. Recently, a second chitinase and a β -(1,3)-glucanase genes have been cloned [De la Cruz et al., 1995; Limón et al., 1995].

In summary, expression of all enzymes from the cell wall-degrading system of *T. harzianum* appears to be coordinated, suggesting a regulatory mechanism involving substrate induction and catabolite repression. Regulation of the expression of the system is most likely at the level

of transcription, as indicated by the repression of enzyme synthesis by 8-hydroxyquinoline, an inhibitor of transcription [De la Cruz et al., 1993] and Northern blot analysis of the available genes [Carsolio et al., 1994; De la Cruz et al., 1995; Flores et al., 1996; Geremia et al., 1993; Limón et al., 1995]. However, whether expression of all genes coding for the cell walldegrading enzymes is switched on by a key molecule produced by the host during *in vivo* interaction remains to be investigated.

The level of hydrolytic enzymes produced differs for each host-parasite interaction analyzed. This phenomenon correlates with the ability of each *Trichoderma* isolate to control a specific pathogen. However, the specificity of *Trichoderma* cannot be simply explained by a difference in enzyme activity, because the nonantagonistic *Trichoderma* isolates produce lower, but significant, levels of lytic enzymes [Elad et al., 1982]. This observation supports the idea that recognition is an important factor in the mycoparasitic activity of *Trichoderma*. The effect of the cell wall-degrading enzymes on the host has been observed using different microscopy techniques. Interaction sites have been stained by fluorescein isothiocyanateconjugated lectins or calcofluor. The appearance of fluorescence indicated the presence of localized cell wall lysis at points of interaction between the antagonist and its host [Elad et al., 1983]. Electron microscopy analysis has shown that during the interaction of *Trichoderma* spp. with either *S. rolfsii* or *R. solani*, the parasite hyphae contacted their host and enzymatically digested their cell walls. In addition, an extracellular fibrillar material was deposited between the interacting cells and a mobilization of the organelles inside the invading cells toward the cellular region in contact with the host has been visualized [Chet et al., 1981]. In response to the invasion, the host produced a sheath matrix which encapsulated the penetrating hyphae and the host cells became empty of cytoplasm [Elad et al., 1983]. The susceptible host hyphae showed rapid vacuolation, collapse, and disintegration [Chet et al., 1981]. *T. harzianum* isolates attack both *S. rolfsii* hyphae and sclerotia [Artigues et al., 1984; Henis et al., 1983]. Electron microscopy also showed that the mycoparasite degraded sclerotial cell walls and that the attacked cells lost their cytoplasmic content. It has been proposed that *T. harzianum* uses sclerotial cell content to sporulate on sclerotial surfaces and inside the digested regions [Chet and Henis, 1985; Elad et al., 1984]. Therefore, it is considered that mycoparasitism is one of the main mechanisms involved in the antagonism of *Trichoderma* as a biocontrol agent. The process apparently includes (1) chemotropic growth of *Trichoderma*, (2) recognition of the host by the mycoparasite, (3) secretion of extracellular enzymes, (4) hyphae penetration, and (5) lysis of the host.

The involvement of volatile and nonvolatile antibiotics in the antagonism by *Trichoderma* has been proposed [Dennis and Webster, 1971a,b]. Indeed some isolates of *Trichoderma* excrete growth-inhibitory substances [Claydon et al., 1987; Ghisalberti and Sivasithamparam, 1991; Sivan et al., 1984]. Claydon et al. [1987] identified volatile alkyl pyrons produced by *T. harzianum* that were inhibitory to several fungi *in vitro*. When these metabolites were added to a peat—soil mixture, they reduced the incidence of *R. solani*-induced damping-off on lettuce. Recently, Ordentlich and Chet (unpublished data) isolated a novel inhibitory substance, 3-(2hydroxypropyl), 4-(2-4-hexadienyl, 2(5H)-furanone, produced by *T. harzianum* that suppresses growth of *F. oxysporum* and may be involved in the biocontrol of fusarium wilts. However, there is not sufficient evidence for their contribution to pathogen suppression and disease reduction *in situ*. A strain of *T. harzianum* (T-35) that controls *Fusarium* spp. on various crops may utilize competition for nutrients and rhizosphere colonization [Sivan and Chet, 1989b]. *Trichoderma* stimulates growth and flowering of several plant species [Chang et al., 1986; Windham et al., 1986]. Thus, the biocontrol ability of *Trichoderma* strains is most likely conferred by more than one exclusive mechanism. In fact, it seems advantageous for a biocontrol agent to suppress a plant pathogen using multiple mechanisms.

B. Perspectives

One of the major problems faced when working with *Trichoderma* spp. is their shaky classification in the species group aggregates established in 1969 by Rifai. However, recent efforts made to establish a better classification system for *Trichoderma* include electrophoretic karyotypes of different species and strains of this genus and their possible variability [Hayes et al., 1993; Herrera Estrella et al., 1993]. In addition, Meyer et al. [1992] used a DNA fingerprinting technique to analyze the nine species aggregates of *Trichoderma*. Muthumeenakshi et al. [1994] used restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) analyses and sequencing of the internal transcribed spacer 1 region in the ribosomal DNA gene block to estimate the intraspecific divergence among isolates of *T. harzianum* and to classify them according to their aggressiveness to *Agaricus bisporus*. This also represents the first step toward distinguishing aggressive strains from less aggressive ones without a bioassay. Another possibility is the use of the mycoparasitism-related genes as molecular probes to identify aggressive strains. However, major efforts should still be made to allow a clear classification of the genus. Recently, we have been able to make a direct correlation between the genome structure of different *Trichoderma* isolates, their in vitro biocontrol efficiency, and their capacity to undergo interstrain hyphal fusion [Gómez I., Chet I., and Herrera Estrella A., manuscript in preparation]. This data together with other cell-to-cell communication studies could have a major influence on the selection of *Trichoderma* isolates for their combined use in the formulation of products for their successful use in the field.

Perhaps the most exciting subject for persons working in biological control with *Trichoderma* is strain improvement. From the moment that genetic engineering of biocontrol strains of *Trichoderma* was made feasible at the beginning of the 1990s [Goldman et al., 1990; Herrera Estrella et al., 1990], enormous possibilities for modifying strains were opened. The first practical use of these techniques was to introduce dominant selectable markers into them to monitor their behavior after release either in soil [Pe'er et al., 1991] or the phylloplane [Migheli et al., 1994]. This was followed by the introduction of foreign genes that could potentially enhance the biocontrol capacity of *Trichoderma*. An example of this is the work of Haran et al. [1993], in which the strong chitinase of the bacteria *Serratia marcescens* was introduced into *T. harzianum* and expressed constitutively. In vitro tests of the ability of these strains to overgrow the plant pathogen *Sclerotium rolfsii* in dual cultures showed wider lytic zones along the contact front between the transformants and the pathogenic fungus than those of the nontransformed strain. Many of this type of genes are available and could be tested in *Trichoderma*. On the other hand, cloning of the genes coding for the different cell wall-degrading enzymes produced by *Trichoderma* will allow us to test their relevance in mycoparasitism through their overexpression and disruption.

However, a very exciting issue for the basic biologists working with *Trichoderma* is the cascade of intracellular events that follow the initial recognition of the host. Studies on this subject would lead to the understanding of a series of signal transduction events that must take place after the initial detection of the host, resulting in gene expression and the regulation of gene expression.

Thus, it is clear that the *Trichoderma* field is still in its childhood and more and more researchers should join the efforts made to obtain natural alternatives for the control of plant diseases.

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Transgenic Plants for the Control of Insect Pests

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I. INTRODUCTION

Insects have always been a major cause of yield loss in agriculture. Many approaches have been used with varying degrees of success to reduce losses caused by insect damage. Natural resistance to insects that provides reasonable protection from some insect pests is available in some crops. Plant breeding can move these resistances into different crop varieties; however, this is frequently a difficult and time-consuming procedure. For other plants, resistance to a given insect pest is not present in the current gene pool of a particular crop. In such instances, breeding alone cannot provide protection against insect damage. Insecticides have been quite successful in reducing crop losses caused by insects. Most insecticides currently used are chemicals, but there are successful biological insecticides, notably microbial sprays using the bacterium *Bacillus thuringiensis* (Bt). Resistance to chemical insecticides has occurred in several insect species. Management of such resistance remains a challenge, and new methods of controlling such species are needed.

The need to control insect pests in agriculture will always exist. As new tools become available, they will be integrated into the pest management system. The ability to introduce new genes into a crop species provides the opportunity to introduce new insect control genes into a crop's gene pool. Once introduced, the new trait can be moved into the other varieties of that crop using traditional breeding. To introduce a new trait into a crop, several components are required: a transformation system for the target crop, a gene encoding an insecticidal protein active against an insect pest of the target crop, and expression of the introduced gene(s). The ability to transform crop species varies from crop to crop, as do effective ways of obtaining insecticidal levels of expression of the introduced gene(s). Various proteins have been identified as possessing insecticidal activity. These proteins include protease inhibitors, α -amylase, lectins, and insecticidal proteins from *Bacillus* species. The proteins produced by the *Bacillus* species possess the most potent insecticidal activities. Some of these proteins, therefore, have been worked with for several years to produce insect-resistant plants. The

early years of this work were confined to dicotyledonous (dicots) plants because transformation systems for dicots were developed before transformation systems for monocotyledonous (monocots) species. As the ability to transform the important crop species has been developed and improved, genes encoding *B. thuringiensis* endotoxins and other proteins with insecticidal activity have been introduced into these species.

The first genetically modified plants to be commercialized with new insect-resistance traits are based on plants expressing a *B. thuringiensis* δ-endotoxin. Other proteins with insecticidal activity are being identified and characterized and will provide valuable options for insect management using transgenic crops. These new traits will provide several new insect management tools, with an increasing number of options becoming available as more insecticidally active proteins are found and as the ability to manipulate pathways for small molecules in transgenic plants becomes routine. Resistance management will be a concern, just as it is with chemical insecticides and naturally occurring resistances. However, this problem can be managed as more options become available and as more is known about the various mechanism by which insects develop resistance(s) to a given active component.

II. EXPRESSION OF *BACILLUS THURINGIENSIS* δ-ENDOTOXINS IN PLANTS

A. Expression of *Bacillus thuringiensis* Genes in Dicots

With the ability to introduce new genes into plants, the δ-endotoxins from *B. thuringiensis* were attractive candidates to be among the first genes transferred. These genes encode proteins with high specific activity toward a narrow spectrum of insect pests [1]. The *crylA* genes from *B. thuringiensis* encode insecticidal proteins of about 130 kDa in size. These proteins are activated in the insect gut by proteases to generate an insecticidally active fragment derived from about the NH₂-terminal half of the protein. Different δ-endotoxins have been expressed in plants using a variety of plant promoters [2–7]. The first experiments using δ-endotoxin genes utilized the native-coding sequences for the *crylA* genes fused with constitutive promoters operable in plants. These constructs were transferred into dicots in which transformation and regeneration systems were functional by using *Agrobacterium-mediated* T-DNA transfer. When the full-length native genes were used, no detectable expression was obtained [2–4]. Because the active part of the molecule is contained in approximately the first half of the protein, and this active fragment is released on proteolytic cleavage (activation) in the insect midgut, truncated versions of the native gene were also used in plant transformation experiments. Constructs tested in tobacco included the full-length gene coding for a 1155-amino acid protein, an NH₂-terminal fragment of *crylA(b)* gene, coding for 610–614 amino acids previously shown to be fully insecticidal, and translational fusions between NH₂-terminal fragments of *crylA(b)* and the neomycin phosphotransferase (*neo*) gene of transposon *Tn5*, which confers kanamycin resistance to plants. Transgenic tobacco transformed with the Bt δ-endotoxin-neo fusions and the NH₂-terminal truncated Bt constructs resulted in mortality to tobacco hornworm (*Manduca sexta*) larvae [2]. Such truncated constructs were successful in producing insecticidal amounts of δ-endotoxin, but typically the levels of expression were low [2,3]. Northern blot analysis of transgenic plants containing native δ-endotoxin genes showed an abundance of smaller than expected RNA bands hybridizing with probes specific for the δ-endotoxin genes [3,8]. In spite of this low level of expression, protection of certain dicot plants from certain susceptible insect pests was obtained, both in the laboratory and in the field [9,10]. The Bt field trials with transgenic tobacco using truncated native Bt δ-endotoxin genes demonstrated that transgenic plants can express sufficient levels of *crylA(b)* endotoxin

to protect tobacco against economic damage from tobacco hornworm and tobacco budworm [9] and tomato fruitworm (*Heliooverpa zea*), which are approximately 40-fold more resistant to Bt than the tobacco hornworm (*Manduca sexta*) [11].

To circumvent the problem of low expression from the native δ-endotoxin genes in plants, synthetic genes have been constructed [12,13]. Different strategies can be employed to design synthetic genes, such as eliminating potential polyadenylation sites, eliminating potential transcriptional stalling sites, eliminating mRNA instability signals, eliminating potential intron splice sites, or optimizing translation by altering codon usage. These strategies can also alter the overall guanine-cytosine (G+C) content of the genes and thereby alter the way they appear to the new host. Native endotoxin genes tend to have a low G+C content, about 37%. Plants in general tend to have a higher G+C content, with maize showing a strong preference for G+C-rich coding regions. Several laboratories have now made partially or completely modified Bt δ-endotoxin genes that have resulted in significant improvements in gene expression in cotton [14,15], tomato [12], and potato [16]. Synthetic Bt genes are a requirement to obtain expression of δ-endotoxins in monocot crops such as corn [13].

Insects that cause considerable damage to cotton include lepidopteran pests, such as the cotton bollworm (*Heliooverpa zea*), tobacco budworm (*Heliothis virescens*), pink bollworm (*Pectinophora gossypiella*) and, to a lesser degree, the beet armyworm (*Spodoptera exigua*) and cotton boll weevil (*Anthonomus grandis*). Transgenic cotton expressing truncated forms of two Bt endotoxin genes, including native and modified sequences, demonstrated the effectiveness of modified sequences [15]. Leaf assays of cotton plants containing the native truncated *crylA(b)* gene failed to demonstrate consistent insect control, whereas the plants containing the highly modified *crylA(b)* gene were toxic to the cabbage looper (*Trichoplusia ni*) and beet armyworm (*Spodoptera exigua*) a species 100-fold less sensitive to *crylA(b)* than *T. ni*. Transgenic cotton plants infested with cotton bollworm (*H. zea*) showed an 87% protection of squares and bolls with a synthetic *crylA(c)* gene and 70–75% protection with a synthetic *crylA(b)* gene, which is three- to fivefold less active against cotton bollworm. A modified truncated *crylA(b)* gene from *B. thuringiensis* var. *kurstaki* Berliner was field tested in three transgenic cotton lines to examine resistance against the pink bollworm (*Pectinophora gossypiella*) [14]. Although pink bollworm entered the bolls, there was a 99.8% reduction in the live insects recovered from transgenic bolls and a 97% reduction in seed damage. The transgenic lines were highly resistant to cotton leaf perforator (*Bacculatrix thurberiella*), and sustained little damage from the beet armyworm (*Spodoptera exigua*). Further studies have shown that transgenic cotton expressing modified *crylA(b)* or *crylA(c)* genes also provide protection against tobacco budworm (*Heliothis virescens*) [17,18].

Transgenic tomato plants expressing a truncated native Bt δ-endotoxin gene were insecticidal against tobacco hornworm in a field environment, but higher levels of Bt expression were needed to control the agronomically important pests, such as the tomato fruitworm (*Heliooverpa zea*) and tomato pinworm (*Keiferia lycopersicella*) [10]. Transgenic tomatoes expressing the native truncated *crylA(b)* δ-endotoxin gene demonstrated significant, although incomplete, control of the American bollworm (*Helioverpa armigera*) in the field [19]. Several versions of the modified *crylA(b)* and *crylA(c)* genes were transformed into both tobacco and tomato to examine the increased expression associated with various sequence modifications [12]. The partially modified *crylA(b)* had 62/1743 bases changed to remove regions with potential plant polyadenylation signals and adenine-thiamine (A-T)-rich regions. The fully modified *crylA(b)* gene had 390/1845 bases changed to remove all ATTAA sequences, most potential plant polyadenylation sites, and regions of potential mRNA secondary structure. The fully modified *crylA(b)* gene also replaced bacterial codons with plant-preferred codons. The partially modified *crylA(b)* gene had 97% homology with the wild-type gene and a G+C

content of 41%, compared with 37% in the wild-type *crylA(b)* gene. The fully modified *crylA(b)* gene had 79% homology with the wild-type gene with a G+C content increased to 49%. The most highly expressing transgenic plants containing the partially modified gene and the fully modified gene increased expression 10- and 100-fold, respectively, compared with the wild-type truncated gene.

Bacillus thuringiensis var. *tenebrionis* produces a δ-endotoxin (CryIII A) with activity against coleopterans such as Colorado potato beetle (*Leptinotarsa decemlineata*). A native *cryIII A* gene was introduced into tomato and potato plants by *Agrobacterium*-mediated transformation [20]. The expression levels in plants transformed with the *cryIII A* gene were very low. A native *crylA(c)-neo* fusion transformed in potato, *Solanum tuberosum*, using an *A. tumefaciens* vector system showed little activity against tobacco hornworm (*Manduca sexta*) [21], potato tubermoth (*Pthorimea operculella*), and European corn borer (*Ostrinia nubilalis*), but was limited to a reduction in feeding [22].

Expression levels have been improved by engineering synthetic *cryIII A* genes using the same criteria used for the synthetic *crylA* genes. A modified version of the *cryIII A* gene was engineered to remove potential polyadenylation sites and A+T-rich regions, with a final G+C content of 49% as compared with 37% in the wild-type gene [16]. The modified *cryIII A* gene was introduced into Russet Burbank potato plants by *Agrobacterium*-mediated transfer. Tests conducted under both greenhouse and field conditions demonstrated complete control of neonate larvae as well as reduced feeding of adults, accompanied by reduced fecundity. Results of several field tests of potato lines transformed with a synthetic *cryIII A* gene of *B. thuringiensis* var. *tenebrionis* were published in 1992 [23–25]. The transgenic potato plants had significantly lower defoliation ratings by the Colorado potato beetle (*Leptinotarsa decemlineata*) than nontransformed plants.

B. Expression of *Bacillus thuringiensis* Genes in Monocots

Transformation of rice with the native *crylA(b)* gene produced no detectable protein, so a highly modified *crylA(b)* gene was designed based on the codon usage of rice genes [26]. The modified *crylA(b)* gene had 66.6% of the codons changed to produce an overall G+C content of 59.2%. Bioassays of transgenic japonica rice showed 10–50% mortality against the striped stemborer (*Chilo suppressalis*) and 45–55% mortality against the leaffolder (*Cnaphalocrosis medinalis*).

The first example of a transgenic cereal plant with increased tolerance to insects was achieved by expressing the CrylA(b) δ-endotoxin using tissue-specific promoters to confer resistance to European corn borer (ECB; *Ostrinia nubilalis*) [13]. ECB typically has two generations per year, and a single borer can cause a 3%–7% yield loss per plant [27,28]. None of the areas of the maize plant where ECB feeds are easily accessible through conventional spray application of insecticides. First-generation larvae feed on whorl tissue before boring into the stalk. Larvae grow and pupate within the stalk to emerge as adult moths. Second-generation larvae feed on pollen, which has accumulated in the leaf axils, and on sheath and collar tissue before boring into the stalk to complete development.

The CrylA(b) δ-endotoxin from *B. thuringiensis* strain *kurstaki* HD-1 has high insecticidal activity against ECB. However, native δ-endotoxin genes from *B. thuringiensis* are not expressed in maize. Their high A+T content poses problems for the maize gene expression apparatus. To solve this problem, a synthetic gene optimized for maize expression using the most-preferred codon from maize for each amino acid was constructed [13,29]. This “maize-optimized” gene encodes the first 648 amino acids of the 1155-amino acid CrylA(b) protein produced by *B. thuringiensis* [30]. The proteins are 100% homologous throughout this 648-amino acid

Table 1 Expression of a Synthetic “Maize-Optimized” *crylA(b)* Gene in Tobacco and Maize^a

	Tobacco		Maize	
	Stable (leaf)	Transient (suspension)	Transient (mesophyll)	Stable (leaf)
35S/native	50	Not detected	NT	Not detected
35S/synthetic	364	5100	6500	160
PEPC/synthetic	NT	560	3700	370

^aValues are in nanograms CrylA(b) per milligram total protein.

NT, not tested.

region. This region contains the entire insecticidally active protein fragment that is produced on activation in the insect midgut. This synthetic gene has a G+C content of about 65% and has about 65% homology with the native *crylA(b)* gene at the DNA level.

When compared with the native gene, the synthetic truncated *crylA(b)* gene produced significantly higher levels of CrylA(b) protein in both tobacco and maize. Table 1 presents a summary of CrylA(b) protein expression levels in both transiently and stably transformed tobacco and maize cells. In stably transformed tobacco, the synthetic *crylA(b)* gene produced about five times the level of CrylA(b) protein as did the native truncated gene. In maize, expression of native Bt δ-endotoxin genes is not seen; however, the synthetic *crylA(b)* gene is expressed at a high level in both transient and stable maize transformants. The synthetic *crylA(b)* gene was fused with a variety of promoters including the cauliflower mosaic virus (CaMV) 35S promoter, the maize phosphoenolpyruvate carboxylase (PEPC) promoter [31], a pollen-specific promoter from maize, and a pith-preferred promoter from maize [13]. Various combinations of these genes were introduced into maize using the Biolistics device. Two of the resulting lines, called event 171 and event 176, were chosen for further analysis in the field. Event 171 was transformed with the synthetic *crylA(b)* gene under control of the CaMV 35S promoter and event 176 was transformed with two versions of the synthetic *crylA(b)* gene, one under control of the maize PEP carboxylase promoter and the other under control of a pollen promoter isolated from maize. The PEPC promoter is expressed in essentially all green tissues of maize, whereas the pollen promoter is specific for pollen expression.

In the field, plants were manually infested with 300 ECB larvae for each of 8 consecutive weeks, resulting in a total of 2400 larvae added to each plant. The first 4 weeks corresponded roughly with a first-generation ECB infestation (ECB1) and the last 4 weeks corresponded with a second-generation (ECB2) infestation. Starting two weeks after the initial larval application each plant was rated weekly for 4 weeks for ECB1 foliar damage by using a modified 1–9 Gutherie scale [32], in which a rating of 1 was reserved for plants with no damage. About 50 days after the initial ECB2 infestation, selected stalks from each of the two events, as well as nontransgenic controls, were harvested. The extent of internal ECB tunneling damage in a 92 cm section of stalk 46 cm above and below the primary ear node was measured. Protection from both first and second generation damage was obtained, even under the abnormally high infestation rate. All transgenic plants from both events were vastly superior to control plants, as assessed by foliar feeding damage and internal stalk damage. Event 176 derivatives had significantly better performance for foliar and internal stalk damage than event 171, but both events were much better protected than the control plants. The average leaf damage rating and mean tunnel length for the best transgenic family was 1.6 and 1.7 cm of tunneling, respectively, as compared to the control inbred with a damage rating of 7.2 and 59.3 cm of tunneling [13].

Transgenic plants containing the synthetic *crylA(b)* gene under control of the PEPC and pollen-specific promoters (line 176) produced over 1000 ng of CrylA(b) per milligram of soluble protein and up to 4000 ng CrylA(b) per milligram of soluble protein in some plants later in the season. Plants with the CaMV 35S promoter driving the synthetic *crylA(b)* gene (line 171) typically showed an overall lower, and much more variable, level of CrylA(b) within a particular maize genotype and also among the genotypes (25–2000 ng CrylA(b) per milligram soluble protein). The levels of CrylA(b) protein in the PEPC and pollen-promoter plants were all approximately equal, both within and among genotypes. Line 176 showed an increase in CrylA(b) expression during the season, with an average twofold increase over the course of the season. This increase in expression was independent of genotype. Line 171 showed a significant decrease in expression during the season, which was also independent of genotype.

Since the 1992 field test of lines 171 and 176, numerous other transformation events have been produced using a variety of maize tissue-specific promoters to express the synthetic *crylA(b)* gene. Several of these events, as well as progeny from event 176, were field tested in 1993 and 1994. New lines tested included various combinations of the *crylA(b)* gene under control of the PEPC, pollen-specific, and a pith-preferred promoter. Tests on the new lines and further testing of event 176 confirm that expression of the CrylA(b) protein in maize provides protection against ECB damage and yield loss. Given its performance in the field, event 176 has been chosen for commercial development. It has continued to perform well under both natural and artificially heavy ECB infestation rates in a variety of field conditions around the world.

The level of protection afforded by the CrylA(b) protein is a considerable improvement over ECB resistance previously available within the maize gene pool. ECB has adapted to maize, and it has doubtless adapted to maize defense mechanisms. To obtain a large increase in resistance, one conceivably should look outside the maize gene pool to find substantial resistance to ECB or other adapted maize pests, such as corn rootworm. After identifying a suitable resistance, one has to introduce that resistance into maize from the distant source. This is not always possible with traditional breeding because one has traditionally been limited to the genes available within a given species. Introduction of new traits into a gene pool using transformation methods allows one to introduce resistances to which a pest has not yet been widely exposed and to which it has not yet developed resistance mechanisms. Exploiting such natural weaknesses will allow breeders to introduce large increases in resistance to pests and then to treat the new traits as part of the species gene pool. For the Bt δ -endotoxins, these new resistances to insect damage behave as single dominant traits.

III. PROTEASE INHIBITOR GENES IN PLANTS

Many plants have evolved natural defense mechanisms against herbivorous insects based on the production of proteinaceous protease inhibitors. In contrast to Bt δ -endotoxins, these proteins have antimetabolic activity against a wide range of insect species. Typically, the protease classes found in the digestive tracts of phytophagous insect pests are either serine proteases, which are the major class of proteases in Lepidoptera, or cysteine proteases, the major protease class found in Coleoptera [33]. Several studies have demonstrated that plant serine protease inhibitors are effective against insect serine protease activity in vitro [34–37] and many have some effect on lepidopteran larvae when incorporated at high concentrations into artificial diets [38–43].

Broadway et al. [44] produced the first evidence that protease inhibitors in plants may have a negative effect on insect growth. In these experiments, levels of potato inhibitors I and II in leaves of wounded tomato plants correlated with reduced growth of beet armyworm

(*Spodoptera exigua*). However, no direct evidence that the protease inhibitors were responsible for this reduced growth was given, as other defensive chemicals could have contributed to the results in this experiment.

Direct evidence that protease inhibitors in plant leaves can function as a defense mechanism against insects was first demonstrated by Hilder et al. [45]. Transformed tobacco plants expressing a foreign cowpea trypsin inhibitor (CpTI) gene were more resistant to feeding by larvae of tobacco budworm (*Heliothis virescens*) than control plants without the CpTI gene. Although there was very little mortality attributable to the CpTI, surviving insect biomass was lower on the highest CpTI expressing plants.

Johnson et al. [46], transformed tobacco plants with genes encoding tomato and potato inhibitor II proteins, which inhibit trypsin and chymotrypsin, and a tomato inhibitor I protein, which inhibits chymotrypsin only. Tobacco hornworm (*Manduca sexta*) larvae, fed leaves from plants expressing the inhibitor II proteins at levels of 52 µg/g tissue, weighed about 10% less than larvae fed leaves from control plants. Plants that expressed 114–332 µg/g leaf tissue caused a 50–64% reduction in growth. Tobacco hornworm larvae fed leaves from plants expressing the inhibitor I protein did not show the same level of growth reduction as did larvae fed leaves expressing inhibitor II. The reduction in growth caused by the presence of inhibitor I was at best about 15%. However, no conclusive evidence of insect mortality was presented.

Although most work carried out to date has used serine protease inhibitors, expression of cysteine protease inhibitors has also been demonstrated. Masoud et al. [47] successfully transformed tobacco with a rice cystatin gene, oryzacystatin-I. The authors speculate that this gene may help in controlling coleopteran insects.

More recently, Orr et al. [48] have shown that potato multicystatin (PMC) isolated from potato tubers has growth inhibitory effects on larvae of the genus *Diabrotica*. However, this growth inhibition is not so apparent during long-term exposure experiments. The authors speculate that multicystatin would be effective in controlling larvae of *Diabrotica* species in transgenic plants, but do not show *in planta* data.

Appreciable effort is being made to engineer protease inhibitor genes into plants. The introduction of specific protease inhibitors into plants that do not produce these proteins may be an effective alternative approach for obtaining crops with resistance to insect attack. However, a major disadvantage of protease inhibitors is the very high levels of protein required for insect mortality. The levels required to kill a significant number of exposed insects has yet to be expressed in a transgenic plant.

IV. OTHER PROTEINS

A. Lectins

Other proteins with insecticidal or antifeeding properties include lectins, α -amylase, and cholesterol oxidase. Lectins are carbohydrate-binding proteins extensively distributed in plants, invertebrates, vertebrates, and bacteria. Lectins found in plants, particularly in seeds, are thought to be involved in defense against bacterial pathogens, fungal attack, and attack from animal and insect herbivores [49]. Wheat germ agglutinin (WGA; *Triticum vulgaris L.*) from wheat, *Ricinus communis L.* from castor beans, and *Bauhinia purpurea L.* from camels foot tree have activity against European corn borer (*Ostrinia nubilalis*). Lectins from poke-weed, *Phytolacca americana L.* also have insecticidal activities against the southern corn rootworm (*Diabrotica undecimpunctata howardi*) [50]. The mode of action of lectin-related insecticidal

activity is not understood but is thought to be a consequence of binding to *N*-acetylglucosamine or *N*-acetylgalactosamine residues on the midgut epithelial cells or the peritrophic membrane, with subsequent disruption of critical cellular functions. The insecticidal activities, or 50% lethal concentration (LC_{50}) of lectins are in the milligram per gram diet range in insect bioassays and are less active than the *B. thuringiensis* δ -endotoxins, which have LC_{50} s in the nanogram per gram diet range. Several of the chitin-binding lectins, such as WGA, a lectin similar to WGA from rice and UDA (*Urtica dioica* agglutinin) from stinging nettle increase the time of larval development of the cowpea weevil (*Callosobruchus maculatus*) at a concentration of 1% (w/w) [51,52]. Since the level of lectin protein required to produce insecticidal activities is higher than that required for insecticidal levels of the Bt δ -endotoxins, lectins must be expressed at a higher level in plants than δ -endotoxins to produce the desired level of protection.

B. α -Amylase Inhibitor

The insecticidal activity associated with the common bean seed has been reported to be due to an inhibitor of α -amylase activity [53]. α -Amylase inhibitor is toxic to larvae of two seed-feeding beetles, the cowpea weevil (*Callosobruchus maculatus*) and the Azuki bean weevil (*Callosobruchus chinensis*). Transgenic pea plants have been engineered that express up to 1.2% α -amylase inhibitor protein in pea seeds and confer resistance to both cowpea weevil and Azuki bean weevils [53]. Azuki bean weevil was more susceptible to α -amylase inhibitor, and concentrations as low as 0.1% (w/w) gave 100% mortality, whereas levels of 0.8–1.0% (w/w) were required for similar cowpea weevil mortality.

C. Cholesterol Oxidase

Cholesterol oxidase isolated from *Streptomyces* has strong insecticidal activity against boll weevil larvae (*Anthonomus grandis* Boheman) [54]. The activity of the cholesterol oxidase protein is in the range of the *B. thuringiensis* δ -endotoxins and has an LC_{50} of 20.9 $\mu\text{g}/\text{ml}$ against the boll weevil. Diet incorporation and histological studies of the boll weevil midgut epithelium indicate that the mode of action of the cholesterol oxidase is from a direct disruption of the midgut cells, rather than an effect related to the enzyme altering critical diet and nutritional requirements. Although cholesterol oxidase does not usually affect the cholesterol in biological membranes, the cholesterol within the boll weevil's midgut epithelium may be subject to oxidation followed by cell lysis and mortality. The LC_{50} of cholesterol oxidase makes it an attractive candidate for production in transgenic cotton for control of the boll weevil.

D. Vegetative Insecticidal Proteins from *Bacillus* spp.

Recently, a new class of insecticidal proteins that are produced during the vegetative stage of growth was discovered in *B. cereus* and *B. thuringiensis* [55]. These new vegetative insecticidal proteins (VIPs) have activity against insect species that are recalcitrant to the known Bt δ -endotoxins, for example, western and northern corn rootworm, *Diabrotica virgifera* and *Diabrotica longicornis*, respectively, and black cutworm, *Agrotis ipsilon*.

The *B. cereus* strain AB78 produces a potent proteinaceous activity against *Diabrotica*. Interestingly, this activity is specific to the two aforementioned *Diabrotica* species and has little activity on the closely related southern corn rootworm, *Diabrotica undecimpunctata*. This unique activity expressed in the roots of transgenic maize offers an effective way of controlling these underground pests, which have been difficult to control with traditional methods.

V. RESISTANCE MANAGEMENT

There has been some concern expressed that use of *B. thuringiensis* δ-endotoxin genes for insect control in transgenic plants will lead to rapid development of resistance to the δ-endotoxins on the part of the insect [56]. Such concerns are based largely on computer models. Slight changes in various parameters can have a dramatic effect on these predictions. Resistance to *B. thuringiensis* δ-endotoxins has occurred both in the laboratory and in nature. Such resistances have typically developed, or been cultivated, using geographically isolated populations of insects and selecting with acute sub-lethal doses of δ-endotoxin. Transgenic plants expressing high levels of δ-endotoxin represent a different type of selective pressure, that is a chronic high-dose exposure. No reports of resistance to chronic high-dose exposure of Bt endotoxins are yet known. Several strategies have been proposed to control, or delay, the onset of such resistance. Which strategy is correct is unclear at this point. Data must be collected to find which of the strategies is likely to aid in resistance management and which may actually expedite the development of resistance. It is likely that development of resistance will be heavily influenced by both the insect and crop in question. European corn borer typically has only two generations per year and feeds on numerous other plant species besides corn. This low number of generations per year, combined with plentiful alternative host plants on which ECB feeds, would make resistance seem a much smaller issue in maize for ECB than for other potential crop-insect combinations.

The rate of discovery of new insecticidal strains of *B. thuringiensis* [57], and the discovery of new insecticidal proteins from such strains is increasing. This increased rate of discovery will provide more active components to be used for resistance management. Also, as the field of agricultural biotechnology continues to progress, new insecticidal components derived from sources other than the *B. thuringiensis* endotoxins will be developed. These components will provide further options for insect control and for resistance management strategies. Prudent use of transgenic plants as part of an overall integrated pest management system, coupled with careful planning, will ensure that resistance does not become a major problem.

VI. SUMMARY

Use of transgenic crops to control insects is a new technology. It provides a new option to be used with the other options available to protect crops from insect damage. Insects will remain a constant source of crop damage and yield loss. Plants producing a δ-endotoxin from *B. thuringiensis* are likely to be the first to be commercialized as insect-tolerant transgenic crops. As new sources of insecticidally active proteins are identified and characterized, new options will become available. These options will allow one to control an insect pest not susceptible to a known Bt endotoxin and will further allow the management of resistance to new traits and chemical insecticides should such a resistance arise. Agricultural biotechnology is in its infancy, and the available technology continues to improve rapidly. More crops can be transformed now, and control of gene expression is becoming better understood. As this technology improves and the number of known insecticidal proteins increases, the options for insect control will increase. Likewise, as various crops are transformed with new resistance traits, these traits will become part of the gene pool of that species and will be available for wider use in a number of varieties created through traditional breeding. Transgenes will become part of the genetic diversity of many crops as important insect control options.

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Biotechnology of Weed Control

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I. WEEDS: A MAJOR PROBLEM IN AGRICULTURE

The farmer's greatest economic input in producing crops is weed control; with inputs in time and capital from plowing and mechanical or manual cultivation along with herbicide, as well as spraying costs. Still, in various places, the farmers still sustain 5–100% yield loss to weeds despite these outlays. The losses from weeds are highest in the developing world where there is a greatest likelihood of inefficient or ineffective technologies, as well as many technologies being presently out of reach of peasant farmers.

Many of the problems have been brought on by the present farming technologies: rare fallowing, much monoculture, and highly intensive agriculture, with high-yield index crops having wimpy competitiveness with weeds. The results of modern crop breeding require excellent weed control. Misguided short-term economics favor monoculture. Herbicides are a cost-effective, soil-conserving adjunct to crop production. Still, it is a bit much to expect herbicides to select between crops and related weeds. Thus, weeds related to crops that were in balance, but naturally resistant to selective herbicides took over in monoculture; legume weeds in soybean; *Abutilon* in cotton; bromes and barleys in wheat. Then there is the problem of resistance; a plethora of new cases have been appearing [1]. There have been the expected cases to the single-target herbicides that have a natural mutation frequency to resistance of 1:1 million, such as the herbicides inhibiting acetolactate synthase (ALS). There has been slightly slower evolution to other single-target nuclear-inherited resistances [e.g., acetylCoA carboxylase (ACCase) inhibitors] and slower yet to chloroplast genome-inherited resistance to photo-system II (PSII) inhibitors. It is especially worrisome that the first examples of resistances have recently evolved in weeds to many groups of herbicides that were thought to be at low risk. This has been occurring in wheat and rice crops around the world—our major sources of food.

Currently, there are three main ways that biotechnology might be of assistance: (1) indirectly, by increasing crops' competitiveness with weeds using exogenous genes; (2) by genetically engineering crops with genes conferring resistance to herbicides; (3) by biotechnologically cultivating and possibly modifying biocontrol organisms. The needs, concepts, and limited successes using these approaches is discussed in the following.

As the methods by which genes are isolated, modified, constructed, and transformed into plants have been discussed in other chapters, this chapter will ignore the sometimes breathtaking wizardry of doing so, and will start from the needs for the biotechnologically derived herbicide-resistant crops (HRC) in specific cropping-weed situations, and how the specific HRCs meet those needs or are contraindicated.

Biotechnology is also used to produce some herbicides (e.g., bilanaphos) and is commonly used to produce chiral precursors for the manufacture of many others, for which only certain analogues are active.

II. BIOTECHNOLOGICAL APPROACHES TO THE PROBLEM

A. Indirect Biotechnological Approaches to Weed Control

Many different approaches have been suggested to increase the competitiveness of crops with weeds. They include engineering genes into the crops to produce "natural" allelochemicals, enhanced nutrient uptake, and increased growth rate or habit.

Allelochemicals are known to be produced by some species and do assist in competition. One has "but" to find the gene(s) for their overproduction and introduce them into crops. This approach has been tested successfully to help the crops compete with other insect and microbial pests, usually by overproducing one or more phytoalexin, or pathogenesis-related (PR) or other proteins. The situation with enzymes against weeds is possibly more complex; it is hard to envisage proteins diffusing from crop to weed at the necessary speed, without being first degraded. Most antifungal phytoalexins require a multitude of synthetic steps from primary pathways to the product (save one known case). It might also be necessary to ascertain how the natural producer remains immune to its toxic emanations—a few more genes. Just because allelochemicals are natural does not mean that they are safe. Strychnine is a plant-produced allelochemical that would not be appropriate. Honesty (if not yet law) would require toxicological and residue studies for allelochemicals similar to those for manufactured pesticides, unless there has been a long history of prior use without seemingly ill effects. Indeed, nicotine, a plant-produced allelochemical insecticide, often used in organic farming, has twice the acute toxicity than the most mammalian-toxic herbicide: paraquat.

Nutrient uptake is a fascinating way to increase competitiveness. Many plant species that were far more innocuous 50 years ago have replaced preexisting weed species. These weeds were far more efficient at utilizing nitrogen, phosphorus, and other fertilizers than the weeds they replaced. Fertilizer use has expanded over the 50 years, along with better drainage and the first selective herbicides [2]. If genes could be introduced into crops that would facilitate better fertilizer utilization, especially of nitrogen-, phosphorus-, and iron-containing compounds, the crops could better compete. Microbial genes are known that have products that rapidly sequester mineral nutrients. Some act to support competition among microbial species; they need "just" to be engineered into crops with root-specific promoters.

Increasing growth rate can add to competitiveness of crops just as bovine growth hormone does with animals. Still, increasing overall height, needed to compete with tall weeds, may require lowering the yield index. The best concept may be to strive for varieties having different leaf morphologies and angles, that will quickly shade out weeds. Mixed morphologies will have to work exceedingly well to convince farmers and buyers that such lines represent "pure" varieties. Much of the changes in growth patterns suggested in the foregoing could be achieved by standard breeding.

If we are to introduce biocontrol agents to control weeds, it will be advisable to add genes for pathogen or arthropod resistance to enhance crop selectivity of the biocontrol agent.

Many of these approaches are long-term, as they require the engineering of more than one gene, often with modified control sequences. Even more problematic is a basic philosophical question: How well will they work? As a part of breeding for increasing yields, we have bred for tastier, less poisonous crops, with higher yield indices. There was also an energy cost to defending; and the cost was in yield. This has been described as the evolutionary “dilemma” of plants “to grow or defend” [3]. Pesticides and fertilizer use can be far more cost-effective in terms of food production than the use of such genes. Environmental constraints on pesticides play in favor of such strategies—but should not if they mean famine. Additionally, the foregoing are generalizations, and there are probably many niches where they are wrong. This has already been shown with pathogen control. Mixed cultivation of barley reduces mildew to the point of not needing fungicides without reducing yield [4]. The engineering of multiple fungal control mechanisms synergistically reduced both infection and the need for high expression of each gene separately [5, 6].

B. Direct Approaches: Adding Genes for Herbicide Resistance

This is a widely reviewed area, with reviews exceeding the number of papers with hard data, including recent ones by this author [7–10], and is the subject of a recent book [11]. Weed control is in a state of flux, with many new problems to be assessed for possible potential uses or needs for herbicide-resistant crops.

1. Many herbicides are being removed from the market, and fewer new ones are being released. HRC are expanding the use range of the remaining compounds by conferring new selectivities.
2. There are legislative demands, especially in Europe to lower the amounts of herbicides applied to fields. There are HRCs that will require less herbicide to adequately control weeds. However, the quantity may not actually be better, as discussed later.
3. As subsidies drop, it may no longer be economical to control weeds with the more expensive herbicides. Replacements can be found by developing HRCs for the lowercost herbicides.
4. Resistance has become rampant to triazine as well as to ALS- and ACCase-inhibiting herbicides already infesting an estimated 6 million hectares (ha). These need replacements, but too often new HRCs are resistant to these chemicals.
5. HRCs may be used to overcome the multiple resistance problems evolving in grass weeds of wheat.
6. HRCs can be used to control weeds that never were problems and never before were controlled by selective herbicides because they had been “held down” by more competitive weeds or by agronomic procedures. In addition to the previous examples, there are perennial weeds, such as the nutsedges (*Cyperus* spp.) that proliferate in conservation (minimum) tillage systems. HRCs help overcome such problems.
7. There are weed problems, especially in developing countries, that were never adequately addressed by industry, and there are no readily usable selective herbicides for their control. This is exemplified by the parasitic broomrapes and witchweeds. HRCs can alleviate these problems.

Examples are presented of both how HRCs assisted in meeting these needs, and also of how HRCs can become a quick, perhaps ephemeral, “chemical fix” for the problems, meeting shortterm, but not the longer-term, needs of agriculture. Many of the problems have come

from an overreliance on chemistry to the point of an addictive, chemical dependency, to the exclusion of other methods of weed control; cultivation, crop rotation, as well as an overdependence on some herbicides to the exclusion of others (i.e., the lack of herbicide rotation).

HRCs can meet critical needs of agriculture (i.e., food production to sustain humanity). Herbicides should not be used as a sole chemical fix to the exclusion of time-proved alternative means of weed control. HRCs should be a part of integrated weed management systems for sane management of our agricultural ecosystems. Indeed, although some HRCs can mitigate or correct past agroecosystem management mistakes, others can exacerbate them, precluding generalizations. For example, (discussed at length later), the HRC imidazolinone-resistant maize may lead to greater resistance problems in maize-soybean rotations, when both crops receive ALS inhibitors, but are excellent at controlling parasitic witchweeds underground, before they do damage. Thus, each HRC must be evaluated in the management system where it is to be used, even when we are discussing the same gene or crop. In many cases resistance management strategies will be needed.

C. Source of Genes

In discussing the safety of genetically engineered organisms, it is considered scientifically unsound to discuss their origin; only the product should be important. With HRCs we can make predictions on whether weeds will evolve resistance to the herbicide used with the HRC, based on the source and the mode of action of the gene product. Some of these genes and their products are summarized in [Table 1](#).

There is good reason to be wary of genes coding for target-site resistances; that is, resistances for which the enzymatic target of the herbicide has been modified. Most of these have come from higher plant sources in which the natural high-mutation frequency to resistance is known (ALS and ACCase). Genes from lower organisms mutated at target sites seem to have fared somewhat better. No mutant phytoene desaturase (giving rise to resistance to norflurazon and related herbicides) or dehydropteroate synthase (resistance to asulam) have been found by selection pressure on higher plants, only microorganisms. HRCs have been generated by using lower organisms' mutant genes [34–44].

Conversely, metabolic resistances, in which the gene product that metabolizes the herbicide comes from lower organisms hold the most promise to a longer use life: HRCs resistant to glufosinate [13–25], 2,4-D [36–38], phenmedipham [45], dalapon [41], and bromoxynil [39–40]. Still, if the gene conferring metabolic resistance comes from a plant, and the herbicide is rare, it is doubted how many other plants can mimic this enzyme. An example—not followed through to fruition—is a pyridate-specific *N*-glucosyltransferase [50]. As most instances of herbicide selectivity in crops are based on the crops' ability to degrade herbicides, it is surprising that more higher plant genes have not been isolated.

III. CASE HISTORIES: PROGRESS AND RETROGRESS WITH HRCs

Instead of looking at genes and products, HRC will be analyzed on the basis of the genes used, their need, and the presumed lifetime of the HRC until weeds evolve resistance.

A. Maize-Soybeans: A Potential Delusion

The major herbicides used in maize— atrazine (and other triazines) and alachlor (or metolachlor)—are under severe pressures, and their use has been banned or curtailed in many

Table 1 Genes, Their Sources and Targets, Conferring Crop Resistance to Herbicides

Gene name	Resistance to	Source of structural gene	Mode of resistance conferred	Crops resistant	Ref.	Likelihood of resistance problems ^a
<i>aroA</i> <i>bar, pat</i>	Glyphosate Glufosinate	Plant mutation Bacterium	Target Site Metabolic	Soybean, maize, cotton, rape Tomato, sugarbeet, wheat, rape, rice, potato, peanut, poplar, alfalfa, <i>Atropa</i> , maize, turf	12 13–25	Moderate Very low
<i>csr1, ahas3r</i>	ALS ^b inhibitors	Plant mutation	Target site	Maize, tobacco, flax, rape, sugarbeet, trefoil	26–33	Very high
<i>sulI</i>	Asulam ACCase inhibitors	Bacterium Tissue culture mutations	Target site Target site	Tobacco Maize	34 35	Moderate High
<i>tfDA</i>	2,4-D	Bacterium	Metabolic	Cotton, tobacco	36–38	Very low
<i>bxn</i>	Bromoxynil	Bacterium	Metabolic	Cotton	39–40	Moderate
<i>deh1</i>	Dalapon Isoxaben Dichlobenil	Bacterium Plant Plant	Metabolic Unknown Unknown	Tobacco None yet None yet	41 42 43	Very low Unclear Unclear
<i>crtI</i>	Pyridazinones ^c	Bacterium	Target	Tobacco	44	Low
<i>pcd</i>	Phenmedipham	Bacterium	Metabolic	Tobacco	45	Moderate
<i>psbA</i>	Atrazine	Plants	Target site	Potato, rape, tobacco	46–49	Moderately high

^aSources: The likelihood is based on the author's intuitive integration of the use history of the herbicide, resistance history, mode of resistance, on such.

^bImidazolinone, sulfonylurea, and triazolopyrimidine herbicides. Not all mutations in the *csr/ahas* gene confer resistance to all ALS inhibitors.

^cAs well as other phytoene desaturase inhibitors with other structures.

countries for purported environmental or toxicological reasons [9]. Additionally, an estimated 1 million ha or more of maize fields in eastern Europe (mainly Hungary) and 30% of Wisconsin maize in the United States are infested with triazine-resistant weeds. Where triazine resistance is rampant, there is a need for a second, postemergence herbicide to suppress the (usually one) uncontrolled species. This opens a market for single herbicides that will replace the triazines as a (predominantly) broadleaf weed killer.

Two groups of herbicides are actively competing to replace the triazines and chloracetamides: the inhibitors of ALS (sulfonylureas, imidazolinones, triazolopyrimidines, and such), and the inhibitors of ACCase, the -fops (e.g., diclofop) and -dims (e.g., sethoxydim). These groups are rather prone to the evolution of resistance. The ease in achieving resistant crops through cell culture, pollen selection, or finding a mutant gene in *Arabidopsis* should have warned researchers that resistance could evolve in the field with equal ease. The same genes should appear at the same high frequencies in weeds. Conversely, there were agronomic reasons to want -fop/-dim resistance in maize. *Sorghum halepense* (shattercane) is a major weed in maize for which there are no selective herbicides, and -fops and -dims would be good, but for how long? *S.halepense* has already evolved resistance to these herbicides in fields of other crops treated with them [51].

The pesticide industry is intent on having an ALS or ACCase inhibitor for each crop in a maize rotation; excellent ones were available for soybeans, the major crop in rotation with maize. Fewer were available for maize, until biotechnology intervened. Four companies are marketing imidazolinone-resistant maize, and the same or related ALS inhibitors can be used in both crops. Still, from both the points of view of cost and the need to use less chemical, the use of the new imidazolinone-resistant maize seems very advantageous. Imidazolinones control *S.halepense* and *Cyperus* spp. (nutesedges) better than the conventional regimens, but the conventional herbicide regimen is thought to be better for controlling *Cirsium arvensis* (Canada thistle) and *Agropyron repens* (quackgrass) [52].

The demands to use less pesticides, when based on quantity of chemical, are specious and misleading. The demands should be based on biological activity and not on grams versus kilograms per hectare. Atrazine and alachlor are both herbicides with long biological lives in the ecosystem, but so are the presently available imidazolinones and many of other ALS inhibitors. Although comparable data on biological life are not available, one of the reasons stated for developing imidazolinone-resistant maize is that there is often remaining phytotoxicity to the maize from the same imidazolinone used in soybeans the year before. Thus, there seems to be little biological difference between the imidazolinone and atrazine despite the differences in actual amount used. The relatively long residual activity of atrazine is often shortened by using less, or by mixing with shorter-lived cyanazine, but there is no comparable short-lived imidazolinone yet available. There are shorter-lived sulfonylurea type ALS inhibitors becoming available that may well be used to replace the imidazolinones. At least some of the imidazolinone-resistant maizes being marketed are also cross-resistant to sulfonylureas. In the United States, imidazolinone-resistant *Xanthium* (cocklebur) has already been found in monoculture soybean fields continuously treated with imazaquin [53]. The much larger areas in maizesoybean rotations have heretofore not seen continuous ALS inhibitors. Mixtures are proposed to prevent resistance problems; the mixing partner may control volunteer imidazolinone-resistant maize in soybeans, and imidazolinone-resistant soybeans in maize. They will have little effect in preventing evolution of resistance in weeds; the mixing partners do not cover the same weed spectra nor do they have the same persistence [54]. For many weeds, it is as if the mixing partner is not there.

Biotechnology and herbicide abandonment are depleting, instead of broadening, the number of target sites for herbicide action for use in maize. The switch from multisite-affecting

herbicides, such as alachlor, and a herbicide with low frequency of resistance, such as atrazine, to single-site, high-resistance frequency herbicides to be used without meaningful rotations or mixtures is ominous. Despite being used even more than atrazine, no weed has been reported to evolve alachlor resistance in maize. In 30 years of use of atrazine alone in monoculture maize, there are more than 6 million ha of resistant weeds. Resistance has been appearing faster and more extensively in weeds with the ALS and ACCase inhibitors than they had with the triazine herbicides. Atrazine resistance was delayed where mixtures and rotations were used; the same quality of mixing partners are not available for the new compounds. Biotechnology should be broadening, not narrowing the spectrum of available herbicide target sites. Still, there is the possibility that an imidazolinone-resistant maize using an imidazolinone herbicide, used in rotation with regular maize using the conventional regimen, especially if used in rotation with other crops, will delay triazine and ALS resistances where they have not yet evolved. The use of imidazolinone-resistant maize where there are already triazine-resistant weeds raises the specter of evolution of multiply resistant weeds (i.e., resistant to both triazines and ALS inhibitors). This could occur very quickly as at the sites where there are triazine-resistant weeds, the weed stands are dense, and the seed banks full of weed seed. Where there are more seeds to choose from, and the herbicide exerts high selection pressure (as do the imidazolinones), and the frequency of resistance is great, resistant populations evolve more quickly than where weed seeds are sparse. Thus, it is strongly advisable to use other means to deplete triazine-resistant weed seed banks *before* using an imidazolinone.

There is a bright spot for the use of imidazolinone-resistant maize. It was predicted [55] and recently found [56, 57] that it is possible to control witchweeds (*Striga* spp.) with imidazolinones in these HR maizes (see discussion in Sec. III.E on parasitic weeds). Still, this may only be a stop-gap measure until a better system is found. Single *Striga* plants produce tens of thousands of seeds so there is good reason to believe that they will rapidly evolve resistance to ALS-inhibiting herbicides. A novel strategy is being tested, based on the ability of target-site—resistant HRCs to withstand ultrahigh doses of herbicide, unlike most crops with metabolic resistance [57]. Instead of treating the whole field with herbicide, seed was treated with a high (local) level of a systemic imidazolinone still giving excellent control of *Striga* [57]. Models have indicated that if only 95–98% of the maize seed are treated, the *Striga* developing on the remainder will dilute any resistance selected, delaying the evolution of predominantly resistant populations. As *Striga* typically halves yields (or worse) the loss of 2–5% of maize plants to *Striga* infection will be bearable. Despite the local high rate of herbicide used, the total amount is low. It might be wise to impose such a resistance management scheme; resistance would evolve (according to the model) in 2–4 years without preemptive resistance management strategies in use [148].

B. Cotton: Needs Being Effectively Met

Cotton growers ignore resistance problems—and do not learn. The industry has been completely wiped out in some countries owing to evolution of insecticide resistance in monoculture. Still, much cotton is grown in monoculture. Resistance problems have evolved, as well as problems caused by weed spectra shifts to weeds that had never been effectively controlled. All weed control in US cotton is with preemergence herbicides, with no ability to later correct for the wrong choice of herbicide with a truly selective postemergence herbicide. There was always poor preemergence control of some weeds such as *Abutilon*. Bromoxynil—as well as glyphosate-resistant cotton varieties have been engineered, with the former released in 1995. Bromoxynil provides postemergence control of problematic annual broadleaf species and glyphosate controls most species including perennials, both cost-effectively. Their use will allow growers to skip

preemergence herbicides when they feel that weed infestations will be below economic thresholds, or allow them to use less expensive equipment, as directed sprays will not be needed postemergence owing to the selectivity afforded by the HRCs [58].

C. Sugar Beets

Sugar beets are widely cultivated in Europe in a heavily subsidized manner, with the cost of production usually much higher than world sugar prices. Sugar beets are not the most competitive of crops, resulting in the need for chemical weed control, as at least within the rows, mechanical or manual weed control is not feasible. Conventional chemical weed control regimens consist of one preemergence and three or four postemergence herbicide treatments using rather expensive herbicides. With 2 million ha in production in the European Union alone, the 8000 tons of herbicide used on sugar beets is a large environmental load. Despite all this herbicide use, weed control is hardly complete, as the conventional herbicide regimen used still gives inadequate control of *Solanum*, *Polygonum*, and *Mercurialis* spp. [52]. The possible use of transgenic glyphosate- [12, 52] and glufosinate- [13, 52] resistant sugar beets can clearly alleviate some of these problems. Less total herbicide is needed, with fewer treatments, at a much lower total cost, while harder to control species are better handled by these herbicides than by the conventional ones. The use of the transgenic crop can also allow control of volunteer forage beets as well as volunteers from older, lower-yielding sugar beet varieties that plague sugar beet growers. Conversely, because of these volunteers, it will be obligatory to use these herbicides in and around the fields to prevent genetic transfer of the resistance genes by hybridization to the volunteer species, at least in seed production areas where the beets are allowed to flower [59].

Sugar beets are among the species most susceptible to the sulfonylurea herbicide chlorsulfuron. Sugar beets are 1000 times more susceptible than wheat and 100 times more susceptible than most weed species [60]. This led to severe carryover problems for sugar beets, as chlorsulfuron tends to persist for exceedingly long times. ALS-level-resistant sugar beets were developed by tissue culture selection [28]. How and whether these will be commercialized, whether for general use or just to be used after ALS-inhibiting herbicides on other crops, is an open question.

D. Worldwide Needs in Wheat

1. Overcoming and Preventing Herbicide Resistances

2,4-D and MCPA were the prime herbicides in wheat for more than 40 years, with a large degree of effectiveness, except for controlling grass weeds. Diclofop-methyl, trifluralin, chlorotoluron, and chlorsulfuron, herbicides affecting different sites of action, were excellent at controlling some of these grass weeds, and chlorsulfuron controls broad-leaf weeds as well. The widespread use of these herbicides hastened the evolution of resistances, often with cross-resistances to all other wheat-selective herbicides [61–65]. The potential needs for HR wheat were first noted 9 years ago [61]. Now the needs are no longer “potential”; the situation with herbicide resistance covers millions of hectares of wheat lands in Australia alone [64]. There is a potential for this situation to worsen. Additionally, weeds such as *Bromus* spp. and wild barley species that were never controlled by selective herbicides are gaining importance in areas where the better grass-selective herbicides are still controlling the competition. All metabolically selective herbicides for which there is published information, are degraded in wheat by monooxygenase(s) [61]. Thus, it is easy for grasses such as *Bromus* spp. to possess similar monooxygenases in advance, or to evolve them, as an evolutionary biochemical mimicry,

as has been proposed as a mechanism used by many grass species [61]. The needs for HR wheat can only be fully understood after the extent of the resistance problems is realized. Thus, resistance to wheat-selective herbicides is described in the following section.

a. *Resistance to Broadleaf Herbicides in Wheat.* 2,4-D and related herbicides, such as MCPA and dicamba, have been the standard for broadleaf control in wheat for 50 years. They have low-selection pressure, both because of imperfect kill and because weeds in many ecosystems can germinate after they have dissipated, diluting resistance [66]. Still, monoculture with 2,4-D in a cool climate with longer 2,4-D persistence and less late germination had its toll. 2,4-D (and MCPA)-resistant populations of *Sinapis arvensis* appeared in limited areas in western Canada [67] and has not spread. This resistance could not be traced to adsorption, translocation, or metabolism, and there is evidence that it may be due to a modified auxinbinding site, having low affinity for phenoxy herbicides [68].

b. *Resistance to ALS Inhibitors.* ALS inhibitors replaced much of the 2,4-D, as they control most broad-leaf weed species and some grass weeds. The first cases of resistance quickly appeared due to the severe selection pressure exerted by the highly persistent chlorsulfuron [62]. The switch to shorter-lived sulfonylureas has not changed the trend where chlorsulfuron had been used [62]. The target-site resistances occurred because of mutation at more than one site on the ALS gene [69]. Not all weeds in wheat have evolved just target-site resistance to sulfonylureas. *Lolium rigidum*, with its ubiquitous resistances to selective herbicides has evolved different methods, in different areas of Australia [64,65].

c. *Resistance to Dinitroanilines.* Trifluralin is widely used in wheat and wheat—rape rotations in western Canada, and resistance has evolved in *Setaria viridis* at many sites [70]. Both the single dominant gene controlling resistance [71] and the far-reaching cross-resistances to other herbicides that act on microtubule formation, strongly suggest a target-site resistance. These include pendimethalin, also used in wheat.

Alopecurus myosuroides and *Lolium rigidum* have evolved cross-resistances to particular dinitroaniline herbicides under the selection pressure of other wheat-selective herbicides as described earlier [63,64].

d. *Triallate Resistance in Wild Oats.* Triallate is a thiocarbamate widely used to control wild oats (*Avena* spp.) in small grains. Thiocarbamates are thought to affect many targets, and thus were considered not to be resistance prone. Nearly 20 years of continuous use yielded their due. Nearly half the nonrandom samples of wild oats from 34 wheat fields in British Columbia were resistant to field rates of triallate, with cross-resistance to difenzoquat, another widely used wild oat herbicide used in wheat [72]. Difenzoquat has a different (but unknown) site(s) of action based on symptomology. Triallate-resistant wild oat populations also evolved throughout an isolated 1200-km² area in Montana dedicated to cultivation of malting barley [73]. The rapid concurrent distribution of resistance may have been due to contaminated seed, as all seed came from the same supplier. These populations also have cross-resistance to difenzoquat.

There are only two other groups of metabolically selective herbicides for wild oats, one of the most pernicious grass weeds of wheat: various inhibitors of ACCase and isoproturon (which provides only marginal control). They are discussed next.

e. *Resistance to ACCase Inhibitors.* Some of these herbicides are degraded by wheat, allowing selective grass weed control. Their use is widespread, especially where more than one grass weed is problematic. Resistance to these herbicides has become almost as widespread as their use, especially in Australia (mainly in *Lolium rigidum* but also *Avena* spp.) [64]. More than

3000 wheat farms are infested with diclofop-methyl-resistant weeds that cannot be controlled by any wheat-selective herbicide. The cross-resistance in *Lolium rigidum* to all other wheat selective herbicides suggests that the weed evolved a biochemical mechanism to mimic wheat. Initially, researchers could not find any diclofop-resistant biotypes with an altered target site, and they were killed by other ACCase inhibitors that are toxic to wheat. The resistant biotypes rapidly degrade other herbicides that they had never previously encountered [64,65], yet they are only slightly more efficient than the wild-type at degrading diclofopmethyl. More recently, a *L.rigidum* biotype was found with resistance to sethoxydim (which is not selective for wheat) having an altered ACCase.

Target-site resistances seem to have evolved (based at least on cross-resistances to many-fops and -dims, including those toxic to wheat) in *Setaria* spp. in the Canadian western plains and the US great plains, *Sorghum halepense* in the United States, *Eleusine indica* in Scotland, *Lolium multiflorum* in the US plains, and *Alopecurus myosuroides* in England. Different *Avena* spp. have evolved resistance to diclofop-methyl use in the United States, Canada, and Australia. The US and Australian strains had different cross-resistances among -fops and -dims, no target-site differences could be found among some Canadian types, and others have not yet been characterized for mode [see 9,67 for citations].

The inhibitors of ACCase are the most widely used herbicides in the developed world for grass control in wheat. Most areas now have their first cases of resistance in problem weeds.

f. Isoproturon Resistance: A New Problem of the Developing World. “Green revolution” dwarf wheat varieties have transformed China and India into the second and third largest producers of wheat in the world. These varieties with their high-yield indices (much grain, little straw) tripled yields, and rendered it economical to introduce fertilizers and irrigation. The self-sufficiency contributed extensively to geopolitical stability. These dwarf wheat varieties cannot compete with grass weeds, and herbicides had to be introduced. India settled on one herbicide, isoproturon, which is now used on about half the wheat in Haryana and Punjab. China introduced these wheats, and then the same herbicides, a few years after India. Simple calculations, based on past experience allowed the prediction of resistance problems [74].

Resistance to isoproturon or its analogue chlorotoluron has been well-documented in England, Germany, Israel, and Spain [63]. The predominant resistant strains have cross-resistance to all wheat-selective herbicides [63]. The first inklings of resistance appeared in India during the 19910–1992 growing season in *Phalaris minor*, the major grass weed in Indian wheat. In 1992–1993, field rates no longer controlled *Phalaris* and a second application was often made, without effect, and many fields had to be cut for fodder [149]. The problem is now quite severe in two districts (0.5 million ha wheat). Control was near zero at seven locations tested by the researchers in Haryana and in considerable areas in Punjab as well [74].

2. Meeting the Needs for New Selective Herbicides in Wheat

Metabolically-selective herbicides, especially those controlling grass weeds, do not have a great future. 2,4-D may last a long while for broadleaf control, unless the sulfonylurea-2,4-D mixtures exert strong selection pressure for a joint metabolic resistance, or if 2,4-D is removed from the market for purported environmental reasons. The only commercial selective herbicide that does not have metabolic resistance is the thiocarbamate prosulfocarb that has placement selectivity in wheat [75]. It has not been reported whether triallate (thiocarbamate)-resistant weeds have cross-resistance to prosulfocarb.

The best herbicide answer to natural and evolved resistance is genetically engineered wheat [61]. The two best already available genes are those for glyphosate and glufosinate resistance. Neither seems to be highly prone to evolved resistance, and when resistance does evolve, it is

probably due to an overproduction of the target enzymes, and the progeny would be very unfit. Both herbicides have low persistence in the environment, and low mammalian toxicity. Still, plants do possess pathways to metabolize glyphosate [76] and glufosinate [77] and evolution of enhanced rates of such pathways might lead to resistance. The efficiency of the glufosinateresistance gene may well have been enhanced by generating a "synthetic" gene for crop resistance in which the preferred codon usage was changed from that of the actinomycete source of the gene, to typical plant codon usage [14].

A gene conferring resistance to the grass-controlling herbicide dalapon has recently been isolated from a pseudomonad and was eventually used to transform tobacco plants [41]. Even though exceedingly high levels of this herbicide must be used, it is relatively inexpensive and is rapidly degraded in the environment, and this gene is being considered for use in wheat. Dalapon does not control broad-leaf weeds, whereas glyphosate and glufosinate control most weed species, which is a disadvantage of dalapon.

The main problem in achieving herbicide-resistant wheat—the inability to transform wheat—has recently been overcome by quite a number of groups using a variety of transformation techniques [71,22]; but this was performed with glufosinate resistance as the selectable marker, not because the researchers perceived the needs. Thus, it finally appears that the genes are available to protect wheat from its enemy weeds (with the help of herbicides), and the technologies are ready to perform the task. The previous sections show how great the needs can be. Present stocks of wheat in the world hover at 1 month's supply. If the transgenic wheat varieties are not readily available in 5-10 years, the oversupply will disappear owing to resistant weeds. Finding suitable gene constructs, genetic-engineering, testing, multiplying, and registering varieties take time, and at the rate resistance is spreading, usually without quarantine and containment to dampen too fast a spread, little time is left.

E. The Needs and Successes in Controlling Parasitic Weeds

Many crops are attacked by higher plant weeds that attach themselves to the growing crop. The parasites remove photosynthate, minerals, and water from the host crop. The most common parasites on field crops are dodders (*Cuscuta* spp.), broomrapes (*Orobanche* spp.) [78], and witchweeds (*Striga* spp.), which devastate among them, most field crops. The areas infested are vast and expanding, especially in tropical and subtropical countries. For example, a survey of 180,000 km² in Nigeria found that 70% of fields were infested with witchweed seeds [79]. In the seven agroecological zones of sub-Saharan Africa, witchweed species are listed as the worst pests affecting agriculture (including insects, diseases, or other) in all except the mountainous and forest regions [80]. Agriculture in developing countries has learned to use dangerous insecticides, but herbicides to a far lesser extent. More than 60% of Third World farmers' time is spent in weeding. The weeds have usually irreversibly depressed yields by the time weeding is performed, and the parasitic weeds cannot be removed. Witchweeds infest the grain crops of more than 100 million people in sub-Saharan Africa and Asia, reducing yields by 50%, and by more in drought years. Broomrapes devastate the legume and vegetable crops in much of northern Africa and elsewhere around the Mediterranean, severely limiting the local production of protein crops for the diet of the inhabitants [78]. Over 30% of Egypt's broadbean-cultivating areas had to be removed from production during a 10-year period because of broomrape infestations [81]. The broomrapes and witchweeds presently seem to have a greater deleterious effect on the health and welfare in these Third World areas than AIDS, when it is expected to peak. Hand and mechanical weeding of other species does not remove attached parasites, and at best, reduces seed set. The lack of rotation or fallowing supports the parasites, especially with the lack of selective herbicides to control them. Why

parasitic weeds are not high on the list of the "worlds worst weeds" [82] is unclear. Crop yields could be doubled without added fertilizer or irrigation if these weeds could be effectively controlled.

There is some marginal natural resistance to herbicides available in some crops, but it is often only phenotypically expressed at specific stages of growth [e.g., 78,83]. These host crops of the parasites are clearly not sufficiently resistant to herbicides for widespread field use without yield loss, especially where uncalibrated backpack sprayers are used, and there is a possibility of underdose with lack of parasitic weed control, or overdose with ensuing crop damage. At times, with some metabolically resistant crops, frequent retreatments are necessary. For example, tomatoes are resistant to the sulfonylurea herbicide rimsulfuron, owing to their rapid metabolism of the herbicide. This herbicide is also rapidly degraded in the soil. Rimsulfuron effectively controls broomrape for only a few weeks, so that 25 g/ha herbicide must be reapplied at 10-day intervals. This action seems to be directly through the soil, as foliar application alone (with a soil barrier) was ineffective [84].

Low rates of glyphosate have often been tried for broomrape control, especially in broadbeans and vegetables [78]. Low rates of glyphosate also suppress the production of induced phytoalexins, that defend against fungal attack [85], and glyphosate-treated broadbeans are often infected with diseases such as chocolate spot. It was proposed that target-site resistances introduced into crops could allow selective control of these weeds [78]. Metabolic resistance to foliar contact herbicides should be of little use to control underground parasitic weeds, because the herbicides are degraded before reaching the parasite. Indeed, this hypothesis was tested using transgenic tomato plants with metabolic resistance to glufosinate [86]. There was no control of *Orobanche* with foliar treatments, even though it is known that glufosinate can be translocated and that it is toxic to *Orobanche* [87]. What is quickly metabolized cannot be translocated to the roots to control the parasites.

The second type of case, where the target enzymes affected by the herbicides are modified in a manner precluding herbicide binding, are of primary interest for controlling parasites. The target-site mutation must be in such a position that the target enzyme can still carry out its normal function. With target-site resistances, the active herbicides remain present in the plant and are translocated to the roots, to be lethally bioaccumulated by the root parasitic *Striga* and *Orobanche* spp., or translocated to stems in *Cuscuta* spp. The herbicide must be one that can affect the parasite. The most common target-site resistance available is target-site triazine resistance, but triazines affect only photosynthesis, which is lacking in the parasites when attached to their crop hosts.

As the parasite species are all dicotyledonous, they are naturally resistant to ACCase inhibitors used for HR graminaceous crops, so they are of no use to use.

1. Successes with Target-Site Inhibitors

Positive preliminary results were achieved with HRCs with three target-site resistances: (1) tobacco plants with a gene encoding a modified ALS gene; (2) oilseed rape plants containing a gene for a modified enolphosphate-shikimate phosphate (EPSP) synthase conferring glyphosate resistance; (3) tobacco plants with a gene encoding a modified dihydropteroate synthase, encoding resistance to asulam. A single foliar application of chlorsulfuron or glyphosate, respectively, to the crops gave full broomrape control without affecting the crops [87]. A foliar application of asulam afforded 70% broomrape reduction [87].

The use of imidazolinone herbicides with the resistant maize was predicted to affect control of *Striga* species [55], with success discussed earlier. In the long-term, the glyphosate-resistant maize scheduled for release in 1997, may well allow longer-term control, until resistance

evolves, than the resistance to the ALS inhibitors. If the ALS inhibitors are used in seed dressings, they will not control the weeds between the maize rows. Glyphosate can be used to control the parasites as well as other weed problems, as it is a total vegetation herbicide. Neither glyphosate nor ALS resistance is an immediate solution, there is much work to be done before such varieties can be released. The maize varieties cultivated in Africa are resistant to diseases that devastate the HR varieties in the United States [57]. Thus, it will be necessary to cross and backcross resistance into local inbreds (hybrids are cultivated on millions of hectares in Africa) as well as local landrace varieties. So far, only the imidazolinone-resistant maize has been made available to African researchers; there has been demurral by the manufacturer to allow testing of the glyphosate-resistant maize.

The transgenic sulfonylurea-resistant flax already available [26] might make flax an interesting trap or catch crop for controlling broomrape, beyond its present use.

a. Cellulose Biosynthesis: A Potential Site. Parasitic weeds must synthesize cellulose *de novo*; cellulose cannot be translocated from the host. Herbicides that affect cellulose might also control parasites when engineered into crops. Separate mutant *Arabidopsis* strains have been isolated that have target-site resistance to isoxaben [42] and diclobenil [43], herbicides inhibiting cellulose biosynthesis. The different mutants have no cross-resistance between the two herbicides, suggesting that different enzymes in the pathway or distant sites on the same enzyme are affected. Resistant crop plants treated with isoxaben or diclobenil might allow parasite control.

Ioxaben and diclobenil are not translocated within the crops, at least not in those crops naturally having non-target-site resistance. There is probably a requirement that the herbicide be taken up directly by the parasites from the soil. It is now known that parasites take up some organic compounds from the soil (such as the herbicide rimsulfuron, see foregoing). If the parasites do take up a cellulose biosynthesis-inhibiting herbicide from the soil, metabolic resistances in crops could be considered for parasitic weed control with those soil applied herbicides.

F. Needs in Rice: Just Appearing

Because of its high yield and value, increased rice production has gone hand in hand with increased herbicide use. A large number of herbicides were developed specifically for rice to keep the weeds to a minimum. In countries such as Japan, where constraints seem minimal, many compounds are used, with over 80% of the 2 million ha receiving more than two sprays, usually consisting of mixtures. Many herbicides are registered solely for rice. There are the beginnings of problems. For example, very safe thiocarbamates needed mainly for grass and sedge control in California were dropped because people drinking purified paddy water downstream could smell the herbicide residues even though they could not be detected instrumentally. The thiocarbamates were replaced by an ALS inhibitor that had to be dropped after 4 years owing to widespread resistance. Meanwhile, populations resistant to older, widely used herbicides, such as propanil and butachlor, have begun appearing [90]. Resistance has also evolved to 2,4-D, molinate, and quinclorac. Thus, no chemical group in use in rice seems immune to evolution of resistance or to encountering environmental problems. Much of the resistance has evolved in highly competitive *Echinochloa* spp., further exacerbating the problem [90]. If rice is engineered for resistance to herbicides, one can expect the genes to naturally transfer into wild and red rices, whenever the herbicide is not used, or into escapes. This may well be considered a constraint. The wild rices are presently not controlled by any herbicides, so the possibility of their control may well be considered an advantage, even if only for a short period. The most appropriate herbicides, considering the weeds and the long-term effects,

might be glufosinate, glyphosate, and dalapon. The last two would be in the price range developing country farmers can afford. Glufosinate resistance has already been transformed into rice.

G. Specialty Crops: Needs Not Being Met

The chemical industry is primarily interested in developing herbicides for a few major crops. Lead compounds are modified until derivatives are found that lack toxicity toward these major crops. The companies register such compounds for only minor uses when they also have a market in major crops. No major efforts are made for minor crops, and minor is considered to be most crops other than maize, soybeans, wheat, rice, and cotton. "Minor" includes high-value vegetable and tree crops (i.e., the definition is based on potential herbicide sales and not on the value of the crop).

Thus, growers often lack good herbicides for many crops. The problem becomes more acute with some machine-harvested vegetables, where weeds can jam sensitive machinery. Hand weeding is often performed instead of hand harvesting. Many minor crops, especially vegetables for which seeds command high prices, can be economically transformed to resistance by seed companies or the public sector, if the available gene constructs discussed in earlier sections are released by industry for such use. Some of these high-value crops have special weed problems, as discussed in the section on parasitic weeds. Considering the value of the crops and the losses engendered by weeds, the costs of generating these HRCs can be recouped by taxes (on income from crop or land), value to national economy, seed price, or other, even though the amount of actual herbicide sold may be negligible.

1. Forestry

It is expected that at least part of the area taken out of crop production will be dedicated to agroforestry—either for short-term multiple cuttings of trees or longer-term afforestation. The greatest weed problems in forestry are in tree nurseries and for the first few years after planting, until canopy closure (or after cutting of regenerable species until after canopy reclosure). Herbicides, often aerially applied, could be a viable alternative to impede weed growth, but only if the trees were resistant [91]. Mechanical cultivation is not economically viable for forestry, at least not in developed countries. Rapid tree growth is important for the purifying effect of trees on the environment and to make forest planting more economically attractive to those who must cover the costs. Herbicides having acceptable environmental and herbicidal characters must be found for introduction of resistance into the trees. Many acceptable candidates seem to be available. The chemical industry is not likely to perform the necessary research and development to obtain HR forest trees, because the market for herbicides would be restricted to one or two applications in new plantings and no applications for the following decade(s) until maturity. Indeed, the transformation of poplar with a gene for glufosinate resistance [15] seems to be a movement in the right direction. Poplars have also been engineered with resistance to ALS-inhibiting herbicides [27]. Because of the rare use of herbicides in forestry, there is little reason not to select for trees resistant to ALS-inhibiting herbicides. The evolution of weeds resistant to this group may be very slow in the context of forestry.

2. The Future of HRCs

There are great needs for HRCs, both in the developed and developing worlds, in specific crops and situations. Although some of those needs are being met by the industries involved, many are not; for examples, the needs of wheat and those in a variety of crops to control parasitic weeds. The reasons stated by the chemical and biotechnology industries for not

pursuing weeds are economic; no large profits from such ventures are perceived. As there would be great national economic profitability from control of grass weeds in wheat or of parasitic weeds, such work must be done by the public sector. There is often public sector willingness, but the author has witnessed examples for which there is considerable reluctance of industry to supply the gene constructs already isolated and used for such purposes. Further problems would ensue with industry when it is time to register herbicide use for such HRCs. This is despite the fact that new-use registration of already marketed herbicides is not very expensive.

There is also a tendency of industry to develop HRCs bearing resistances to already widely used herbicides, increasing the chances of evolution of herbicide resistances. This is clearly already a critical problem with the single target-site herbicides affecting ALS and ACCase. Despite the chemical industry's setting up of committees to deal with these resistance problems, they seem to have no teeth, as newer resistant crops keep coming on market despite their dubious long-term potential.

Thus, there seems to be a mismatch between the needs of agriculture for certain HRCs where there are few good alternatives, and the immediate goals of the biotechnology industries to meet these needs of the farmers. This is unfortunate, as there are many mouths to feed on this planet.

IV. BIOCONTROL OF WEEDS

There are many organisms that kill weeds. This chapter will not cover "classical" biocontrol agents: organisms that failed to move with a weed when it invaded new territory. Classical agents include imported insects and diseases, even though there is an element of biotechnology in the rearing and culturing of these organisms before their one-time release. This review will not cover manual weeding nor the use of grazing animals, even though semantically this is also biocontrol. Only microbial agents are covered that must be continually in biotechnological production.

A. Why Bioherbicides?

Despite the many herbicides available, there are still cases where there are needs to control single weed species. This is where biocontrol agents with their high specificity can be considered. HRC are also appropriate as an alternative in many such cases. These needs for biocontrol agents include the following:

1. Weeds that have evolved resistance to a still-favored herbicide. Typically, only a single weed (initially) evolves resistance under monoherbicide monoculture, and the herbicide still adequately controls all other weeds.
2. There are many examples in which an especially high rate of a herbicide is needed to control one weed species and the rest of the weed spectrum can be controlled by lower rates. If this single weed species could be controlled by nonchemical means, then lower, more economical, and less potentially polluting rates of the herbicide could be used.
3. There are often problems with single weed species, where herbicides cannot economically or easily be used. These include (1) pasture weeds, such as spurge and bracken, for which herbicides can be uneconomical; (2) urban situations with allergenic weeds, but spraying with chemicals is unacceptable for any of a variety of reasons; (3) illicit narcotic "crops" (that fit the definition of weeds as they are deleterious to humanity), which need high aerial treatment to provide a modicum of protection for the applicators from the wrath of the criminals, yet not damage nearby crops.

Table 2 Examples of Inoculum Levels of Mycoherbicides

Pathogen	Major target weeds	Level used (spores/cm ²)	Ref.
<i>Fusarium lateritium</i>	<i>Abutilon</i> and <i>Sida</i> spp.	7,500	93
<i>Colletotrichum coccodes</i> f.s.	<i>Solanum ptycanthum</i>	3,000	94
	<i>Abutilon theophrasti</i>	20,000	95
<i>C. gloeosporioides</i>	<i>Aeschynomene virginica</i>	600	96
<i>C. orbiculare</i>	<i>Xanthium orbiculare</i>	2,000	97
<i>C. dematum</i>	<i>Crotalaria spectabilis</i>	10,000	98
<i>Alternaria macrospora</i>	<i>Anoda cristata</i>	200	99
		1155 kg/ha ^b	100
<i>A. cassiae</i>	<i>Cassia obtusifolia</i>	500 ^a	101
<i>Phoma aquilina</i>	<i>Pteridium aquilinum</i>	40,000	102
<i>Cercospora rodmanii</i>	<i>Eichhornia crassipes</i>	11 kg/ha ^c	103
<i>Puccinia expansa</i>	<i>Senecio</i> spp.	500,000 ^d	104
<i>Fusarium udum</i>	<i>Crotalaria spectabilis</i>	10,000 ^a	98

^aCalculated from stated spore concentration and a typical spray volume of 115 L/ha, unless stated as "to run off" and then 1000 L/ha.

^bGranular formulation containing 10⁵ spores/g.

^cBlended mycelium.

^dPer seedling at three-leaf stage.

In all these examples biological control is appropriate because it can potentially afford selectivities unavailable cost-effectively with presently available chemicals (except with HRC). Pathogens are often specific to single-host species, and it is such pathogens that can have the necessary selectivity between crop and weed host. There are many reports about finding mycoherbicidal agents, as well as excellent books on the biocontrol of weeds [92], but commercial successes are limited. Typically, such nonclassic agents just make plants mildly ill, but do not kill them, and a truly "inundative" approach must be initiated; only with heavy inoculation are weeds sometimes controlled (Table 2).

1. Reasons for High Inoculation Levels

From an evolutionary standpoint the inundation (see Table 2) is understandable; if an organism was hyperpathogenic, both it and the weed host might have become extinct or at least the weed would be diseased enough to render it just another wild, but not weedy, species. Thus, ways must be found to circumvent the need for heavy inoculation, unless one accepts it to be a "given," as many do. Most industrial efforts have been to produce and formulate the inocula less expensively, instead of finding ways to use less.

There are two groups of reasons for the need of such gargantuan inundations of fungal material: (1) There can be inherent problems in getting the organism to the right place and keeping it alive until an adequate infection is established. There are problems in getting soil and stem pathogens to their inaccessible targets. Leaf and stem pathogens often need long dew periods (6–18 h) to germinate on and become established in the weed. Such optimal conditions do not often exist in nature, which is why many epidemics are sporadic and weather-related. (2) Despite a pathogen being "compatible" with the weed, it does have to overcome the weed's inherent defense mechanisms. This is a "quantitative" battle (i.e., a function of the amount of soldiers [inoculum] used). After the attack, the weeds initiate various defenses to

suppress the spread of invading pathogens, including pathogenicity-related (PR) proteins (chitinases and glucanases as well as other stress-related proteins)—the chitinases and glucanases degrade fungal cell walls, preventing penetration and growth in the tissue—phytoalexins, which are nonprotein, secondary metabolites of various chemistries that poison the fungus; physical barriers composed of polymers such as suberin, lignin, callose, or mixtures of some or all of these. Often papillae of callose rapidly form, blocking the advancing fungal hyphae. Such papillae can contain small amounts of lignin. Traces of lignification can severely suppress degradation of carbohydrate polymers such as callose and cellulose by fungal enzymes [105]. Different species initiate different defenses, and the levels of induced and preexisting defenses vary during weed growth and development. The outcome is that different quantities of inocula are needed at different stages of growth, a major complication to using biological control agents. Similar problems of developmental variability in susceptibility often exist with herbicides, but usually not to the same quantitative extent.

One way to render bioherbicides cost-effective is to assist them in overcoming the foregoing barriers. This can be done by adding chemicals, biologicals, or genes that will synergistically perform the task.

B. Synergizing Mycoherbicides by Formulation

Getting a biocontrol agent to the right place and keeping it there is the task of formulants. Initially, bioherbicides that attack roots were spread at rates of hundreds of kilograms per hectare together with the solid substrate on which they were cultured. Organisms for infecting weed leaves and stems were formulated in water, sometimes with some thickening agents to keep spores from settling out, some detergent to prevent clumping, and sticking agents to hold the inoculum on the leaves and stems.

The need for extended dew periods was addressed by developing formulants having an oilbased component and an emulsifier, such that water and medium would surround the propagule (spore or cells) with an oil wrapper to prevent loss of water [106]. One such invert emulsion was very efficient in lowering the inoculum level [107], but also abolished selectivity [108]. Organosilicone surfactants also show promise [109].

Several novel solid substrates, and pelleted or beaded formulations are discussed in Boyette et al. [106] for preplant or preemergence soil applications. They decrease inoculum requirements from gargantuan to very large. The biocontrol of root-attacking parasitic weeds is a special case [110]. Some success was achieved at the greenhouse level in treating transplant plugs of tomatoes with a pathogen of *Orobanche* before planting out. Presumably the pathogen continues growing along the rhizoplane, attacking the parasite as or after it attaches to roots because *Orobanche* was infected on roots that formed long after inoculation [110]. The ideal general situation would be to coat crop seeds with a weed-specific pathogen that would spread in the soil near the developing crop, and kill nearby weeds.

C. Synergizing Mycoherbicides by Suppressing Weed Defenses

Chemicals, especially herbicides, have been used, some seemingly “off the shelf,” to ascertain if they will synergize biocontrol agents [111–113]. Random testing of each putative biocontrol agent in this manner with the few hundred registered herbicides seems like a daunting task. Still, there are occasionally important recent successes—for example, the PSII-inhibiting herbicide ioxynil at sublethal levels synergized the control of bracken ferns by *Ascochyta pteridis* [114]. There are also many nonherbicidal chemicals that also might prevent the production of induced defenses in the weeds. A little forethought should clearly allow testing of only the more relevant compounds as synergists. This requires an understanding of the

defenses being used by the pathogens. This approach can work successfully, at least at the laboratory level. For example, the attack of a model legume weed *Cassia obtusifolia* (a great problem in soybeans) by *Alternaria cassiae*, a rather specific pathogen, induced the synthesis of a large number of phenolic compounds. Most did not inhibit pathogen growth, but one was clearly a phytoalexin. This compound was isolated and its structure determined to be a dihydrochromone [115]. Part of this compound was demonstrated as coming from the shikimate pathway, a pathway inhibited by glyphosate [116]. Leaves treated with a spore suspension containing sublethal doses of glyphosate produced negligible amounts of phytoalexin, and the intensity of infection increased. The number of conidia required for equal infection also dropped more than fivefold when glyphosate was used [116].

Many weeds produce callose when attacked by specific pathogens. Various chemicals inhibit callose synthase in vitro [117], but do not penetrate plant cuticles. Specially synthesized analogues that penetrate cuticles increased infectivity of a strain of *Colletotrichum coccodes* that is specific for *Abutilon* [118]. Such synergists could even be envisaged to be used to increase the host range of nonspecific pathogens, when the crop does not use callose synthesis as part of its defense mechanisms. This line of research continues to show promise, and more of these compounds are being synthesized to optimize structure.

D. Biologically Synergizing Biocontrol Agents

1. Genetic Manipulation

Organisms can be potentially modified to increase pathogenicity by transformations with genes for virulence from other species, by increasing the endogenous expression of genes, or by transfer of genes from other organisms by protoplast fusion [119,120]. Increasing virulence, especially by gene transfer, requires extreme care owing to environmental effects from inadvertently increasing the host range to other crops. Researchers are considering ways to engineer "fail-safe devices" into their pathogens. Conversely, highly virulent, not too specific organisms can be "disarmed" to allow them to be used where there is some crop selectivity. For example a widespread broad-spectrum pathogen *Sclerotinia* was mutated to both auxotrophy and to be sclerotia minus (i.e., it could reproduce only vegetatively in culture, and only when the missing nutrients were added). The mutant pathogen can still infect weeds having the missing nutrients, but "commits suicide" when the weed dies [121].

Genetic manipulation of mycoherbicides to fungicide resistance will often be called for. This would allow farmers to treat crops with a fungicide of the types, and at times that would otherwise have suppressed the biocontrol agent. There are alleles for resistance to many fungicides in most fungal populations, and with a little selection pressure in the laboratory, resistant strains can be isolated.

2. Interorganism Synergies

Just as herbicide mixtures often synergistically provide cost-effective weed control, the use of more than one organism can effect better biocontrol. How some of these work is unclear. Some show ingenuity and mimic nature (e.g., the use of arthropods to spread microorganisms) [cf. 122]

There is a possibility that scientists have overextended the meaning of Koch's postulates (that to be considered a pathogen, an organism must always be present in infected lesions, be isolatable in pure culture, cause disease when reinoculated into a healthy plant, be reisolatable) into Koch's "axiom," despite contradictions with nature. The pathogenic *Agrobacterium* disappears from crown galls. Some organisms are pathogenic only when a subsidiary,

nonpathogen is present. Many microorganisms are typically found in diseased lesions, not just the primary pathogen. Most are there for a free meal, but some may enhance the pathogenicity of the primary pathogen. This was clearly shown with a rust that infected, but did not control, a burr; a *Colletotrichum* spp. that also by itself was ineffective, enters the rust lesions, killing the weed [123]. Far more research is needed to elucidate synergisms by such facilitating organisms.

E. The Future of Biocontrol

It was discussed earlier how it should be possible to render biocontrol agents cost-effective by synergizing them to allow the use of lower inoculum levels. There are theoretically two to five orders of magnitude for improvement (see [Table 2](#)). The improvement of one order of magnitude would render many biocontrol agents cost-effective. Impermanent synergies (the use of chemicals and adjuvants) may have advantages over many of the permanent synergies (many genetic manipulations, or organism mixtures that may reproduce in concert). It may be valid to fear both the effects of changed host-specificity, and also the formation of "persistent residues" of biocontrol agents. Impermanent synergies provide weed control when applied, and the level of the organism should later dissipate, just as environmentally sound chemical herbicides dissipate.

Resistance can evolve to biocontrol agents just as it has to chemical pesticides, and resistance management strategies preventing overuse should be initiated. Resistance has already evolved to a biocontrol agent used to control a pathogen in greenhouses [124].

V. CONSTRAINTS TO BIOTECHNOLOGICAL WEED CONTROL

A vocal consortium has become organized against the commercialization of HRC as well as the use of genetically modified biocontrol agents, using a large number of reasons to prevent their dissemination [125–131]. The most vehement have referred to HRCs as a capitalist conspiracy of monopolistic seed and chemical companies [127], and have even viewed HRCs as male chauvinist as they would displace female weeder. They view HRCs as a way of increasing pesticide usage, clearly not in accordance with organic gardening. HRCs would, they say, limit crop diversity, and there is a fear of such genes moving into weeds.

The first two claims are rather trivial; just as there has been tremendous competition between herbicide manufacturers to sell their varied products, similar competition should remain rampant with HRCs. This is already becoming apparent with the competition in the marketplace for sales of the imidazolinone-resistant maize varieties.

Most of the manual weeding in 19th century Europe was performed by women; they were happy to lose this chore to mechanical equipment. The women and girls performing the weeding in developing countries will similarly be happy to lose the onerous, degrading task to HRCs and herbicides. European women have found far more rewarding pursuits to fill their time, and there is no doubt this will happen elsewhere. Literacy among women in developing countries is far below that of men; they are expected to hand weed instead of going to school.

Herbicide use is not without its dark side. Herbicides allowed cultivation of marginal lands that otherwise would not have been used to be put into food production, especially in Europe, lands that should have been left to wildlife. Minute quantities of herbicides often appeared in water, although only rarely at levels for which regulatory concern is mandated. Foodstuffs are far cleaner from herbicides than from other pesticide residues [132]. Part of these problems is due to an overreliance on a few groups of herbicides. There are at least 16 distinct groups of herbicides based on known targets or similar symptomologies of action. Farmers primarily use herbicides from only five of these groups. There are few resistance

problems from the two widely used types that do not have a known target for action, and may well have a multitude of targets: the phenoxy-type (e.g., 2,4-D) and the chloroacetamide type (e.g., alachlor) [133–135]. Resistance is common with herbicides having single biochemical targets in the weeds. HRC with resistance to lesser used, nonresistance-prone herbicides can increase the diversity of chemicals used.

A. The Morality of Using Tools

Farmers have to be able to use all weapons that will allow them to stay ahead of the weeds, to “confuse” the weeds, and to delay evolution of large weed populations. These weapons include crop rotation, tillage, breeding, herbicide rotation, finding new herbicides, *and* the use of genetic engineering. Genetic engineering is just another tool, albeit a powerful one, to assist in breeding. Similar to all tools, it can be used elegantly or destructively. Obviously, it is immoral to propose the destructive uses. The plow can be used constructively and destructively, depending on the direction it is used on a slope. Should we ban all plowing, or should we promote erosion-preventing contour plowing?

Earlier in this chapter a variety of needs for HRCs was discussed. When a need is met by transformation in one variety, local seed companies and breeders can easily perform the crosses and backcrosses required to introduce the useful resistance into indigenous varieties to prevent loss of genetic diversity. Single-gene transfers are not expensive to perform. Companies competent in biotechnology will shorten the time of transferring these genes into other varieties by marker techniques, such as restriction fragment length polymorphism (RFLP). At worse, the crosses and backcrosses into local varieties could take about 4 years with low labor costs. Because just plant-specific pathways are involved, the herbicides should have very low mammalian toxicities. The doubling of yield, when the only input is a one-time purchase of seed, and an annual purchase of herbicide is clearly in the realm of affordable economics, almost everywhere [150].

The problems of wheat were discussed earlier. Mechanical cultivation to control weeds in standing wheat is not an alternative. One alternative is selective herbicides that are not metabolically or target-site selective. Only one such herbicide seems to be available—prosulfocarb—which has placement selectivity [75]. The wheat must be drilled at a precise depth to prevent being damaged. Examples were given of engineering to previously not widely used herbicides that would fit the bill for wheat—with genes available. If such resistances were already in wheat, farmers would have more options and could rotate herbicides. If different herbicides were used, there would be even less likelihood that any one would ever be found above allowable thresholds in the food chain. Varying herbicides intelligently can keep most weeds below threshold levels and allow skipping the use of herbicides in certain years.

B. Misuse of Genetic-Engineering

Any powerful tool can be misused. The effect of some genetic-engineering programs for herbicide resistance is to increase the use of certain groups of herbicides that may be already used too heavily. There seems to be a PSII inhibitor for each crop in rotation. The same is true for ALS and ACCase inhibitors. There was nothing that could be done to stop the companies from marketing chemical analogues for use in each of the crops in a rotation; nor can one legally stop them from genetically engineering toward the same contraindicated end. Logic alone should have stopped both efforts as being against the long-term interests of agriculture. Besides the longer-term effect of effecting the rapid evolution of resistant weed populations, there would have been immediate negative effects. For example, not all crop seeds are harvested; some shatter to the ground and germinate the following year in rotational crops. These

"volunteer" weeds, as the plants coming from the last crop are euphemistically called, are often controlled with herbicides. If both the volunteer weeds and the new crop are resistant to the same herbicides, a problem can ensue. This author has severe reservations about engineering resistance to herbicides when preemergence use is planned. Preemergence herbicides must be used before the crop is planted, before the farmer knows if there are really weed problems that need a herbicide that season. Thus engineering resistance to postemergence treated, contact herbicides is preferable.

Besides crops becoming weeds, these are cases where genes could in theory transfer from transgenic crops to weeds by cross-pollination [136–140]. Most crops do not have weedy relatives to pollinate. Some have weedy relatives with a high degree of genetic incompatibility for which there is a minute likelihood of a gene being transferred. In these circumstances care is called for. If there is a high natural mutation rate to resistance, then resistance may evolve by natural selection and not through pollination. Extreme forethought should be used when there is easy cross-pollination, as between sorghum and johnsongrass, between oats and wild oats, barley and wild barleys, sunflowers and wild sunflowers, and rapeseed and related crucifers [136–140]. There still could be an overriding agronomic justification for conferring crop resistance, even in these worst cases. For example, in rapeseed in which it has already been shown that gene transfer to wild species is a (remote) possibility [138,139], weed control could be effected where none was previously possible [140]. Rapeseed oil and meal are rendered unusable for human and animal consumption when contaminated with seeds of some of its weedy relatives. The wild relatives can be annihilated only by using HRC rape varieties [140]. The long-term risk of having the genes move to the wild varieties by cross-pollination is considered to be outweighed by the present possibility of having an uncontaminated crop [140]. In describing the possibilities of transfers, the detractors commonly state that gene transfer is common within genera, and warn against growing transgenic plants wherever there are wild relatives. Plant breeders wish there was more truth to such generalizations, as there are many genes from wild relatives that they would like to cross into crops, but rarely publish their negative results. One outstanding recent publication showed that potatoes could be crossed with two wild European *Solanum* spp. [141], but only when the wild species were emasculated. All the seeds derived from such crosses gave rise to sterile plants [141]. Where the wild relatives do not exist, or where they flower in a different season, these problems of crosspollination also become moot. Simple sanitation, use of herbicide, and some common sense can limit the problems, delaying such gene transfers. Even when a wild species does become herbicide-resistant, that does not necessarily mean that the wild species will automatically become a pernicious weed [142].

There has been much argument about whether the genetic engineering of herbicide resistance into forest trees is a misuse. Many thought that quick reforestation because of lack of weed competition would be a benign if not a positive use of herbicide resistance. This may not be true on slopes, where total clearing of vegetation promotes erosion. Here the choice of herbicide is important, to prevent misuse. One should engineer resistance to a selective herbicide that does not control certain less-competitive (but ground-covering) weeds. Resistance to glufosinate- and ALS-inhibitors has been (separately) engineering into poplar varieties [15,27]. Glufosinate has no soil residual activity, and there are ALS-inhibitors with short residues. Their use on a slope in certain seasons would not be a misuse. Their use just before heavy rains or before slope revegetation could be a misuse. One could also, in theory, engineer resistance to a compound that does not kill weeds, but severely stunts their growth, while retaining ground cover. Other possible misuses are discussed at length by Duke et al. [143].

C. The (Putative) Moral Debate

There are some that are against genetic engineering in general [125,126,129,131] and more that are more specifically against engineering herbicide resistance into crops [127,128]. Their arguments often sound moralistic, which is confusing, as intuitively it seems moral to help feed the world, especially when it can be done without increasing the area of land being tilled, and the compounds used were ascertained safe by toxicologists. Life with minor limitations is preferable to starvation. Thus, it was enlightening to see an analysis of the moral claims by a professional moral philosopher in an article entitled: "We have not yet identified the heart of the moral issues in agricultural biotechnology" [144]. After following the arguments against genetic engineering for herbicide resistance, the author states: "...it is difficult to find any genuinely *ethical* issues in them." He later summarizes that "if we are to fashion, at least from the philosophical point of view, a powerful argument against...herbicide resistance, it is going to have to be general enough to call other technologies into question, including some we have accepted as benign. When one reads the critics of biotechnology, one suspects that they are interested in making some very wide ranging points about our technological society." The analysis concludes "if the moral discussion about biotechnology is to advance, it is going to have to stop being applied sociology and applied philosophy, and turn to a serious examination of basic social and political philosophy" [144].

The detractors from the development of HRCs have never bothered to review the needs for such crops, as if agriculture was having no problem keeping up with population growth. One clearly cannot morally deny such technologies from the starving farmers with crops infested by parasitic weeds, or from wheat farmers in India who have infestations of herbicide-resistant *Phalaris*.

The detractors have also used the arguments that such technologies would actually make farmers poorer by increasing yields and thus lowering prices [145]. Similar arguments were used about hybrid maize when it was introduced during the great depression. The first farmers to use it had a high relative profit, but as it was accepted there was indeed a drop in price owing to overproduction. As the markets grew, the price increased to good profitability for all. This would certainly be true with wheat, which is easily shipped. Too often, detractors invoke economic models based on increases in production of fresh milk, a commodity that cannot be shipped long distances and thus has an almost inflexible market size.

For parasitic weeds in the Third World, the economic scenario should be different. With undernourished people, or with a system where food must be imported to meet demands, the markets are far more flexible to enhance production. The added nutrition and income per farm family and their better health should clearly assist the economy. The argument that the use of HRC would cause massive unemployment in the agricultural sector [131] is somewhat specious, considering the present ineffectiveness in removing parasitic weeds. The doubling of yield will increase the need for harvesters, and the added income will allow the channeling of resources to more productive enterprises. The argument that such technologies cannot benefit poor peasants has been analyzed and tested in the field and shown to be quite inaccurate with far more costly inputs as fertilizers [146]. Productive agriculture will do more for preventing migration to the cities or worse, moving into nature reserves in the Third World than unproductive agriculture.

The only moral arguments against genetic engineering of herbicide resistance that seem to hold up to logical analysis are limited to examples for which such resistances are contraindicated, as described in the section on misuse. If cases of misuse are not judged amoral, they are at least cynical. Even here one must be careful, because good might come from bad. Increasing the spectrum of ALS-resistant crops (whether by designing selective

chemicals or by genetic engineering) for the developed world is contraindicated. Resistance evolves too quickly and with herbicides for all major crops in one group, rapid evolution is almost guaranteed. The same gene, in crops allowed selective control of parasitic weeds [56,57,87]. This gene that seems bad for the developed world may double yields in the underdeveloped world—until the parasitic weeds evolve resistance.

D. Who Should Perform the Research and Development?

The need for HRCs are those of agriculture. The farmers already having the needs are incapable of performing the research to provide the solutions. One might think that all the major needs pertaining to genetic engineering would be addressed by industry. This might happen in some cases, but not in many others. The chemical industry limits its major efforts to a few main target crops for herbicides. The biotechnology and seed industry prefers crops for which sales can be controlled through hybrids. The detractors from these biotechnological solutions are afraid of collusion between seed companies and the chemical companies who often own the seed companies [126,127,130]. This corporate constellation will act only when they perceive rapid, adequate return on investment. They are unlikely, then, to meet the needs of Third World agriculture or even forestry, because they do not perceive sufficient sales to justify the investment.

Even the needs of wheat may not be handled with major investment by seed companies, as hybrids are not cultivated. Industry legitimately has to make a fair return on investment and cannot be interested in all agricultural problems, yet weed problems impinge on the economy as a whole. This leaves the public sector to address the needs. Here we have a “Catch-22”; the public sector is increasingly dependent on the private sector for research funds; with the result that the public sector’s research priorities are now less targeted to needs of producing food, than to the needs of their benefactors. This is exasperated by the pressures being applied by the detractors of this technology to prevent governmental funds from being used in such research [127–129]. They have applied strong political pressures to prevent such funding in the United States and have stooped to knowingly overinflate the extent and amounts of public funding for such research [126–130], as has recently been pointed out [143]. One can discuss the moral issues arising from using such disinformation to support one’s agenda. One answer to get needed genes into crops would be for farmers’ groups to support more research, as they do in many countries through levies on sales of their commodities. Grower’s lobbies should well understand the long-term needs in weed control, and make sure they are addressed.

Third World parasitic weed control needs with resistant crops are even less likely to be met by industry. Industry seems to undervalue the market potential for herbicides, even though insecticides are widely used in agriculture in developing nations. The developing countries can expect little help from international organizations such as the Food and Agriculture Organization of the United Nations (FAO); a minuscule part of the Plant Protection Division of the FAO deals with weeds; even though weeds are the major plant protection problem in large parts of the Third World [80]. The detractors from these technologies are working especially hard to keep engineered crops out of the Third World. None of their publications discuss the plant protection needs in the underdeveloped world and how meeting these needs might alleviate starvation or even just the dependency on the developed world for nutritional largesse. Parasitic weeds and their effects are never mentioned by the detractors. If we want to keep the farmers from encroaching on badly needed wildlife preserves or forests, they must be helped to increase yield on their weed-infested land. If the amounts of herbicide required

to control parasitic weeds in crops are near as low they seem to be [54,78,87], then industry will not be interested. The prospect of not having repeat sales after an original seed sale will not draw the enthusiasm of private seed companies. Only public research can meet this need. Thus, the recommendation to "urge the FAO of the U.N. to develop restrictions on the export of herbicide-tolerant plants" [127] instead of supporting their use for developing a more sustainable agriculture, seems misanthropic if not amoral.

Total sustainability is a utopian goal, unattainable with present human populations. Those propounding totally sustainable agriculture often mean "organic farming," but are hypocritically not loath to erosion-inducing cultivation. Some have even lobbied to raise toxin allowances from fungal contaminants in organically grown foods, because current thresholds cannot be met without fungicides. Greater sustainability is not utopian, but requires hard tradeoffs. For example, the Indian rice farmer must control weeds and supply nitrogen. Controlling weeds with many herbicides kills the nitrogen-fixing cyanobacteria (blue green algae), requiring exogenous fertilizer application. The genetic engineering of herbicide-resistant cyanobacteria being performed in India [147] seems to be a better solution than buying more fertilizer, having weedy rice, or starvation. A small amount of a sound herbicide together with engineered herbicide-resistant nitrogen-fixers seems more sustainable than large amounts of fertilizer.

The public sector must become more involved to ensure that the best resistances are engineered into the most-needed crops. The greater the public sector involvement using public funds, the less the likelihood that this powerful biotechnological tool will be misused. The research and development costs may not be that great if the private sector makes the currently available gene constructs coding for resistance readily accessible to the public sector. The public sector should also be looking for genes for resistance to inexpensive, ecologically sound herbicides that may have been avoided by the private sector. If a herbicide is selective in some crop or is biochemically degraded in the soil, the genes are there to be isolated. They should be put into crops the farmers need. The pressures exerted on governments by antichemical and antigenetic-engineering lobbies to eliminate public research into engineered herbicide resistance in crops should be countered by the farmers who will need the crops. So far they have not been.

Groups such as the FAO must do more to meet the plant protection needs of the developing world. If weeds are as great a problems as local scientists estimate, the FAO has an obligation to meet these needs with the best tools available for local conditions. They should better explore how biotechnology can help meet these needs in the short- and longer-term, in the most cost-effective, as well as sustainable ways possible. They and other governmental, intergovernmental and nongovernmental groups should carefully evaluate and weigh the moral as well as other arguments for and against these technologies, along with the needs of their constituents.

ACKNOWLEDGMENTS

The many who supplied ideas, comments, feedback, and unpublished material are gratefully thanked. None but the author is to be held responsible for the views expressed herein. The author's and colleagues' research on HRC and mycoherbicides for use in parasitic weed control is supported by grant HNE-0158-G-00-3060-00 from the trilateral U.S.-Israel-Egypt U.S. Agency for International Development (A.I.D.) program. The author holds the Gilbert de Botton Chair of Plant Sciences.

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Biotechnology of Biofertilization and Phytostimulation

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I. PROBLEM DESCRIPTION

A. Economic Importance

To sustain the world population in the year 2020 it will be necessary according to United Nations (UN) estimates from 1989, to increase agricultural production by 100%. The role that the use of effective fertilizers can play in increasing agricultural production is fully recognized. A clear relation has been established between the increasing yields of cereals, especially in developed countries over the past 70 years, and the introduction of high-yielding varieties, better pest control, and the increase in fertilizer consumption (i.e., nitrogen, phosphorus, potassium). It is generally assumed that an input of 1 kg of fertilizer produces up to 10 kg of additional cereal, at least for the initial fertilizer applications [1,2].

B. Plant Growth-Limiting Compounds

1. Nitrogen

The most common nutrient limiting the production of agricultural crops is nitrogen. The earth's atmosphere is 80% molecular nitrogen; it contains 4×10^{15} metric tons (t) of this element. However, plants can utilize nitrogen only in the combined mineral form (fixed nitrogen), such as ammonium (NH_4^+) or nitrate (NO_3^-).

Up to the 19th century, crop yields obtained in cultivated fields were generally low. With the advent of modern agriculture, the natural nitrogen cycle, chiefly the process of biological nitrogen fixation, was no longer able to provide for the nitrogen needs of the plants. Nitrogen fertilizer produced industrially by the Haber-Bosch process ($>80 \times 10^6$ t were produced in 1995) seemed capable of supplying these increasing demands. For both more-developed and less-developed countries, however, capital and energy costs of production by the Haber-Bosch process have become significant: 500–700 million US dollars to establish a plant and approximately 20 billion US dollars economic cost per year [3,4].

The increasing demand for fixed nitrogen in modern agriculture could be solved by the enhancement and extension of plant growth promotion and nitrogen fixation. Agriculturally important legumes are estimated to account for about one-half (80×10^6 t/yr) of all nitrogen fixed by biological systems. Although legumes have had a major role in food production throughout history, the total world area currently cultivated with these plants is approximately 15% of the area used for cereal and forage grasses, the main source of food in the modern world [2].

The production of meat, alcohol, and sugar partly depends on the availability of cereal and forage grasses. To obtain high crop yield, especially when using highly productive cultivars, it is necessary to apply nitrogenous, phosphorus, and potassium fertilizer in larger amounts. For example, a crop of irrigated sweet corn (*Zea mays*) in Israel is usually fertilized with 240 kg of nitrogen per hectare (ha) to obtain a yield of 20–25 t/ha of fresh grain [2]; irrigated wheat (*Triticum*) fertilized with 120 kg of nitrogen per hectare yields 6–7 t/ha of grain.

Individual protein consumption of the 5.3 billion people on Earth averages about 70 g/day of protein, or 23 million t/annum of nitrogen (N). To maintain this level of intake as the Earth's population doubles over the next 40 years, will necessitate a doubling or tripling of crop production, despite a significant deterioration of much prime agricultural land and will require the use of large areas now considered marginal. Judicious management of N, P, K, and water in the environment will be essential [1].

2. Phosphorus

Long-term fertilization has improved the phosphorus status of much of Europe's arable land to the extent that over large areas only maintenance application is now required. But the early development of plants often benefits from some phosphorus application. In other parts of the world, phosphate deficiencies are not uncommon.

Phosphate is supplied to cropped land at application rates ranging from a few kilograms phosphorus per hectare to 35 kg/ha or more [4].

3. Potassium

Potassium is a common element. The Earth's crust contains about 2.3% potassium. It is one of the three major crop nutrients, with an essential role in physiological processes, such as water uptake, osmotic regulation, photosynthesis, and enzyme action. An adequate potassium supply is necessary for ensuring crop resistance to lodging, disease, and drought.

The main soil sources of potassium are clay minerals. Much of soil potassium is present as part of insoluble mineral particles and inaccessible to plants. Only the slow process of weathering can liberate such potassium. Fertilization is required to ensure that crops get a sufficient supply of soluble potassium and that the soil reserves of accessible potassium are not depleted. Usual application rates for potassium are between 40 and 170 kg/ha. Potassium binds to the surface of clay particles: this reduces leaching. But organic and light sandy soils can have little potassium-binding capacity. Heavy application of potassium (notably with manures) on such soils can result in leaching losses. There are regions (e.g., in The Netherlands) where groundwaters now have potassium concentrations above the maximum specified in the European Communities' (EC) water directive. This states that drinking water should not contain more than 12 mg potassium per liter, and preferably less than 10 mg [4].

4. Water

Water is the principal constraint on crop yield on much arable land. Although rainfall is adequate for crop needs in some regions, others have erratic or sparse rain. Currently 15% of the world's total arable land is irrigated, but this produces 36% of the total crop yield.

Crops must have adequate water supply to utilize nutrients properly. Where growth is severely water-restricted, fertilization is of limited value. Water and nutrient management, therefore, are connected.

Irrigation can be expensive, its management complex, and equitable water distribution difficult to achieve. Drip irrigation methods could be a solution to maintain high yield while using water more conservatively.

C. Use of Microbes for Fertilization and Phytostimulation

Various soil microorganisms that are capable of exerting beneficial effects on plants or antagonistic effects on plant pests and diseases either in culture or in a protected environment have a potential for use in agriculture and can lead to increased yields of a wide variety of crops. However, this ability does not always ensure that the release of the organisms into an environment, such as soil, will produce the desired results [6].

Microbial groups that affect plants by supplying combined nitrogen include the symbiotic N₂-fixing rhizobia in legumes, actinomycetes in nonleguminous trees, and blue-green algae in symbiosis with water ferns. In addition to supplying combined nitrogen by biological nitrogen-fixation, free-living nitrogen-fixing bacteria of the genus *Azospirillum* affect the development and function of grass and legume roots, thereby improving mineral (NO₃⁻, PO₄³⁻, and K⁺) and water uptake. Other microorganisms that are known to be beneficial to plants are the phosphate solubilizers, plant-growth-promoting pseudomonads, and mycorrhizal fungi.

Indirect effects on crop yield can be obtained by inoculation with microorganisms capable of reducing damage caused by pathogens and pests. These groups include biocontrol agents of soilborne pathogens such as *Pseudomonas*, *Agrobacterium radiobacter*, and *Trichoderma*, bacterial and fungal insecticides, nematode-trapping fungi, microbial herbicides, and microbes that compete with ice-nucleating bacteria, thereby preventing frost damage to leaves. These agents will not be treated in this chapter, but in [Chapter 11](#).

The use of these microorganisms is of economic importance to modern agriculture as they can replace costly mineral fertilizers and improve water utilization, lowering production costs, and reducing environmental pollution, while ensuring high yields. The potential benefit of manipulating agricultural systems through modification of the rhizosphere and phyllosphere microflora is evident.

Technical problems involved in the successful inoculation of agricultural crops include the delivery of sufficient inoculum to the target, the economical production of large quantities of microorganisms, the promotion of extended shelf life, and the development of convenient formulations [5].

This chapter will deal with microbiologicals that can be used as biofertilizers and phytostimulators. Major limiting factors for crop growth can be water, carbon, nitrogen, phosphate, and other minerals.

D. Environmental Constraints

In the more developed countries, fertilizer use is inefficient. It is estimated that only 50% of the applied nitrogen fertilizer is used by plants, with most of the remainder lost by either denitrification or leaching [2]. The concentration of the toxic nitrate has increased in water reservoirs in the vicinity of heavily fertilized fields. Denitrification of nitrate produces about 90% nitrogen gas and 10% nitrous oxide, the latter being a greenhouse gas with energy reflectively 180 times that of carbon dioxide. The increase in nitrous oxide content in the troposphere during the 1980s correlates more strongly with increased use of fertilizer nitrogen than with the increase in combustion of fossil fuel [3].

E. Political Decisions

Until recently, subsidies and legislation in Europe were designed to increase agricultural production, assure farmers a fair income, and to keep food prices at a reasonably low level. Today, food production in the Western World is at a sufficient level. Moreover, the excessive use of chemicals has resulted in health hazards (e.g., owing to leaching of toxic NO₃ into groundwater and volatilization of N-oxides into the environment). The European Union has adopted its "Common Agricultural Policy" with price cuts for key products, incentives for setting aside agricultural land, and for a reduction in chemical input. This policy of sustainable agriculture (i.e., keeping the soil constantly in good shape) will result in a strongly decreased chemical input and, therefore, in a more extensive agriculture. Farmers are faced with environmental taxes and the need to produce less yield per hectare. A recent policy of the Dutch government is even to give very fertile farm land, sometimes polders, "back to nature."

II. ROLE OF BIOTECHNOLOGY

A. Biotechnological Approaches

Basically two kinds of approaches can be taken for the application of microbial fertilizers or phytostimulators. First, a large number of strains are screened on selected crop plants under laboratory or greenhouse conditions, (e.g., for their capabilities to improve germination, seedling vigor, root elongation, root branching, nitrogen fixation [acetylene reduction], and legume nodulation). The screening is usually carried out in pouches or petri dishes with filter paper and sterile nutrient solution, making possible the selection of promising microbial strains. However, this procedure does not predict performance in soil. Therefore, selected strains are further tested in pots in soil and finally under field conditions. It is necessary to demonstrate, using Koch's postulates, that the selected microorganism is responsible for the observed effect on the plant. The best strain(s) will be developed into a product. *Bradyrhizobium* and *Rhizobium* inoculants were developed in this way.

Another approach consists of trying to understand *why* certain strains exert beneficial effects. This understanding will provide notions for improvement of strains and screening procedures and of the inoculant production or storage process. A clear advantage of the latter approach is that it will result in qualitatively superior products. However, the disadvantage is that this approach is so expensive that it is not feasible for most agroindustrial products. Occasionally, the questions on mechanisms have been studied by academic institutions. In the following we will elaborate on a few examples of microorganisms of which the mechanisms of beneficial action have been studied in more detail (e.g., *[Brady]rhizobium* as a nitrogen-fixing bacterial symbiont and *Azospirillum* as a bacterial phytostimulator).

B. Use of Specific Microorganisms

1. *Bradyrhizobium* and *Rhizobium*

a. *Mechanism.* Biological nitrogen fixation (BNF) accounts for 65% of the nitrogen currently used in agriculture and will be increasingly important in future crop productivity, especially for sustainable systems, small-scale operations, and marginal land utilization [1]. *Rhizobium* and *Bradyrhizobium* bacteria are responsible for most of the BNF. These bacteria are able to invade the roots of their leguminous host plants, where they trigger the formation of a nodule. In this organ the bacterium develops into a differentiated form, the bacteroid, which is able to

convert atmospheric nitrogen into ammonia. The latter compound can be used by the host plant as a nitrogen source. In exchange, the host plant provides the bacteroid with dicarboxylic acid carbon sources. This plant-bacterium symbiosis is host-specific in the sense that on a particular host plant only one or a limited number of rhizobia are able to generate nitrogen-fixing nodules. For example, pea, vetch, and lentil can be nodulated only by *R. leguminosarum* bv. *viciae*, whereas clover is nodulated by the very similar *R. leguminosarum* bv. *trifolii*. Economically, the most important of these symbioses is the combination soybean-*Bradyrhizobium*. The latter bacterium was previously known as *R. japonicum*.

The molecular basis of some of the crucial steps of this microbe-plant interaction is known. The bacterium contains nodulation (*nod* or *nol*) genes. Most of these genes are inactive in the absence of the plant. However, root exudate components, most of which were later identified as flavonoids [7], activate the positive regulatory protein NodD. This results in transcription of the inducible *nod* genes. Their products are required for the biosynthesis and secretion of a novel series of compounds, lipochitin oligosaccharides (LCOs) or Nod-factors [8,9]. They consist of a chitin oligomer with an N-linked fatty acid at the nonreducing end (Fig. 1). This molecule acts as a major host-specific factor. Specific moieties added to the sugar backbone structure, or a specific fatty acid, determine the host range [10] (for a recent update see Table 1). Purified host-specific LCOs are able to induce several nodulation-related effects [11–13], including the induction of nodule primordia. The latter structures are indistinguishable from those induced by the homologous bacterium in the first stage of nodule organogenesis [9]. Therefore, the major function of the *nod* genes seems to be to produce a hormone-like substance that locally reprograms plant development. The genetic information for nitrogen fixation is also present in the bacterium, namely in the *nif* and *fix* genes. Also the plant contributes to the nodulation process by producing several nodule-specific proteins, designated as nodulins. Synthesis of some of these nodulins can also be induced by purified LCOs [14].

Specific LCOs are major determinants of the host range of nodulation. In contrast, flavonoids usually do not act as host-range determinants, although exceptions to this rule exist [15]. During evolution the *nodD* gene seems to have fine-tuned its interactions with the flavonoid inducers of its host plant(s). Spaink et al. have constructed a chimeric *nodD* gene consisting at the 5'end of the *nodD1* gene of *R. meliloti* and at the 3'end of the *nodD* gene of *R. leguminosarum* bv. *trifolii*. This hybrid gene, termed *nodD604*, does not need flavonoids for activation; therefore, it does not cause a limitation of the host range. This flavonoid-independent transcription activation (FITA) *nodD* gene is even able to confer (pseudo)nodules on tropical leguminous plants. In contrast to most other *nodD* gene products, NodD604 is insensitive to so-called anti-inducers of nodulation present in exudates. Finally, *nodD604* not only confers superior nodulation properties, but also superior nitrogen fixation properties [16].

The third factor known to be crucial for host range is the plant lectin. Diaz et al. [17] showed that transfer of the pea lectin gene to hairy roots of white clover resulted in nodulation

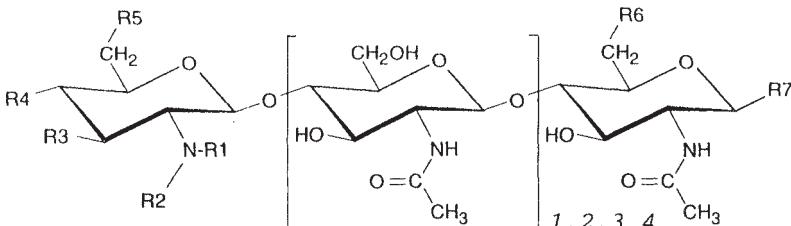


Fig. 1 General structure of rhizobial lipochitin oligosaccharide signal molecules. R1-R7 represent substituents that can vary in structure (see Table 1).

Table 1 Structural Variations of Rhizobial Lipochitin Oligosaccharides

Species	Substituents of lipochitin oligosaccharides ^a							<i>n</i>
	R1	R2	R3/R4	R5	R6	R7		
<i>R. meliloti</i>	H	C _{16:1} , C _{16:2} , C _{16:3} , (ω-1)OH-C _{18:0} , (ω-1)OH-C _{20:0} , (ω-1)OH-C _{22:0} , (ω-1)OH-C _{24:0} , (ω-1)OH-C _{26:0}	H	Ac, H	S	H		1,2,3
<i>R. I. bv. viciae</i>	H	C _{18:0} , C _{18:1} , C _{18:4} C _{16:0} , C _{16:1}	H	Ac	H, Ac	H		2,3
<i>R. I. bv. trifolii</i>	H	C _{18:0} , OH-C _{18:0} , C _{18:1} , C _{18:3} , C _{20:1} , C _{20:2} , C _{20:3} , C _{20:4}	H	Ac	H	H		1,2,3
<i>R. etli</i>	Me	C _{18:0} , C _{18:1}	Cb(O-4), H	H	AcFuc	H		3
<i>R. loti</i>	Me	C _{18:0} , C _{18:1}	Cb(O-4), H	H	4-O-AcFuc	H		3
<i>R. tropici</i>	Me	C _{18:1} , C _{16:1} , C _{16:0}	H	H	S, H	H, Man		2,3
<i>R. fredii</i>	H	C _{18:1}	H	H	Fuc, 2-O-MeFuc	H		1,2,3
<i>R. sp. GRH2</i>	H, Me	C _{16:0} , C _{16:1} , C _{16:2} , C _{18:0} , C _{18:1} , C _{18:2} , C _{20:1}	H	H	H, S	H		2,3,4
<i>R. sp. NGR234</i>	Me	C _{16:0} , C _{18:1}	Cb (1 or 2), H	H	2-O-MeFuc, 2-O-Me,3-O-SFuc, 2-O-Me,4-O-AcFuc	H		3
<i>B. japonicum</i>	H	C _{16:0} , C _{16:1} , C _{18:1}	H	Ac, H	2-O-MeFuc	H		3
<i>B. elkanii</i>	H, Me	C _{18:1}	H, Cb	Ac, Cb, H	2-O-MeFuc, Fuc	H, Gro		2,3
<i>A. caulinodans</i>	Me	C _{18:0} , C _{18:1}	H	Cb, H	H, D-Ara	H		2,3

^aPositions of R1-R7 are depicted in Figure 1.

Abbreviations: Ac, acetyl; D-Ara, D-arabinose; C, carbon; Cb, carbamoyl; Fuc, fucose; Gro, glycerol; H, hydrogen; Me, methyl; Man, mannose.

Source: Adapted from Refs. 96 and 97.

and nitrogen fixation of the transformed clover roots by *R.leguminosarum* bv. *viciae* bacteria, the guest bacteria of pea which normally are unable to nodulate white clover.

b. Results. Inoculants containing cells of *Bradyrhizobium* or *Rhizobium* have been commercially available for a century. Usually these preparations contain combinations of three to five strains. The major problem with the application of such inoculants is that only 5–20% of the nodules are occupied by the inoculant bacteria, the remainder by indigenous (brady)rhizobia, most of which fix less N₂ than the selected inoculant strains.

A possible breakthrough in this area has been reached by Tikhonovich et al. who bred a new pea cultivar that can be nodulated only by the efficient nitrogen-fixing *R.leguminosarum* bv. *viciae* strain A1 and not by indigenous bacteria [18]. The highly efficient combination of the novel pea cultivar and *R.leguminosarum* strain A1 is presently being commercialized. It would be intriguing to see how this extreme form of host-specificity is reflected in the structure of the microsymbiont's LCOs.

Another approach to suppress the activity of indigenous rhizobia has been to use the combination of anti-inducers and inoculant bacteria carrying FITA *nodD604* [16]. The rationale was that anti-inducers [19] would suppress nodulation by indigenous bacteria, whereas the inoculant strain, because of the presence of FITA *nodD604*, would not be sensitive. The idea failed because some indigenous rhizobia were found that carry anti-inducer-resistant forms of *nodD*.

Knowledge of the molecular basis of symbiosis has been used to increase the nitrogen-fixing activity of inoculant *R.meliloti*, the guest bacterium of alfalfa. The knowledge included the facts that *nifA* is a major nitrogen-fixation regulatory gene and that the genes *dctABD* are involved in dicarboxylate transport from the plant to the bacteroid. After inoculation with modified *R.meliloti* bacteria, which were provided with an extra copy of both of these DNA fragments, the alfalfa biomass was 12.9% higher than after inoculation with the parental strain. The soil of this test site contained low concentrations of nitrogen and organic matter. At sites where soil nitrogen or resident rhizobial populations were high, no significant effect of the recombinant DNA bacterium was observed [20].

A study of Polonenko et al. [21] suggested that *B.japonicum* inoculants may be improved by the addition of other soil bacteria, predominantly pseudomonads, which enhance *B.japonicum*-induced nodulation and plant growth. The basis of this enhancement is unknown, but biocontrol of pathogens or phytohormone production are likely possibilities.

Current inoculant formulations and applications are adapted to the needs of the grower, especially of soybean. Preparations of *R.meliloti* are available with a constant shelf life over a 24-month period [5].

2. *Azospirillum*

All azospirilla are nitrogen-fixing bacteria with nitrogenase properties comparable with those of other nitrogen fixers. It has been postulated that biological nitrogen fixation by *Azospirillum* in association with roots may contribute significant amounts of nitrogen to the plant, thereby potentially saving valuable nitrogen fertilizers. Indeed, greater nitrogen fixation activities, as measured by the acetylene reduction assay, were detected in inoculated plants than in noninoculated controls [22]. Higher nitrogen fixation rates were detected near or at flowering under conditions of high temperature and soil moisture. However, incubation of plants in a ¹⁵N atmosphere and utilization of the ¹⁵N-isotope dilution technique showed that only a very small proportion of the apparently fixed N₂ was incorporated into the plant [22]. It has not been possible to demonstrate nitrogen gains in inoculated plants above those initially present in soil. The foregoing measurements have shown that BNF by *Azospirillum*-root associations

in the field contribute some nitrogen to summer grasses and cereals (1–10 kg¹ of nitrogen per hectare), in itself a very positive phenomenon. However, the amounts fixed are of less agronomical significance than expected [22]. By using an *A. brasiliense nifH-gusA* fusion, the expression of the *A. brasiliense* nitrogen reductase structural gene was monitored during the association of wheat roots in in vitro experiments [23]. Associative *nifH* expression was very low under these conditions. Enhanced bacterial *nifH* induction was observed in the presence of an additional carbon source or when the oxygen tension was lowered to microaerobic levels, indicating that both oxygen and the availability of energy sources are required [22,23].

Despite their N₂-fixing capability, the increase in yield caused by *Azospirillum* inoculation is mainly attributed to an improvement in root development and thus increases in the rates of water and mineral uptake [24]. It is generally assumed that *Azospirillum* enhances the root development by the production of plant growth-promoting substances, such as auxins, cytokinins, and gibberellins [25]. The theory that *Azospirillum* exerts its effect through the production of plant growth-promoting substances is further strengthened by the morphological changes observed in inoculated roots. *Azospirillum* enhances cell division in the root tip [26]; increases the number, diameter, and length of lateral roots; enhances root hair appearance [6,27]; and increases root surface area [24,28].

Phytohormone synthesis by *Azospirillum* is proposed to influence the host root respiration rate and metabolism and root proliferation, with a concomitant mineral and water uptake in inoculated plants [25]. *Azospirillum* is capable of producing indole-3-acetic acid (IAA) by multiple IAA biosynthetic pathways: amino transferase, indole-3-acetamide, and by a tryptophan-independent pathway(s) [29–32]. The production of gibberellin (GA), GA₃, and iso-GA₃ in cultures of *A. lipoferum* was demonstrated by gas chromatography-mass spectroscopy (GC-MS) with selected ion monitoring.

In corn seedlings inoculated with *Azospirillum*, relatively higher amounts of free (compared with conjugated forms) active IAA, indole butyric acid, and gibberellin [25,33] were detected when compared with noninoculated controls. In the noninoculated roots seedlings, GA₃ was found after hydrolysis of a fraction expected to contain glucosyl conjugates (with less phytohormone activity) [33].

It appears that the presence of *Azospirillum* in the rhizosphere affects the metabolism of endogenous phytohormones in the plant. It is not known, however, whether this phenomenon is due to free phytohormone production by the bacterium or by the elicitation or activation of conjugate phytohormone hydrolysis by the root tissue.

No mutants completely lacking production of IAA have so far been isolated; however, when comparing low-IAA- with high-IAA-producing strains, a reduced ability in wheat root system promotion (root hairs and lateral roots) was observed [34].

By evaluating worldwide data accumulated over the past 20 years on field inoculation experiments with *Azospirillum* [35], it can be concluded that this bacterium is capable of promoting the yield of agriculturally important crops in different soils and climatic regions, using various strains of *A. brasiliense* and *A. lipoferum* and cultivars of different species of plants. However, it is difficult to estimate accurately the percentage of success that is due to *Azospirillum* inoculation. The picture emerging from the extensive data reviewed is that of 60–70% successes with statistically significant increases in yield in the order of 5–30%. This estimate is in agreement with Okon *et al.* [36] and Wani [37] from experiments in Israel and India, respectively. Sumner [38] has presented 32 references relating to positive effects in the field and 7 citations (mostly on wheat) of failures to obtain yield increases. He concluded that the responses have been quite substantial, well in excess of the likely costs of inoculation, making this technique highly attractive to the farmer. Furthermore, Fages [39] proposed that a well-focused strategy of field experimentation could demonstrate an acceptable consistency

of agronomic results; for example, the results with sorghum in Israel [40,41] and the results with maize in France [39].

3. Interaction of *Azospirillum* with the *Rhizobium-Legume Symbiosis*

Positive effects of combined inoculation with *Azospirillum* and *Rhizobium* have been reported for different legumes. A possible cause for this enhanced susceptibility of the plants to *Rhizobium* infection following *Azospirillum* inoculation could be the greater number of epidermal cells that differentiate into infectable root hairs. Itzigsohn *et al.* [42] demonstrated with Fahraeus slides that *Azospirillum* increases the number of root hairs and their diameter, whereas no increase in the total number of infection threads was observed. The effect that *Azospirillum* has on nodulation and on the specific activity of nodule N₂-fixation, leading to growth promotion, may be attributed to the following causes: early nodulation, an increase in the total nodule number, and a general improvement in mineral and water uptake by the roots [43].

Beneficial growth response under gnotobiotic conditions was achieved in most cases following the application of *Azospirillum* in the right number and ratio, before or after inoculation with *Rhizobium*. Therefore, it is reasonable to conclude that *Azospirillum* exerts its effects through the host plant, and not through direct interaction with *Rhizobium*.

Field inoculation with *A. brasiliense* strain Cd increased nodule dry weight (90%), plantgrowth parameters, and seed yield (99%) of naturally nodulated *Cicer arietinum* L (chickpea). In *Phaseolus vulgaris* (common bean), inoculation with *R. etli* TAL182 and *R. tropici* CIAT899 increased seed yield (13%), and combined inoculation with *Rhizobium* and *Azospirillum* resulted in a further increase (23%), whereas plants inoculated with *Azospirillum* alone did not differ in yield from uninoculated controls, despite a relative increase in shoot dry weight [44].

Azospirillum clearly promotes root hair formation in seedling roots. In experiments carried out in a hydroponic system, *Azospirillum* caused an increase in the secretion of *nod* geneinducing flavonoids, as observed by *nod* gene-inducing assays of root exudates fractionated by high-performance liquid chromatography (HPLC) [44,45].

4. Azotobacter

The nitrogen-fixing bacterium *A. paspali* was first described by Döbereiner and Pedrosa [46] and has been isolated only from the rhizosphere of *Paspalum notatum*, a tetraploid subtropical grass widely distributed in South America [46]. Isolates are characterized as obligately aerobic, which give rise to opaque colonies with entire or undulate margins. The bacteria are pleiomorphic, ranging from long filaments to cocci. Typically, N₂ fixation occurs at pH 6.5–9.5, growth at 14°–37°C. Green fluorescent pigments are produced on iron-deficient medium.

Oxygen is known to be a factor in influencing N₂ fixation because high O₂ concentrations probably inactivate nitrogenase. Estimates of maximal nitrogenase activity were obtained at P_{O₂} of about 0.04 atm, on roots removed from the soil and less than half of that under anaerobic conditions or in air. Most of the activity was localized on the roots and was not removed by vigorous washing in water. Inoculum of *A. paspali* declined rapidly in Brazilian soil, even in the rhizospheres of *Penicillium notatum*, were it normally thrives under natural conditions; decline was less rapid in potting compost [47]. *A. paspali* improved the growth of *P. notatum* by fixing atmospheric N₂ in the rhizosphere.

Inoculation with 5×10⁸ cfu ml⁻¹ of *A. paspali* under gnotobiotic conditions in petri dishes increased root hair formation in canola roots 24 h after inoculation [48]. By the ¹⁵N-isotope dilution technique, Boddey *et al.* [49] reported that *A. paspali* fixed at least 11% of the

nitrogen utilized by *P.notatum* cv. Batatais when the bacterium was under microaerobic conditions. Maximal N₂ fixation was obtained at a Po₂ of 0.04 atm [46]. Alternatively, Barea and Brown [47] were able to obtain large increases in plant growth for a variety of dicotyledonous and monocotyledonous plants growing in pots in natural soil incubated with *A.paspali*. By adding inorganic nitrogen, they were able to eliminate N₂ fixation as a source of plant growth. They concluded that the plant growth promotion was bacterially mediated by the production of plant growth factors (indole acetic acid, gibberellins, and cytokinins).

Treatment of seedling hypocotyls and roots of several plant species with cultures of *A.paspali* changed plant growth and development and significantly increased weight of shoot and roots. Morphological changes of root tips were already observed 5 days after inoculation. After 21 days, the main effect was on the root surface area. Plant growth promotion was dependent on the inoculum size, indicating that, for any given plant growth condition, there is an optimal number of *A.paspali* for a positive effect on the plant [50].

5. Mycorrhizae

Mycorrhizae are fungi that are so closely connected to the roots that they are considered an extension of the root system. The vesicular-arbuscular mycorrhizal (VAM) fungi, which are members of the class *Zygomycetes*, order *Glomales*, form mycorrhizae with plant roots. The VA mycorrhizal fungi are obligate symbionts and are not host-specific. They occur in about 80% of plants [51]. The VA mycorrhizal fungi grow primarily inside the root, but the network of extraradical fungal hyphae form an extension of the effective root area of the plant, which increases the absorption and translocation of immobile nutrients.

Most of the beneficial effects of VAM fungi are related to increases in the effective root surface area, thereby increasing the ion uptake of the plant [52]. Positive growth responses to mycorrhizal development can be expected when the concentration of some nutrient is extremely low in the aqueous phase, but some solid or unavailable form exists in reserve [53]. Although it has been demonstrated that many elements (e.g., P, S, Zn, Cu, Ca, N, K, Sr, and Cl) can be taken up by mycorrhizal hyphae and transported to the root, most experimental work has been concerned with phosphorus uptake and, to a lesser extent, nitrogen [52]. Field inoculation of crop plants with VAM fungi is very much dependent on field conditions. The potential for increasing plant growth and yield by inoculation will very much depend on the probability of natural inoculation by the indigenous fungi and the level of available nutrients, especially phosphorus. Other factors that influence successful field inoculation will be the selection of the correct fungal isolate for the crop host, and inoculum type (e.g., spores, infected root pieces), formulation, and placement [54].

Although pathogenic biotrophs generally exhibit a high degree of host specificity, VAM fungi show little to none, indicating that the colonization of roots by these fungi is not influenced by the vast array of constitutively produced plant metabolites that may serve to prevent fungal infection, as well as somehow able to overcome a large variety of induced resistance mechanisms.

The VAM fungi are not considered to induce typical defense responses in host plants [55]. Nevertheless, transient increases in the activities of the normal pathogen-response proteins chitinase [56] and peroxidase [57] were detected in leek roots during early stages of colonization by VAM fungi. Furthermore, soybean roots colonized by *Glomus mosseae* or *G.fasciculatus* accumulated more of the isoflavanoid phytoalexin glyceollin 1 than nonmycorrhizal roots [58]. Faba bean roots infected with *G.intraradix* contained elevated levels of the nonflavonoid acetylenic phytoalexin wyerone, but the amounts did not reach those measured in host-pathogen

interactions [59]. In alfalfa, during early colonization of plant roots by *G.intraradicis*, isoflavonoid phytoalexin defense response transcripts are induced and then, subsequently, suppressed [60,61]. Thus, although infection by mycorrhizal fungi appears to initiate some plant defense responses, these do not seem to reach their full potential, which would probably have prevented colonization.

The role of flavonoids as signal molecules in the establishment of the mycorrhizal plant is unclear, but some flavonoids enhance germination and hyphal growth of VAM fungi [62,63] and promote VAM fungal colonization of white clover roots [64]. Likewise, both rhizobial nodulation factors, and several of the flavonoids known to accumulate in response to the nodulation factor, promoted VAM colonization of soybean roots [65], suggesting a flavonoid-mediated stimulation of mycorrhizal colonization. Moreover, all *Myc*' pea mutants induced by chemical mutagenesis were also *Nod*', suggesting that a common early infection event is required in both types of symbioses. Further indications that colonization of alfalfa roots by mycorrhizal fungi affects flavonoid metabolism is that nodule distribution on mycorrhizal roots is significantly different from that on nonmycorrhizal roots [66].

Fungal colonization is limited when high phosphorus concentrations are available [67]. High phosphorus concentrations inhibit intraradical fungal growth [68], possibly through phosphorus-mediated physiological alterations of the roots [69]. Induction of plant defense genes may be one factor in reducing colonization. Phosphorus, when applied to cucumber leaves, induces the expression of chitinase and peroxydase both locally and systemically [70].

6. Mycorrhization Helper Bacteria

The symbiotic establishment of mycorrhizal fungi on plant roots is affected especially by bacteria of the rhizosphere. Some of these bacteria consistently promote mycorrhizal development. This notion has led to the concept of mycorrhization helper bacteria (MHBs) [71]. It seems likely that the use of MHBs can improve the effect of mycorrhizal inocula. Garbaye has listed five possible explanations to explain their activity [71]:

1. MHBs may improve the receptivity of the root to mycorrhizae formation (e.g., by producing auxin or by producing plant cell wall-softening enzymes such as endoglucanase, cellobiose hydrolase, pectate lyase, and xylanase). Replacing the living bacterial cells by filtrates of bacterial cultures or by solutions of the enzymes had the same effect [71].
2. MHBs can interfere with the plant-fungus recognition and attachment mechanisms, which are the first steps of the interactive process, leading to the symbiosis.
3. MHBs stimulates the growth of the fungus in its saprophytic, pre-symbiotic stage in the rhizosphere soil or on the root surface. Indeed, a highly significant correlation was observed between the ability of bacterial isolates to reduce or promote the mycelial growth of *Laccaria laccata* and their effect on mycorrhiza formation.
4. MHBs modify the rhizosphere soil (e.g., by altering the pH or the complexation of ions).
5. MHBs trigger or accelerate the germination of spores, sclerotia, or any other dominant propagules specialized in the conservation and dissemination of the fungus in the soil. In nature this is often the first step leading to mycorrhiza formation.

An European consortium has been established with the goal of testing these hypotheses, thereby increasing the feasibility of inoculation with the combination of mycorrhizae—MHBs.

C. Bacterial Stimulation of Water and Phosphate Uptake

1. Water

Mild water deficits affect development of the root system. The abilities of plants to absorb both water and mineral nutrients from the soil is related to their capacity to develop extensive root systems. Plants are known to wilt more rapidly in water-logged soils, as a result of decreased hydraulic conductance in the roots. Inoculation of sorghum in the field with *Azospirillum* led to 25–40% increase in hydraulic conductivity, compared with the control. This could be explained by observed increases in the total number and length of adventitious roots of *Sorghum bicolor*, ranging from 33 to 40% over inoculated controls [41].

2. Phosphate

Phosphate deficiency can be diminished in crops by utilization of bacteria that act directly as phosphate solubilizers in the rhizosphere, indirectly by bacteria that stimulate root activities; the root excreting organic acids that help solubilize phosphate and at the same time increase phosphate uptake [40,72], and by mycorrhizal associations.

Insoluble inorganic compounds of phosphorus are largely unavailable to plants, but many microorganisms can bring the phosphate into solution. Species of *Pseudomonas*, *Mycobacterium*, *Micrococcus*, *Bacillus*, and *Flavobacterium* are active in the conversion. These bacteria grow in media with $\text{Ca}_3(\text{PO}_4)_2$ and apatite as sole phosphorus sources. Not only do the microorganisms assimilate the element, but they also make a large portion soluble. The many phosphate-dissolving microorganisms in the vicinities of roots may appreciably enhance phosphate assimilation by higher plants [73].

Inoculation of plants with *A. brasilense* significantly enhanced (30–50% over controls) the uptake of H_2PO_4^- by maize in hydroponic systems [72] and by 10–30% in the sorghum and wheat field [40,74]. The increases in phosphorus uptake could be derived in this case by increased root respiration. In inoculated plants [25], respiratory energy is the driving force behind biosynthetic reactions and transport processes.

Maize root cell-free extracts from seedlings inoculated with *A. brasilense*, at a concentration of 10^7 cfu/plant, contained elevated levels of enzymes related to the tricarboxylic acid cycle, the glycolysis pathway, and the breakdown of organic phosphate. Enzyme activity increases of 13–62% over the uninoculated controls were observed [25].

D. Prospects of Microbial Fertilization of Specific Major Crops

1. Rice

Rice is the most important food crop of the developing world: stable food for over 2 billion persons in Asia and for hundreds of millions in Africa and Latin America. To feed the ever-increasing population of these regions, the world's annual rice production must increase from the present 460 million t to 560 million t by the year 2000 and to 760 million t by 2020. Because the future growth in rice production has to come from the same or even a reduced land area, enhancement in productivity (yield per hectare) must be attained. Nitrogen is the key input required for rice production. Super high-yielding rice genotypes with potential grain yields of 13–15 t/ha require a nitrogen supply of about 400–700 kg/ha. Over the past two and a half decades, rice farmers have become increasingly dependent on chemical fertilizers as a source of nitrogen. However, spiraling increasing costs, limited availability and low-use efficiency demand an increasing nitrogen supply aided by microorganisms [75].

It is in this context that BNF-derived nitrogen assumes importance, because the submerged soils on which more than 85% of the world's rice is grown provide two of the most favorable conditions for BNF: namely, optimum oxygen tension and a constant and regular supply of carbon substrate. Diazotrophs can be broadly divided into two existing BNF systems: (1) those that supply exogenous BNF, such as phototrophic cyanobacteria in symbiosis with *Azolla*, and heterotrophic and phototrophic rhizobia in symbiosis with aquatic *Sesbania* and *Aeschynomene* species; (2) indigenous nitrogen-supplying diazotrophs, including heterotrophic-phototrophic bacteria or cyanobacteria in soil-plant-flood water [75].

Azoarcus is a slightly curved gram-negative, rod-shaped diazotroph isolated from the root interior of Kallar grass [76]. The cells fix nitrogen micro-aerobically, grow well on salts of organic acids, but not on carbohydrates, and on only a few amino acids. This bacterium is able to systematically infect roots of both Kallar grass and rice [77]. Evidence was obtained for the involvement of cell surface-bound glucanases in the infection process [77]. Nitrogen fixation by *Azoarcus* is extremely efficient (i.e., specific nitrogenase activity was one order of magnitude higher than values found for bacteroids). Such hyperinduced cells contain tubular assays of internal membrane stacks which can cover a large proportion of the intracellular volume. Because an *nifK* mutant is unable to induce such membrane stacks, it has been proposed that these structures are functional membranes related to highly efficient N₂-fixation [77].

2. Sugarcane

The sugarcane crop is one of most important crops in tropical regions of the world. Between 13 and 15 million ha of this crop are harvested worldwide, being principally used for the production of sucrose and a smaller fraction for alcohol production. In Brazil, with 4 million ha of this crop, about 68% of the total sugarcane harvested each year is used for the production of alcohol, the most important liquid biofuel used in this country. Apart from the adaptation of the sugarcane varieties to growth in mostly low-fertility soils in Brazil, the BNF associated with this crop plays an important role in its yield and the energy balance [78].

In Brazil, approximately 10 billion L of ethanol are produced annually. This permits the replacement of 200,000 barrels oil per day and, therefore, has a major influence on the economy of the country. The Brazilian alcohol program, which resulted in an increase in the sugarcane area from 1.5 million ha in 1975 to 4 million ha today, has not only created about 1 million new jobs, contributing to employment in the rural areas, but also this biofuel, used alone or mixed with gasoline, has eliminated the use of tetraethyl lead to raise the octane rating of normal gasoline, and reduced the emission of other polluting gases (hydrocarbons, NO_x and CO) and, hence, contributes to the reduction of environmental pollution [78].

Although sugarcane accumulates large quantities of nitrogen in its tissues (100–250 kg ha⁻¹ yr⁻¹), in Brazil the sugarcane crop rarely responds to nitrogen-fertilizer application, even when growing on soils with very low nitrogen availability, and other crops, such as maize, normally need considerable nitrogen fertilization. This observation stimulated researchers to investigate this phenomenon and results suggested that plant-associated BNF could be playing an important role in nitrogen nutrition of this crop. In 1975, from a study carried out using ¹⁵N₂, significant incorporation of labeled nitrogen by sugarcane plants was observed. Later experiments, set up under controlled conditions, applying both nitrogen balance and ¹⁵N-isotope dilution techniques, it was confirmed that the associative BNF can contribute nitrogen at more than 150 kg ha⁻¹ yr⁻¹, which can represent more than 60% of the total nitrogen accumulated by the plants, and that some varieties are more efficient than others [78]. A long-term experiment was carried out from 1983 to 1992 with the variety CB 45-3 under commercial plantation conditions. The total nitrogen balance of the soil-plant system indicated that the

BNF contribution to the crop was between 39 to 68 kg ha⁻¹ yr⁻¹, which represented up to 70% of the total nitrogen accumulated by the plants.

In the last decade, two new nitrogen-fixing genera were identified [79] and, because of their occurrence principally within plant tissues, they have been called *endophytes*, instead of endorhizosphere-associated bacteria, a term used until recently for root interior [79]. Diazotrophic endophytes have an enormous potential for use because of their ability to colonize the entire plant interior and locate themselves within niches protected from oxygen competition by most other bacteria or other factors so that their potential to fix nitrogen can be expressed at the maximum level. These properties may be the reason for the high nitrogen fixation observed in sugarcane plants [78]. Among the endophytic diazotrophs found associated with sugarcane are *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*. Studies on the infection and colonization process of sugarcane by these bacteria have been carried out [80]. In both cases, the xylem vessels were seen filled with these bacteria and nitrogenase promotion has been observed with antisera raised against the FeMoCo subunit of nitrogenase in leaves of sugarcane infected with *Herbaspirillum* spp. [81]. *A.diazotrophicus* has been found mainly associate with sugar-rich plants, such as sugarcane, sweet potato, and Cameroon grass, that propagate vegetatively. In addition, it was recently isolated from coffee plants in Mexico. The species *H.seropedicae* is much less restricted than *A.diazotrophicus* because it has been isolated from many other graminaceous plants, including oil palm trees and fruit plants [81] and seems to be transferred mainly through the seeds.

E. Colonization

To function as a biofertilizer or as a phytostimulator, a microbe must be present at the right site and the right time at the place of action. This process, called colonization, can be considered as the delivery system of the microbe's beneficial factor(s). In fact, lack of success with biocontrol bacteria is often correlated with lack of colonization [82,83]. Despite its importance, not only for the beneficial effects of bacteria, but also in pathogenic interactions, relatively little is known about the mechanism(s) behind colonization. De Weger et al. [84] showed that the presence of flagella is required for efficient potato root colonization by *Pseudomonas fluorescens* biocontrol strain WCS374. Although the role of flagella was not confirmed in all systems [85], they also play a crucial role in nodulation by *Rhizobium* [86,87]. The role of flagella in colonization may be due to their function in chemotaxis towards root exudate nutrients. Vande Broek and Vanderleyden [88] showed that *Azospirillum* mutants impaired in motility and chemotaxis exhibit a strongly reduced wheat root colonization ability.

The second factor shown to play a role in rhizosphere colonization of potato is the O-antigen of the bacterial cell surface component lipopolysaccharide (LPS) [89]. The mutants survive well at the site of inoculation, but were not recovered from the deeper root parts. Mutants lacking the O-antigen are still motile.

More recently, a gnotobiotic system was developed to screen random transposon mutants for their ability to colonize the 7-day-old tomato root tip after inoculation of germinated seedlings with a 1:1 mixture of one mutant and the parental strain *P.fluorescens* WCS365 at day 0. The results showed that mutants unable to produce amino acids or vitamin B₁ are defective in root tip colonization and, also, when applied alone. Apparently, the root produces insufficient amounts of these compounds to allow normal growth of the mutant cells [90]. Another factor that was correlated with efficient colonization was growth rate. Several poorly colonizing mutants appeared to grow more slowly, as tested in laboratory media, than the parental strain, suggesting a causal relation [90]. We are now analyzing the composition of the major tomato exudate compounds: sugars and organic acids. Subsequently, several mutants

defective in the utilization of one of these compounds will be tested for their colonizing properties to test whether, as expected, root colonization also depends on growth on major exudate carbon sources.

Interestingly, most of the mutants that appeared to be defective in tomato root colonization are nonmotile, lack the O-antigen of LPS, are poor growers, or are auxotrophic, confirming the results previously mentioned. However, also novel mutants were found [91]. Given their frequency, and assuming that the mutations are randomly distributed over the assumed 6000 *Pseudomonas* genes, we predict that at least approximately 35 so far unknown genes are involved in colonization. When we realize that screening in the gnotobiotic system does not allow one to screen for mutants in genes that are important for colonization, especially in the presence of biotic factors absent in the gnotobiotic system, it is evident that colonization is an extremely complex and poorly understood process.

We are planning to analyze the nucleotide sequence of the DNA regions flanking the transposon. Analysis of the first novel mutant showed that the transposon resides in a gene encoding a sensor kinase. Upstream from this gene we found a gene encoding a response regulator [92]. These genes show strong homology with genes of so-called two-component systems, which function in sensing of environmental signals and subsequent transduction of this message to the cell interior, finally resulting in activation of a certain process. We conclude that apparently *pseudomonads* sense a stimulus (from the plant?) which, through the two-component system, activates a bacterial trait that is crucial for colonization. It is clear that the analysis of the novel collection of 12 mutants will, in the near future, shed light on the molecular basis of the root colonization process.

Although their general applicability has not been demonstrated, the present data on the analysis of colonization-impaired mutants can be translated in simple guidelines for the inoculant industry: make sure that your (gram-negative) bacterium is motile, grows fast, also in minimal medium, and make sure that its LPS produces a ladder pattern on sodium dodecyl sulfate-polyacrylamide electrophoresis gels [93].

III CONCLUSIONS, FUTURE DIRECTIONS, AND PROSPECTS

A. Present Situation

Inoculation of crops with microbial fertilizers or phytostimulators has been successfully applied. The best example is (*Brady*)*rhizobium*, which has been sold for a century as an inoculant. The policy of many governments to reduce chemical input stimulates further research in and application of microbial products. A recent example is that the Dutch seed firm S & G Seeds BV sells radish seeds only in the form of seeds coated with *Pseudomonas* biocontrol bacteria (i.e., the product Biocoat). For inoculation to become more successful, several major bottlenecks have to be overcome.

1. Results in the field and, to a lesser extent, in greenhouses are not always consistent.
2. Inoculant microbes tend to loose competition with the indigenous microflora.
3. Shelf life can be a problem, especially for nonsporulating bacteria.

B. Application of Biotechnology to Improve Microbial Inoculants

The major bottlenecks for the successful application of inoculant microbes is our poor understanding of which bacterial traits are involved in the beneficial effects of inoculant

bacteria, and how these traits are influenced by environmental factors. In the following we indicate several topics that require further elucidation.

1. Molecular Rhizosphere Physiology

Molecular biologists understand in detail how bacteria grow under laboratory conditions. The challenges for the next decades is to understand how microbes grow and survive *in situ*. What factors are limiting growth in the rhizosphere (e.g., nutrient limitation, toxic products)? What is the influence of abiotic factors, such as temperature and drought? Which genes are specifically expressed under rhizosphere conditions? Which of these have essential functions in growth and survival? Can these genes be used to increase the rate of appearance of bacterial beneficial activity immediately after rehydration of planted seeds or for increasing the shelf life of coated seeds?

2. Colonization and Beneficial Traits

For optimization of the success of inoculation it is essential to understand which traits are involved in these processes and how their expression is influenced by environmental factors. For example, of the *Pseudomonas* two-component system, which plays a role in colonization (see Sec. II. E) it is crucial to identify the stimulus, to identify the colonization trait that is finally activated by this stimulus, and how this process can be influenced by environmental conditions.

3. Competition of Inoculant Microbes with Indigenous Biotic Factors

Several results indicate that the success of inoculation severely suffers from competition of inoculant microbes by biotic factors. Soybean nodules resulting from seeds treated with inoculant *Bradyrhizobium* are, in only 5–20% of the cases, occupied by inoculant bacteria. The colonization of wheat roots by *P. fluorescens* strain WCS365 is over 100-fold inhibited by field soil in comparison with the use of X-ray-irradiated field soil [94]. It is important to identify the factors responsible for the latter competitive effect. If bacteria appear to play a role, is this then due to (an) individual strain(s), due to a consortium of strains, or due to the recent growth history of the bacteria?

4. Better Inoculant Strains

Once the mechanisms of action of beneficial bacteria are known, screening specifically aimed at such a mechanism can be developed. Similarly, once we understand more of the influence of environmental factors, we can screen for better strains by choosing better sites for sampling the bacteria. Considering that only about 1% of the soil bacteria can be cultivated, progress can also be expected in the cultivation of bacteria that so far could not be cultivated, and the subsequent screening for beneficial isolates. The performance of inoculant bacteria can be improved by genetic modification (e.g., the *R. meliloti* strains mentioned under Sec. II.B.1.b). Because the application of recombinant DNA microbes is subject of extreme scrutiny, one can wonder whether large-scale application of genetically modified microbes is a feasible option.

5. Endophytes

Some bacteria exert their beneficial effect inside the plant (e.g., *Rhizobium* and *Azoarcus*). Up to the moment of internalization, endophytes are subject to the same competitive factors

as other bacteria. However, once inside, the competition from other bacteria is absent or strongly decreased. Therefore, the use of endophytes for inoculation may be advantageous. However, knowledge of which traits are required to make a bacterium an endophyte is completely lacking.

6. Environmental Factors

An understanding of these factors is of crucial importance. Fundamental research can reveal some of the important factors, as illustrated by the following example. In cases where sugarcane plants respond to nitrogen fertilizer application, the same response has sometimes been observed by substituting the nitrogen fertilizer by molybdenum application, which may be because this micronutrient is essential for the synthesis and activity of nitrogenase.

C. Application of Biotechnology to Create Novel Combinations of Crops and Beneficial Microbes

During the last two decades many new nitrogen-fixing bacteria have been isolated and identified, including species of the genera *Azospirillum*, *Herbaspirillum*, *Acetobacter*, and *Azoarcus* [95]. The greater part of these diazotrophs have been isolated from tropical regions, especially in Brazil, and they have been the main source for groups in the world working with associative diazotrophs. Other associative nitrogen-fixing bacteria have been identified, but probably because of their low number, or restricted occurrence, they are not well explored [75]. The interest in the association of diazotrophs with graminaceous plants reinforces the importance of the biological nitrogen fixation process to sustainable agriculture systems where low inputs of nitrogen fertilizers are desirable.

Rice suffers from the mismatch of its nitrogen demand and its nitrogen supply. Fertilizer nitrogen in flooded rice soil is highly prone to loss through ammonia volatilization. The possibility of nitrogen-fixing rice has been discussed for a long time. An old dream is to engineer rice to establish a symbiosis with *Rhizobium*. Alternatively, DNA fragments encoding the *nif* and *fix* genes should be transferred to and expressed in rice in such a way that the oxygensensitive nitrogenase complex would be protected from oxygen. Now that genes encoding nodulins and Nod factors and their host specificity are available, such an approach could be attempted. We believe, however, that this would be technically too complex. Moreover, it is very likely that we do not know all factors required to create a nodulating rice plant. Rather, the recent success obtained with *Azoarcus* in nitrogen fertilization of rice makes the latter approach a much more promising alternative.

Herbaspirillum rubrisubalbicans and *Burkholderia* spp. are also nitrogen-fixing endophytic bacteria found in association with sugarcane, cereals, and other plants of agronomic importance. *H.rubrisubalbicans* was recently indentified among *Pseudomonas rubrisubalbicans* strains, a species considered a mild phytopathogenic agent caused mottle stripe disease in some susceptible varieties of sugarcane grown in countries other than Brazil [79]. Although it was thought that *H.rubrisubalbicans* would be restricted to sugarcane, it was recently isolated from rice plants and fruit plants. *Burkholderia*, a novel nitrogen-fixing bacterium, has been isolated from several plants, including sugarcane, sweet potato, cassava, cereals, and more recently, fruit plants. The role of these two new endophytic nitrogen-fixing bacteria in their associations with plants is not yet known, although they may exercise the same functions as the other endophytes.

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Lignin-Degrading Fungi: Mechanisms and Utilization

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I. INTRODUCTION

Lignocelluloses are defined as plant or wood cell walls in which cellulose is intimately associated with lignin. In addition to these compounds, lignocellulose contains other polysaccharides, commonly called hemicelluloses. Lignocellulosic waste is that portion of the entire plant kingdom that is not properly employed for the welfare of human beings—mainly crop residues and wood and forestry wastes. The general chemical composition of these two groups differs basically in its cellulose and lignin portions. Crop residues contain 30–45% cellulose and 3–13% lignin. Wood residues contain higher amounts of both cellulose (45–56%) and lignin (18–30%) [1–3].

Lignocellulosic waste abounds in most parts of the world. The net productivity of dry biomass owing to photosynthesis by all plants on earth is estimated to be close to 150×10^9 tons/yr, of which 11% is represented by the primary production of materials with food or feed properties. Wastes of various kinds (e.g., inedible plant parts, wastes generated during the harvesting season, wastes during handling and processing), suitable for consideration as sources of raw materials, may amount to more than 13×10^9 tons/year. In Europe, for example, the amount of unexploited lignocellulosic by-products is immense. Cereal straw, the major byproduct, shows a yearly surplus of 24×10^6 tons [4]. Most of the straw is either burned in the field, creating environmental problems, or wasted by being ploughed back into the soil. Additional amounts of ligninous wastes are produced by the paper industry.

Lignin degradation by fungi or their specific enzymes has been the focus of numerous biotechnological studies [5,6]. The fungal kingdom constitutes a promising group of microorganisms for use in upgrading lignocelluloses and transforming recalcitrant xenobiotic compounds. Different fungal species can either modify or completely degrade all the major components of wood. Fungi that only modify the lignin and utilize the exposed polysaccharides are named *brown-rot* fungi, based on the appearance of the degraded substrate. Wood-rotting

basidiomycetes that completely mineralize lignin are called *white-rot* fungi, and they are considered to be the most efficient lignin degraders in nature [1].

II. CLASSIFICATION OF WHITE-ROT FUNGI

Lignin biodegradation by white-rot fungi is an oxidative process, probably involving enzymes such as lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases [1,7–10]. This process has been studied extensively over the past two decades. In the 1970s the main focus was to define laboratory conditions under which white-rot fungi, and especially *Phanerochaete chrysosporium*, maximally mineralize lignin. In the 1980s, the biochemistry of lignin-modifying enzymes was predominantly studied, owing to the discovery of ligninolytic peroxidases. In 1983 and 1984, two extracellular enzymes, LiP and MnP, were discovered in *P.chrysosporium* [11–13]. They were the major components of the lignin degradation system in this organism. The discovery of the enzymes involved in lignin degradation by *P.chrysosporium* led to their biochemical, biophysical, and physiological characterization. This work has served as an indispensable background for studies on lignin degradation by other fungi [9]. In the 1990s, in addition to detailed studies on the molecular biology of the lignin-modifying peroxidases, major lines of research have involved applications of enzymes in biopulping and pulp bleaching and a search for the enzymes responsible for lignin degradation in more preferential lignin degraders (i.e., fungi that degrade larger amounts of lignin relative to carbohydrates). This has led to a reassessment of the biotechnical potential of white-rot fungi other than *P.chrysosporium* and investigations of their ligninolytic enzyme systems [8]. Many white-rot fungi, with the notable exception of *P.chrysosporium*, produce an extracellular laccase [1,5,7,14]. Another essential component of the ligninolytic system was first identified in 1987 [15], and is composed of extracellular H₂O₂-producing enzymes, such as glyoxal oxidase [15–17] and arylalcohol oxidases (AAO) [18–21]. An array of ligninolytic enzymes have been isolated and are currently under extensive study. White-rot fungi can be divided, according to their typical production patterns of the more common extracellular enzymes, into three main groups [22]:

A. Lignin-Manganese Peroxidase Group

Phanerochaete chrysosporium, which is the most studied white-rot fungus, is a typical representative of the LiP-MnP group. *P.chrysosporium* is a very efficient lignin degrader, and some strains possess industrially appreciated properties, such as selective lignin degradation in biopulping [23]. The usual way to determine lignin-degrading ability (i.e., cultivation of fungi in an oxygen atmosphere and measurement of ¹⁴CO₂ evolution from [¹⁴C]-synthetic lignin) favors fungi that produce LiP. The ability of many fungi in the family Corticiaceae (*Phanerochaete*, *Phlebia*) to readily produce LiP and MnP makes these fungi superior lignin degraders under laboratory conditions. Some of these fungi are also very efficient white rotters in nature. The presence of a LiP gene in a fungus is the only reliable proof of the presence of LiP [8]. To date, molecular analyses of LiP genes have been carried out in *P. chrysosporium* (several groups, for a review see [9]), *Phlebia radiata* [24], *Coriolus (Trametes) versicolor* [25], and *Bjerkandera adusta* [26], among others.

B. Laccase-Manganese Peroxidase Group

Several fungi that are efficient lignin degraders in nature and especially suitable for preferential lignin degradation, apparently do not produce LiP. The MnP-laccase combination seems to be most common among wood-inhabiting fungi [27] and especially white-rot fungi. Recent

studies have shown that *Panus tigrinus* (Bull. ex. Fr.) Sing (syn. *Lentinus tigrinus* Bull. ex. Fr. (Fr.) [27], *Dichomitus squalens* (Karst.) Reid (syn. *Polyporus anceps* Peck) [28], and *Rigidosporus lignosus* (K1) Imaz. [29], among others, produce MnP and laccase but not LiP. *Phlebia breviospora* Nakas. and *Ceriporiopsis subvermispora* [30] also represent white-rot fungi that produce only MnP and laccase, but not LiP. However, in these two fungi, LiP-like genes have been found [30]. Edible fungi, such as the oyster mushroom *Pleurotus ostreatus*, and others may possess yet another ligninolytic system involving laccase and AAO.

C. Lignin Peroxidase-Laccase Group

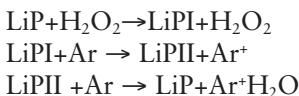
Only two fungi have been described to secrete LiP and laccase, but not MnP, even under elevated Mn(II) concentrations [31,32]. However, these fungi degrade DHP slowly. The slow rate of degradation suggests that fungi that lack MnP activity may be rather inefficient degraders of lignin to CO₂ [8].

III. MAJOR LIGNINOLYTIC ENZYMES: PROPERTIES, CATALYTIC MECHANISMS, EXPRESSION, AND APPLICATIONS

A. Lignin Peroxidase

Lignin peroxidase has been purified by a combination of anion-exchange chromatography, gel filtration, fast protein liquid chromatography, and isoelectric focusing [9,33,34]. The enzyme is present as a series of glycosylated isozymes with pIs ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa. Each isozyme contains 1 mol of iron heme per mole of protein [9,33,34].

LiP catalyzes the H₂O₂-dependent oxidation of a wide variety of nonphenolic lignin model compounds, synthetic lignins, and aromatic pollutants [5,7,35–39]. Its ability to oxidize aromatic compounds with redox potentials beyond the reach of horseradish peroxidase (HRP) and many other peroxidases is unique. The catalytic cycle of LiP is similar to that of HRP [40–42]. The primary reaction product of LiP with H₂O₂ is the two-electron-oxidized state compound I, LiPI. As with HRP, LiPI is reduced back to the native enzyme by two singleelectron steps with compound II, LiPII, as an intermediate. In the process, an aromatic reducing substrate is oxidized to an aryl cation radical (Ar⁺). These cation radicals may then decompose chemically [1,5]:



Multiple LiP isozymes of *P. chrysosporium* are encoded by families of structurally similar genes that exhibit complex patterns of regulation, all of which cross-hybridize to various extents [43–45]. The LiP genes of *P.chrysosporium* are differentially expressed according to the conditions encountered during growth. The existence of allelic variants has complicated the identification of LiP genes, but analysis of single basidiospore cultures has allowed alleles to be differentiated from closely related genes. Relatively little attention has been paid to the ascomycete-like imperfect fungi and actinomycetes, which are very abundant in lignin-rich environments. However, several lignin-depolymerizing and LiP-producing actinomycetes have been described [46,47]. The cloning and expression of a LiP gene from *Streptomyces viridosporus* in *S.lividans* [47] and the effects of this recombinant on biochemical cycling and ecomicrobiology in the soil were studied [48].

The LiP production by white-rot fungi and its regulation by carbon-nitrogen and oxygen levels have been extensively studied over the last decade [11,7,9,33,49–53]. The production of LiP in shaken cultures is enhanced by veratryl alcohol [54]. This observation was followed by a report that immobilized *P.chrysosporium* can be efficient in producing extracellular LiP [55]. High yields of LiP were obtained with nylon web [49,55], polyurethane foam [56], and porous poly(styrene-divinylbenzene) [57] as carriers.

Despite much detailed work on the reaction mechanisms of LiPs, there is little evidence that they can actually cleave polymeric lignin. Experiments with low molecular weight lignin model compounds provide strong support for a ligninolytic role, but cannot prove it. Investigations with lignin itself as an LiP substrate have, with the exception of some preliminary data, failed to show ligninolysis, and some researchers have concluded that LiPs do not cleave lignin in vivo [58]. On the other hand, the oxidation of ¹⁴C- and ¹³C-labeled lignins by a purified isozyme of *P.chrysosporium* has been described [59]. It was suggested that LiP catalyzes the initial steps of ligninolysis by *P.chrysosporium* in vivo, but LiP alone is not sufficient for lignin mineralization [59,60]. It was concluded that H₂O₂ and veratryl alcohol concentrations in the medium, as well as O₂ concentration, are all obligatory in LiP-catalyzed ligninolysis. LiP does not appear to be involved in the bleaching of kraft pulp by *Coriolus versicolor*. The enzyme is not detected during bleaching [61], nor is fungal bleaching enhanced by the addition of exogenous enzyme [62]. The difficulties linked particularly to the definition of optimal conditions for the in vitro enzymatic depolymerization of lignin are increased by the relative instability of the enzyme. Indeed, LiP is rapidly inactivated by excess H₂O₂ [9] and low pH [63], which conflicts with the facts that LiP activity requires H₂O₂ and increases with decreasing pH. It is usually accepted that one of the powerful tools for enzyme stabilization is immobilization. Immobilization of LiP by covalent coupling on CNBr-Sepharose 4B seriously affected catalytic parameters, but provided noticeable stabilization of the enzyme against acidic pH and high temperatures [64].

B. Manganese Peroxidase

Magnesium peroxidase has also been purified to electrophoretic homogeneity [9,65]. The enzyme exists as a series of glycosylated isozymes with pIs ranging from 4.2 to 4.9 and molecular masses ranging from 45 to 47 kDa. Similar to LiP, each isozyme contains 1 mole of iron per mole of protein [34,42].

MnP catalyzes the H₂O₂-dependent oxidation of lignin and lignin derivatives [60]. The oxidation of lignin and other phenols by MnP is dependent on free a manganous ion [34,42]. The primary reducing substrate in the MnP catalytic cycle is Mn(II), which efficiently reduces both compound I (MnPI) and compound II (MnPII), generating Mn(III), which subsequently oxidizes the organic substrate. Organic acids, such as oxalate and malonate, which are secreted by *P.chrysosporium* [66], stimulate the MnP reaction by stabilizing the Mn(III) so that it can diffuse from the surface of the enzyme and oxidize the insoluble terminal substrate, lignin [66,67]. The Mn ion participates in the reaction as a diffusible redox couple, rather than as an enzyme activator [67,68].

Similar to LiP, the MnP isozymes are encoded by multiple genes [69]. The first *mnp* cDNA (*mnp1*), from strain OGC101, was cloned by using polyclonal antibodies raised against purified MnPI [70]. The expression of MnP in white-rot fungi is regulated by nutrient nitrogen, H₂O₂, chemical stress, and molecular oxygen (at the level of gene transcription) [28,70–72]. MnP activity is dependent on the presence of Mn(II) in the culture medium [60,70–74].

MnP has been detected and isolated from pulp-bleaching cultures of *P.chrysosporium*, *P.sordida*, and *C.versicolor* [62,75,76]. Enzyme production depended on the density of conidial

inoculation, a factor which, in turn, determined the shape of the pellets. Decolorization and MnP production were obtained only when the fungus was grown in the form of a fluffy pelleted material [77]. The hyperproduction of MnP and LiP through the control of fungal morphology, associated with high shear forces, oxygen transfer, and the presence of proteases in the extracellular medium, has been studied [73,78].

Although in vitro experiments with purified MnP and LiP have shown that only MnP has decolorizing activity, this was limited to about 26%. Full in vivo decolorization, which reaches over 80%, therefore, may depend on the production of other enzyme components by the fungus [77]. MnP, produced by *C. versicolor* during pulp bleaching, demethylated and delignified kraft pulp [79]. In vitro bleaching of an unbleached hardwood kraft pulp was performed with MnP from the fungus *P. sordida* YK-624 [75]. When the kraft pulp was treated with partially purified MnP in the presence of MnSO₄, Tween 80, and sodium malonate with continuous addition of H₂O₂ at 37°C for 24 h, pulp brightness increased by about 10 points and the kappa number decreased by about 6 points as compared with untreated pulp. Pulp brightness was also increased by 43 points to 75.5% by multiple (six) treatments with MnP combined with alkaline extraction. These results indicate that in vitro degradation of residual lignin in hardwood kraft pulp with MnP is possible. In a recent work, MnP supported the slow oxidation of phenanthrene to 2,2'-diphenic acid in a reaction that required Mn(II), oxygen, and unsaturated lipids. Phenanthrene oxidation to diphenic acid by intact cultures of *P. chrysosporium* occurred to the same extent as oxidation in vitro and was stimulated by Mn. These results support a role for MnP-mediated lipid peroxidation in phenanthrene oxidation by *P. chrysosporium* [80].

C. Laccase

Laccase is a multiple enzyme, produced by a large number of fungi [81–83]. The enzyme occurs in both inducible and constitutive forms, with possible differences in activity [84]. The most studied laccases appear to be from *Agaricus bisporus*, *Podospora anserina*, *Rhizoctonia praticola*, *Coriolus (Trametes) versicolor*, *C. hiristus*, *P. ostreatus*, and *Neurospora crassa* [82,83,85,86]. The biological functions of these enzymes are still unclear; they have been implicated in different processes. Several fungal laccases have been purified and characterized [87–89].

Laccase is a multicopper blue oxidase that catalyzes the one-electron oxidation of *ortho*- and *para*-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical [5]. It can catalyze the alkyl-phenyl and Cα-Cβ cleavage of phenolic lignin dimers [5]. It also catalyzes the demethoxylation of several lignin model compounds [1,7]. Its oxidation activity is accompanied by the reduction of molecular oxygen to water. An artificial laccase substrate S 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) acts as a mediator, enabling the oxidation of nonphenolic lignin model compounds that are not laccase substrates on their own [90].

During recent years, laccase gene or cDNA sequences have been described from several sources, of which the first to be reported was the ascomycete fungus *N. crassa* [91]. Subsequently, the sequences from another ascomycete, *Aspergillus nidulans* [92], *Cryptococcus neoformans* [93], and the lignin-degrading basidiomycetes *C. hirsutus* [94], *P. radiata* [95], *A. bisporus* [96], and PM1 [88] were described.

Laccase activity in fungal cultures can be increased by the addition of different aromatic compounds to the media [81]. Platt et al. [97] reported increased laccase production by *P. ostreatus* after supplementing various phenolic substrates to the fungal cultures. Plant extracts also promote extracellular laccase activity of *Pleurotus* spp [98–101]. Different

laccase-producing fungi excrete different forms of laccase owing to supplementation of aromatic compounds, such as toluidine, vanillic acid, *p*-hydroxybenzoic acid, and aniline to the fungal media [102,103].

Isolated laccase 1 from *T. versicolor* was used to demethylate pulp and to reduce its kappa number [104]. The possibility of using immobilized laccase for the enzymatic removal of phenolic compounds from must and wine has been tested on a laboratory scale [105]. Laccase was covalently immobilized by activated carbon for industrial phenolic effluent treatment [106]. Other applications of laccases have been recently reviewed [82].

IV. POTENTIAL APPLICATIONS OF LIGNIN-DEGRADING FUNGI

A. Upgrading Wastes

Lignocellulosic residues are not high-value feeds. However, the potential to transform these wastes via solid-state fermentation (SSF) into valuable products is remarkable. SSF involves the growth of microorganisms on moist solid substrate in the absence of free-flowing water. The necessary moisture in SSF exists in an absorbed or complexed form within the solid matrix, which is likely to be more advantageous because of the possible efficiency of the oxygen transfer process. In SSF the water content is very low and the microorganism is in contact with gaseous oxygen in the air [107]. The direct use of lignocellulosic residue as animal feed, or as a component of such feeds, represents one of its oldest and most widespread applications and, as such, it plays an important role in ruminant diets [108]. Several authors have examined this possibility under different culture conditions and substrate pretreatments [109–114]. The ability of 45 fungal strains to degrade wheat straw and beechwood was studied [115]. Degradation patterns were defined in terms of chemical evolution of substrates and changes in lignin and polysaccharides. *C. versicolor* exhibited marked lignin degradation and increased substrate digestibility, but it caused high weight losses and gave rise to similar decay patterns on both substrates. Preferential degradation of lignin was produced during straw transformation by *Pleurotus eryngii* [115]. A significant increase in *in vitro* dry-matter digestibility of wheat straw by *Pleurotus* was reported [109]. Lignin content was decreased by 51% during the incubation period (90 days). Similar results were reported by Martinez et al. [116,117] and Zadrazil [118], the latter showing an increase of 23–32% in *in vitro* digestibility following fermentation by *P. eryngii*, *sajor-caju*, *P. ostreatus*, and *P. serotinus*. Lignocellulose degradation and activities related to lignin degradation were studied in the SSF of cotton stalks by comparing two white-rot fungi, *P. ostreatus* and *P. chrysosporium* [14]. *P. chrysosporium* grew vigorously, resulting in rapid, nonselective degradation of 55% of the organic components of the cotton stalks within 15 days. In contrast, *P. ostreatus* grew more slowly, with obvious selectivity for lignin degradation, resulting in the degradation of only 20% of the organic matter after 30 days of incubation. Preferential degradation of lignin was observed for other *Pleurotus* species, grown on wheat straw [116,117]. Lignin degradation by *P. ostreatus* was studied under SSF in chemically defined medium [119] containing various levels of Mn. Degradation of [¹⁴C]-lignin prepared from cotton branches to soluble products, as well as its mineralization to [¹⁴CO₂], were enhanced by the addition of Mn. The effect of malonate of lignin mineralization was most marked during the first 10 days of SSF, in a treatment with 73 µM Mn [120]. Preferential degradation of lignin was also enhanced by the addition of Mn(II) to cotton stalks at an Mn concentration of 30–620 µg/g. This effect was most apparent when comparing mineralization rates of [¹⁴C]-lignin with those of [¹⁴C]-cellulose. Enhancement of selectivity was also demonstrated by analyzing the residual

organic matter at the end of the fermentation period using crude fiber analysis. The cellulose fraction in the original matter was 1.8 times higher than lignin. This ratio (cellulose/lignin) increased during the 32 days of SSF, from 2.5 in the control to 3.3 following the addition of Mn to the medium. In vitro digestibility of fermented cotton stalks was 53% of the dry matter. Amendments of Mn to the cotton stalks resulted in digestibility of 65.4% of the dry matter with 620 µg Mn. Enhancement of selective lignin degradation could be the result of either increasing the activities of ligninolytic enzymes or producing Mn(III), which might preferentially degrade aromatic structures in the lignocellulosic complex. The influence of the growth of two white-rot fungi, *P. ostreatus* and *Lentinus edodes*, on the composition and digestibility of corn straw (*Zea mays*) was evaluated [121]. *P. ostreatus* and *L. edodes* showed different patterns of extracellular enzyme release, which included phenol oxidases, cellulase, and xylanases. The best results in terms of delignification and increases in digestibility were obtained in *L. edodes* cultures. When the two fungi were grown under submerged fermentation in the presence of a water-soluble lignin-rich copolymer, a crude filtrate containing phenol oxidases but no cellulolytic enzymes was obtained.

B. Biochemical Pulping with White-Rot Fungi

Pulp-and paper-making technologies undergo constant improvements because of market demands and new developments in research [23,62]. Pulp production by means of mechanical and chemical processes and bleaching treatments is an energy-demanding process that causes environmental pollution. The need for sustainable technologies has brought biotechnology into the realm of pulp- and paper-making. Suitable biological pretreatment, defined biopulping and biobleaching, in conjunction with less intensive conventional treatments could help solve many of the problems of currently used processes. Xylanases are already used in pulp mills to facilitate subsequent chemical bleaching. However, with xylanases, the biological oxygen demand (BOD) of the wastewaters is high, and the coloring of the water by the solubilized lignin becomes a serious concern. The biotechnological approach using ligninolytic enzymes as a pulping reagent resulted in easier pulping, with less consumption of chemicals and energy. However, to develop an industrial process with the aid of a biological approach, it is necessary to optimize uniform degradation of the biomass to achieve high reproducibility of the results, an incubation period suitable for industrial processes, and a biodegradation efficiency and selectivity for lignins and cellulose. The incubation period required with white-rot fungi is often more than 2 weeks [122]. Works in this field are aimed at elucidating the effects of fungal species and growth conditions on the biopulping process [23,62,116,123,124].

Because there has been renewed interest in annual plants that are used to produce cellulose for pulp, and wheat straw is an abundant waste material for the near future that has to be disposed of in some way, its suitability as a source of biopulp for paper-making was studied using nine different fungi [124]. Corn straw [121], treated with crude filtrate of *P. oestreatus* and *L. edodes* and subjected to paper-pulping, produced paper handsheets with improved technical properties (freeness, breaking length, and burst index). Wheat straw, treated with an exoenzyme mixture obtained from the SSF of straw by *L. edodes*, resulted in an energy savings of 50% for the refining treatment of the pulp and in better paper qualities [124].

C. Mushroom Production

Several of the white-rot fungi that can utilize lignocellulose are edible mushrooms [125]. These saprophytic basidiomycetes have been successfully cultivated at a commercial level worldwide using lignocellulosic wastes as the main substrate for their cultivation [126]. *A. bisporus*, known as the “button mushroom,” *L. edodes*, and *P. ostreatus*, known as the

"oyster mushroom," are only three examples of this agricultural-biotechnological crop [127–129]. This review focuses on the edible mushrooms of the genus *Pleurotus* and their potential to utilize and upgrade lignocellulosic wastes during SSF. *Pleurotus* spp. are widely distributed and cultivated in many countries throughout the world. *Pleurotus* cultivation is gaining popularity in Europe, America, and the Far East, and from the standpoint of annual production, it has become the third most important mushroom [108]. The substrate is usually partially shredded, mixed with water (up to 70%) and placed in containers, such as bags, trays, or frames. However, unlike *A. bisporus*, no composting or casing layer is required. Because *P. ostreatus* is a wood-degrading fungus, it was first cultivated on logs [130]. Today it has become common practice to prepare *Pleurotus* substrate from shredded wheat straw, which can be supplemented with protein-rich materials, such as alfalfa meal or soybean flour. Because *Pleurotus* spp. can decompose lignocellulose efficiently without chemical or biological pretreatment, a large variety of lignocellulosic wastes can be used and recycled. Some examples of the agricultural wastes studied as substrates for *Pleurotus* spp. have been recently reviewed [108].

D. Bioremediation

The need to remove toxic pollutants from both terrestrial and aquatic ecosystems has heightened interest in biotechnological approaches to degrading aromatic organohalides from contaminated environments, especially *in situ*. Several cases of environmental contamination have been well documented [131]. Degradation of such compounds by nonligninolytic microorganisms occurs intracellularly, and the limited bioavailability of the compounds is a key factor in the slow rate of biodegradation by these organisms. Ligninolytic enzymes and mediators are active extracellularly, thus white-rot fungi may serve as better candidates for the bioremediation of highly apolar pollutants, as compared with nonligninolytic microorganisms. The best-studied organism in this group is *P.chrysosporium* [9,132,133]. Many reports [9,134–139] have shown that the ligninolytic system of *P.chrysosporium* is actively involved in the degradation of recalcitrant aromatic pollutants. Common intracellular systems, such as that of cytochrome P450 monooxygenases, appear to be involved in phenanthrene degradation by *P.chrysosporium* [140]. However, intermediates resulting from phenanthrene degradation in actively ligninolytic cultures cannot be attributed to the activity of monooxygenases [37]. *C. versicolor*, *Bjerkandera* spp. and *P. ostreatus* may be more promising than *P.chrysosporium* in their ability to degrade and mineralize toxic chemicals and polycyclic aromatic hydrocarbons (PAHs) [141–143]. Some examples are the degradation of atrazine by *P.pulmonarius* [144] and mineralization to $^{14}\text{CO}_2$ of [^{14}C]catechol, [^{14}C]phenanthren, [^{14}C]pyrene, and [^{14}C]benzo[a]pyrene by *P. ostreatus* [145]. Several papers describing field evaluations of other lignin-degrading fungi for the bioremediation of soils, and the degradation of aromatic pollutants have been recently published [141,146–150]. However, the pathways and mechanisms of degradation and the identity of the enzymes involved have not been elucidated.

Some hazardous organic compounds are soil contaminants, so methods for using these fungi to decontaminate soil through either land farming or composting technologies are being developed [151].

VI. CONCLUDING REMARKS

Large-scale practical implementation of the potential of biotechnology based on the unique properties and enzymatic system of the white-rot fungi is currently limited. One exception is the traditional cultivation of edible mushroom. Other areas, covered here and in other recent

reviews [10,133,139,152], could be of enormous benefit to various industries and to the environment. To promote the utilization of white-rot-based technologies, two approaches may be taken:

1. Such process could be used to replace those that are now performed chemically or physically, but with greater selectivity and specificity, lower energy requirements, or other benefits specific to the process; these may include decreased generation of pollutants, reduced cost for facility construction, and others.
2. Novel processes could be developed that would solve problems that are not covered by conventional or economical treatments.

Examples of the first category can be found mainly in the pulp and paper industry and of the second in in situ remediation and in the biosynthesis of unique biochemicals. The ubiquitous and important role of the ligninolytic system in natural habitats as well as the huge efforts in the exploitation of its biotechnological potential holds space for optimism.

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Bioremediation: Pesticides and Other Agricultural Chemicals

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I. INTRODUCTION: TERRESTRIAL CARBON CYCLE

Bioremediation is increasingly being considered as a less-expensive alternative to physical and chemical means of degradation of organic pollutants [1,2]. It deals, by definition, with substances that are anthropogenic, distributed in nature, and recalcitrant (i.e., they are not degraded within short times in the soil). Since several reviews on single aspects and the techniques as a whole [e.g., 3-7] have appeared in print during the recent years, we will concentrate on a general understanding of the mechanisms by which bioremediation operates.

The ultimate model for cycling of the carbon moiety of chemicals in nature is the terrestrial carbon cycle: Carbon dioxide and water are used for the synthesis of biomass which, in turn, is mineralized by microorganisms and animals to carbon dioxide and water again, the whole cycle being driven by energy from sunlight. The main compartments of the terrestrial carbon cycle are [8]

Carbon dioxide in the air: 720 billion tons (t) of carbon

Aboveground biomass (plants and animals): 1,000 billion t of carbon

Soil organic matter: 1,700 billion t of carbon

The annual rate of biomass production is 120 billion t of carbon, the annual mineralization of biomass and soil organic matter is the same, about 60 billion t of carbon coming from the aboveground biomass, and the same amount of carbon is mineralized in the soil. The terrestrial cycle is in equilibrium with the marine carbon cycle, with no net loss or gain on each side, and the amounts of carbon fossilized from terrestrial ecosystems are negligible. This means that we have a closed cycle in which net biomass production is completely balanced by net carbon mineralization. Each compound that has been introduced into this cycle by any organism obviously must have been eventually converted to carbon dioxide and water, otherwise the cycle would have come to an end, owing to its rather rapid turnover (30 years) compared with the geological time since when it is in existence (350 million years).

In the recent history, man-made chemicals, mainly pesticides, have been introduced into this cycle, which apparently are *recalcitrant*; that is they are not compatible with the terrestrial carbon cycle and accumulate in soils and food chains and pose extreme environmental problems (see, for instance, the DDT-story [9]). It is obvious that the terrestrial carbon cycle cannot cope with these new chemicals, although it is able to mineralize millions of different organic compounds. Therefore, we have to look carefully into the mechanisms, by which this cycle operates. This, in the end, will give us means for both remediation of recalcitrant chemicals already present in our environment and help us design chemicals that are more compatible with the cycle altogether.

II. BIOCHEMICAL MECHANISMS RESPONSIBLE FOR MINERALIZATION IN THE CARBON CYCLE

A. Intracellular Reactions That Conserve Energy: "Classic Biochemistry"

The biochemical strategy that a cell uses for the mineralization of organic compounds, with the ultimate goal to convert as much of its energy to a usable form (i.e., ATP), can best be demonstrated by the digestion and finally mineralization of starch, the main staple food of human beings. Starch is a polymer composed of several thousand molecules of glucose that are linked together by an ether bond. The first and chemically rather simple step is the hydrolysis of the ether bond, using water as the splitting agent. This yields the monomer glucose, which is composed of six carbon atoms. To get from this molecule to carbon dioxide, which has only one carbon atom, obviously five carbon-carbon bonds have to be cleaved. Carbon-carbon bonds are rather stable and the repertoire of possible chemical reactions of the cell, compared with that of an organic chemist, is rather limited: the cell uses water as a solvent and pH values between 4 and 8. In addition the energy changes accompanying the reactions in the cell must be within certain boundaries, otherwise the enthalpy of the reaction would injure the cell structure. This limits the cellular chemistry to aldol reactions, Claisen-ester condensations, and decarboxylation reactions of β -ketoacids. In these reactions, nucleophilic carbanions react with electrophilic C atoms, usually the sp^2 -hybridized carbonyl carbons of ketones and aldehydes [10]. Because of these restrictions, the conversion of even moderately complex chemicals such as glucose to CO_2 , water, and energy needs about 30 single reactions, each of which is catalyzed by a different enzyme [10]. This strategy of small steps yields the maximal available energy from the metabolized molecule for the cell. However, it has the disadvantage that it needs highly specific catalysts (i.e., enzymes) that are made at a very high cost.

It is obvious that this strategy of mineralization of organic compounds is profitable for cells only if the compound in question is present at a sufficient high concentration, otherwise the costs of supplying the catalysts would be higher than the gains from metabolizing the substrate. The textbooks on molecular genetics provide deep insight into the mechanisms of how the cells make their enzymes only when they are needed, otherwise the information on how to make these enzymes is not activated.

It is easy to deduce that this method for mineralization of organic compounds in a certain organism must be restricted to relatively few compounds that occur with a certain reliability in its ecological niche, otherwise even the costs of maintaining the information for making the catalysts in the cellular DNA will become too high. In addition, the catalysts in use are so specific that they react at a reasonable rate only with one single compound. These two considerations mean that a given population of (micro)-organisms usually are limited in their metabolic activity to a certain spectrum of organic chemicals. Any additional compound will

not be mineralized by this type of metabolic strategy unless either new organisms are introduced into the system, or the enzyme spectrum of the organisms is changed by mutation.

B. Radical Reactions

An alternative metabolic pathway for the synthesis and degradation of molecules has been developed in nature for lignin, the substance that gives the lignified plant cell wall its pressure stability and represents quantitatively the second most important molecule in the biosphere (Fig. 1). The molecule contains no bonds that can be cleaved hydrolytically under conditions prevalent in cells. The strategy for its degradation, therefore, is based on free radical reactions, which are catalyzed by extracellular enzymes. The following enzymatic activities have now been characterized that are supposed to be able to apply changes to native lignin:

1. Lignin Peroxidase

Tien and Kirk [11] and Glenn et al. [12] reported independently the isolation of a peroxidase from media of the white-rot fungus *Phanerochaete chrysosporium*, which catalyzes the degradation of the side chains and the ring of lignin model compounds. Lignin peroxidase has the ability to oxidize nonphenolic aromatic substrates with high redox potential. It is a glycoprotein with a molecular weight of about 41 kDa, which contains one iron-protoporphyrin IX per molecule. The enzyme has a very low substrate specificity and catalyzes rather different reactions with lignins and lignin-type model compounds [13]:

1. Oxidation of phenyl alcohols to the respective aldehydes
2. Cleavage of C α -C β -bonds in dimeric lignin model compounds
3. Hydroxylation of the aliphatic double bonds in styrene
4. Polymerization of phenols and lignin
5. Oxidative cleavage of aromatic ether bonds

The first reaction is always the elimination of one electron and the formation of a radical cation [14–16].

2. Manganese Peroxidase

Manganese peroxidase (MnP) is an H₂O₂-dependent heme-glycoprotein (MW about 46 kDa) that also contains iron-protoporphyrin IX. It catalyzes the manganese^{II}-dependent oxidation of a large variety of phenoles and phenolic lignin model compounds. The reaction, however, does not take place, directly, and the first reaction always is the oxidation of Mn^{II} to Mn^{III}. The Mn^{III} forms a complex with an organic acid, diffuses away from the enzyme, and oxidizes the phenolic compounds [17,18].

3. Polyphenol Oxidase (Laccase)

Polyphenol oxidases are widely distributed in plants and fungi and can oxidize all kinds of phenolic compounds [19]. They have the most complex active center of all copper enzymes, with three different types of copper needed for each reaction [20]. The first reaction again is the reduction of the enzyme by elimination of an electron from the substrate and the formation of radical cations [21]. These primary substrate radicals react further in a nonenzymatic way.

Laccases play very important and different functions in fungal metabolism and life cycle that go far beyond lignin metabolism:

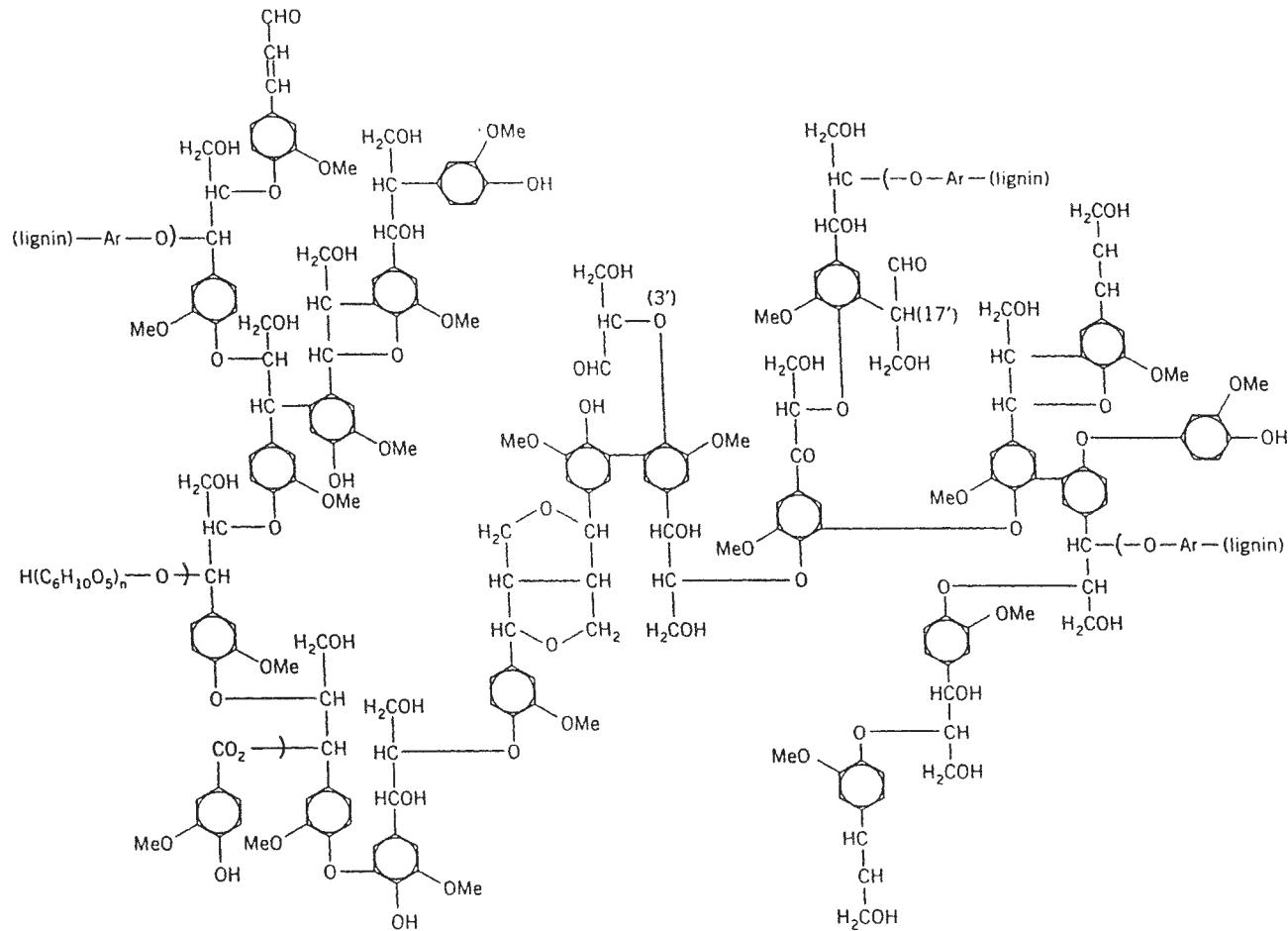


Fig. 1 Formula of lignin.

1. Enzymatic catalysis of the formation of adhesives from phenols during the formation of cell walls of spores [22,23] and rhizomorphs [24]
2. Detoxification of toxic phenols [e.g., 25–27]
3. Catalysis of the reduction of oxygen during cyanide-resistant respiration [28]

All these enzymes have some features in common: they are extracellular and the first reaction that takes place is always the elimination of an electron from a substrate, which can either be lignin itself, a metal ion, or a low molecular aromatic compound such as veratryl alcohol. After this initial reaction, very complex reactions follow, which involve both depolymerizations and polymerizations [29] and eventually lead to the complete mineralization of the lignin molecule. It must be assumed that only part of this mineralization actually takes place inside the cell. The most important reactions must take place outside the cell.

Because the aboveground biomass is composed of more than 95% lignocellulose and the cellulose in this complex is wrapped in lignin, which must first be cleaved before cellulose degradation can take place, the reactions leading to lignin degradation must be considered as key steps in the mineralization of biomass.

III. MECHANISMS FOR FORMATION OF SOIL ORGANIC MATTER, ITS DEGRADATION, AND ITS DYNAMICS

A. Facts To Be Considered

Bioremediation, by definition, is concerned with the fate and, if necessary, removal of unwanted organic chemicals from soils. Therefore, it is necessary to review the mechanisms that operate in the cycling of organic soil matter. Owing to its complexity, scientists still are far from being able to understand the reactions that lead to the formation and finally the degradation of soil organic matter [for recent reviews see 30–36].

The following facts, however, have emerged as a basis that is accepted by most scientists working in this field:

1. Soil organic matter is a highly complex and little-defined array of covalently bound aromatic and aliphatic molecules [35,37].
2. Most of the nitrogen present in soils is bound to the humus complex in a form that protects it from rapid microbiological degradation [31,38].
3. The different organic compounds present in litter and root debris exhibit very different decay characteristics. Simple aliphatic compounds may usually decay rather fast; under laboratory conditions the first-order decay constant of glucose is about 1 day [35; own unpublished observation]. The decay rates of aromatic compounds are considerably slower. The first-order decay constant for lignin under the same conditions as reported for glucose was 1 whole year [35]. Rather long residence times must be assumed for cutin and suberin [39]. During the microbial degradation of the litter, the labile, mostly common aliphatic macromolecules are degraded and lost, whereas refractory compounds or biopolymers, such as lignin, cutin, and suberins, are enriched and transformed to humus [40].
4. In soils even very reactive aromatic compounds of low molecular weight are relatively rapidly incorporated into the humus fraction instead of being metabolized [41–43].
5. During its residence time in soils, the organic matter is subject to constant metabolic changes that lead both to CO₂ mineralization and the transformation to compounds that are even more resistant to microbial attack [44].

6. Although the organic matter of soils is rather recalcitrant against microbial degradation and may have rather long residence times of more than 1000 years, even in aerated soils, the bulk of carbon is finally metabolized and mineralized to carbon dioxide [1,45]. The humic material is in a dynamic state of equilibrium, its synthesis being compensated for by gradual mineralization of existing material [31]. If this were not true, the carbon cycle of the biosphere would have already ceased to function a long time ago.

B. Mechanisms of Humus Formation

Although still controversial discussions may be found in the literature, the relevant textbooks and major reviews agree with the following general view of humus formation:

The first step is the breakdown of the organic polymers of the plant material to monomers or other low molecular weight compounds; these then are subsequently polymerized by radicalmediated processes. During this process, the more readily degradable aliphatic (i.e., carbohydrate) moieties of the litter are preferentially mineralized, whereas the aromatic constituents of the plant material are enriched. This can at best be seen by analysis of undisturbed soils or composts by CPMAS NMR-spectrometry [46,47].

The oxidation of degradation products of the aromatic plant constituents—the polyphenols to the corresponding quinones—is believed to be a key step in the formation of soil humic matter [48]. This reaction may be a spontaneous chemical autoxidation, or an oxidation catalyzed by microbial enzymes, such as laccases, polyphenol oxidases, and peroxidases. The aromatic rings that serve as building blocks for the humic acid core may originate either from lignin degradation [49] or transformation, or from the metabolism of soil microorganisms [35,50–55].

From the literature it is evident, that at least two pathways exists, by which phenolic substances can be transformed to humic acids: the abiotic oxidation, catalyzed by minerals or metal salts; and the enzymatic oxidation by laccase and other oxidative enzymes.

The abiotic polymerization of phenolic compounds has been shown [56–58]. The same group was able to demonstrate the catalytic effects of certain oxides of Mn, Fe, Al, and Si to polymerize phenols to polymers[59].

Owing to the pioneering work of Haider and Martin, ample evidence has been accumulated for the notion that phenol oxidases (i.e., peroxidases or polyphenol oxidases) [laccases] have an important function in the structural assembly of the humic acid complex [60–63]. Martin et al. [64], for instance, described the decomposition of ¹⁴C-labeled catechol and its incorporation into the stable organic fraction of the soil and could relate both phenomena to the action of phenol-oxidizing enzymes. Since then, the cross-coupling of phenolic compounds relative to its importance to humus formation, has been studied extensively by several laboratories using both peroxidases [65–68] and laccase-type phenol oxidases [69–72]. Suflita and Bollag [73] showed that the transformation of phenolics to humic acid-type substance could also be achieved *in vitro* by enzymes extracted from soils.

For the enzyme laccase from white-rot fungi during the polymerization of polyphenols, lignin was used as a model phenol, and the strength of the binding is extremely high [74,75]. The authors were able to use this reaction for the production of wood composites, such as particle boards. The vigor and chemical reactivity of the phenolic radicals can at best be demonstrated by the fact that even amino acids and amino sugars, such as glycine, lysine, cysteine, and glucosamine, are incorporated into the humic acid-type substances and, after incorporation, they became recalcitrant to rapid microbial attack in soils [76].

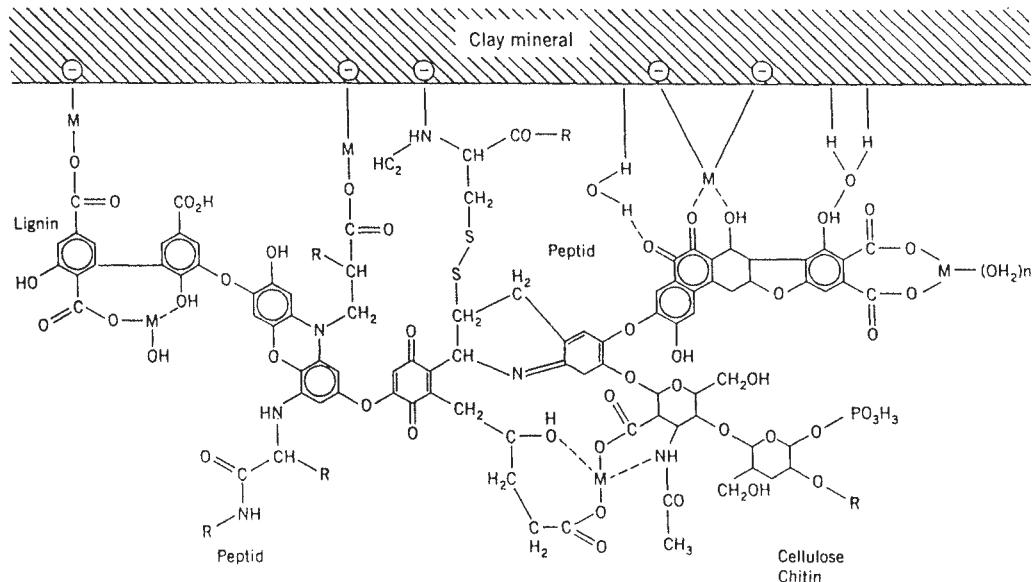


Fig. 2 Formula of humic substance. (From Ref. 10.)

From the evidence accumulated so far, it is reasonable to assume that the unspecific polymerization of phenoles and lignins is the basic mechanism by which the core of the humic acid-type soil substances are formed. These reactions are catalyzed either by radical-donating enzymes, such as peroxidase or laccase; ions or oxides that readily exhibit charge transfers, such as Mn or Fe; or even oxides from aluminum or silicon and minerals present in the soil. The origin of the aromatics may either be the aromatic moiety of the litter and root organic substances, or metabolites from litter-decomposing microorganisms. From the chemical background of this reaction, it is obvious that a free phenolic group is needed for this polymerization, a fact that can be extracted from virtually all the studies quoted in the foregoing.

C. Mechanisms of Humus Degradation

There is little doubt that all the organic material present in soils eventually is degraded and undergoes a constant turnover [37,45]. Zech and Kögel-Knabner [77] describe "humic substances as a complex mixture of different plant- and microbial-derived compounds, which exist in soils in a continuum of degradative stages." However, the organisms and enzymes responsible for this process are still more or less obscure.

From the structural models of humic acids (Fig. 2), it appears to be obvious that the degradation of this compound is bound to be catalyzed by enzymes that must have activities similar to those described for the lignin-degrading white-rot fungi. Zech and Kögel-Knabner [77] demonstrated evidence for the assumption that the same biochemical mechanisms that are employed during lignin degradation by white-rot fungi also operate during humus formation in soils. The major differences between the degradation of humus in the soil and the wood degradation are presumably the partial pressure of oxygen, which is much higher in decomposing wood than in soils [13] and a lower concentration of radical-forming enzymes. These and perhaps other factors also may be responsible for the slow rate of humus disintegration in most soils.

IV. BIOREMEDIATION USING CLASSIC BIOCHEMISTRY

A. The Influence of the Chemical Structure on Its Possible Degradation

The fate of any anthropogenic chemical that is introduced into the terrestrial carbon cycle is mainly determined by its chemical structure. For the intracellular degradation by bacteria or other microorganisms, the following rules of thumb have been published [78]:

1. **Chain length:** Below a chain length of 12 carbon atoms, aliphatic chains are degraded more rapidly with increasing chain lengths. Longer aliphatic chains, especially polymeric compounds, such as polyethylene, are more or less completely recalcitrant.
2. **Chain branching:** The more branches a chain has, the less degradable it is. Quarternary C atoms are more resistant to degradation than tertiary ones, and both are much more recalcitrant than secondary C atoms and unbranched chains.
3. **Chlorination:** The more chlorine atoms a molecule contains, the more resistant it is to aerobic degradation. Under reductive conditions, however, highly chlorinated compounds may be dechlorinated.
4. **Double bonds:** Saturated compounds are more easily degraded than the corresponding unsaturated ones.
5. **Substitutions:**
 - a. Compounds with oxygen-containing functional groups, except ketones, are more easily degraded than the unsubstituted alkanes or olefines. Amides and amino acids are less recalcitrant than the corresponding amines. Ketones, dicarboxylic acids, and nitriles are relatively resistant to degradation.
 - b. Aromatic rings substituted with -OH, -COOH, -NH₂, and OCH₃ are more easily degraded, those substituted with halogens, nitro- or sulfonic acid-groups behave more recalcitrant.
 - c. Methylation of carbohydrates results in a decrease of degradability [81].
6. **PAHs:** These compounds become more recalcitrant with increasing numbers of benzene rings in the molecule. With more than three rings, no appreciable degradation can be expected under normal conditions in natural soils [79].

Other criteria that may be used for prediction of biodegradability may be the water solubility, melting point, refractivity, density, log K_{ow} , or some other descriptors of hydrophobicity, or the molecular topology, electronic, and steric parameters of the molecule [3].

Characteristic for bacterial degradation (i.e., metabolism by classic biochemical pathways) is always the fact that even very small changes in the molecule will lead to very pronounced changes in their biodegradability. An example for this is given in [Figure 3](#). In addition, any chemical modification of natural compounds, such as, for instance, methylations, will lead to a reduction of their biodegradability [81].

B. Xenobiotic Compounds as Growth-Substrates for Microorganisms

1. Exploitation of the Indigenous Microflora

Owing to its high biomass (between about 700 and 12,000 kg ha⁻¹ surface soil) [82] and diversity, the indigenous microflora have a rather high potential for detoxification or organic compounds introduced into soils. Even “a contamination with crude oil is by far not so dangerous as is commonly thought. In animated and well aerated soils a complete degradation

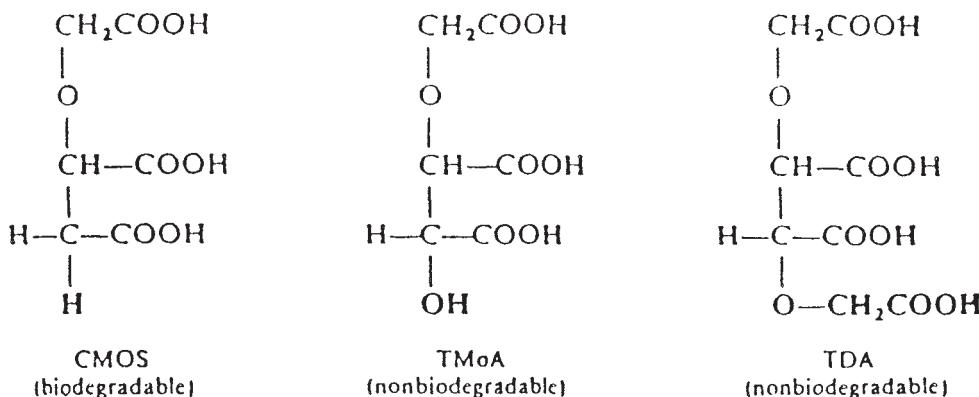


Fig. 3 Example for a high specificity of microbial degradation of aliphatic compounds: Whereas CMOS (carboymethyloxysuccinate) is biodegradable, the hydroxylated derivate (TMoA; tatrylmonoacetate) and its acetate (TDA; tatryldiacetate) are not degradable. (From Ref. 80.)

occurs rather fast. Only in the case of massive contaminations and under anaerobic conditions exists the danger of groundwater pollution." [83]. Therefore, it can be expected that chemicals, that either have structures that at least partly resemble molecules that are present in nature, or are in principle, easily degradable according to the rules given earlier, will be decomposed at decent rates by the indigenous microflora without posing too many problems. When special enrichment procedures are employed, organisms that degrade even complicated chemical structures, such as alachlor can be isolated from such soils [84]. A crucial requirement for such spontaneous biodegradation is the presence of a rich microbial biomass [85].

For many compounds, especially for structurally simple agricultural chemicals, a lag phase has been observed before their degradation starts [3]. The time period before actual degradation starts varies enormously for different chemicals, ranging from hours to many months. This acclimation is not observed, however, after a second application of the chemical. Acclimation is interpreted as the enrichment of organisms that can degrade the added chemical and use it for energy and biomass production. Depending on the substance in question and the usually unknown composition of the soil microflora, the time during which this enrichment takes may vary and is not easy to predict.

In polluted soils, acclimation can be a highly desirable process, because it will be the cheapest method of bioremediation, whereas in agricultural lands it might not be appreciated if a pesticide that is added to the soil disappears too soon. To avoid this phenomenon, either crop rotation can be applied, implying the use of different pesticides with each different crop, or special extenders applied that protect the pesticide against degradation [3].

If the conditions are not favorable for the spontaneous degradation of the pollutants, measures can be taken for the activation of the existing microflora. If the soil cannot be removed, percolation with oxygen and inorganic nutrient (nitrogen and phosphorus) or an electron acceptor (nitrate) can greatly accelerate the rate of toxicant degradation [86,87]. If the soil can be taken from the site, the simplest method available for activation of the indigenous microflora is the process of landfarming (88). Here the soil is formed into mounds that are turned over at certain intervals. During this process, the soil is aerated and necessary substrates can be added. It must be considered, however, that with this treatment volatile compounds will be released into the air and any measured remediation may be only the result of transfer of this compound into the air.

2. Addition of Specially Grown Bacteria

Another approach, using organisms that grow on the contaminants, is the use of specially cultivated bacteria. Suitable bacterial strains are isolated by a series of enrichment cultures, grown on a cheap food base, such as wheat bran, and added to contaminated soils, which also receive treatments such as the ones described in the foregoing. Several processes using specialized bacteria added to contaminated soils are already successfully marketed in Europe [89,90].

All bacterial processes have in common that the organisms use the xenobiotic substances as a substrate for the generation of energy and biomass production. These organisms, however, are not able to cope with highly recalcitrant compounds such as DDT and lindane, PAHs with more than four condensed rings (with the exception of very expensive technologies [e.g., 91], PCBs, or polychlorinated dioxins. For the remediation of these compounds obviously other means have to be employed.

However, it must be kept in mind, that soils that are polluted by xenobiotics very often are not in any condition to support a rich microbial life. At industrial sites, the soils usually are highly compressed, allowing no oxygen to move into the soil. In addition, most sites have not maintained their original soil profile, but have been filled with gravel and other debris. Thus, the establishment of an active indigenous soil microflora is not possible, especially because no input of biomass (litter and fine roots) to the soil is taking place at an industrial site. The high remediation potential of the microflora for agricultural or forest soil cannot be expected to operate on typical former industrial sites.

V. BIOREMEDIAL BY RADICAL REACTIONS

An alternative approach for bioremediation is the use of free radical-mediated reactions that are used by white-rot fungi and are in operation during composting and in the humus dynamic of soils.

A. The Influence of the Chemical Structure on Its Radical-Mediated Degradation

Free radical-mediated degradation of organic material is characterized by a very low specificity of the enzymes involved. This can at best be shown by the different chemicals that are, even simultaneously, degraded by white-rot fungi (Fig. 4). Although the basic mechanism is a radical one, there are definitely differences in the rates by which the various compounds are transformed:

1. More highly condensed aromates are degraded at a slower rate than smaller compounds.
2. A high degree of chlorine substitution decreases the rate of degradation, especially in PCBs and dioxins.

The basic structural requirement for a degradation by the radical pathway seems to be the presence of aromatic compounds that are available for electron elimination. This can be demonstrated by the following example: whereas polystyrene is not available for degradation by white-rot fungi [92,93], the copolymer with lignin is readily decomposed [93].

B. The Use of White-Rot Fungi for Bioremediation

The most efficient producers of extracellular enzymes that nonspecifically oxidize aromates by electron elimination known so far are white-rot fungi. They generate such a high redox

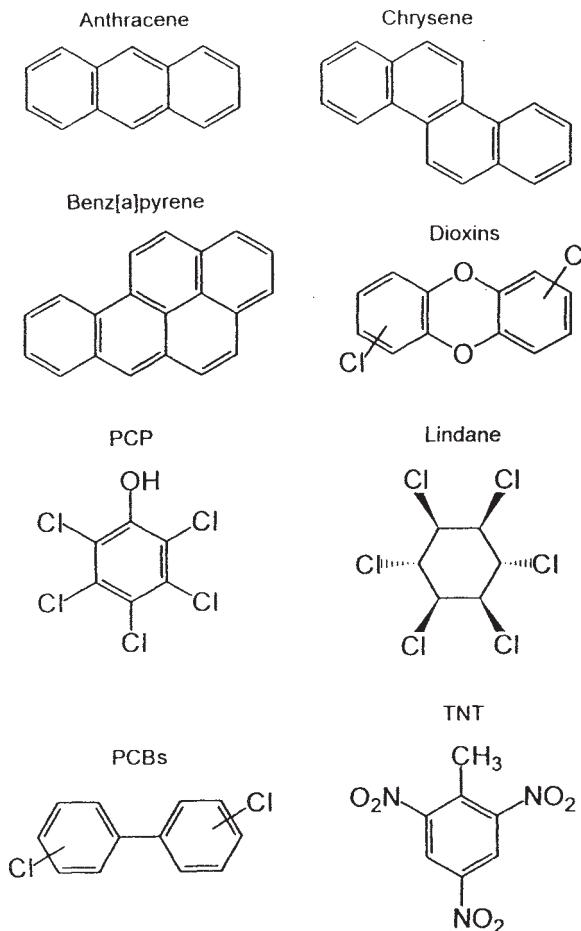


Fig. 4 Examples for compounds degraded by white-rot fungi.

potential, that this metabolic approach was termed “enzymatic combustion” [94], which means that they “burn down” all available aromatics present in the proximity of the mycelia, without obtaining any immediate return from this action. Numerous experiments have shown that in liquid culture white-rot fungi are able to degrade mixtures of even very highly condensed PAHs, including the carcinogenic benz[a]pyrene, the first publication appearing as early as 1985 [95].

The degradation of lignin, the metabolic pathway that is used in this bioremediation strategy, however, is not primarily geared to provide metabolites or energy for the mycelia [96], but only to obtain access to the more easily digestible carbohydrate substrates and to the little nitrogen that is bound to the lignin [97,98]. Thus, the xenobiotic substance also cannot be used by the fungus as the sole nutrient. Therefore, it has to be supplied with a nutrient source in addition to the toxic compound. This was under sterile conditions achieved by adding straw to the soil [99]. For nonsterile soil treatment, the supplementation of sawdust or other lignocellulosics with potato pulp has proved to be the most efficient and cheapest way for growing the fungi in large quantities and introducing them into the soil [100,101]. [Figure 5](#) gives a schematic view of the envisaged technical process.

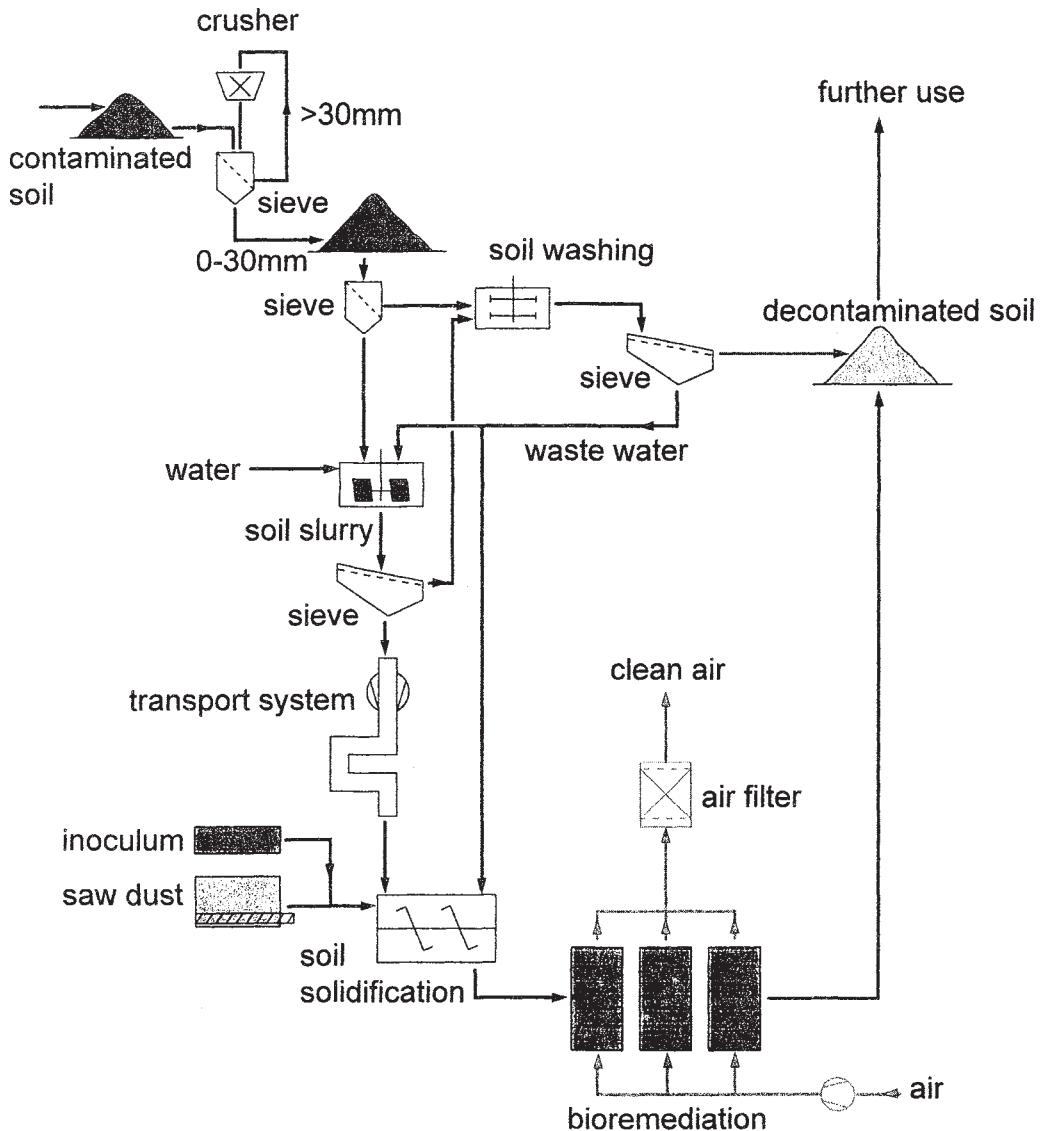


Fig. 5 Process scheme for the remediation of soils by white-rot fungi under nonsterile conditions.

The spectrum of substances that have been successfully degraded in soils from contaminated sites using this method includes virtually all important xenobiotics (see Fig. 4) [102]:

1. **Polycondensed aromatic hydrocarbons (PAHs):** We have shown that the fungi are able to grow in soils and also to degrade, although at a lower rate than in liquid culture, the PAHs [103,104]. In field experiments, we were able to transfer these results in principle to a larger scale and could remediate some 80 t of contaminated soil down to a PAH-concentration that was tolerable according to the relevant regulations [104].
2. **PCBs:** If PCBs were added to sawdust, several white-rot fungi were able to more or less completely decompose all congeners with three or four chlorine atoms within 6

weeks. Congeners with five or six chlorine atoms were degraded to more than 50%, except for the 2,2',4,4',5,5'-hexachlorobiphenyl (Ballschmitter No. 153), which was not degraded at all [105], presumably owing to its highly symmetric array of chlorine atoms. When asked to try this method with a soil that had been treated unsuccessfully with bacteria for 2 years, we could achieve good degradation for PCBs with up to six chlorine atoms in the molecule [105].

3. **TNT:** For TNT excellent degradation was achieved by this technique in soils. Within 4 weeks, 90% degradation was achieved by treating a soil from a site of an old munition plant [102].
4. **Lindane and DDT:** Soils contaminated with these compounds can also be remediated by our treatment with white-rot fungi (Fig. 6).

From the data obtained worldwide so far, it is obvious that the low substrate specificity of enzymes from white-rot fungi can be utilized for the remediation of recalcitrant aromatic xenobiotic compounds in soils. The fungi are able to attack, transform, and at least partially, mineralize a wide spectrum of compounds having a variety of chemical structures.

The final fate of the compounds treated this way depends on their chemical structure and, presumably, on the enzymes that are involved. So far four different possible chemical pathways have to be considered:

1. **Complete mineralization to CO₂ and water:** This is the optimal fate one would like to see when applying bioremediation. During the usual time frames by which bioremediation is applied, this only happens to part of the carbon atoms of the toxic compounds.

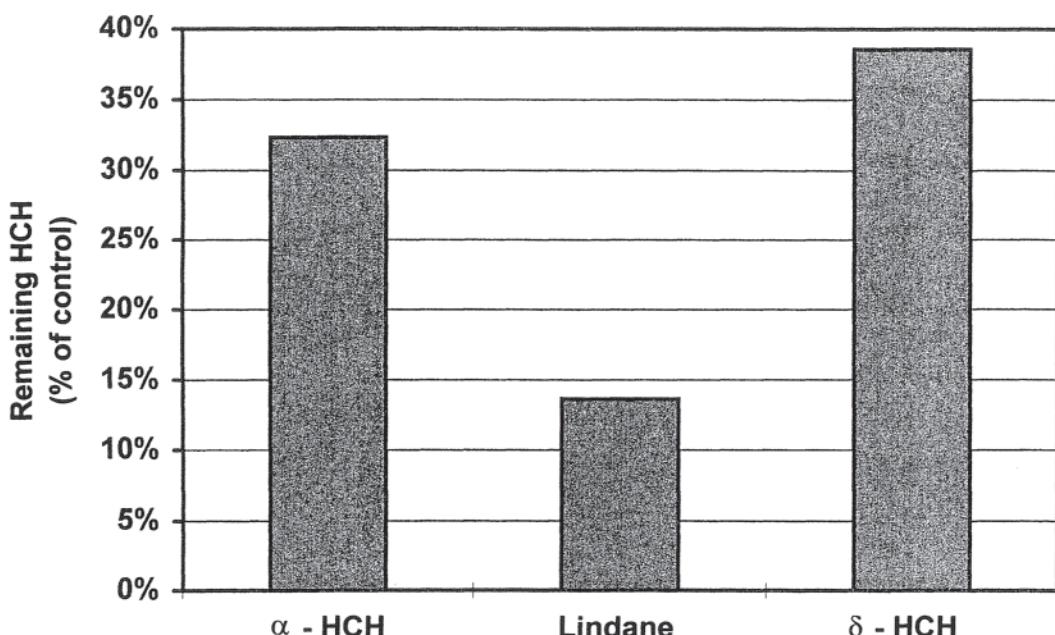


Fig. 6 Degradation of lindane and other isomers of hexachlorobenzene by the white-rot fungus *Pleurotus ostreatus*.

2. **Incorporation into fungal or microbial biomass:** After oxidation of the aromatic compounds, molecules are formed that are much more easily metabolized than the parental ones. These newly formed compounds can be funneled into the metabolism of the soil microflora and converted to microbial biomass. In our experiments, no deadend products have yet been observed for PAHs and PCBs degraded by fungi, and, there was no formation of toxic intermediates, as could be seen by toxicity test (Ames test and others) [105].
3. **Polymerization:** Definite polymerization reactions occur during the degradation of lignin. Whether a certain compound is either degraded or polymerized by a certain enzyme is dependent on the chemical structure of the very molecule. We have shown that rather small changes in the structure of dimeric lignin-model compounds (i.e., exchange of substituents on a ring) determine whether that compound is either polymerized or degraded [106]. The same principle holds for the fate of even rather similar xenobiotics. By the use of radioactive labeled compounds we have shown, for example, that in the case of anthracene, the compound is mainly degraded, whereas, under the same conditions, benz[a]pyrene is polymerized to a great extent (105).
4. **Incorporation Into the Soil Matrix:** Another reaction that is usually neglected is the incorporation of the compound, or its transformation product, into the organic matrix of the soil. This pathway is not surprising, considering that all reactions catalyzed by the lignolytic enzymes of the white-rot fungi go through radical intermediates, which are very reactive. Therefore, it is not surprising that at least part of the reactions take place with the organic matter of the matrix, leading to a fixation in the form of an insoluble and not readily accessible part of the humus structure.

The typical distribution of the radioactivity of labeled compounds after treatment with white-rot fungi is given in Table 1.

The results obtained with the white-rot fungi so far indicate that the bioremediation of highly recalcitrant compounds is possible by this treatment, provided they are accessible to the fungi. Apart from scale-up problems, which we expect to be solved in the near future, the largest obstacle that has to be overcome is the bioavailability of the compounds for the fungal enzymes [102]. Owing to their aromatic nature, the most important xenobiotics firmly bind by van der Waals bonds to aromatic structures in the soil, especially coal dust particles, which are a normal soil constituent in old gas works or other sites that may need bioremediation. It is not yet completely clear how much the clay-humus complex itself may also contribute to the reduction of bioavailability.

Table 1 Distribution of the Radioactivity of Labeled Compounds After Treatment for 72 Days with White-Rot Fungi

Compound	Compartment ^a					
	Undergraded		Extractable (organic, solvent)		Water- soluble	Organic matrix
		CO ₂				
Anthracene	<1	19	31	15		18
Benz[a]pyrene	<1	17	20	12		35
PCBs (¹⁴ C-label)	48	10	18	11		12
PCBs (³⁶ Cl-label)	26		14	10	39	11

^aData given in percentage of total recovery.

Source: Ref. 105.

If the compounds are bioavailable, however, the treatment with the white-rot fungi results in the remediation of the soil. Part of the xenobiotics are completely mineralized or incorporated into the microbial biomass. Compounds that are not mineralized immediately, are transformed to much less toxic compounds that are fixed into the organic matter of the soil and eventually will take part in the regular humus dynamics. In view of the chemical reactions that take place in this process, it is rather safe to expect no further toxic effects from xenobiotica that are transformed and fixed by the fungi to the organic matrix of the soil. They will be oxidized again and eventually totally mineralized by the reactions of the humus cycle.

C. Composting

The degradation of organic matter during composting can be viewed as a quick-motion film of the events leading to the mineralization of organic matter during humus formation and the humus cycle. During composting, both the classic biochemical pathways of decomposition of organic molecules and the radical-mediated reactions are operating. Owing to the high organic matter content of the compost, the elevated temperatures during its operation, and the high C/N ratio at the beginning of composting (which leads to autolysis cycles [107] with liberation of intracellular peroxidases), a milieu is created in which at least part of the radical reactions induced by white-rot fungi are also operating. Even lignin, when artificially added to the composting process, is also degraded there [108].

In view of these conditions, it appears to be not too surprising that during composting at least simple aromatic xenobiotics are degraded. Among these compounds are TNT and other explosives [109,110] and pesticides such as diazonin, chlorpyrifos, isofenphos, and pendimethalin [111]. Because the real science of composting is still at the beginning and many means of optimization and control are available [112, 113] that have yet to be tested for their influence on the process of bioremediation, many more xenobiotic compounds can be expected to be degraded at reasonable rates during composting, provided the right conditions and controls are applied. Thus, in the long run, composting can be expected to at least partially supplement the fungal solid-state fermentation techniques for remediation of recalcitrant xenobiotics in soils.

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Marker-Assisted Selection in Animal Biotechnology

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I. THE NEED FOR GENETIC IMPROVEMENT IN ANIMAL AGRICULTURE

A. Extensive Agriculture: Selection for Adaptation

Initially, and in many parts of the world even today, animal agriculture was extensive in nature. That is, it was based on the ability of domesticated animals to gather plant materials of little value to humans (natural pasture, mast, assorted waste products of human activity) and convert these to food of high nutritional quality: meat, milk, eggs. Going out into the field demands adaptation to local climatic and sanitary conditions. Consequently, as domesticated animals followed humans in their dispersion, innumerable locally adapted "native" breeds and landraces developed.

B. Semi-Intensive Agriculture: Selection for Responsiveness

The productivity of native breeds depends primarily on the environmental conditions of a given year. Accordingly, most native breeds can adjust productivity over a relatively wide range. For example, the fat-tailed Awassi sheep of the Middle East, can produce from 50 to 500 L of milk, depending on the level of nutritional inputs. Within the range of response, the marginal returns on additional nutritional inputs are very great, because basic requirements for body maintenance and reproduction have already been provided. For this reason, it became economically attractive in some localities, to shift to a semi-intensive mode of agriculture, by providing additional nutritional inputs to the animals, in the form of high quality roughage or grain. When this is done, however, the response capacity of the native animal is quickly reached; at which point further inputs do not provide further response. This is frustrating, because the farmer has potentially much more to give, but the individual animal cannot make use of the resources provided.

In the regions where this situation arose, natural selection for adaptation was supplemented by artificial selection for responsiveness. In a few localities, such selection resulted in animals that, given adequate inputs, can attain productivity levels well beyond those of the usual native breed. Thus, under the best conditions, native cattle will not exceed 2000 L of milk a year, but the Damascene breed in Syria, and the Gir breed in India, when fed appropriately, are able to reach production levels of 4000–5000 L a year. Comparable processes took place in much of Europe, where many local breeds can reach these levels.

In a few European cattle breeds, continued selection for productivity under fully intensive management has produced cows that are even more responsive, yielding upward of 10,000 L under suitable inputs, primarily grain. Similarly, highly selected layer crosses can produce 280 eggs weighing 60 g in 300 days; a native breed, under the same conditions, will produce 200 eggs weighing 50 g. Modern broilers will reach a body weight of 1500 g at 42 days; the original “dual-purpose” breeds of 50 years ago reached this weight at 120 d.

This has led to three tiers for most species of livestock: native breeds, highly adapted to local conditions, but having only limited ability to respond to additional inputs; semi-improved breeds, also well adapted to their local conditions and capable of moderate response to additional inputs; and highly improved breeds adapted specifically to the climatic and sanitary conditions of Western Europe, but with great capacity to respond to additional inputs.

C. The Need for Further Genetic Improvement

The market economy of the West, exerts inexorable pressure for increased productivity of animal agriculture. At the same time, population growth and increase in income throughout the world is leading to increased demand for animal products, which can be met only by intensification of animal agriculture, often based on imported grain. For this to be economically feasible requires replacement of existing native and semi-improved breeds with modern, highly responsive animals. Thus, animal breeders face two very different challenges. One, is to further increase the productivity of the existing, most highly improved breeds. The other is to provide highly productive animals that are adapted to the climatic and sanitary conditions of the tropical and subtropical regions of the world.

II. GENETIC VARIATION AND SELECTION

A. Genetic Variation

All existing breeds, including the most highly productive, retain a store of within-breed genetic variability for most traits of agricultural importance. The store of genetic variation across the species as a whole is much greater, because many potentially useful genetic variants may be found in only one or a few of the many breeds and landraces the species comprises. In large part, therefore, the biological basis for achieving the goals of genetic improvement, already exists. Current improvement of the highly productive European breeds, relies exclusively on within-breed genetic variation. However, as this is exhausted by selection, sources of genetic variation presently scattered through the species as a whole, may become progressively more important. For improvement of adapted local breeds, it would appear attractive from the first to cross European and native breeds, and from the mixture, develop individuals combining the productivity of the European breeds with the adaptation and disease resistance of the native or semi-improved breeds.

In principle, all that needs to be done is to gather up the desired alleles at the genes affecting the traits of interest, and combine them in a single individual. Moreover, sexual reproduction, with independent assortment and crossing over, continually produces new combinations of

parental alleles among the individuals born in each generation. The task of the breeder is to identify those individuals that carry the combinations of alleles most closely approaching the desired goal; and allow only these individuals to produce the next generation. Through this process, termed *artificial selection*, the proportion of individuals carrying favorable combinations and the value of the most favorable combinations, will increase from generation to generation.

B. Selection

The ability of the breeder to recognize those individuals carrying favorable alleles or allelic combinations is often very limited. Classic selection is based on phenotype; in many instances phenotype is not expressed (e.g., sex-limited traits such as milk and egg production in males) or difficult to measure (e.g., feed efficiency, carcass composition, disease resistance, and climatic adaptation). Even when expressed, phenotype is an inaccurate indication of genotype [29]. This is because genetic variation in productivity depends on allelic variation at a number of loci (genetic variation is “polygenic”), and gene expression at these loci is strongly affected by environmental factors. In this case, trait variation and genetic variation are said to be “quantitative” in nature; and the individual loci affecting trait expression are termed *quantitative trait loci*, or QTL [33]. Furthermore, productivity and adaptation are composite traits, consisting of many components (e.g., egg number and egg weight). There are often negative genetic correlations between some of these components (e.g., between egg number and egg weight; milk quantity and milk protein percentage). This weakens the effectiveness of selection for each of the individual components [26,29].

To overcome some of these limitations, animal breeders employ methods based on the fact that any individual shares part of its genetic makeup with its close relatives—parents and grandparents; uncles and aunts; sibs, cousins, and progeny. Consequently, information on phenotypic expression of a trait in the relatives of an individual can provide information about the genotype of that individual. These methods are variously termed *pedigree* selection, *family* selection, or *progeny testing*, depending on the type of relative involved [29]. Sophisticated statistical method are now available for extracting every bit of genetic information from the phenotypic information available on an individual and its relatives [122]. Information on different traits is then combined in an optimal manner that takes account of genetic correlations among them. Nevertheless, even when optimal use is made of these methods, the rate of genetic progress is far below that which would be possible if perfect genetic information were available. Furthermore, attempts based on these methods, to combine productivity of European breeds with adaptation of native breeds, have almost uniformly failed. This was due to the inability of selection to cope simultaneously with the many traits involved. Particularly troublesome is the necessity to evaluate individuals simultaneously for productivity and for adaptation to severe climatic stress or disease challenge, which reduces productivity. Classic methods of selection are also unable to recognize the presence in native or semi-improved breeds of rare favorable allelic variants at one or more of the QTL affecting productivity or product quality [103,104]. Such variants are termed *cryptic*, because their presence is hidden by the generally low productivity of the local breed, owing the presence of unfavorable allelic variants in the majority of the relevant QTL.

III. MARKER-ASSISTED SELECTION

Beginning in 1980, technological and scientific developments have combined to provide a method for identifying genotype for productivity and adaptation directly at the DNA level,

without going through expressed phenotype. This opens the way for development and implementation of genetic improvement programs that are based on selection at the DNA level. In such programs, variants in DNA structure at specific chromosomal loci, serve as "markers" for the presence of alleles having positive or negative effects at the QTL that affect trait expression.

Selection of individuals carrying favorable alleles at QTL, based on direct evaluation of their DNA is termed *marker-assisted selection*, or MAS [92,98,101,100,114]. MAS aims to ameliorate the weaknesses of classic selection by supplementing or substituting selection at the DNA level for selection on the basis of phenotypic trait value. As compared with selection on the basis of phenotype, MAS can be more accurate, because it is not affected by environmental factors, is not limited to one sex, and does not require expensive or destructive testing. MAS can be carried out at an early age, reducing generation interval; it can include all individuals, and even individuals that are not born, by genotyping at an embryo level, thus increasing selection intensity. Even in the case of negative genetic correlations between productivity traits at the population level, individual QTL may be found that have favorable effects on one trait, without associated negative traits on the other [119]. MAS will enable selection to be focused on these QTL, avoiding part of the loss in selection intensity associated with index selection [26].

Because MAS examines allelic variation at individual QTL, it may be able to uncover cryptic favorable allelic variants in local breeds for incorporation into improved breeds. Because of its great accuracy and other advantages, MAS may even enable the successful prosecution of breeding programs based on crosses between European and local breeds. MAS will permit identification and rapid introgression of a few desired favorable QTL alleles from resource populations to recipient populations for rapid improvement of specific production traits in adapted landraces, and for transfer of specific monogenic or polygenic resistance loci from landraces to improved commercial populations [6,103].

IV. UNCOVERING GENETIC VARIATION AT THE DNA LEVEL

Genetic variants at the DNA level, termed *DNA polymorphisms*, fall into two main categories: polymorphisms involving base-pair substitutions or small structural changes (deletions, additions, or inversions); and polymorphisms involving variation in the number of tandem repeats of simple, short motifs in tracts of DNA consisting of the same simple motif repeated many times. DNA polymorphisms of both types are detected by a variety of methods, as described in the following.

A. Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms (RFLPs) were the first class of DNA level markers to be uncovered [5,11]. These are revealed through the use of *restriction endonucleases*, enzymes that cleave the DNA molecule internally at particular sites defined by a specific small sequence of nucleotides, yielding a series of DNA fragments. The length distribution of the fragments depends on the relative location of adjacent restriction sites, and will be specific to the particular enzyme-DNA combination used. When total genomic DNA of a higher plant or animal is digested in this manner, a great many fragments, of many different lengths are obtained, giving a continuous DNA "smear" when the fragments are separated according to size by gel electrophoresis. A particular fragment, however, can be located on the gel in a procedure termed *Southern analysis* [108], involving *in situ* transfer of the DNA smear from the gel to a solid support (generally a nitrocellulose or nylon filter) and hybridization to an appropriately

labeled DNA probe that is complementary to part or all of the DNA sequence within the fragment. Following hybridization, an autoradiograph of the filter is produced and inspected for the location of the bands indicating bound radioactivity. Each such band represents the location of restriction fragments sharing sequence homology with the probe.

Many changes in DNA sequence or microstructure occurring in or near the locus that is homologous with a given probe will change the size of the fragment carrying the sequence complementary to the probe. Changes in fragment size will cause corresponding changes in the location of the labeled band. When DNA from individuals carrying different allelic variants in the "probed" region is subjected to these procedures, different band distributions are obtained on autoradiography, demonstrating a restriction fragment length polymorphism.

B. Variable Number Tandem Repeat Loci

The RFLPs are typically diallelic. For utilization within segregating populations, diallelic markers have many limitations owing to difficulty in tracing particular marker alleles from generation to generation. These limitations can be avoided, to a large extent, by the use of polyallelic markers [54,59,92]. In this case most individuals will be heterozygous at the marker locus, and alleles can be traced from parent to most offspring. Consequently, great interest was aroused when a highly polyallelic human RFLP, characterized by a large number of alleles was uncovered [125]. Probes detecting other such loci were found subsequently [78]. DNA sequencing showed that allelic variation at these loci was due to the presence in the genome of tracts of DNA, consisting of tandem repeats of a short (9- to 60-bp) DNA motif. These tracts are termed *minisatellites*, by analogy with the tandem-repeat structure of satellite DNA [57]. Not all minisatellites are polymorphic; but when present, polymorphism takes the form of variation in the number of tandem repeats of the recurrent motif; hence, the designation variable number tandem repeat (VNTR) loci [78].

C. Microsatellites

The eukaryote genome also contains exceedingly numerous dispersed tracts consisting of very short motif repeats; for example, poly(TG), poly(AT), poly(CAC), and so on [42,112]. With the advent of the polymerase chain reaction (PCR) [89,121], specific microsatellite tracts contained within a stretch of unique sequence DNA could be individually amplified using a pair of unique flanking oligonucleotides, and examined for length variation in sequencing gels (capable of detecting length variation of a single nucleotide). When amplified microsatellite sequences are examined in this way, they are often found to exhibit a marked degree of VNTR polymorphism, similar to that shown by minisatellite sequences [71,111,115]. Because of their great numbers and even distribution in the genome, and the ease with which they can be scored, microsatellites were rapidly recognized as the marker of choice for mapping studies [7].

D. Random Amplified Polymorphic DNA Markers

When genomic DNA is amplified by means of the PCR reaction, using a short oligonucleotide primer, a small number of amplification products are obtained, which can be discerned as individual bands on gel electrophoresis [16,120,123]. These represent genomic sequences that by chance alone happened to be flanked by sequences complementary to the given oligonucleotide primer in reverse orientation. Oligonucleotides having a specific and known nucleotide sequence are used in any particular PCR amplification reaction, but the oligonucleotide sequence for any specific amplification is simply chosen at random. When

the band pattern produced by a given oligonucleotide is compared across a sample of individuals from the same or different population, occasional bands are polymorphic (i.e., present in some individuals, but not in others). This polymorphism is termed *random amplified polymorphic DNA* or RAPD.

Detection of polymorphisms using RAPD methodologies is rapid and less laborious than using RFLP or microsatellite techniques, does not require the use of cloned probes or radioactive labeling, and is independent of prior DNA sequence information. An important limitation is that allelic differences at RAPD markers are generally expressed as presence versus absence of a particular band, so that homozygotes and heterozygotes cannot be distinguished. Occasionally, however, allelic differences between RAPD amplification products are expressed as bands of different lengths; or products having similar lengths can be shown to differ on subsequent digestion by restriction enzymes. Also, informative RAPD bands can readily be cloned and converted to locus specific probes for RFLPs or SSCP [17,75,83].

E. Single-Strand Conformation Polymorphisms

Under nondenaturing conditions, single-stranded DNA has a folded conformation that is stabilized by intrastrand interactions. The conformation and, therefore, the mobility, is dependent on the sequence. Nucleotide substitution causes a conformation change of the single-stranded DNA, which changes its electrophoretic mobility [43,80,81]; hence, the designation *singlestrand conformation polymorphisms* (SSCPs). In its most useful form the method involves amplification of a DNA segment of a sequenced gene, using a pair of labeled oligonucleotide primers that uniquely flank the amplified segment. The amplification product is denatured in alkaline solution, and electrophoresed on a neutral polyacrylamide gel. After transfer to a nylon membrane, the mobility shift caused by a single nucleotide substitution of a single-stranded DNA fragment is detected by a radioactive or fluorescent signal. By using this method, most single-base changes in fragments of up to 200 bp in length can be detected as mobility shifts. Thus, given a known and sequenced gene, the SSCP methodology provides a means for systematically identifying virtually all polymorphic points within the gene and its surrounding control regions.

V. SPECIES GENOMIC MAPS

The methods used to uncover DNA level polymorphisms are random. That is, the polymorphisms uncovered can be located anywhere in the genome. For maximum usefulness, therefore, after polymorphic DNA level markers have been uncovered, they must be mapped relative to one another. This requires that they be jointly segregating in the same set of families. By genotyping parents and progeny of each family for a given pair of markers, it is possible to determine whether they exhibit linkage or independent transmission; and if they are linked, to determine the proportion of recombination between them. Because DNA level polymorphisms in animal populations are often segregating throughout the species, it is possible to use any existing set of two-generation families for mapping purposes. If one or both parents are heterozygous for both markers, information on linkage and recombination will be provided by the distribution of marker genotypes among the offspring.

DNA samples can be stored indefinitely, and only minute quantities of DNA are required to score DNA level polymorphisms. Thus, once DNA samples have been obtained from a useful set of families, it is not necessary to go out and obtain new DNA samples from a new set of families for each newly uncovered polymorphic DNA marker that was to be mapped. It is sufficient to simply genotype the existing families relative to the new marker. Furthermore,

mapping a new marker requires obtaining information on its joint segregation relative to other markers segregating in the same family. However, as a laboratory continues to genotype its families for additional polymorphic markers as they are uncovered, information on the segregation of more and more markers accumulates in the same set of families. Consequently, when a new polymorphic marker is uncovered, it can be mapped simply by genotyping it on the existing families. The accumulated information on the segregation of the previously genotyped markers is then used to identify those markers with which it is in linkage, and also the degree of recombination between the new marker and the closest previously genotyped markers. It is not necessary to genotype again any of the previously genotyped markers.

From this it was only a step to deciding on a set of reference families that would be used by the entire community of geneticists involved in generating a marker map for a particular species. In this case, although any set of families can serve for mapping polymorphic markers, it is useful that the parents of the families be as heterozygous as possible, and that the families be as large as possible. Consequently, the set of families used for cattle mapping consists of several large full-sib families produced by multiple ovulation and embryo transplantation [3,10]. For the most part, the parents of each family are themselves each the products of a cross between the two subspecies of cattle: *Bos taurus* (European-type cattle) and *B. indicus* (Zebu-type cattle). For chicken mapping, two large backcross families are used [14,21]. Each family was produced by crossing a single male with a female of a highly inbred line, and then back-crossing an F₁ male to a single female of the same inbred line. For swine mapping, crosses were carried out between the Meishan pig of China and a European pig [40]. For all of these sets of reference families, DNA from the parents and offspring are distributed to all laboratories that are engaged in mapping the species. All new polymorphisms are genotyped on the parents and progeny. The data are sent to the central data banks, and mapping is carried out relative to all previously mapped markers.

At present, almost all markers added to the maps are RFLP or SSCP markers based on known cloned genes, or anonymous microsatellite markers, usually of the poly(TG) type. Markers based on known genes are important for tying together maps of different species [79]. Markers based on microsatellites, are important for their convenience in genotyping, and for providing maps of virtually unlimited marker density [7]. Complete maps are available for the major livestock and poultry species [3,10,15,20,87].

VI. ASSOCIATING DNA LEVEL POLYMORPHISMS WITH VARIATION AT QTL

There are two active routes presently being explored that can lead toward the ultimate goal of associating DNA level variation with QTL allelic variation. The *candidate gene* approach, is based on exploration of variation in trait value in relation to DNA level variation in known genes that are thought to be directly involved in the physiology and development of the trait in question. Once such associations are found, selection would be for the known gene structural variant that has a positive effect on trait value. The *linkage-mapping* approach, involves mapping QTL to specific chromosomal regions through linkage to anonymous DNA level marker loci; establishing coupling linkage associations between specific alleles at the marker loci, and positive or negative alleles at the QTL; and basing selection on the marker alleles. With the linkage-mapping approach, along with establishment of coupling relationships between marker alleles and QTL alleles and their utilization for MAS, attempts will ordinarily be made to locate the QTL in the chromosomal region to progressively narrower intervals, until suitable Candidate genes can eventually be identified. At this point, the linkage-mapping approach merges with the candidate gene approach.

A. Candidate Genes

The candidate gene approach has scored a number of notable successes. These include associations between electrophoretic variants at the kappa-casein and lactalbumin loci in cattle, and milk protein production and cheese making qualities [12]; between RFLP variants at the estrogen receptor in swine, and fertility [88]; between SSCP variants at the bovine growth hormone locus, and milk protein percentage [64]. In chickens, endogenous viruses, as a group, give many indications of being associated with genetic variation in productivity [32,51,53] and are being intensively investigated; and RFLP variation at chicken homeobox genes has been reported to affected onset and rate of lay [52].

Simulation studies show that for relatively small closed populations, only a few different variants can be maintained at any given gene [A.Genizi, personal communication]. Many agricultural breeding populations are of this nature. Even in dairy cattle, because of the small number of elite sires used to produce each generation of young sires, effective population numbers are small, even though commercial dairy cattle populations are enormous. Consequently, a useful approach to exploring association of DNA level variation at candidate genes with trait variation is to define several polymorphic marker sites at the candidate gene, and use these to identify all of the distinct structural variants present at the gene in the population under investigation [64]. To the extent that structural variants at the candidate gene also represent functional variants, a simple analysis of variance, with structural variant as the independent variable and trait value as the dependent variable, will identify association of DNA structural variants with trait value.

The candidate gene approach is very attractive, because it is relatively easy to implement, and the results, when positive, can readily be used for MAS in all populations carrying the favorable structural variant of the candidate gene. However, because only about 2000 genes have been cloned to date, from among the 100,000 or so estimated functional vertebrate genes, it follows that the great bulk of QTL are probably found among the remaining 98% of genes. Furthermore, even when many more genes have been identified and characterized functionally, their relations to quantitative trait variation may not be obvious. An example in point is the relation of allelic variation at the apolipoprotein E locus with incidence of Alzheimer's disease [19]. The trail from gene to syndrome, is still obscure, and certainly would not have been postulated a priori. For these reasons, even maximal exploitation of the candidate gene approach will probably not provide access to the totality of genetic variation in the main traits of importance to animal agriculture. By default this leaves the linkage-mapping approach, which does have this ultimate capacity.

B. Marker-Based Linkage Mapping of QTL

1. QTL Mapping in Crosses Between Inbred Lines

The rationale underlying linkage mapping of QTL is best illustrated in a cross between inbred lines [94,105]. Consider two inbred lines differing at a particular marker locus M, such that line 1 has the genotype *MM* and line 2 has the genotype *mm*. If the chromosomal region in the vicinity of the marker locus is one that does not contain any QTL affecting the quantitative trait under analysis, the expected mean trait value of *F*₂ individuals carrying the *MM* genotype and those carrying the *mm* genotype will be the same. It may be, however, that one of the QTL, denoted A, affecting trait value is located in the same chromosomal region as the marker M, so that the genotype of line 1 is *MA/MA* and that of line 2 is *ma/ma*. Here, except for recombination, the *M* marker allele in the *F*₂ will be in coupling with the *A* QTL allele, and the *m* marker allele will be in coupling with the *a* QTL allele. Alleles at all other QTL will

tend to be distributed equally between individuals receiving the *M* as compared with the *m* allele at the *M* marker locus. Thus, a comparison of the mean trait value of *MM* and *mm* genotypes in the *F*₂ population should reflect only the quantitative effect of the *AA* genotype, as compared with the *aa* genotype. A significant difference in trait value between *MM* and *mm* genotypes in the *F*₂ population will, therefore, be indicative of a QTL in the vicinity of the *M* marker. Numerous experimental results based on this design have been reported in plant species [see, e.g., 27,58,84,109,117,119,128,129].

2. QTL Mapping in Outbred Populations

In outbreeding populations, such as poultry or dairy cattle, marker alleles and QTL alleles can be expected to be at or close to linkage equilibrium, unless marker and QTL are very closely linked. In this instance, coupling relations between marker allele and QTL allele will differ among the chromosomes segregating in the population. That is, some chromosomes will carry the combination *MA*, some *Ma*, some *mA*, and some *ma*; and individuals in the population will carry all possible two-way combinations of these. Because only double heterozygotes (*MA/ma* or *Ma/ma*) are informative for linkage, this means that when diallelic markers are used, most individuals in the population will not be informative for linkage studies. Even in this situation, however, there will be some double heterozygous individuals; and QTL mapping is based on the specific relations between marker alleles and QTL alleles in the progeny of such individuals. When polyallelic markers are used, the proportion of double heterozygotes is much larger.

In a full-sib design, a large full-sib family is formed by mating two individuals. If one parent is heterozygous at the marker, and the other homozygous, there will be two marker genotypes among the progeny. If both parents are heterozygous for the same marker alleles, there will be three marker genotypes among the progeny; four, if a polyallelic marker is used and the two parents are heterozygous for different marker alleles. In all of these instances linkage is expressed as a difference in trait value among progeny having different marker genotypes. The statistical power of analyses based on single full-sib families is limited, because the parents of any single family, even if heterozygous at the marker may not be heterozygous at the QTL. Also, except for some fish species, the number of progeny in a single full-sib family is far fewer than required for useful statistical power. Thus, demonstration of linkage will usually require combining information from several families. There are various statistical designs that will do this effectively [63,106], and experimental results have been reported [1,86].

In a half-sib design, a large half-sib family is formed by mating a single individual, usually a male, to many mates. If the common parent is heterozygous at the marker, the progeny can be divided into two groups, according to the marker allele transmitted from their common parent. The analysis then proceeds exactly as for a full-sib family [106,118], and many experimental results have been reported [see, e.g., 2,8,34,36,39].

3. QTL Mapping in Crosses Between Outbred Populations

A special design that combines some features of a cross between inbred lines, can be employed for mapping in crosses between populations that share the same segregating marker alleles, but are at or close to fixation for alternative alleles at QTL affecting the trait of interest [4]. This design is particularly appropriate for mapping QTL involved in disease resistance, adaptation, and productivity in crosses between native breeds and European improved breeds; for mapping QTL involved in differences between experimental selection lines, or between commercial breeds selected for widely different agricultural characteristics (e.g., layer and broiler breeds in poultry; milk and beef breeds in cattle).

4. Reducing Genotyping Costs

Establishing marker-QTL linkage can require genotyping and phenotyping very large numbers of individuals [105,106,118]. The number of genotypings required to establish marker-QTL linkage, however, can be reduced in a number of ways. First, relatively few markers per chromosome are sufficient to provide power near that provided by an infinite number of markers [23]. Thus, the most efficient designs are those that combine a large number of progeny with a relatively sparse marker spacing [25]. This can be combined with sequential sampling, for early identification of markers that are not in linkage with a QTL, so that these markers need not be genotyped on the full sample [77]. Because most markers will not be in linkage to a QTL, this can reduce total genotyping by about one-half. Second, for any particular trait, most of the statistical information relative to marker-QTL linkage, is found among the progeny at the two tails of the population distribution. Thus, linkage can be established by selectively genotyping these individuals only [24,66,67]. Furthermore, among the extreme individuals, almost all information relative to marker-QTL linkage is carried simply by the relative frequency of the parental marker alleles in the two tails [22]. In many instances allele frequency in a group of individuals can be determined by pooling their DNA samples and determining allele frequency in the pool through densitometry [61,82]. This procedure, termed selective DNA pooling [22], can reduce the amount of genotyping required for marker-QTL linkage studies by an order of magnitude or more.

5. Estimating Map Location and Gene Effect of the QTL

The observed mean difference between marker genotypes, due to linked QTL, will depend both on the actual effect of the alleles at the QTL, and on the degree of recombination between marker locus and QTL—the greater the degree of recombination, the smaller the market-associated effect [105,106]. When a complete genome map is available for mapping, so that a QTL can be located within an ordered set of marker loci, methods for obtaining QTL map location relative to the adjacent markers, and for obtaining unbiased estimates of gene effect at the QTL including dominance, are available [41,48,49,54–56,62,63,66,72,116].

VII. FINE MAPPING OF QTL

A. Confidence Interval of QTL Map Location

The foregoing procedures when applied to populations of reasonable size (some few hundred or few thousand animals, depending on the design) and using a map with 20-cM marker spacing, can place a QTL within a confidence interval of about 10–20 cM, depending on the size of the experiment and on the QTL effect [23,113]. Simulation studies show that for given population and gene effect, marker spacing equal to the confidence interval of map location, provides close to maximum information. That is, marker spacing closer than this does not further decrease the confidence interval. Remarkably, however, if marker spacing is adjusted appropriately, the minimum confidence interval decreases linearly with increase in population size [A. Darvasi, personal communication]. This is because both population size and marker density are increasing simultaneously. This means that a twofold increase in population size (from 1000 to 2000 individuals, say) can yield a twofold reduction in QTL confidence interval (from 20 to 10 cM, say), provided marker spacing is adjusted accordingly. A variety of methods are available for this purpose [17,69,70,75,83,90,110], and have been successfully applied for fine mapping in plant species [9,73,85,110]. Thus, the first step to fine mapping is simply

to increase both experimental population size and marker density in the chromosomal region of interest.

B. Fine Mapping by Linkage Disequilibrium

Once the QTL has been located to a 5- to 10-cM confidence interval by linkage mapping, the most promising approach for fine mapping in animal species, may be one that is based on saturating the chromosomal region covered by the confidence interval with markers, and searching among them for a marker or marker combination (haplotype) showing population-wide linkage disequilibrium with the QTL [38,93,98]. Simulation studies show that disequilibrium in a large natural population (effective number >100) will occur primarily for linked loci separated by no more than 1–2 cM [38; A. Genizi, personal communication].

C. Synteny Mapping

Once a QTL has been located to a haplotype covering a 1- or 2-cM-chromosomal region, the general synteny of mammalian chromosomes [124] should provide an indication of the totality of genes present in the region. Although a 1-cM stretch of chromosome might include dozens of genes, only a few of these will be attractive candidates. These can then be assessed for QTL nature as described earlier. This approach has been applied successfully in several instances [19,31,44,76].

VIII. MARKER-ASSISTED SELECTION

A. MAS Based on Population-Wide Linkage Disequilibrium

When population-wide association has been established between a marker locus or marker haplotype and a tightly linked QTL, selection for the marker allele or haplotype coupled with the favorable QTL allele, can proceed as though a simple mendelian gene were involved. Ultimate genetic progress owing to selection for a “known haplotype” of this sort, will depend primarily on the effect and initial frequency of the favorable QTL [91,92,93]. MAS of this nature will be most useful in those situations where classic selection is least useful, and will be more effective as the quantitative effect associated with the marker is known more exactly [37,65,91–93,126,127].

B. MAS Based on Linkage Mapping

A more interesting situation is when marker and QTL are more loosely linked, as would be expected for the relatively loose marker-QTL linkages uncovered in the first step of a QTL-mapping program. Consider, for example, a QTL that has been mapped to an interval of 10 cM. The distribution of possible QTL locations throughout the confidence interval is approximately normal. Consequently, utilizing a marker at the center of the interval, means that, at most, the marker will be 5 cM from the QTL, and on the average it will be only 1.5 cM from the QTL. Thus, even relatively gross mapping can provide markers that are tightly linked to QTL, and that will remain associated with them over a number of generations. For this reason, even this level of mapping can provide information for useful application of MAS.

Here, however, the population may be at or close to linkage equilibrium between marker and QTL alleles. Consequently, for application of MAS, specific-coupling relations between marker alleles and QTL alleles need to be established for the chromosomes under selection at

the start of the MAS program; and these associations must be periodically reconfirmed. Because this situation is the one we will most likely be facing over the next decade or so, we will explore its application to MAS in the context of three very general breeding situations [114]: (1) within population selection, exemplified by preselection of candidate bulls for progeny testing; (2) Cross-population introgression of a polygenic trait, exemplified by the trypanotolerance trait of the N'Dama cattle of West Africa; and (3) selection to improve crossbred performance, exemplified by a commercial layer cross.

In each example, we assume that prior QTL-mapping experiments have located the important QTL to chromosomal regions of 10–20 cM, so that a marker placed at the center of the region will be within 5–10 cM of the major QTL-affecting trait value, or a pair of markers can be found bracketing the region of interest. In each instance, then, there remain to be defined: (1) a method by which marker allele-QTL allele coupling will be established for the individuals of the breeding population among which MAS will be carried out; and (2) the manner in which the marker-QTL coupling information will be incorporated into the breeding program.

1. Within Population Selection: Candidate Bulls for Progeny Testing

The reproductive life of dairy cows under modern intensive farming is short—only 3 or 4 years on the average, so there is little opportunity for selection among females. Through widespread artificial insemination, however, selection can be very intense among the males. The traits of primary importance in dairy cattle genetic improvement are all related to milk production. These include liters of milk per year, the percentages of milk fat, and milk protein, milk somatic cell count (mostly leukocytes, an index of susceptibility to udder infections), and udder shape and attachment. All of these are sex-limited. Consequently, males are chosen on the basis of the performance of their dams and of their half-sisters, the daughters of their sire. Since this is relatively inaccurate, males so chosen are progeny-tested before entered into widespread use. This requires mating the young sires to sufficient cows to produce 50–100 daughters. Only after these daughters are evaluated is the male returned to widespread service as a proven sire. Each proven sire will produce many hundreds or even thousands of daughters. The very best (“elite”) of the proven sires will also be mated with the very best (“elite”) cows, producing the next generation of young bulls.

Because production of daughters for progeny testing competes directly with utilization of proven sires, the number of daughters that can be allotted for progeny testing is limited. This limits the number of candidate sires that can be progeny tested each year. Consequently, particularly through multiple ovulation and embryo transplantation, it is possible to raise many more males from the elite bulls and elite cows than can be progeny-tested. This provides an opportunity for marker-assisted preselection of the candidate bulls for progeny testing; a situation that has been extensively analyzed [13,30,35,47,74,91,92,95].

Marker-assisted selection can play a role here in the following manner. Both the elite sires and the elite dams are the progeny of the proven sires of the previous generations. Thus, for each QTL assigned to a chromosomal region in the population, each candidate bull will receive 0, 1, or 2 chromosomes that derive from his maternal or paternal proven grandsires. For QTL for which the proven grandsire is heterozygous, however, we would like to retain for progeny testing, those grandsons that received favorable alleles at the QTL. This requires that specific-coupling relations between marker alleles and QTL alleles be established. This can be achieved by implementation of a “daughter” design [118] at all chromosomal regions known to contain QTL segregating in the population, among the many phenotyped daughters of the proven grandsire (ordinarily over 1000). The entire marker-QTL evaluation procedure can be carried out very easily by using milk as a source of DNA for genotyping [68], and by

carrying out selective genotyping with DNA pooling for each trait under selection [22]. In this way, a determination of marker allele-QTL allele coupling can be made in all of the proven grandsires, with a small number of genotypings, yet with statistical power almost equivalent to that provided by complete individual genotyping of each daughter.

This information can now be used for selection among the grandsons of the proven sire, out of his elite sons and daughters. Biometric calculations show that with polyallelic markers, this procedure could increase the rate of genetic progress by about 30% in the first generations in which it is instituted [59], rising to 40–50%, as information on market-QTL-coupling relations in the greatgrandsires of the candidate bulls accumulates in the population.

Fairly soon a point will be reached at which there is sufficient information on marker-QTL couplings in the ancestors of the candidate bulls, to eliminate the progeny-testing step altogether, and shift completely to MAS of young sires. The best MAS-selected young sires would be used extensively as soon as they can produce semen. Their many daughters would be used to establish new marker-QTL couplings, and to reconfirm the marker-QTL couplings that were determined in the previous generations. In this way the supply of information on marker-QTL couplings would be continually replenished and maintained at a high level. This breeding scheme would dramatically reduce generation interval, and should lead to a doubling or more of the overall rate of genetic progress (unpublished calculations).

2. Cross-Population Introgression: Trypanotolerance

Application of MAS for efficient introgression of polygenic traits from one population to another has been extensively analyzed at a theoretical level. MAS can help here by retaining the desired QTL in the face of continued backcrossing, and by selectively eliminating unwanted chromosomal segments from the donor population [33,45,46,50,103,104,107]. Trypanotolerance is a case in point [99]. Although the entire central region of tropical Africa is off-limits to livestock production owing to the prevalence of tsetse-transmitted trypanosomiasis, at the Northern border of the tsetse dominated regions several trypanotolerant breeds have developed. Prominent among these are the N'Dama cattle of West Africa. These are a very small, Jersey-type animal, reaching a maximum of some 1500–2000 L of milk per year under optimal management, as is typical for most native breeds of cattle. Because they are small, N'Dama cattle (*B. taurus*) are not suitable for draught purposes, and for this and perhaps other reasons, it has been observed that Zebu-type cattle (*B. indicus*) are invariably preferred by local herdsmen, and will replace N'Dama, in full, or in part (through crossing) whenever this is permitted by levels of tsetse infestation. On the general principle that herdsmen know what they are doing [R. Bar-Anan, personal communication], it may be useful to specifically introgress trypanotolerance from N'Dama to Zebu; or larger mature body size from Zebu to N'Dama. In the more distant future, it may be useful to introgress trypanotolerance into European beef or dairy breeds, to obtain quantum increases in beef or milk production.

In an introgression program, MAS will allow selection for trypanotolerance without the necessity for challenge, and will allow control of all loci affecting trypanotolerance. This will shorten the period from the first intercross to fixation of the trypanotolerance trait in the recipient. Furthermore, MAS can be carried out against those parts of the donor genome that are unrelated to the trypanotolerance trait, in this way reducing the number of backcross generations required for reconstitution of the recipient traits.

To carry out MAS in this situation, it is necessary to first map the trypanotolerance loci (in trypanotolerance introgression program); or trypanotolerance loci and also QTL affecting desired donor traits (in a program for introgression of desired donor traits into the N'Dama). The genetic situation here is one where, for the most part, donor and recipient share the same

marker alleles, but are probably at or near fixation for alternative alleles at the traits of interest (i.e., trypanotolerance and, say, mature body weight). The appropriate mapping procedure for this situation has been described for an F_2 design [4], but can be readily adapted to a backcross design. Depending on dominance, a backcross to the recipient breed would be the appropriate mapping design for an introgression program because here the first backcross generation can serve both as a mapping population and as the first generation of the introgression program.

Following the mapping exercise, to carry out MAS it is necessary to couple particular donor marker alleles with particular donor trait alleles (trypanotolerance and body size, in our example). When the intention is to use the backcross-mapping population as the first step of the introgression program, however, establishing marker allele-donor allele coupling must also be carried out as part of the mapping exercise. This can be achieved by using only a small number (say, two) donor males to initiate the introgression program and then choosing only a single F_1 male to represent each of the donor males. The purpose of this is to strongly limit the number of donor alleles at each chromosomal region, and to increase the likelihood of uncovering unique or near-unique donor alleles and haplotypes in chromosomal regions carrying the desired donor traits. Each F_1 male is then used to produce a large number of progeny. This should allow specific coupling relations to be established between donor marker alleles and donor trait alleles in the first backcross generation. Coupling relations established in these males would be used to implement MAS in the first and subsequent backcross generations, but would also be tested and reconfirmed in each generation. Inbreeding can be minimized by careful choice of backcross males and recipient females. This procedure should also allow unique or near-unique donor alleles or haplotypes to be identified in chromosomal regions that do not carry desired donor traits. These markers can then be used to select against these donor chromosomal regions, in this way hastening the overall recovery of recipient genotype for all but the desired donor traits.

3. Improving Cross Performance: Layer Chickens

The challenge of improving cross performance, is most acute for laying strains of chickens. All commercial layers are currently strain or breed crosses. The economic value of these crosses depends on the genetic makeup of the two parent strains relative to QTL having codominant genetic effects, and on the degree for which they are complementary for QTL having dominant or overdominant effects. Theoretical analysis [98; J.Arthur personal communication] suggests that at some of the QTL, favorable codominant QTL alleles present in high frequency in one parental line, have simply been lost in the other parental line. Fixation of the unfavorable QTL allele in one of the parental lines represents a permanent loss in the potential productivity of the cross, because for a codominant locus, even if the favorable allele is at fixation in the other parental line, the commercial cross will be heterozygous and will have intermediate value at that locus. Selection on the basis of phenotype alone is unable to specifically transfer favorable codominant alleles between the parental lines, and is relatively ineffective at increasing the frequency of favorable dominant alleles within a given parental strain. Thus, once a superior egg-laying cross has been identified through extensive testing, it is exceedingly difficult to improve it further by within-line selection at a phenotypic level.

MAS can be of assistance here as a means of introgressing complementary favorable codominant alleles from one parent line to the other; and as a means of increasing the frequency of complementary dominant loci within each of the parent lines. Implementation of such an MAS program, will require prior mapping of the two parent lines relative to one another to identify QTL at which they differ, and to classify the gene effects at these QTL as codominant,

dominant, or overdominant [109]. For the critical loci that can make the major contribution to cross improvement, the two parental lines will be at or close to fixation for alternative alleles. Consequently, they can be analyzed as for lines that share alleles at the marker loci, but differ at the QTL of interest [4]. Analysis as a double backcross would be most effective in this instance, because it would immediately provide the two reciprocal backcross populations for cross-introgression of complementary additive alleles.

ACKNOWLEDGMENTS

This research was supported by grants from the Israel Academy of Sciences, the United States-Israel Binational Science Foundation (BSF) and the United States-Israel Binational Agricultural Research and Development Fund (BARD). Thanks also to Sijne van der Beek for providing a preprint of her PhD thesis discussion and bibliography.

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Embryo Manipulation for Biotechnology in Domestic Animals

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I. EMBRYO PRODUCTION

The production of bovine embryos is achieved using either in vitro maturation, fertilization, and culture, or superovulation, artificial insemination, and transcervical uterine flushing. The meiotic maturation of oocytes occurs in vitro, or in the follicle in vivo. After fertilization the zygote and preimplantation embryo growth occurs in culture medium in vitro, or in the oviduct and upper uterine horn in vivo. The development of embryos from in vitro production (IVP) and in vivo multiple ovulation and embryo transfer (MOET) form normal fetuses and calves after transfer into the uterus of a suitable recipient.

A. Oocyte Maturation

The nuclear maturation of germinal vesicle stage oocytes requires chromosomal condensation of the dictyate nuclear stage of the first meiotic prophase. Diakinesis occurs, followed by metaphase I, anaphase I, and then telophase I when the first polar body is extruded. The oocyte is arrested at metaphase II ready for fertilization by a spermatozoon [1,2]. Maturation of oocytes in vitro (IVM) involves the recovery of germinal vesicle stage oocytes from antral follicles within the ovary. These oocytes undergo germinal vesicle breakdown (GVBD) by 10 h after removal from the follicle, with GVBD usually occurring approximately 4 h after aspiration. Diakinesis and chromosomal condensation occur between 8 and 17 h, with the highest frequency at 14 h after oocytes are aspirated from antral follicles. The addition of follicle-stimulating hormone (FSH) to the maturation medium delays diakinesis for 3 h. Anaphase I and telophase I occur at 15–18 h, and mature metaphase II oocytes are found at 15–24 h, with the highest frequency at 20 h. In the presence of FSH the highest frequency for the appearance of metaphase II oocytes is at 23 h [1–3].

Maturation of germinal vesicle stage oocytes involves changes within the cytoplasm of the oocytes. Nuclear and cytoplasmic maturation can occur in oocytes from antral follicles larger

than 0.5 mm in diameter. Before reaching this size, the growing oocyte does not contain sufficient RNA to resume meiotic maturation [4]. Cytoplasmic maturation before metaphase II arrest involves RNA synthesis from the transcription of DNA. Approximately 10–15% of total RNA [5] is polyadenylated in the cytoplasm and requires no nuclear involvement to maintain further translation [6]. Messenger (m)RNA composes 15% of the polyadenylated RNA. Transfer (t)RNA makes up 20–25%, and ribosomal (r)RNA 60–65% of the total RNA [5]. The RNA encodes proteins that are essential to the progression of the cell cycle, such as cyclins and Mphase-promoting factor (MPF) [4]. Actin is translated and polyadenylated during the germinal vesicle stage, but deadenylated during maturation. Conversely, hypoxanthine phosphoribosyltransferase (HPRT) is translated and polyadenylated during meiotic maturation that leads to metaphase II arrest [7]. Other genes that are translated during oocyte maturation are associated with the regulation of meiotic events and oocyte metabolism. The protooncogene *c-mos* is expressed during oocyte maturation until metaphase II arrest. The product of *c-mos* is a serine (Ser)-threonine (Thr) protein kinase and a component of cytostatic factor (CSF) that stabilizes active MPF during metaphase II arrest. This prevents progression through the meiotic cell cycle [8,9]. The *c-mos* gene product also regulates protein kinases associated with cyclic-AMP (cAMP) secondary messenger systems [10] and centrosome phosphorylation that controls cytoskeletal dynamics by shortening microtubules [11]. Another protooncogene expressed during meiotic maturation is *c-kit*, which is a tyrosine kinase receptor and forms a ligand with stem cell factor (SCF). These gene products are found on the surface of a variety of germ cells, including oocytes [5].

Protein synthesis in oocytes is required for meiotic maturation [3]. The inhibition of protein synthesis in bovine oocytes with cycloheximide [3] or puromycin [12] at various time during IVM prevents GVBD, chromosomal condensation, and metaphase I [3]. The extrusion of the first polar body is sensitive to the inhibition of protein synthesis induced by puromycin treatment before metaphase I [12]. The inhibition of protein synthesis after metaphase I allows meiosis to progress to metaphase II; however, abnormalities are observed [3]. Nuclear membrane breakdown at GVBD is less sensitive to the inhibition of protein synthesis and phosphorylation than the extrusion of the first polar body [12].

The synthesis of proteins in GV stage oocytes before IVM, is greater than for metaphase II oocytes after maturation. Protein synthesis in oocytes also differs according to the morphological classification [13]. Oocytes that have poor, or no associated cumulus before maturation synthesize less protein than oocytes with an obvious cumulus mass and distinct corona radiata [13]. Active protein synthesis is associated with GVBD and the extrusion of the first polar body after telophase I [14]. The changes in protein synthesis are also associated with protein phosphorylation.

The inhibition of phosphorylation with 6-dimethyl aminopurine (DMAP) reversibly prevents GVBD [15]. During GVBD, the phosphorylation of 50- and 60-kDa proteins increases, whereas the phosphorylation of a 19-kDa protein decreases [14]. The production of mRNA is also necessary for events early in meiotic maturation. The inhibition of transcription and mRNA synthesis with α -amanitin [16] or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazo(DRB) [17] prevents both phosphorylation and decrease GVBD. The inhibition of transcription does not affect meiotic maturation events after GVBD [16].

The oocyte is connected to the surrounding cumulus cells and corona radiata by gap junctions. The gap junctions exist between cumulus cell processes and the cortex of the oocyte. During IVM, gap junctions disconnect so that cellular communication is prevented and the oocyte loses direct connection with its environment [18]. When the corona radiata of germinal vesicle stage oocytes appears as a dark ring, GVBD occurs later than when the corona is not distinguishable. The absence of corona appears with rapid GVBD and cumulus cell elongation

with increased arrest at diakinesis and anaphase I [1,19]. Failure of these oocytes to complete nuclear maturation may be due to the absence of FSH in the maturation medium. The use of FSH facilitates nuclear maturation of oocytes [1].

The organization of organelles within an oocyte changes during maturation. After the onset of maturation the nuclear membrane becomes ruffled, GVBD then occurs, and the mitochondria become spread throughout the oocyte. As the nuclear status of the oocyte approaches telophase I and the extrusion of the first polar body, membrane-bound vesicles fuse together and the mitochondria cluster with lipid droplets and endoplasmic reticulum (ER). At metaphase II the cortical granules begin to move to the oocyte cortex, the Golgi complexes and smooth (S)ER decrease in size, and vesicles are found near the center of the oocyte [18,20].

The secondary messengers that control the events of oocyte maturation are driven by changes in cAMP. The concentration of cAMP in an oocyte can be increased with the addition of cAMP or its derivatives to the medium, or by preventing the pathways that phosphorylate cAMP to ATP. An increase in the concentration of cAMP decreases GVBD [21] and the resumption of meiosis [22]. Alternative pathways exist for the metabolism of cAMP so that GVBD is not always completely inhibited. One pathway is the cumulus cells that surround the oocyte. The removal of cumulus affects meiotic maturation [21]. Cumulus cells have a high concentration of cAMP that signals the oocyte to bypass the inhibitory effect of cAMP inside the oocyte [23]. The signals that are controlled by mRNA synthesis, protein synthesis, phosphorylation, and cAMP are thought to be complex cell cycle control proteins that initiate and regulate the meiotic maturation [24].

The gonadotropins FSH and luteinizing hormone (LH) are required during IVM to improve the developmental competency of the embryos after fertilization. The proportion of oocytes that are fertilized after maturation without LH or FSH is similar to those matured with LH and FSH. However, the development of embryos to the blastocyst stage is enhanced when oocytes are matured with gonadotropins [25]. Maturation of oocytes *in vivo* relies on the LH surge to induce the resumption of meiosis. Superovulation of animals with FSH increases the number of developing follicles that may ovulate, but this treatment can induce abnormalities, such as premature chromatin condensation [26].

B. Sperm Capacitation

Spermatozoa undergo a series of changes before fertilization that allow the acrosome reaction, gamete fusion, and fertilization [27]. The changes that occur *in vivo* in the female reproductive tract, or are induced *in vitro* in the laboratory, are collectively termed capacitation. Morphological assessment of spermatozoa is required to visualize the acrosome reaction that occurs when the outer acrosomal membrane fuses with the plasma membrane on the head of spermatozoa [27]. The acrosome reaction also induces hyperactivated motility of spermatozoa. Capacitation of spermatozoa is not assessable by morphological examination. Capacitation can be assessed only by the ability of spermatozoa to fertilize oocytes, or indirectly, by the proportion of acrosome-reacted sperm and the ability of an external stimulus to induce the acrosome reaction [27]. The oviductal fluid of estrus cows increases capacitation [28], as assessed by increased acrosome reaction rates and the penetration of oocytes. A heparin-like glycosaminoglycan is a factor that accelerates capacitation. Capacitation of spermatozoa *in vitro* is achieved using heparin [29], which binds specifically to bovine sperm and induces capacitation in the presence of extracellular calcium [27]. Washing spermatozoa with heparin induces the acrosome reaction and decreases returns to estrus after artificial insemination [30]. Calcium ionophore (A-23187) is also able to induce capacitation [31]. This confirms the role of Ca^{2+} during the capacitation of spermatozoa.

Capacitation is reversed by glycoproteins, sterols, and seminal fluid, or inhibited by glucose, which prevents an increase in pH by glycolysis. The addition of compounds that influence cAMP can reverse the inhibition of capacitation by glucose. The presence of phosphodiesterase inhibitors increases cAMP, decreases the acrosome reaction and fertilization rates [32,33], and induces hypermotility [34]. Changes in cAMP affect the phosphorylation of protein kinases that are associated with capacitated sperm [35]. This implicates both calcium and cAMP as secondary messengers in the control of capacitation of spermatozoa before fertilization [27].

C. Fertilization

Fertilization occurs after spermatozoa have bound to the zona pellucida following the passage between the remaining corona radiata and cumulus cells that surround the oocyte. The acrosome of the capacitated spermatozoa contain the enzyme hyaluronidase that digests the cumulus cell matrix that is conjugated with hyaluronic acid [36] and facilitates the movement of spermatozoa toward the oocyte. At this time the cumulus cells have large Golgi complexes, rough endoplasmic reticulum (RER), polyribosomes, lysosomes, and lipid droplets. Some spermatozoa are phagocytosed by cumulus cells [37]. The zona pellucida is composed of glycoproteins, the most abundant of which is ZP3 that is the species-specific sperm receptorbinding site for capacitated spermatozoa and induces the acrosome reaction [1]. Only capacitated and acrosome-reacted spermatozoa can penetrate the zona pellucida of cattle oocytes [38].

Spermatozoa begin to penetrate the zona 6 h after insemination when the cortical granules in the oocyte are clustered in the cortex of the oocyte [37]. Sperm fuse with the plasma membrane to fertilize the oocyte 8 h after insemination. Intracellular calcium ion (Ca^{2+}) concentrations are elevated to 870 nM, then undergo multiple Ca^{2+} pulses that decrease in magnitude every 15–29 min [39]. Changes in the Ca^{2+} concentration is a sign of oocyte activation [36] and is regulated by inositol triphosphate (IP_3) that modulates Ca^{2+} channels in ER and sarcoplasmic reticulum. Heparin, the compound used to capacitate spermatozoa, inhibits IP_3 activity, but does not prevent increases in Ca^2 [40]. The cortical granules in the oocyte remain clustered until 2–3 h after the sperm has fused with the oolemma, when the contents of the cortical granules are exocytosed into the perivitelline space [37]. This causes transient pulses of increased Ca^{2+} concentrations [36] that result in a modification of the zona pellucida ZP3 [5] to prevent further penetration of spermatozoa.

After fertilization, the sperm nucleus begins to decondense in the oocyte cortex [38] as protamines that supercoil spermatozoa DNA are replaced with histones [36]. A microtubule aster is formed in association with the junction of the sperm head and tail [41]. The second polar body is emitted, and the nuclear envelope begins to form from cisternae of ER around the decondensing metaphase chromosomes. The DNA condenses, and the pronuclei are clearly visible 16–20 h after insemination [37,38]. The pronuclei possess agranular nucleoli [38] as DNA synthesis occurs [42]. A microtubule aster is situated between pronuclei [41], and a microtubule network extends from the male pronucleus and contacts the female pronucleus [43]. As the pronuclei move to the center of the zygote, the centriole remains between the pronuclei and is attached to the microtubules that originate from the male pronucleus. At 24 h after insemination, the nuclear envelope of the pronuclei is broken down, and the chromosomes condense and mix, the centrosome divides and oppose each other on a bipolar spindle formed from the microtubule array. The sperm aster is reduced and new asters form at the poles of the spindle, these asters occupy the entire cytoplasm during cytokinesis [43]. The cAMP-dependent protein kinases (PKA) are involved in cytokinesis [44]. The inhibition

of protein synthesis during fertilization does not prevent the events described previously [45]. This suggests that Ca^{2+} and cAMP are the primary messengers that control events during fertilization and zygotic development.

D. Embryo Development

Preimplantation bovine embryo development progresses from the first cleavage division to the hatching of a blastocyst from the zona pellucida 6–7 days later [46]. One day (24 h) after sperm penetration, most *in vivo* fertilized zygotes have reached the two-cell stage, whereas most IVP embryos have cleaved by day 2 (48 h) after insemination. Embryos produced *in vivo* have four blastomeres by day 2 and 8–16 blastomeres by day 3 (72 h). In contrast, IVP embryos complete the second cell cycle and have four blastomeres by day 3. From this stage the embryo undergoes asynchronous cleavage, so by day 4 (96 h) *in vivo* the embryos may have between 8 and 64 cells, whereas IVP embryos lag by 1 day. The exact time of fertilization is known for IVP embryos. However, the time of fertilization for *in vivo*-produced embryos varies with ovulation after superovulation. This results in slight differences in developmental stage between the embryo types. After fertilization *in vitro*, the first cell cycle takes 30 h, whereas the second and third cell cycles take only 13 h. The fourth cell cycle takes between 21 and 30 h and is associated with a decrease in protein synthesis. Although mRNA synthesis profiles begin to change from maternal to zygotic at the late four-cell stage, the complete onset of embryonic transcription does not occur until the 8- to 16-cell stage [47].

The polarization of the blastomeres begins during compaction of the embryo when the thread-like microvilli on the membrane surface of blastomeres remain distinct at one pole and disappear at the other. Polarization of the blastomeres is the signal that the blastomeres are dividing into two populations that become either the inner cell mass (ICM) or the trophoblast of the blastocyst [48]. By day 5 (120 h) *in vivo* the embryo forms a compacted morula with approximately 32 cells. The blastomeres adhere to each other and flatten so that the individual blastomeres are not distinguishable. At this stage, gap junctions appear and facilitate cell-to-cell coupling between the blastomeres. Gap junctions are less obvious in IVP embryos [49]. Midbodies from residual cleavage furrows may imitate gap junctions and provide an alternative form of cellular communication [46]. By day 6 (148 h) hatched blastocysts are the most advanced developmental stage *in vivo*, so that by 7 days (168 h) after fertilization *in vivo* viable embryos will be between the 16-cell and a hatched blastocyst stage [46]. Although, at 7 days after fertilization a 16-cell embryo may be viable, its developmental potential is considered retarded.

The formation of a blastocoel occurs when a compacted morula undergoes cavitation. The outer cell layers are epithelial trophectoderm. These cells are polarized and represent the first differentiated cell type. The primitive endoderm develops from the inner cell mass (ICM) that constitutes the cell lineages that are included in fetal development. Cavitation relies on the active uptake of Na^+ and K^+ by the trophoblast and the maintenance of water in the blastocoel. The difference in ion concentration gradients facilitates the movement of water into the blastocoel. The zonula occludens are tight membrane contacts between the trophectoderm cells that prevent leakage of water from the blastocoel. Further cell contacts are created by the uvomorulin group of cell adhesion molecules. This provides a Ca^{2+} -dependent zonula occludens associated with the ZO-1 protein [50]. Growth factors are able to influence blastocyst development by modulating Na^+ and K^+ ATPase activity. Insulin-like growth factors (IGF-I and IGF-II) increase RNA, protein synthesis, and cytokinesis [50]. The most likely mechanism for the effect of growth factors is on the secondary messengers of active ion pumps [50].

The usual endpoint for experiments investigating preimplantation embryo development in cattle is the development of a blastocyst 7 days after fertilization. However, viable embryos are able to form blastocysts up to 9 days after fertilization [51]. There is a difference in the sex ratio of the blastocysts formed on day 7, 8, and 9. Male embryos appear to develop more rapidly to the blastocyst stage than female embryos [51]. The differences in embryo viability between embryos collected *in vivo* [52] and those cultured *in vitro* are associated with fewer cells in of the ICM of IVP embryos [53].

The culture of cattle embryos *in vitro* does not adequately simulate the oviduct and uterine environment of the female reproductive tract. The use of IVM and *in vitro* fertilization (IVF) facilitates the study of preimplantation cattle embryo culture *in vitro*. The development of IVP embryos can vary according to the category of the antral follicle from which the oocyte originated [54] so the culture conditions that are applicable to IVP embryos may not be optimal for the culture of *in vivo*-produced embryos.

The culture of embryos to the blastocyst stage can be achieved using somatic cells under coculture conditions, during which the embryos are placed into medium with a variety of somatic cells. The cell types include bovine oviduct epithelium, buffalo rat liver cells [55], granulosa cells [56], and cumulus cells [57]. Embryos can also be cultured with trophoblast vesicles [58] or in the ligated oviducts of sheep [59]. The embryonic development of viable cattle embryos is not dependent on the type of coculture cells used.

Many culture media have been used to grow cattle embryos *in vitro*. The most commonly used media can be broadly classified into the simple salt solutions, such as Charles Rosenkrans medium 1 (CR1) [60], and synthetic oviduct fluid (SOF) [61], or the more complex media such as Menezo's B2 (B2) [62] and tissue culture medium 199 (TCM 199) [63]. Each medium that is used for embryo culture has specific conditions that facilitate embryo development. For example, the use of TCM 199 is most useful when used in conjunction with a somatic cell coculture [55].

Growth factors are not normally present in embryo culture media except by the inclusion of those contained in serum when it is used as a source of protein. When testing the effects of these proteins a chemically defined salt-based medium is used. The addition of proteins, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), the IGFs, nerve growth factor (NGF), the transforming growth factors (TGF), and transferrin do not affect bovine embryo cleavage [64]. Only transferrin increases blastocyst development, although it is not absolutely required for embryo development. Other reports indicate that TGF and FGF in combination increase blastocyst development [65] and that fibronectin is required for blastocyst development [66]. Even though the effects of exogenous growth factors on embryo development are conflicting, molecular studies have shown that mRNA for growth factors is produced during embryo development. Studies on the role of growth factors during embryo development show that insulin and IGF-I move to the ICM and are internalized. The ICM produces TGF that binds to EGF receptors on trophoblast cells. Similarly, insulin and IGF-I share receptors and are found in the oviduct [67]. Clearly, the role of growth factors is important in pre-implantation embryo development, although the mechanisms and absolute requirement for growth factors during development have yet to be determined.

The assessment of embryo metabolism during preimplantation development provides information about the developing IVP embryo. The study of energy substrates during embryo development *in vitro* provides an insight into the nutrient requirements of the oocyte and embryo during the extended period of *in vitro* culture. The use of noninvasive techniques for measuring nutrient uptake and embryo metabolism [68] provide a guide to the requirements of the embryo only in the *in vitro* environment that the measurements are taken. The metabolism of the embryo changes with different media and metabolism assays because the

metabolism of cattle oocytes during IVM follows different pathways under different conditions [69]. The metabolism of glucose through the Embden-Meyerhof and pentose phosphate pathways is low and does not change during IVM. However, the metabolism of glucose through the Krebs cycle is initially low, but increases during meiotic maturation. The metabolism of pyruvate, glutamine, and glycine increases during IVM. The utilization of pyruvate reaches a peak at 12 h after the onset of maturation when glutamine metabolism increases. The uptake of glycine increases steadily during IVM [69].

The energy substrates in the medium used to culture embryos *in vitro* have an effect on the development of the embryo [60,70]. The presence of excess glucose in the culture medium causes the *Crabtree effect* during which mitochondrial respiration is inhibited by enhanced glycolysis [71]. The presence of glucose in culture medium inhibits development of bovine embryos to the morula stage [73] and the removal of glucose from the culture medium allows development to the blastocyst stage in the presence of fetal calf serum [72]. The ratios of glucose, lactate, and pyruvate, along with the use of amino acids and vitamins are beneficial to the development of cattle embryos to the blastocyst stage [73]. The use of media for the culture of preimplantation cattle embryos is under constant investigation. The trend to move from the technically demanding complex media and coculture systems to the simple salt solutions is based on the increased use of simple media to determine the requirements of cattle embryos during development *in vitro*.

E. Periimplantation Embryo Development

The stages of cattle embryo development after the hatching of the blastocyst are not able to occur *in vitro*. Once an embryo has reached the blastocyst stage, optimal embryo development must occur *in vivo*. The cattle blastocyst hatches 8-10 days after fertilization when a combination of enzymatic and physical forces thin the zona pellucida and cause it to tear so that the embryo may escape [46]. The embryo continues to expand and the ICM migrates to the outside of the spherical embryo and becomes the embryonic disk (ED). The embryo begins to elongate at 12-14 days after fertilization, so that by day 16 it is between 0.6 and 60.0 cm in length. Elongation of the embryo is variable and depends on the uterine environment for the development of the embryo. This stage of development is characterized by vast differences in embryonic growth that can remain static, or is stimulated to increases in length of up to 45 mm/h [46].

During elongation, the embryonic cell lineages differentiate. The ED develops into embryonic ectoderm, mesoderm, and endoderm, while the trophoblast develops into the trophectoderm that consists of ectoderm, mesoderm, and endoderm. The embryonic mesoderm forms an outer layer that lines the trophectoderm and becomes the chorion. The inner layer of embryonic mesoderm covers the endoderm to create a wall for the yolk sac. The allantois develops at approximately 20 days after fertilization between the two mesoderm layers, termed the exocoelos. At this time the elongated embryo attaches to the wall of the uterus [46].

The application of reproductive biotechnologies to cattle has resulted in the production of calves from oocytes that have been collected from antral follicles, then matured, fertilized, and cultured *in vitro* [74]. Various modifications to this have allowed the oocytes to be frozen, then thawed before *in vitro* fertilization [75], the oocytes can be collected from prepubertal calves [76], or the embryos are allowed to develop without a zona pellucida [77]. These techniques provide scope for a variety of applications of reproductive biotechnologies to the production of cattle.

II. BACKGROUND TO REPRODUCTIVE BIOTECHNOLOGY

The use of reproductive biotechnological techniques enables research into more advanced manipulations such as nuclear transfer. The background of cell mechanisms that are applicable to nuclear transfer are taken from a wide range of scientific fields including cancer research and reproductive and molecular biology. The cell cycle of oocytes and embryos is controlled by basic signals that are elucidated through an understanding of the molecular pathways of cells. This permits the artificial control of oocytes and embryos within the limits that allow normal development. Most of the discussion in this section comes from scientific experiments from areas other than cattle embryology. The information remains relevant because the topics are crucial to all cell biology, especially during experimental manipulation.

A. Parthenogenetic Activation

Parthenogenesis is the creation of an embryo from only a female gamete [78]. Oocytes that have passed the first meiotic metaphase, or are at metaphase II arrest can be parthenogenetically activated [79,80]. Oocytes can spontaneously activate after aging, or are parthenogenetically activated after receiving an external stimulus. Parthenogenetic activation induces elevated Ca^{2+} concentrations [81], cortical granule exocytosis, translation of oocyte mRNA, pronuclear formation, and the initiation of DNA synthesis [78].

The parthenogenetic activation of oocytes with an electrical stimulation results in 30% development to the blastocyst stage and induces elevations in intracellular Ca^{2+} concentrations to greater than 1000 nM [81]. This is the same magnitude as the Ca^{2+} concentrations stimulated by the penetration of a fertilizing spermatozoon [82]. The activation of oocytes stimulates a pulsatile increase in Ca^{2+} concentration that acts as a secondary messenger to cause CG exocytosis by the calmodulin and Ca^{2+} -dependent protein kinase C (PKC) pathways [79]. The Ca^{2+} concentration in parthenogenetically activated oocytes is critical to the success of parthenogenone development. A nonphysiological Ca^{2+} concentration results in partial parthenogenetic activation that is characterized by irregular formation of pronuclei and a third meiotic metaphase arrest [83,84]. The exocytosis of CG releases protons that induce kinase activity [78] and generate changes in the zona pellucida by modifying the ZP2 to ZP2_f zona pellucida-associated glycoproteins [85].

Oocytes matured *in vitro* are more susceptible to parthenogenetic activation than those matured *in vivo*. Approximately 80% of parthenogenones are haploid and have emitted the second polar body, the remaining are diploid or tetraploid [86]. When the kinase inhibitor DMAP is used during ionomycin treatment for parthenogenetic activation, the second polar body is not emitted, the parthenogenones develop one pronucleus and 21% develop to the diploid blastocyst stage [87]. When cattle oocytes are parthenogenetically activated with ethanol and then treated with cytochalasin B, the extrusion of the second polar body is inhibited, two pronuclei form and 14% develop to the blastocyst stage [88]. When oocytes are parthenogenetically activated by treatment with the Ca^{2+} ionophore (A-23187) followed by cycloheximide treatment, the second polar body is emitted and parthenogenones develop as haploid blastocysts [89].

The electrical potential of oocytes changes from negative to positive during normal fertilization [78]. The use of an electrical stimulus parthenogenetically activates oocytes with 70%, 13%, and 2% of oocytes forming one, two, and three pronuclei, respectively. When these parthenogenones are treated with cytochalasin B, 80% of the oocytes formed two pronuclei [90]. After transfer, approximately 30% of bovine parthenogenones are capable of inducing extended estrus cycles. Parthenogenones exist as uterine vesicles that may persist

until 42 days after parthenogenetic activation. Embryonic structures without a heart beat have been observed up to day 35 [87,88].

The age of the oocytes relative to the start of maturation influences parthenogenetic activation. Aged oocytes are more susceptible to parthenogenetic activation by treatment with the Ca^{2+} ionophore. Oocytes that are 18 h after the onset of maturation gain the ability to be parthenogenetically activated, and those at 30 h have the greatest response to stimuli by forming pronuclei [91].

Parthenogenetic activation of oocytes alters the histone H1 kinase activity associated with the cell cycle. A single electrical pulse causes an initial decrease in H1 kinase activity; however, this activity returns after 6 h. Multiple electrical pulses rapidly and nonreversibly decrease the H1 kinase activity [82].

B. Cell Cycle

The cell cycle progresses from one cell division to the next and is broadly classified into metaphase and interphase [92]. After a cell has duplicated its chromosomes, it divides. The two diploid daughter cells then progress through DNA replication and cell metabolism. Interphase is divided into the first gap phase (G_1), when the cell prepares for DNA synthesis that occurs during the S phase of the cell cycle. During the second gap phase (G_2), the cell prepares for a reductional mitotic division during M phase [93]. During G_1 the cell can reversibly rest in G_0 , a phase when the cell cycle is quiescent and the cell undergoes growth or differentiation [92].

The M phase is divided into distinct events. During prophase the chromosomes condense and form chromatids and the spindle begins to form. The breakdown of the nuclear envelope occurs during prometaphase when the spindle microtubule fibers attach to the kinetochores at the centromere of the chromosomes. The chromosomes become aligned between the two poles of the spindle at metaphase. The chromosomes separate into chromatids at the centromere and move toward the spindle poles during anaphase. During telophase the spindle disappears, chromatids decondense, the daughter cell nuclear envelope and a cleavage furrow forms, cytokinesis occurs, and the cytoplasm is divided to form two approximately equal-sized cells [93].

The length of the cell cycle in cattle embryos changes as development progresses. After an oocyte is penetrated by a sperm, meiosis is completed within 2 h and pronuclei form within 4 h. The length of G_1 is 6-7 h, finishing at approximately 11 h after sperm penetration. The duration of S phase is 8 h, and G_2 lasts for 4-6 h. The M phase and cytokinesis is completed in 2 h, ending at 24-27 h after fertilization [94]. The second cell cycle is faster than the first because the G_1 and G_2 phases are shorter. The length of G_1 is 2 h and S phase lasts 8 h, G_2 and M phase combined last 2-4 h. The entire second cell cycle lasts approximately 15 h. The cleavage of blastomeres during the third cell cycle is asynchronous because the length of the cell cycle varies from 9 to 15 h as embryonic transcription begins. The fourth cell cycle lasts 20-30 h when the blastomeres become polarized. The changes in the length of the cell cycle at different embryonic stages occurs because of the disappearance of G_1 and G_2 phases during the second and third cell stages that correspond with a decrease in protein synthesis [47].

An understanding of the cell cycle is particularly important in the manipulation of oocytes and embryos when the meiotic and mitotic cell cycles interact. The events of the cell cycle are driven by the MPF group of protein complexes. The MPF complex is a 34-kDa protein product of the cell division cycle (*cdc2*) gene ($p34^{cdc2}$) and is also known as the first member of a group of cyclin-dependent serine-threonine (Ser-Thr)-dependent protein kinases (CDK) [92]. The

known CDKs (CDK1-7) are a group of catalytic subunits that contain approximately 300 conserved amino acids, that are inactive when monomeric and have varying degrees of activity, depending on phosphorylation as a dimer with a cyclin [95]. The cyclins have eight groups (A-H) [96] and contain a cyclin box of approximately 100 conserved amino acids that are specific to CDKs and elicit specific effects during the cell cycle. The cyclins are characterized by an oscillating appearance in cells. Some of the CDK and cyclin combinations can be measured by an assay for histone H1 kinase activity [97].

The inactivation of MPF after M phase involves the ubiquitin-mediated degradation of cyclin that is controlled by CDK1 [95]. MPF can be inhibited and activated by phosphorylation changes at different sites. When a CDK is phosphorylated by the CDK-activating kinase (CAK) gene product on the Thr-160 site, a T loop that blocks protein binding moves to facilitate the binding of a cyclin [95]. When a CDK is complexed with a cyclin its MPF activity is also controlled by phosphorylation. The activity of MPF is inhibited by phosphorylation of the Thr-14 and tyrosine (Tyr)-15 sites near the ATP-binding site by the p107^{wee1} kinase. The phosphorylation of the Thr-14 and Tyr-15 sites follows the cyclin levels during G₂ when MPF is inactive. The activation of MPF during M phase occurs when the Thr-14 and Tyr-15 sites are dephosphorylated by the p80^{cdc25} phosphatase. During G₁, the MPF complex consists of CDK4 or CDK6 with the cyclins D1, D2, and D3. These cyclins bind the retinoblastoma protein (pRb) and inhibit transcription factors such as E2F [98] that control the onset of DNA synthesis and the onset of S phase.

The overexpression of the G₁ cyclins causes the length of G₁, the cell volume, and the requirement for mitogens to decrease [98]. The expression of G₁ cyclins is affected by the transcription factors *c-myc* and *c-ras* that interact with pRb to control the entry into G₀ [96]. The interaction of *cdc25* with *c-ras* during active glucose uptake by the cell [99] implicates cell metabolism with the control of the cell cycle. The MPF complex can be inhibited by CDK inhibitors (CKI) that bind the phosphorylated Thr-160 site and inhibit MPF activity. The CKIs are controlled by factors such as the protooncogene *p53*, TGF- β , and interleukin-2 (IL-2). The pRb is also controlled by *p53*, and prevents E2F activity. When E2F activity increases at the same time as *p53* the cells undergo apoptosis [98]. The CKIs are subject to ubiquitin-mediated degradation at the G₁ to S phase transition and prevent DNA synthesis until DNA repair and correct ploidy are achieved [95].

Entry into S phase occurs when the MPF complex consists of CDK2 and cyclin E. As cyclin E is degraded, CDK2 complexes with cyclin A during S phase and the proliferating cell nuclear antigen (PCNA). This complex combines with DNA polymerase γ and controls DNA replication [98]. During M phase, CDK1 forms a complex with cyclin B. The MPF activity of this complex is controlled by phosphorylation by CDK7-cyclin H and CAK or dephosphorylation by *cdc25* initiating prophase [100]. This activates ubiquitin-mediated cyclin B degradation and the beginning of anaphase. The destruction of cyclin B stops as G₁ cyclins are transcribed and the cell cycle progresses [100].

Entry into M phase is promoted by the type 2 protein phosphatase (PP2) that dephosphorylates *cdc25*. The activity of MPF during G₂ is stabilized by the phosphorylation of MPF by *wee1*. During M phase, *cdc25* dephosphorylates MPF which activates the complex. Exit from M phase is prevented by p13^{suc1} that inhibits MPF activity by binding the CDK1-cyclin B complex [92]. The type 1A protein phosphatase (PP1A) inactivates *cdc25* and promotes the exit from M phase [100].

The initiation of meiosis is connected with the *c-mos* protein kinase when cells progress from prophase of meiosis I into metaphase. Cellular *c-mos* initiates mitogen-activating protein (MAP) kinase that stimulates CSF generation, causing metaphase II arrest [96].

C. Cell Cycle Control

Once a basic understanding of the cell cycle is obtained, various methods can be used to manipulate it. Throughout the cell cycle, checkpoints exist that represent the weak points of the cell cycle. These checkpoints are the G₁ to S phase intersection and M phase. The progression through the cell cycle is dependent on the completion of DNA synthesis during S phase [101]. Similarly, the condensation of chromosomes during M phase is reliant on DNA repair during the progression through G₂ to M phase. At M phase *cdc25* becomes associated with PP1 and activates MPF activity and mitotic events [102]. The cell cycle checkpoints during a normal cell cycle involve the interaction of CDKs and cyclins, the cascades of phosphatases and kinases, and the secondary messengers cAMP and Ca²⁺ [103,104]. Treatment of cells with prostaglandins or TGF- β arrest the cell cycle at the beginning of S phase [98,105].

Chemicals directed at a vital event in the cell cycle are used to control the checkpoints. The chemical forskolin prevents the metabolism of cAMP by inhibiting adenylate cyclase activity; this increases cellular cAMP [106]. The activation of PKA by elevated cAMP affects the cell cycle by PKA kinase activity on the phosphorylation of cell cycle complexes [106]. When cells are treated with the chemical aphidicolin to inhibit DNA synthesis, the cell cycle stops at the beginning of S phase [107,108]. The cell cycle is stimulated to progress, without the completion of DNA synthesis after caffeine treatment [109] that inhibits phosphodiesterase activity and increases cAMP, which activates PKA and dephosphorylates MPF [110]. The chemical genistein inhibits topoisomerase II and tyrosine kinase activity, which arrests the progression of the cell cycle at the G₂ and M phase interface [111]. The PCC and the onset of M phase can be induced with the PP1A and PP2 inhibitor okadaic acid [112,113], which induces spindle lengthening and microtubule disruption [114]. Okadaic acid also inhibits cell cycle progression during oocyte maturation [115]. The chemical nocodazole inhibits microtubule polymerization, which reversibly stops the cell cycle in M phase [116]. The protein kinase inhibitor DMAP inhibits phosphorylation of cell cycle proteins and facilitates the exit from M phase, through G₁ to pronuclei formation and S phase in mouse oocytes [117]. The use of DMAP also prevents the effects of okadaic acid [118].

The possibilities for manipulating the cell cycle are expanding along with the understanding of the mechanisms that normally control the normal cell cycle. Although not all the studies mentioned have been performed in cattle embryos, the basis for manipulation of the cell cycle is understood.

D. Transcription and Methylation

The transcription of DNA into RNA provides the basis for the translation of RNA into proteins that regulate cell function and cell-to-cell interactions. Before embryonic transcription begins in preimplantation embryos, the synthesis of proteins by translation relies on mRNA from maternal stores within the oocyte [119]. The transcription of mRNA in cattle embryos has been detected in late four-cell embryos [120,121]. However, the control of embryonic development by proteins derived from embryonic transcription occurs from the 8- to 16-cell stage [42,120,122].

The 8- to 16-cell cattle embryo at the third and fourth cell cycle is characterized by an increase in the length of the cell cycle. The transcription of DNA into mRNA is achieved by transcription factor-mediated RNA polymerase III recognition. The presence of excess histones during the second cell cycle represses transcription [124] by inhibiting the binding of transcription factors and RNA polymerase to DNA by increasing the folding of chromatin [125]. The RNA polymerase promoter *c-myc* increases with the change of transcription from

the maternal to embryonic genome [126] and correlates with the decrease in competition between chromatin assembly and transcription complexes [124].

The phosphorylation changes that occur during the cell cycle affect the binding affinity of transcription factors to DNA. Phosphorylation mediated by PKA and PKC is involved in the activation of transcription factors [127]. The kinase activity of MPF stimulates the production of a transmembrane protein from the ER that signals the DNA transcription by transcription factors [128]. The activation by PKA of the transcription factor associated with the cAMP response element-binding protein (CREB) is by phosphorylation of a serine residue on the CREB. The CREB then binds a DNA sequence on which cAMP response elements (CRE) initiate transcription [127]. Transcription factors are also controlled by mitochondrial communication with the nucleus. When the Krebs cycle is limited, the mitochondria regulate interaction with the glyoxylate cycle by activating transcription factors that allow citrate from the glyoxylate cycle to enter the Krebs cycle [129].

Transcription that is controlled by cAMP can be mediated by the effect of growth factor and hormones on adenylate cyclase [130]. The transcription of growth factors during cattle preimplantation embryo development shows that mRNA exists for IGF-II, PDGF-A, TGF- α , TGF- β , and the receptors for insulin, IGF-I, IGF-II, and PDGF- α . The mRNA for these growth factors is derived from both oocyte and embryonic transcription. The mRNA for FGF comes from transcription during oogenesis, while mRNA for interferon- τ (INF- τ) exists from transcription during the blastocyst stage. Preimplantation cattle embryos do not transcribe insulin, EGF, or NGF [131]. Even though the addition of these growth factors to embryo culture media does not effect development, the synthesis of growth factors by the embryo shows they have an important role during embryonic development. Insulin and IGF-I share the same receptor and stimulate DNA, RNA, and protein synthesis; glucose transport; and metabolism when the receptors are present in compacted embryos [132]. The growth factors TGF- α and EGF share the same tyrosine kinase receptor [133]. The binding of TGF- β requires two receptors. The type I receptor is bound only when the type II is present, and the type II Ser-Thr kinase receptor will signal only when both receptors are present [134]. The TGF- β complex is implicated in the differentiation of embryonic cells and in progression through the cell cycle [98]. The transcription mRNA for IFN- τ occurs at the blastocyst stage of cattle embryos, but the protein is not translated into active protein until the expanded blastocyst stage [135]. This shows that although transcription of DNA into mRNA occurs at relatively fixed times during development, the translation into protein does not follow exactly the same pattern.

The regulation of transcription of some specific genes, for which correct expression is critical to normal development, is controlled by imprinting of DNA [136]. Imprinting describes the different effects that the maternal and paternal genomes have on embryo and fetal development. Experimentally produced parthenogenetic and gynogenetic embryos develop into fetuses with poor extraembryonic tissues derived from the trophoblast. In contrast, androgenetic embryos develop fetal membranes from the trophoblast, but the fetuses develop poorly [137]. These imprinted effects on the parental genomes are independent of cytoplasmic effects, although the experimental embryos can be rescued by constructing chimeras with normal blastocysts [137].

Genetic imprinting is controlled by methylation of cytosine-guanosine dinucleotides (CpG sites) of DNA. The level of methylation changes during development [138] when increased methylation usually causes a decrease in expression of specific genes [136]. Imprinting is established during gamete maturation, and the methylation pattern of the maternal genome is present during oogenesis. Certain CpG sites termed *Alu* repeat sequences are not methylated on sperm DNA, but are densely methylated on DNA from somatic tissue [139]. The DNA

around the CpG sites of sperm is methylated, although male and female primordial germ cells, germ cells from the fetal ovary, and adult testes, are not methylated [140]. The methylation of the paternal genome undergoes changes after fertilization and throughout embryonic development [138]. Total methylation remains high during embryo development and begins to decrease to 10% of the original total after blastocyst formation. Parthenogenenes are demethylated more slowly than normal embryos [141].

The control of transcription by imprinting involves methylated DNA being bound by nuclear methyl-CpG-binding proteins that prevent the interaction with RNA polymerase II (142). Methylated DNA also directly prevents the binding of transcription factors such as E2F and *c-myc* [143]. When DNA methylation is minimal, transcription is possible when the activity of the promoter is high. When methylation of a gene is high, transcription is inhibited under any circumstance [142]. The relative methylation density of a promoter region for a particular gene controls the level of transcription of the target gene [144].

The control of the methylation is by DNA methyltransferases, which methylate CpG sites that are found at high levels in preimplantation embryos [145]. Most of the methyltransferase activity in early embryos is found in the cytoplasm. Methyltransferase forms granules during embryo compaction when nuclei contain large amounts of methyltransferase. Nuclei of blastocysts contain low amounts of methyltransferase that corresponds with the low methylation status of DNA in blastocyst-stage embryos [145], the high levels of embryonic transcription, and a high proportion of blastomeres in the S phase of the cell cycle [146]. Methylation levels increase at the time of fetal attachment and implantation [147].

The artificial demethylation of DNA is achieved with the chemical azacitidine (5-azacytidine). This allows transcription from previously methylated alleles [144]. When methylation is disrupted in transgenic mice with a disrupted methyltransferase gene, development is affected and the fetuses are small [143], similar to androgenetic embryos [137] and chromosomally abnormal XO fetuses with a paternally derived X chromosome [148].

The abnormal development of parthenogenetic embryos is due to the failure of X chromosome inactivation in blastocysts and the absence of extraembryonic tissues [149]. The inactivation of X chromosomes during embryonic development is associated with high levels of methylcytosine and DNA methylation [143]. Critical genes on the inactive X chromosome are densely methylated, whereas the same genes on the active chromosome are not [147]. During S phase, DNA replication of the paternal copy of imprinted genes is replicated before the maternal copy because of methylation and inactivation of the X chromosome and other imprinted genes [147].

Imprinted genes that are expressed from the paternal genome include IGF-II and small nuclear ribonucleoproteins (snRNP), whereas those expressed from the maternal genome include IGF-II receptor and H19 [147]. The IGF-II gene has a relatively small methylated site on the paternal chromosome and is expressed from the transcriptionally active paternal allele. The H19 gene is densely methylated on the inactive paternal allele, including the promoter [147]. Transcription of the IGF-II gene is facilitated by selective methylation [150] on the active allele that controls transcription by preventing the binding of transcription repressors. The inhibition of transcription by dense methylation prevents transcription factor binding [147].

Although IGF-II and H19 are both imprinted and situated on the same chromosome, the expression of the two genes differs during development [151]. The IGF-II gene is expressed in the trophectoderm of the embryo, and a gradual increase in methylation of the gene is completed late in gestation, rather than at embryo attachment when methylation generally increases [147]. The expression of IGF-II ceases with the increase in methylation in the neonate [152]. Unlike all other fetal tissue, both IGF-II alleles are expressed in the choriod plexus and leptomeninges, and the H19 gene is repressed [151]. When IGF-II is experimentally disrupted

and is paternally inherited, the progeny are growth-retarded. When the disruption is maternally inherited the progeny are normal [152]. When two paternal alleles of IGF-II are expressed in chimeras, the birth weight of the fetuses is increased by approximately 50%. Also, the expression of IGF II in androgenetic embryos is higher than that in parthenogenetic embryos [153]. The differences in growth do not explain the developmental differences between androgenetic, parthenogenetic, and normal fetuses.

E. Totipotency, Embryonic Stem, and Germ Cells

A totipotent cell is able to differentiate into all the cell lineages that constitute an organism. A totipotent zygote can be produced after intracytoplasmic sperm injection (ICSI) into an unfertilized oocyte [154] that mimics fertilization. Similarly, the first haploid stage of spermatogonia, the round spermatids, can be introduced into an unfertilized oocyte and may produce viable progeny [155,156]. Haploid, parthenogenetic, and androgenetic embryos are not able to produce viable progeny [137,157,158].

The nucleus plays an important role in the maintenance of totipotency [159], and the combination of haploid parthenogenetic and androgenetic nuclei result in viable progeny [160]. Biparental gynogenetic and androgenetic embryos do not develop into normal progeny [137]. Paternally and maternally derived diploid homozygous female progeny can be produced [161] after manipulation of a normal zygote. This shows the requirement for the combination of paternal and maternal genomes at some time. When normally fertilized zygotes are treated with cytochalasin B and one pronucleus is removed, the developing embryos are diploid female homozygotes that are totipotent and develop into normal progeny [161]. The animals produced are not identical because of crossingover during meiosis [162].

Identical animals can be produced from bisected or split embryos [163,164]. Totipotency of the blastomeres that constitute the developing embryo is confirmed by the production of monozygotic twin, triplet, and quadruplet calves from single blastomeres isolated from eight-cell embryos [165]. The experimental induction of tetraploidy prevents totipotential development of embryos. However, tetraploid embryos can be saved when the blastomeres are incorporated into a normal embryo to create a chimera. An increased proportion of tetraploid cells in a chimera increases the incidence of developmental abnormalities [166]. Tetraploid fetuses are growth-retarded, with neurological abnormalities and enlarged nuclei [167]. The activity of some enzymes are also increased [168].

The construction of chimeras removes the absolute requirement for totipotency because a number of cells are aggregated to form the chimeric embryo. Chimeras are produced by the aggregation of blastomeres [169] from embryos up to the eight-cell stage [170] and produce chimeras with up to eight different parents [171]. Alternatively, cells can be injected into the blastocoele cavity, near the ICM of a blastocyst stage embryo [172]. Chimeras are also obtained after the transfer of the nucleus from a blastomere of an eight-cell embryo into an enucleated two-cell blastomere [173]. Chimeras do not test the totipotency of blastomere nuclei. When a blastomere nucleus is introduced into an enucleated oocyte by nuclear transfer, only totipotent nuclei will allow normal fetal development and the birth of viable progeny [174].

Stem cells have the potential to provide large numbers of genetically identical totipotent cells. Embryonic stem cells (ES) are isolated from the ICM of a blastocyst stage embryo. ES cells remain pluripotent after extended culture in vitro [175]. Cattle ES-like cells have been isolated and are pluripotent [176], but have not yet proved to be totipotent [176,177]. The establishment of ES totipotency has been enhanced by the production of 55-day fetuses after nuclear transfer [178]. Other stem cell types are isolated from neural [179] and hematopoietic [180] tissue using a series of cell selection criteria. The totipotency of these cell types is unknown.

Embryonic stem cells differentiate into many cell lineages, including hematopoietic cells [181,182]. This suggests that ES cells are placed above other stem cell types in a hierarchy. Neural stem cells differentiate into neurons and glia cell lineages [180], whereas hematopoietic stem cells differentiate into erythroid and myeloid cell lineages that constitute the circulatory system [183].

An alternative source of large numbers of genetically identical nuclei is primordial germ cells (GC) that are isolated from the genital ridge of fetal cattle and ovaries [184] and male fetal germ cells [185]. Because GC are able to differentiate into ES cells [186], the pluripotency of GC is partially established. The failure of GC to produce viable progeny after nuclear transfer in mice [185] may be due to a mixed population of GC that are undergoing differentiation.

The establishment of cell totipotency is accomplished after nuclear transfer of the nucleus into an enucleated oocyte. The cell is proved to be totipotent when live progeny are produced. Blastomeres from preimplantation embryos are totipotent and remain so after serial recycling of the blastomeres to produce multiple generations of nuclear transfer calves [187]. The ICM also contains totipotent cells that differentiate after five passages [188]. These have been compared with granulosa cells that are not totipotent or able to produce viable calves [189]. When ES cells derived from the ICM are used for nuclear transfer-viable progeny are not produced [178,190]. When cells are isolated from the ED and used for nuclear transfer lambs have been produced [191,192]. This shows that the ED cells are totipotent, so the ES cells that are derived from ICM cells, should remain totipotent under certain in vitro culture conditions.

The totipotency of GC, ES, ICM cells, and blastomeres depends on the differentiation status of the cells. Proliferation and differentiation of GCs are induced by cAMP [193]. As previously described, changes in cAMP within cells are associated with the cell cycle and transcription. The addition of cAMP to GCs increases the mitotic index [193]. The survival and proliferation of GCs requires SCF, which is the ligand that binds the kinase receptor for the protooncogene *c-kit* [194]. The *c-kit* gene is expressed in various cell types, including spermatogonia, oocytes [5], GCs and hematopoietic stem cells in postimplantation embryos [194,195]. Leukemia inhibitor factor (LIF) increases the support of GCs by somatic cells that produce SCF [194]. The support of GCs' growth by somatic cells is achieved by preventing apoptosis of the GCs [196]. The differentiation of totipotent cells leads to cell lineages that constitute the endoderm, mesoderm, and ectoderm of embryos and fetuses [197]. The differentiation of cells occurs relative to the position of the cells in the embryo and is correlated with changes in protein synthesis within the cells [197]. The effect of the developmental regulator activin induces blastomeres to differentiate into five cell types [198]. The expression of inhibin and follistatin change with the differentiation of embryos from the blastocyst stage. In contrast, the expression of activin does not change with differentiation [199], even though activin can induce differentiation.

III. THE COMBINATION OF BIOTECHNOLOGIES FOR NUCLEAR TRANSFER

The nuclear transfer procedure involves the substitution of the maternal genome of an oocyte with a totipotent diploid nucleus from a developing embryo. The removal or destruction of the maternal genome involves enucleation of the oocyte. The introduction of the donor nucleus is achieved by directly inserting the nucleus into, or fusing a blastomere with, the enucleated oocyte. Successful nuclear transfer was first achieved in the northern leopard frog (*Rana pipiens*) [200], the South African frog (*Xenopus laevis*) [201], in laboratory animals *Mus*

musculus [202,203], and in domestic animals *Ovis aries* [174] and *Bos taurus* [204]. Nuclear transfer has the potential to produce identical progeny that would provide genetic stability for scientific experiments, or allow uniformity within the population of agricultural animals. For this potential to be realized the current limitations of the nuclear transfer procedure must be overcome. The major limitations are oocyte enucleation, fusion of a blastomere with an oocyte, the synchrony of the cell cycle, and the source of large numbers of totipotent nuclei. These factors are critical components for increasing the efficiency of success in producing nuclear transfer embryos and viable progeny.

A. Oocyte Enucleation

Enucleation of in vivo-derived [204] and in vitro-matured [205] cattle oocytes is achieved by biopsy of the first polar body and the adjacent cytoplasm that contains the metaphase spindle and chromosomes [204]. Alternatively, the oocytes can be bisected so that one-half is enucleated [206]. When oocytes are treated with the vital DNA fluorescent stain Hoechst 33342, the metaphase II chromosomes can be observed when the oocyte is irradiated with ultraviolet (UV) light. This allows a high proportion of oocytes to be successfully enucleated. The development of the oocytes is not inhibited when irradiated for less than 10 s [207]. The use of Hoechst 33342 can be combined with the biopsy technique so that the karyoplast removed from the oocyte is subjected to UV light [208]. This prevents the detrimental effects of UV light on membrane integrity, mitochondria damage, and protein and DNA synthesis [209]. Oocyte enucleation is labor-intensive, requires skilled technicians, and expensive equipment.

The efficiency of oocyte enucleation is influenced by several factors. When the method of enucleation involves biopsy of half of the ooplasm with the polar body, more than 50% of the oocytes will be enucleated [204]. When the oocyte is stained with Hoechst 33342 and the metaphase chromosomes are identified using UV light, almost 100% of the oocytes are enucleated [207]. When the oocytes are allowed to age before being enucleated, the proportion that are enucleated by either of the foregoing methods decreases [210].

Alternative methods of oocyte enucleation also involve removing or destroying the maternal nucleus. This is achieved in *Rana* by removing the nucleus with a pin [200], whereas the enucleation of *Xenopus* oocytes is achieved by subjecting the animal pole of the oocyte to UV radiation [211]. The chemical enucleation of mouse oocytes [212] uses the inhibition of protein synthesis in combination with etoposide, a chemical that inhibits topoisomerase II. This prevents chromatid separation during the first meiotic division, and 96% of oocytes become enucleated when the metaphase I spindle and chromosomes are expelled into the first polar body. Unfortunately, chemical enucleation is not a reliable method for the enucleation of cattle oocytes [212].

Somatic cells are enucleated when they are centrifuged while attached to a glass cover slip in the presence of cytochalasin B [213]. The enucleated cells produced viable somatic cell hybrids when used for nuclear transfer [214]. Mammalian oocytes may be enucleated after centrifugation in a Percoll density gradient; however, the treatment does not result in a consistent proportion of enucleated oocytes [215]. The enucleation of cattle oocytes using centrifugation [216] provides the potential for large-scale production of nuclear transfer embryos.

B. Activation

The development of nuclear transfer embryos depends on the cell cycle stage of the blastomere and enucleated oocyte when the embryonic cells are fused together. The synchronization of rabbit blastomeres in G₁ with colcemide and aphidicolin before fusion increases the successful

development of nuclear transfer embryos [217]. Synchronizing the cell cycle prevents PCC of the DNA in the donor nucleus [218]. The synchronization of the cell cycle for nuclear transfer in mice relies on the timing of ovulation with human chorionic gonadotropin (hCG). When donor blastomeres are isolated from embryos immediately after a cleavage division, a higher proportion of the nuclear-transfer embryos develop to the blastocyst stage and have a higher cell number than when the blastomeres are isolated from embryos later in the cell cycle stage [219,220].

The cell cycle stage of cattle embryos is asynchronous after the four-cell stage [206], when approximately 80% of blastomeres are in S phase at any one time [221]. The synchronization of blastomere and enucleated oocyte cell cycle stages requires either chemical control of the blastomeres, or activation of the oocyte so that the cell cycle matches the S phase blastomeres. The ability to parthenogenetically activate cattle oocytes increases with increased age of the oocyte [91]. The activation of enucleated oocytes decreases the MPF activity of the oocyte so that it matches that of the blastomere [221]. This simple method of synchronizing the cell cycle prevents PCC, nuclear envelope breakdown, and DNA rereplication during nuclear-transfer embryo reconstitution [222]. This results in an increase in the development of nuclear transfer embryos to the blastocyst stage from 8 to 24% [223].

Various types of parthenogenetic activation treatments have been attempted during the nuclear-transfer procedure, including electrical [206], ethanol [224], fertilization, ionophore, and temperature [225]. However, the most recent methods of oocyte activating that involve treatment with protein synthesis [89] or kinase inhibitors [87] have not been assessed as treatments for the activation of enucleated oocytes during nuclear transfer.

C. Fusion

The fusion of cells is achieved by biological, chemical, or physical methods. Biological fusion of cells uses Sendai virus, a group I parainfluenza myxovirus that is incubated in the allantoic fluid of hens eggs [226]. The allantoic fluid is collected and diluted before the infectious nature of the virus is inactivated with UV light [226] or β -propiolactone [227]. Polyethylene glycol (PEG) is used for the chemically induced fusion of mammalian cells [228].

Membrane fusion, using a virus or chemical, is caused by an increase in the fluidity of the membrane lipid bilayer after a disruption allows the integration of membrane proteins and mixing of the lipid molecules [228]. The fusion of cells is temperature-dependent. Sendai virus uses the agglutination properties of the allantoic fluid to bind the virus envelope to sialic acid receptors on the cell surface. Cell agglutination is facilitated by phytohemagglutinin [229], and membrane contact occurs after PEG treatment as the cells shrink when water is removed by the hydrophilic PEG molecules [230]. Virus-mediated cell fusion occurs when the viral envelope between two cells fuses to both cells. A virus bridge is created after the viral fusion protein has opened a membrane fusion pore [231]. The fusion of cells by PEG occurs after the membrane is disrupted and cytoplasmic bridges form between the cells [230]. When embryonic cells are treated with PEG, 82% become fused [232]. When Sendai virus is used, 95% become fused [227]. Embryo development is poor when fusion is achieved with Sendai virus or PEG.

Electrofusion has the advantage over other fusion techniques because all aspects of the procedure can be controlled [233]. Electrofusion has been used most extensively for the fusion of embryonic cells for nuclear-transfer in cattle [204]. The aggregation of cells for electrofusion is achieved with an alternating current (AC) that generates dielectrophoretic charges on the two cells and causes tight membrane contact. The fusion of cells is achieved by reversible membrane breakdown in the membrane contact zone after a short, high-intensity pulse [233].

The dielectrophoresis effect describes the behavior of neutral charged cells that become polarized in an electric field and attract each other. The cells move to the position of highest intensity in the electric field at which the point that membranes contact remains parallel to the electrodes generating the AC field. The field strength for dielectrophoresis is specific for each cell type. If the field strength is too high, the cytoskeleton of the cells becomes damaged and, in extreme cases, the cytoplasm streams from the cell. The frequency of the electric field is particular to cell types. When the frequency is not correct, the cells rotate and prevent stable cell membrane contact [233].

The electrofusion of cells occurs after the membrane undergoes reversible breakdown. This occurs when the lipid bilayer membrane quickly becomes highly polarized. The membrane becomes permeabilized, and membrane resistance is decreased. If the field strength exceeds the critical threshold of amplitude or pulse duration the membrane is mechanically destroyed [233]. The breakdown of membranes is facilitated by increased temperature and decreased diameter of the membrane. When temperature is controlled, the membrane diameter can be manipulated by the voltage difference across the membrane, or by increased turgor pressure that increases the cell volume as the membrane stretches and becomes thinner [233]. As the membrane thins, pores in the membrane become larger. Electrically induced membrane breakdown causes pores to be generated in the opposing membranes so that channels are formed and materials can be exchanged between the cells. This is termed membrane fusion and is different from cell fusion because it is reversible [233]. When a critical voltage is reached the membrane fusion allows true cell fusion that is indicated by cell organelle mixing.

The electrodes that apply an electrical field and pulses for cell electrofusion have a range of designs that create a particular disturbance in the electric field of the electrofusion chamber [234]. The most common electrofusion chamber consists of two cylindrical parallel wires that are covered with fusion medium so that the cells to be electrofused can be placed between the wires and subjected to electric treatment. This type of electrofusion chamber is advantageous because the strength of the electric field can be calculated precisely and the electric field is almost homogeneous [234].

Media for the electrical manipulation of cells are nonelectrolyte solutions of glucose, histidine, mannitol, sorbitol, or sucrose [233]. The sugar solutions are used to maintain an isotonic environment for the cells. Various additions have been made to fusion medium that increase the conductivity of the media [234]. Zimmerman fusion medium (ZFM) includes Ca^{2+} and Mg^{2+} , K_2HPO_4 , glutathione, and bovine serum albumen (BSA) in a sucrose-based fusion medium [235]. The use of ZFM results in up to 100% electrofusion over limited numbers [204], while successful electrofusion can be as low as 61% [187]. When mannitol, supplemented with MgSO_4 and CaCl_2 , is used for fusion medium, successful electrofusion varies between 54 [210] and 84% [236]. The success of electrofusion depends on the type of cell used to provide the donor nucleus. When ICM cells were used, successful electrofusion was 49% [237]. In contrast, when IVP morulae were used, electrofusion was 84% [236]. Even though electrofusion has the potential for high success through the control of the physical parameters, this potential has not been realized. The use of electrofusion for nuclear transfer is widespread; however, the method for optimization of electrofusion is not clear, nor is there complete understanding of the interactions of enucleated oocytes and donor blastomeres in an electric field.

D. Embryo and Fetal Development

Successful nuclear transfer requires the production of viable progeny that indicates the donor nucleus is viable and totipotent. Blastomeres are isolated in medium without Ca^{2+} and Mg^{2+} [236] or with cytochalasin B [187] or EDTA [210] after the zona pellucida is removed with

pronase or micromanipulation. Alternatively, the blastomeres are individually removed from the embryo using micromanipulation [204,207]. The nuclear-transfer embryos produced with blastomeres can be recycled, and viable progeny can be produced up to the third nuclear-transfer generation [187]. Nuclear-transfer embryos are also produced from ICM cells with totipotent nuclei. The isolation of ICM cells requires immunosurgery to remove the trophoblast cells using antibovine serum [237] and are disaggregated using a fine-bore pipette [189]. The ICM cells cultured in suspension for up to 2 months [188] remain totipotent and produce viable progeny after nuclear transfer. Only blastomeres from eight-cell to morula-stage embryos and ICM cells have produced embryos and live calves after nuclear transfer.

The manipulation required for the production of nuclear-transfer embryos is relatively simple, even though highly skilled technical expertise is necessary to achieve success in the manipulation of oocytes and embryos. The events that influence the development of embryos and fetuses are more complex. After electrofusion, nuclear-transfer embryos develop in surgically ligated sheep oviducts or in vitro culture media. Embryos that are temporarily grown in sheep oviducts are placed into agar cylinders [238].

The cleavage of nuclear-transfer embryos can be assessed only when the embryos are cultured in vitro. When oocytes are activated before electrofusion, 61% cleave and 24% develop to the morula and blastocyst stage [223]. When oocytes are aged before electrofusion, 77% of nuclear-transfer embryos cleave, but only 5% develop to the blastocyst stage [210]. This is similar to the development of nuclear-transfer embryos in vitro (22%) when oocytes are activated before electrofusion to decrease the MPF activity [221]. When nuclear-transfer embryos are not activated and are cultured in sheep oviducts, 18 [204] to 25% [207] developed to the blastocyst stage and 26% resulted in a pregnancy [205]. When nuclear-transfer embryos are produced from fresh, frozen, and IVP embryos, 19, 37, and 11% developed to the blastocyst stage after in vitro culture, respectively [31]. When oocytes are activated before electrofusion with fresh, frozen, and IVP donor blastomeres, 75, 64, and 70% of nuclear-transfer embryos cleaved, respectively. After culture in vitro, 30, 7, and 23% of the nuclear-transfer embryos developed to the blastocyst stage, respectively [236]. When IVP embryos are used in nuclear transfer, 33% developed to the blastocyst stage in vitro [57]. When nuclear transfer embryos are produced from ICM cells 5-22% developed to the blastocyst stage [188,237]. The culture of multiple generations of nuclear-transfer embryos in sheep oviducts resulted in 30, 17, 36, and 22% development to the blastocyst stage of first-, second-, third-, and fourth-generation nuclear-transfer embryos, respectively [187].

The pregnancy and calving rates from nuclear-transfer embryos is currently lower than for normal embryo transfer [239]. Initial attempts at the production of nuclear-transfer calves resulted in 38% pregnancy and 15% live births [204]. An initial pregnancy rate of 42% [240] decreased to 33% live births and 23% pregnancies, 60 days after transfer. Embryonic loss is apparent after nuclear-transfer pregnancies, with 26% pregnancies at 60 days after transfer, but only 22% resulted in live births [205]. This is consistent with the result of 35% pregnancy at 35 days after transfer that decreased to 12% at 65 days after transfer and 8% at 85 [31], and a pregnancy rate of 63% at 21 days after transfer, which decreased to 30% at 90 days. This resulted in 30% of transferred embryos producing a viable calf [236].

The use of ICM cell in nuclear transfer also resulted in a relative low-pregnancy rate of 23%, with 16% live births [237]. After the use of ICM cells for nuclear transfer, a pregnancy rate of 27% resulted in 50% of pregnancies producing viable calves [189], whereas a pregnancy rate of 49% decreased to 12% calves born [188].

The pregnancy after transfer of recycled, multiple generation, nuclear-transfer embryos varies between 17 and 36% and does not depend on the embryo generation [187]. The production of live calves decreases from 10% after the first generation to 2 and 3% for the

second and third generation, respectively. No calves have been produced from the fourth and subsequent nuclear-transfer embryo generations.

The calves produced from nuclear-transfer embryos have some abnormalities. An abnormally high birth weight of some calves has been reported. A calf of 47 kg (103 lb) required considerable force to deliver [31] and was heavier than the mean birth weight of 39 kg (86 lb) for 11 nuclear-transfer calves [236]. However, calves up to 70 kg (155 lb), with 32% of births requiring cesarean section and 14% requiring considerable force to deliver [240], show the extent of the excessive growth abnormalities.

Calves from nuclear-transfer embryos produced from ICM cells also have high birth weights of up to 73 kg (160 lb) [237]. In comparison, normal birth weights of 34-39 kg (75-86 lb) [188] are also produced when ICM cells are used to generate nuclear-transfer embryos. A proportion of calves from recycled multiple generation nuclear-transfer embryos also have high birth weights, with first and second nuclear-transfer generation calves having birth weights of approximately 74 kg (160 lb) [187].

The development of nuclear-transfer embryos is not the same as MOET or IVP cattle embryos. The development of preimplantation nuclear-transfer embryos *in vitro* is approximately the same as embryos produced after IVF. However, the peri- and postimplantation development and pregnancy rates are drastically decreased for nuclear-transfer embryos [189,225]. Also, a proportion of calves have excessively high birth weights [187,188,237].

The treatment of the donor nucleus during the nuclear-transfer procedure and after electrofusion with an enucleated oocyte may stimulate the abnormalities observed in cattle. Abnormalities in DNA content are induced by high MPF activity in oocytes that are not activated before electrofusion. This causes PCC and rereplication of DNA [222]. Such abnormalities would not be expected to result in pregnancy. Even when the cell cycle of blastomeres and oocytes is synchronized, pregnancy rates decrease during gestation and high-birth-weight calves are produced. The effects of UV light on protein synthesis and intracellular damage [209] may induce some developmental abnormalities; however, nuclear-transfer embryos produced without subjecting the oocyte to UV light also have low-calving rates and excessive growth abnormalities.

The ability of the oocyte cytoplasm to reprogram donor blastomeres differs between oocytes [202], and it may change as protein synthesis in the enucleated oocytes changes over time [241]. Glucose phosphate isomerase (GPI) activity remains maternally derived in nuclear-transfer embryos produced with trophoblast cells, whereas embryos produced from ICM cells exhibit a mixture of maternal and donor embryo-derived enzyme activity [202]. The development of microtubule asters after electrofusion occurs with either one or two asters developing in the cytoplasm [43] that are contributed from the donor blastomere. This suggests that the blastomere has some effect on the developmental competence of nuclear-transfer embryos. Reprogramming of the blastomere, as indicated by stage-specific, cell surface antibody expression is the same in all nuclear-transfer embryos [208], suggesting that all donor blastomeres are reprogrammed to some degree. Transcription of DNA is altered after electrofusion [242]. The nucleolar precursor body of a blastomere from a morula stage embryo changes after electrofusion with an enucleated oocyte. Embryonic transcription almost ceases as the nucleolar precursor body becomes less electron-dense, indicating a reversion to early cleavage-stage embryos [242]. The alteration of transcription after nuclear transfer is supported by the inhibition of the expression of small nuclear ribonucleoproteins (snRNP) using pig blastomeres [243]. When transcription is prevented, the snRNPs are not detectable in embryos because RNA is not being processed.

Nuclear transfer stimulates the synthesis of transcription, requiring complex (TRC) cAMP-dependent proteins in mouse embryos that are not normally expressed until the embryonic transcription begins at the two-cell stage [244,245]. This suggests that the donor nuclei are not fully reprogrammed to a zygotic transcriptional status. The synthesis of 18% of total proteins is reduced in nuclear-transfer embryos [246], although some proteins remain transcriptionally active after transfer, most of these would not normally be expressed in a one-cell embryo. Other proteins are repressed after nuclear transfer and then fail to be expressed normally as embryo development continues [246].

Many proteins exist that would inhibit fetal development if modified or incorrectly expressed. The failure of protein expression could cause the gradual loss of pregnancies throughout early gestation. For example, inappropriate expression of lipoprotein receptor-related protein that is essential for embryo implantation [247], or the *Ped* gene that interacts with major histocompatibility (MHC) complexes and influences embryo and fetal development [248], could prevent normal events during fetal development.

The production of high-birth-weight calves may be associated with the high amounts of mRNA for IGF-II in nuclear-transfer embryos when compared with normal cattle embryos [249]. The IGF-II gene is imprinted in mice [152], so nuclear changes during nuclear transfer may affect the methylation of embryonic DNA and alter the expression of specific genes that results in developmental abnormalities. The expression of IGF II normally occurs after implantation when it acts as a mitogen that affects the mesoderm, gut, placental, and central nervous system development [152]. The paternally inherited X chromosome in XO causes the development of heavy mouse fetuses [148]. Since the X chromosome is inactivated by hypomethylation, the implication for methylation as an inducer of birth defects is enhanced. Also, the incorporation of paternally imprinted androgenetic cells in an embryo cause an increase in the size of mouse fetuses [136].

The control of fetal weight depends on the size of the placenta and fetal cotyledons and the effect on nutrient supply and endocrine regulation [250]. The levels of fetal prolactin, insulin, and IGF II are positively correlated with fetal growth [250]. Prolactin decreases the diffusion of water from the fetus, whereas insulin increases the supply of glucose to the fetus [250]. The role of insulin in fetal growth is supported by the heavier weight of babies born to diabetic mothers [250]. Insulin affects the fetus by regulating the synthesis of adipose tissue, but has a low effect on cell proliferation and is a weak analogue of IGF-II. Fetal IGF-II is a potent mitogen, stimulating cell proliferation, amino acid uptake, and mediating the effect of growth hormone (GH) [250]. The effect of GH is to increase growth and the efficiency of nutrient utilization and increase protein synthesis in the presence of insulin [250]. Before parturition, fetal prolactin, testosterone, and cortisol levels increase as GH levels decrease. The birth weight of calves is related to perinatal death after complications such as dystocia, stillbirth, and hypothermia [251]. The average calf weight at parturition is 38.5 kg (84.7 lb) over all breeds, with gestation measuring 283 days [251]. Factors other than nuclear transfer that influence birth weight are genetic or environmental. Genetic effects on calf birth weight include breed, heterosis, the sire and dam, inbreeding, calf sex, and genetic abnormalities. Environmental effects include maternal body weight, age nutrition and nurturing ability, the position of the fetus in the uterus, lactation status, fetal number, gestation length, and seasonal influences [251].

Investigations into nuclear transfer have provided methods for the production of embryos and viable calves. The mechanisms and developmental effects that occur owing to the manipulation of cattle embryos by nuclear transfer are unknown.

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Successful Gender Preselection in Farm Animals

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I. INTRODUCTION

Predetermining the sex of offspring has been of interest to mankind since virtually the beginning of time. Theories abound as to how one could predetermine sex. Many theories have their origins from the Greek philosophers. Some thought that females developed on the left side of the uterus, males on the right. Even as late as the 18th century there were those who thought that sperm from the right testes produced more male young, whereas sperm from the left testes produced more females. Coincident with theories for gender control in humans, numerous theories emerged and were applied to livestock. The keenest interest has always focused on in vitro manipulation of sperm before insemination, instead of determining the sex of the embryo. This is of the most interest because sex determination is then based on the chromosomal content of the fertilizing sperm.

This chapter will deal primarily with the successful development of a method to preselect the gender of offspring of mammals, which is based on the inherent difference in DNA content between the X- and Y-chromosome-bearing spermatozoa. Numerous reports have appeared in the scientific literature over the past 75 years purporting to skew the sex ratio in one direction or another. The pioneering experiments of Lush [1] sought to separate X from Y sperm based on density centrifugation. These efforts still continue today, although considerable research effort has been expended to develop other sexing methods. Generally speaking, most of these efforts fall under the heading of "physical separation" methods. None of the physical separation methods have been validated with offspring born that demonstrate a significantly skewed sex ratio. In recent years, nearly all such methods have been tested using DNA content for differentiating X from Y sperm by flow cytometry. No method has shown anything but a 50:50 sperm sex ratio [2–4]. In no case has there been evidence to support the claims made for various physical separation procedures or other non-DNA-based separation procedures. Only methods based on X and Y sperm DNA difference have demonstrated a skewing of the sex ratio (5–7) in offspring of rabbits, swine, and cattle, respectively. For

comprehensive reviews of the various physical X and Y sperm separation methods the reader should consult several excellent reviews [8–12].

II. BASIS OF X AND Y SPERM SEPARATION

A. Chromosomal Constitution

Individual sperm DNA content is the only known and scientifically validated and measurable difference between X- and Y-chromosome-bearing sperm. The Y-chromosome is smaller and carries less DNA than the larger X-chromosome. Autosomes on the other hand are also carried by X- or Y-bearing sperm, but are identical in DNA content. Although this has been known since the turn of the century, its usefulness for X-Y sperm separation was not fully clarified until Moruzzi [13] demonstrated through physical measurement of the X- and Y-chromosomes of numerous species that those species with larger differences in X- and Y-chromosome measurements may be especially good candidates for the application of some type of sexing methodology.

B. DNA Difference in X and Y Sperm

The ability to measure DNA content in individual X and Y sperm on a large scale became a reality only with the development of flow cytometry. The DNA content of individual sperm was measured by Gledhill et al. [14], who sought to determine mutations caused by radiation exposure. However, Gledhill and his group were not able to distinguish the difference in DNA between X and Y sperm because of what is now termed a DNA artifact. Numerous advances in sperm analysis and sorting for DNA has paralleled advances made in improving flow cytometric instrumentation. This coupled with cellular staining and the use of fluorochromes to identify various organelles has advanced the area significantly. The successful adaptation of flow cytometry for sperm DNA analysis and sperm sorting can be traced to the need to overcome the orientation artifact by orientating sperm heads to the laser beam [15], resulting in the differentiation of X and Y sperm in a sample of semen [16,17]. This was followed by improved stain and preparation protocols [18] that resulted in fertilization using intracytoplasmic sperm injection (ICSI) of sorted sperm nuclei [19,20] and sorting of viable X and Y sperm capable of producing offspring [5–7,21].

III. FLOW CYTOMETRIC SORTING OF X AND Y SPERM

A. Flow Cytometry

Development of flow cytometry was a remarkable advance that originated with the work of numerous investigators, particularly at Los Alamos and Lawrence Livermore National Laboratories [22,23]. Although development was stimulated by the need to measure blood cells for disease diagnosis, its application was broad enough to encompass a unique cell, the spermatozoa. Flow cytometers are generally used both as analytical tools (DNA content) and as cell-sorting instruments [for review see Ref. 12]. Commonly, ploidy levels of many cell types are determined for cancer research and diagnosis. Also, depending on the specificity of the probe-dye combination selected, many cellular components other than DNA can be determined. Fluorescent-labeled antibodies, probes, and other dyes can also be used to measure cellular morphology, activation states, and function. If equipped as a cell sorter the system can be used to flow-sort specific populations for further functional studies. These selected population of cells, or sperm in our case, are highly purified by flow cytometric cell sorting.

With sperm, as with other cells, one is able to sort two populations at once. By placing a sort window around each X and Y population, two purified populations are recovered during the sorting process.

B. Resolution of X and Y Sperm

Resolving X- and Y-bearing sperm by using flow cytometric DNA analysis of them is difficult. The compactness of chromatin in the morphologically flat, ovoid-shaped sperm head, which is characteristic for domestic livestock, causes a high index of refraction. The difference in refractive index between the sperm head and the surrounding sheath fluid, coupled with the flat shape of the sperm head, results in preferential emission of light in the plane of the cell (from the edge of the sperm head). Because of these properties, the orientation of the sperm head relative to the excitation laser beam and the optical detectors is critical for resolution. The basic configuration of a flow cytometer is shown in Figure 1. The flow cytometer-cell sorter system that is shown has been modified specifically for sorting sperm. The two modifications consist of a forward fluorescence detector that replaces the normal light-scatter detector and a beveled, wedge-shaped, sample injection needle, which replaces the normal cylindrical injection needle [17]. The modified flow cytometer-cell sorter is essential for attaining

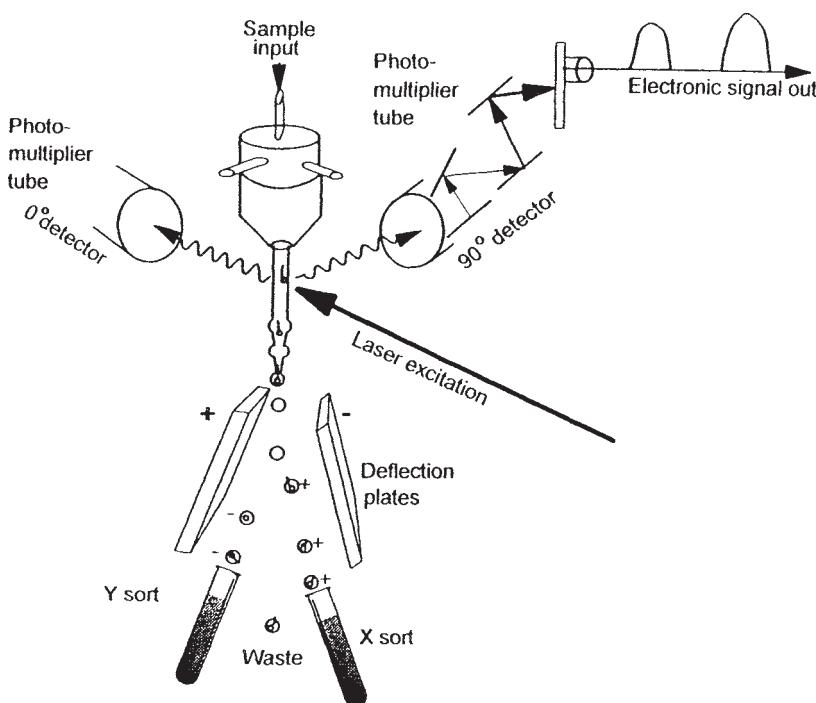


Fig. 1 Schematic of an orthogonal sperm sorting system: A modified flow cytometer-cell sorter is used to analyze and separate X- from Y-chromosome-bearing sperm. The standard sample injection needle is beveled at the exit orifice to properly orientate (as measured by the 90° detector) a higher percentage sperm for X- or Y-chromosome determination by an additional 0° fluorescence detector. Individual sperm (within a droplet) are then sorted by deflecting their respective carrier droplet either to the left (Y-bearing) or to the right (X-bearing). Sperm are routinely sorted and collected in each direction at 100/s. (Modified from Ref. 12.)

separate populations of X- and Y-chromosome-bearing sperm based on DNA content difference and for reanalyzing sorted sperm to determine the proportions of X or Y sperm in a given sorted sperm sample [5].

Differentiating the amount of DNA present in the X and Y sperm can be accomplished on virtually all commercial flow cytometer-cell sorters that have been manufactured in the past 15 years. To date, flow cytometer-cell sorter systems manufactured by the Coulter Corporation, Hialeah FL (EPICS V; EPICS 750 series, ELITE) and the Becton Dickinson Co., Mountain View CA (FACS Vantage; FACStar Plus; FACStar; FACS 440) have been modified for sperm sorting. Each system was modified to provide for a beveled sample injection needle and a fluorescence detector located 0° to the laser, as described by Johnson and Pinkel [17]. Recently, an Ortho Cytofluorograf cell sorter has also been modified for sperm [J. Aten, personal communication].

C. Sperm-Sorting Technology Applied to Sperm Heads

Development of the Beltsville Sperm-Sexing Technology for preselecting the gender of offspring originated using only sperm heads (sperm tails were removed by sonication) [17]. The sperm tails caused too much variability in how the sperm head was presented to the laser beam ([Fig. 2](#)) even with the use of the beveled needle to control orientation. Two key developments occurred to prepare the way for separation of two viable populations of sperm. The first was the application of Hoechst 33342 as the vital stain of choice for gaining uniformity of stain of the DNA in the sperm head. It not only had advantages of greater membrane permeability, but it bound to the DNA more uniformly, and it was nontoxic to sperm motility [24]. Second, the use of Hoechst 33342 allowed the elimination of harsh treatment conditions to obtain stain penetration that was characteristic of earlier stains such as 4-6-diamidino-2-phenylindole (DAPI), which maintained the general integrity of the sperm membranes. These advances [18] led to the sorting of X and Y sperm heads with high purities [25]. In a subsequent report, the integrity of the DNA in the sperm head under the milder-staining and milder-sorting conditions was illustrated with evidence that the pronucleus of the developing embryo could progress normally [19,20]. Throughout this process of development, the flow cytometer's analytical capability was used to monitor not only the proportions of X or Y sperm in any semen sample [4], but also the X-Y purity in the resulting sorted populations [19,25]. This reanalysis of sorted sperm for DNA content was validation that the putative X-Y populations were indeed separated into X and Y sperm.

D. Sperm-Sorting Technology Applied to Intact and Viable Sperm

The success of the technology applied to sperm heads brought the consideration that perhaps intact sperm could be orientated to such an extent that X and Y sperm could be isolated. Intact sperm were capable of being orientated (20–30%), but not at the high proportions that sperm heads could be orientated (80%). When sorting the intact sperm the resolving power of the bimodal X-Y sperm populations was compromised, leading to somewhat lower purities of the sorted populations. Sorting living sperm is one thing, maintenance of sperm viability is another. Stain, incubation temperatures, high-dilution rate, and atmospheric shock, all associated with cell sorting, have a negative affect on an otherwise normal sperm. At the outset, sorted sperm died very quickly after collection either on a slide or in a tube. A key element in overcoming the problem was the application of bovine serum albumin (BSA) to the polycarbonate collection tube to reduce the static charge and loss of sperm. Another element to maintain survival was the placing of a small amount of the Test-yolk semen extender

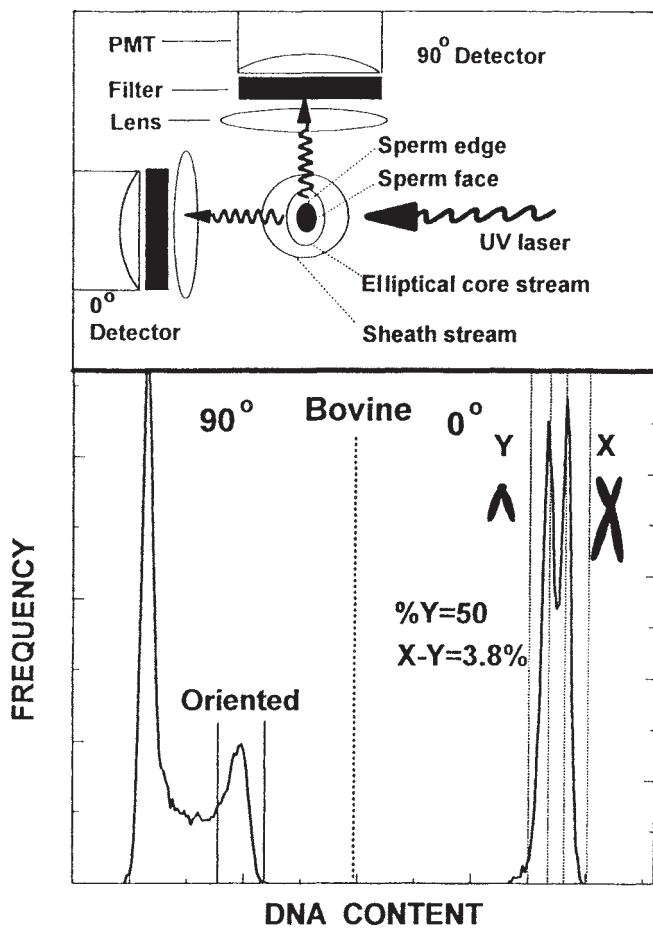


Fig. 2 Two fluorescence detectors (0° and 90°) are used to differentiate X and Y sperm. The top schematic shows these two detectors in relation to the sperm cell and the incident excitation source (laser beam). The histograms show the resulting distributions from each detector. Properly oriented sperm with their bright edge toward the 90° detector fall within the orientation gate of the 90° histogram. The sperm within the orientation gate are analyzed for fluorescence by the 0° detector and exhibit a bimodal X and Y distribution. Sort windows, shown superimposed on each peak, are used to separate the X and Y sperm.

in the bottom of the sort collection tube. This feature allowed sperm to be projected during the sorting process into the collection tube and continue swimming into test yolk component at the bottom of the tube. In the Test-yolk component sperm remain viable over the 1 or 2 h that it takes to sort a full tube. These developments resulted in the first pregnancies achieved with rabbit sperm sorted for DNA content [5]. The resulting litters showed significant shifts in the sex ratio. Sperm sorted in this way was also used to successfully impregnate swine [6], sheep [11], and cattle [7,21]. In recent years we have applied the technology to humans [26]. It is now being used to produce pregnancies in humans [27]. The recent birth of a daughter to a mother who is a carrier of a sex-linked disease was the first pregnancy to go to term in humans [S. Black, personal communication] using this technology.

1. Beltsville Sperm-Sexing Technology

The refined technology as it is currently being used involves the use of an aliquot of semen (15 million sperm) that is diluted to 1 mL with a semen extender appropriate to the species. To this sample, Hoechst 33342 is added at a concentration of 7.12 μ M. The solution is incubated at 32°–39°C for 30–60 mins, depending on the species of sperm. The sperm is flow-sorted, (see Fig. 2) as described earlier, into polycarbonate microcentrifuge tubes (0.6 mL) previously treated with 1% BSA to coat their interior. If the sperm are to be used for insemination by surgical means [5,6] or for in vitro fertilization (IVF), Test-yolk extender is added to the bottom of the collection tube (50 μ L). The percentage of egg yolk in the test extender can range from 2 to 20%. Twenty percent egg yolk is suitable for insemination, whereas much lower percentages are better for use with IVF procedures [21,28].

IV. VALIDATION OF THE SEXING PROCESS

A. Flow Cytometric Reanalysis of Sperm for DNA

An available laboratory method for determining the proportion of X to Y sperm is a critical factor in ensuring the purity of the particular sorted sperm population. This method uses aliquots of sorted sperm, sonicating to remove the tails, and adding Hoechst 33342 to maintain staining uniformity. This flow cytometric reanalysis of the sorted sperm heads is a good validation technique. The proportion of X and Y sperm are determined, based on the DNA difference, and histograms are analyzed by computer fitting to double gaussian peaks [18]. Representative histograms of unsorted sperm from several species are shown in Figure 3. Note the species specificity of DNA difference between the X and Y population.

B. Evaluating Sorting Process with Single Sperm Sorting and Molecular Genetic Analysis

A qualitative determination of the presence of the X- or Y-chromosome in an individual sperm can be made by using the allele-specific polymerase chain reaction (PCR). By analyzing several hundred individual sperm, a quantitative determination of the proportion of X and Y sperm in a given population can be made [29]. Single sperm determined to be X or Y by flow cytometric analysis are deposited into each well of a 48-well plate. The wells are shallow enough that microscopic verification of the wells' contents after single sperm sorting can be done. The wells also serve as the first-round amplification vessel for PCR. Specific X and Y products from a second amplification are separated by agarose gel electrophoresis, product bands are identified and counted, and the resulting purities are determined. Results from 100 amplifications of each X and Y sperm sort showed 94% purity for X and 90% purity for Y. These purities are consistent with results obtained by flow cytometric reanalysis of sorted sperm [29].

C. DNA Probing of Sexed Embryos

Embryos produced from IVF with sorted sperm are frozen by standard methods and stored for gender analysis by PCR. After thawing and incubation with proteinase K, the DNA is probed using primers derived from a multiple repeat bovine Y-chromosome-specific sequence [7,21].

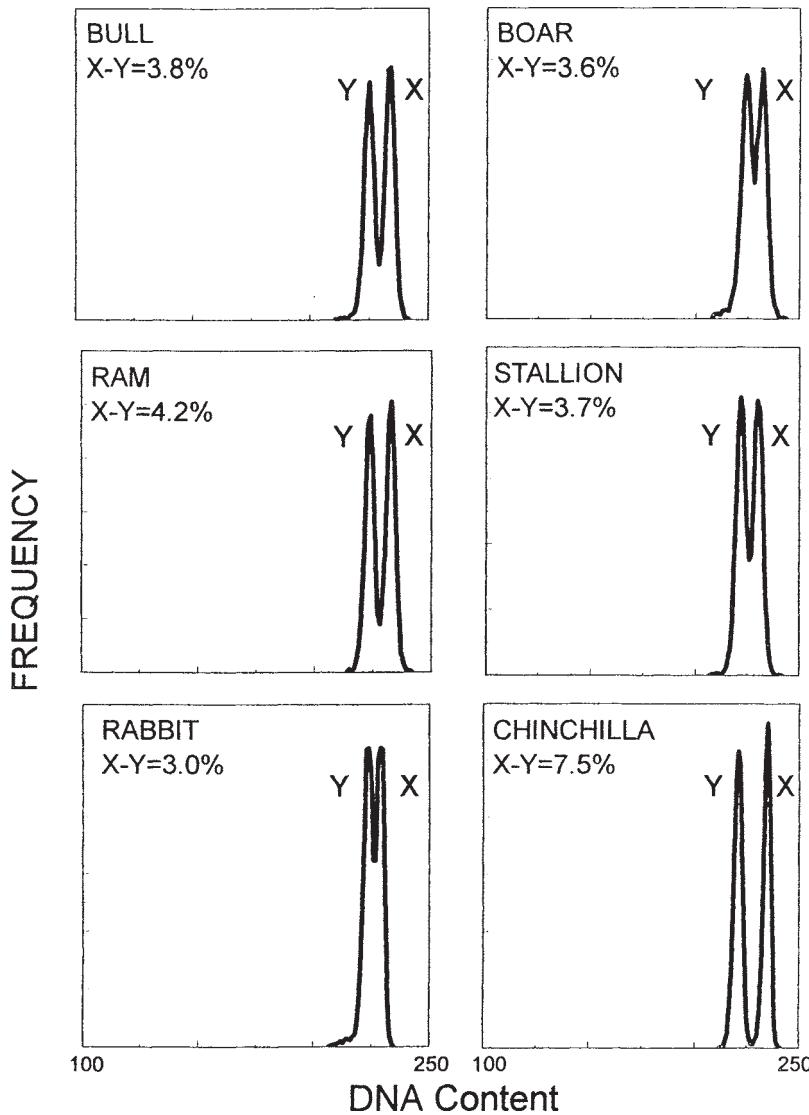


Fig. 3 All mammalian sperm yet analyzed contain a different amount of total DNA between the X and Y sperm. These histograms illustrate the DNA content difference between the X- and Y-bearing sperm of six animal species. (From Ref. 11.)

D. Fluorescence In Situ Hybridization of Sorted Populations

Application of fluorescence in situ hybridization (FISH) to determination of X and Y proportions is fairly straightforward with human sperm because of the availability of commercial microsatellite centromeric DNA probes. Our initial work on the human was validated using FISH [26]. Sperm (X or Y) were sorted onto slides, fixed with methanol and acetic acid; exposed to biotinylated DNA probes for X- or Y-chromosomes and incubated; the signals indicating an X- or Y-chromosome were then counted using fluorescence microscopy. This technique is particularly helpful when the X-Y DNA difference (<3.0%) is small and sperm

DNA reanalysis by flow cytometry is inconsistent. It also provides a method of validation that is different from the original separation method (cell sorting).

E. Validation Through Sex Determination of Offspring

The ultimate test for any sexing procedure is if the offspring are of the predicted sex. Typically litter-bearing animals give the quickest results because of the within-animal comparison. However, without the aforementioned laboratory validating techniques, this would be the only determination of phenotypic sex. The amount of time it takes to obtain key birth results tends to militate against relying on this validation until one is fairly certain of the sex ratio outcome.

V. PRODUCTION OF SEXED PROGENY

A. Sperm Sorting

1. Rates and Purities of X and Y Sperm

Rates at which viable X and Y sperm can be produced approximate 3×10^5 to 5×10^5 sperm per hour. The rate is slow because increased flow rate of the sperm through the system tends to increase the coefficient of variation of the analysis. It is also essential that the sperm be properly orientated to the laser beam to properly differentiate their X and Y sperm DNA content. Only 10–20% of the sperm that enter the system can be sorted into X and Y populations. The lower rate is based on the need to achieve a high purity of the individual sperm populations (the greater the purity the narrower the sorting windows and the slower the sorting rate). Although one cannot achieve 100% X or Y purity (under normal-sorting conditions), one can approach 95% purity with livestock sperm [11]. Virtual 100% purity is possible with some species in which the DNA difference is much greater, such as in the creeping vole (*Microtus oregoni*; 12.5% [30]) and the Chinchilla langier (7.5% [25]). Higher purities can also be achieved if sperm that have been sorted once can be passed through the system and sorted a second time; however, this is not practical (unpublished observations).

2. Methods of Insemination

The highest purity attainable in using flow sorting is primarily attributable to four factors: (1) the genetically determined DNA content difference between the X- and Y-chromosome-bearing sperm; (2) proper configuration and sensitivity of the flow system to allow selection of a properly orientated population of sperm; (3) uniform and consistent binding of the fluorochrome to the DNA in the sperm head; and (4) the width of the selected sort windows. At the rate of 3×10^5 to 5×10^5 sperm per hour, one cannot economically produce enough sperm for artificial insemination (AI). Application of the Beltsville Sperm-Sexing Technology in practice requires that it be used either for intratubal (ITI) or intrauterine (IUI) insemination, intracytoplasmic sperm injection (ICSI), or for IVF. Use with IVF requires that IVF procedures be optimized for that species. This has been done in cattle [7,21]. However, swine IVF has still not been developed to a high enough efficiency [28] and requires more research. Recent results using IVF are encouraging with the birth of two litters of pigs that were produced from sexed embryos transferred on day 2. Both litters were 100% female [31]. For the human, IVF, ITI, and ICSI are currently being applied [27]. It is now clear that IVF offers the most feasible means of applying the current sexing technology to gender preselection in cattle. In addition, several fold improvement in sorted sperm production could increase the applicability for ultrasound-guided insemination in cattle where smaller numbers of sperm can be used.

The use of low numbers of liquid-stored sperm and artificial insemination in cattle suggests that even at the current sexed sperm production rates, there may be applicability for AI of 1×10^5 to 2×10^5 per insemination [32]. Studies to test this theory with sexed sperm are in progress (unpublished data).

A sperm-sorting facility in close proximity to the IVF and embryo culture laboratory enhances the efficiency of the two systems, because survival of the sorted sperm tends to be shorter than nonsorted sperm. Normal protocols call for sorting the sperm over a 2-h period, achieving approximately 7×10^5 to 8×10^5 sperm for use in IVF. The sperm are concentrated and coincubated with the eggs within 1 h of the end of the sort [7,33]. Exposing sperm to the stain, the subsequent sorting process, and to the sort collection medium appears to capacitate the sperm obviating the need for several hours of capacitation time before fertilization. This represents a period of about 4–6 h from the time of semen collection to the time of fertilization. After the sorted sperm are collected, they are centrifuged to concentrate, and washed before placement with the eggs. Only ova recovered from slaughterhouse ovaries have been used for IVF by flow-sorted sperm. Standard techniques have been used to in vitro mature the ova before coincubation with flow-sorted sperm [7,21,33].

B. Sperm Sorting for IVF: Standard Protocol

The standard protocol developed for sorting sperm and subsequent IVF involves the preparation of 30 million sperm by staining and diluting to 2 mL in extender. Sorting over a 2-h period will use about 65% of the sperm prepared. This results in the accumulation of approximately 7×10^5 to 8×10^5 sorted X sperm and the same number of sorted Y sperm with sort windows set to achieve about 90% purity of either X or Y sperm. Increasing the sort window by 15% results in a 10% decrease in purity (>80%). This can result in about a 20% increase in sperm that can be sorted over a 2-h period. Sperm motility and acrosomal integrity of sorted sperm compare well with the original sample. Cleavage rates range from 60 to 80% when using approximately 2000 sperm per egg [21]. [Figure 4](#) illustrates the effect on purity of the sorted sperm on setting sort windows and eliminating as much of the overlapping population as possible.

C. Development: Embryo, Fetus, and Offspring

Reduced litter size in does and sows inseminated with flow-sorted X or Y sperm [5,6] led us to evaluate early embryonic development to determine the effect on flow-sorted sperm. McNutt and Johnson [34] found a significant influence of sorting on development of embryos flushed at 42 h after surgical insemination. Embryos had reached the 8- to 16-cell stage, whereas embryos from the controls (unstained, unsorted sperm) had already progressed to the early morula stage. The effect seen early in development was carried through pregnancy because there were significantly fewer fetuses from flow-sorted sperm inseminations than for control-unsorted-unstained sperm inseminations. Overall, these studies confirmed that flow-sorted sperm have a definite effect on the early development when such sperm are used for fertilization. However, it is also clear that affected embryos never go to term.

Offspring born from all species, using flow-sorted sperm, have shown no morphological abnormality whatever. Numerous matings have been conducted with both sexed animals and nonsexed animals. Progeny have been produced through the F₂ generation in both rabbits and swine. The founder male and female were the result of sexed sperm. No reproductive or morphological abnormality was observed. Many boars produced from sexed semen have been used over periods of years to provide semen under stud conditions for experiments.

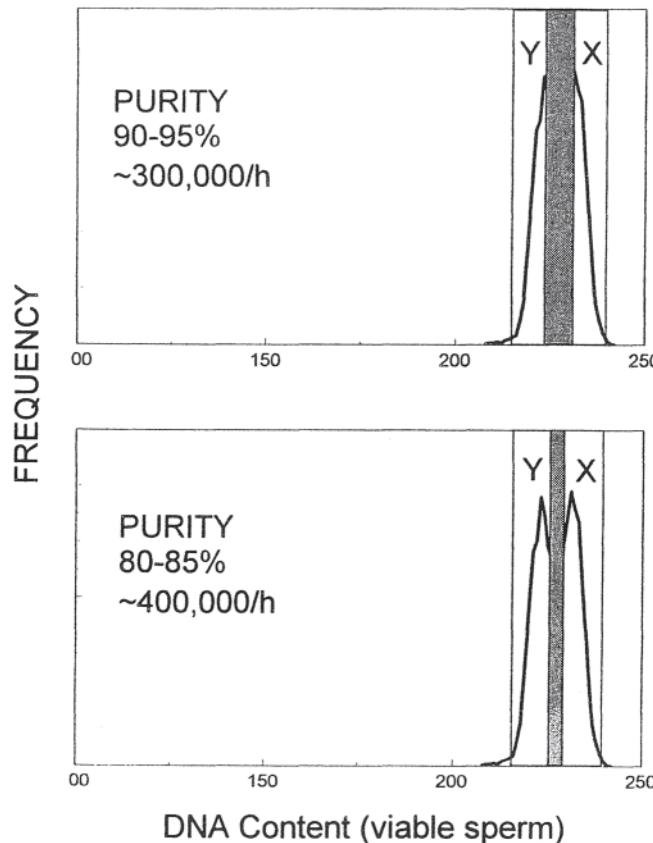


Fig. 4 The small difference in DNA content between X and Y sperm in conjunction with the resolving limits of flow cytometric analysis and sorting do not allow complete resolution of the X and Y populations. This overlap lessens the resulting sort purities from that obtained if the two populations could be completely resolved. Thus, sort window placement can affect both the recovery rate and purity of the sorted sperm. By excluding most of the overlapping region (top histogram) a higher purity can be obtained, but at the expense of slower recovery.

being conducted. Semen production and quality has been equal to that of control boars (unpublished data).

D. Application of the Technology to Livestock

Validation of the technology's efficacy has been conducted in rabbits and swine directly with sorted sperm. The sex ratios of the litters produced from females surgically inseminated with sorted X or Y sperm supported the results of reanalyzing aliquots of sorted sperm populations [5,6]. No practical, on-the-farm testing, of the technology has yet been done in swine. This must await the further improvement of IVM and IVF procedures. Until that happens, the only feasible way to use sexed sperm for swine is through surgical insemination. A summary of the overall results obtained in the various surgical insemination studies is presented in [Table 1](#).

Once proof that the technology was valid and consistent across species, it was applied to cattle through IVF. The first study [21] was conducted at Cambridge, England and resulted in nine twin transfers of sexed embryos produced from IVF. Four cows calved, producing two

Table 1 Summary of Data on Offspring After Surgical Insemination or Transfer of Sexed Embryos Produced from Sorted X and Y Sperm

Treatment	Number females inseminated/ ET	Number kindled/ farrowed/ calved	Number born	Actual %		Significance (deviation from 50:50)
				Male	Female	
Cattle^a						
Sorted X	4	2	3	0	100	**
Sorted Y	5	2	3	100	0	**
X	0	0	4			
Sorted Y	106	35	37	10	90	**
Swine^b						
Sorted Y	8	4	37	68	32	*
Sorted X	10	5	34	26	74	**
Unsorted stained	11	5	40	43	57	NS
Unsorted unstained	7	5	46	42	48	NS
Rabbit^c						
Sorted Y	16	5	21	81	19	**
Sorted X	14	3	16	6	94	**
Sorted (X+Y recomb)	17	5	14	43	53	NS

Source: ^aRefs 7, 21; ^bRef. 6; ^cRef. 5.

sets of twins and two single calves. Offspring were of the predicted sex based on DNA reanalysis and embryo sexing using PCR.

After the demonstrated success of the initial study, a field trial was designed in which only Y sperm were sorted with a minimum 90% purity based on reanalysis of sorted sperm populations for DNA. Sexed embryos were produced and cultured to days 7 to 8. Grade 1 blastocysts were frozen for later direct transfer to recipient cows. Twin transfers were conducted on 11 farms in Scotland, resulting in 41 calves from 35 calvings. Of the total born, 37 were males and 4 calves were females for a 9:1 ratio [7]. A more detailed summary is included in Table 1. These results along with those with swine and rabbits confirm the efficacy of the sorting technology as an effective means of skewing the sex ratio of livestock progeny.

Although virtually all the sorting and production of offspring have arisen from freshly ejaculated semen, widespread application is dependent on the ultimate routine sorting of previously frozen semen as well as the freezing of sperm after sorting. Procedures for both are in development to maximize the efficiency of the technology when used in this way. Preliminary studies indicate that sperm that have been sorted after freezing will produce normal healthy embryos [Cran et al., unpublished data]. Postthaw recovery of sperm frozen after sorting indicate approximately 30–40% survival based on motility estimates (unpublished observations).

VI. CONCLUSIONS

The ability to preselect the sex of progeny in livestock has been validated on the basis of live births, reanalysis of sorted sperm for DNA content, and of the resulting embryos. Utilization of the flow cytometric-sorting method to separate X- and Y-bearing sperm can be used in conjunction with IVF for the production of sexed embryos to be transferred to eligible recipients for the duration of gestation. This semipractical sexing method, although impractical for

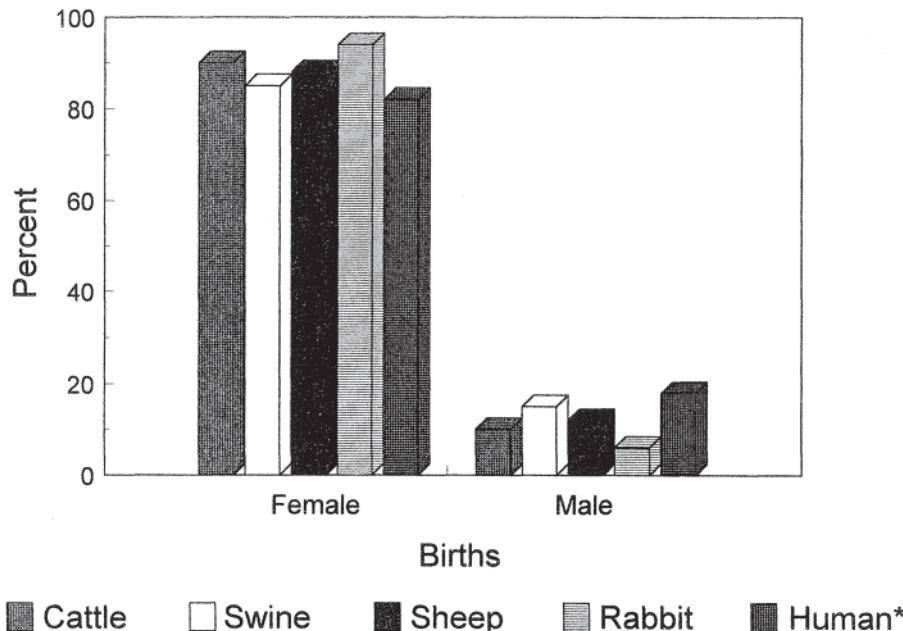


Fig. 5 Sperm from many mammalian species have been separated into separate X and Y sperm populations. This bar graph summarized the skewed sex ratios from animal species that were also verified by live birth sex ratios. Human data is based only on sperm-sort purities. Generally, livestock and other animals can be sorted to purities of 85–95%. The very small difference in DNA content (2.8%) between X and Y human sperm so far limits their purity to 75–85%. *No birth data is currently available for humans.

some production systems and with regular AI, could be used to provide a more flexible progeny-producing option in many livestock operations. The use of the technology for gender preselection in humans is currently being clinically tested. Figure 5 illustrates the current capacity to skew the sex ratio of mammalian semen when subjected to the Beltsville sperm-sexing technology.

Flow cytometric sorting of sperm for reproductive purposes requires significant commitment in expertise and resources to apply the systems commercially or in research situations. Research to improve the efficiency of sorting more sperm per hour is continuing. Current research involving the removal of dead sperm from the sorting process through the addition of the dead membrane permeant dye propidium iodide, may result in a more efficiently sorted population [35]. Slit-scan optics developed for chromosomal sorting applied to the sperm-sexing technology shows potential for enhancing the efficiency of the sorting process [36]. High-speed sorting, as developed for mammalian chromosomes [37], may have application to the sperm-sorting process if the need to orient sperm to the laser beam can be minimized, thus releasing the sperm to flow faster. We continue to search for a sperm, sex chromosome-specific marker (surface protein) that can be exploited in the development of an antibody to be used to tag sperm under affinity chromatography protocols or for use with micromagnetic bead separation systems. Initial results to carry this to completion have proved unsuccessful [38,39]. Antibody-based X-Y sperm separation procedures would lend themselves to relatively easy application in semen-production systems.

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Transgenic Livestock

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I. INTRODUCTION

Agriculture is a multibillion dollar industry in the United States alone [1]. It is no wonder that livestock species have been considered obvious candidates for genetic engineering. When Palmiter et al. [2] reported that transgenic mice grew twice as large as their nontransgenic littermates, discussions of higher-producing, more efficient livestock immediately ensued. Transgenic technology provided a tool with which the genetics of animals could be altered much more rapidly and with greater precision than with selective breeding programs. It soon became evident that genes could be transferred across diverse species, and that gene products, tissue specificity, and timing of gene expression could be altered through genetic engineering. For a variety of reasons, to be discussed later, visions of genetically engineered livestock have yet to become reality. Although some of the initial enthusiasm has been tempered by economic and biological limitations, most of the initial goals of transgenic livestock are alive and well. Applications of transgenic livestock other than agriculture production have arisen, and it is almost certain that they will see commercial application before any agricultural applications.

The definition of transgenic animals has evolved since Jon Gordon [3] used the term in 1980. This chapter will focus on animals in which transgenes were introduced into preimplantation embryos by pronuclear microinjection, with the intended consequence of producing germline transgenic animals, as opposed to somatic cell transgenic ones. For the purpose of this chapter, livestock species will be restricted to pigs, cows, sheep, and goats. In some cases, sheep and goats will be treated synonymously because of the similarities in their reproductive physiology.

II. TRANSGENIC LIVESTOCK PROJECTS

Only a very brief summary of the 37 gene constructs that have been tested in livestock will be presented here. The reader is referred to two excellent reviews that list those constructs and their consequences [4,5].

A. The Transgene

The power of transgenic technology is derived from the introduction of genetic information with new functionality. The strategy for building a transgene (fusion gene) involves selecting a genetic regulatory element (often called a promoter, but usually containing both an enhancer element and a promoter) that will determine the tissue in which the gene is to be expressed and the time and magnitude of expression. In some instances, the regulatory element can act as a switch, allowing the transgene to be turned on and off at will. The second part of the gene construct consists of a DNA sequence encoding the desired protein (often referred to as the structural component of the transgene). For example, in the first transgenic livestock experiment [6], we wanted to increase the levels of circulating growth hormone in a controlled manner. The gene construct used to accomplish this consisted of the regulatory element of a metallothionein (MT) gene fused to the coding sequence for growth hormone (GH). Metallothionein is an inducible liver enzyme, and its gene is usually quiescent (turned off) until a threshold level of circulating zinc or cadmium triggers transcription. Therefore, it was expected that the MT-GH fusion gene would be silent until the animals were fed zinc. In those experiments GH expression could be induced, but usually, the transgene could not be turned off completely. New more complex inducible approaches are now being tested [7,8]. These new systems rely on tetracycline or its analogues to activate or repress transgene expression. It is too early to know if these strategies, will yield more tightly regulated gene expression than the MT system. However, if they are not, the general paradigm on which the new systems are based will probably lead to improved inducible systems.

B. Applied Transgenic Projects

The vast majority of original research reports have focused on growth enhancement. Growth hormone (GH) was the structural gene employed in 13 of those publications and the gene for growth hormone releasing factor in 4. Other structural genes tested include IGF-1, cSKI, and an estrogen receptor. The regulatory elements derived from MT genes from various species, were most frequently used, appearing in nine of the growth-related fusion genes. Long terminal repeats (LTR) from two retroviruses, murine leukemia virus (MLV) and human respiratory syncytial virus (RSV), and a sequence from cytomegalovirus (CMV), a DNA virus, served as regulatory components of transgenes, as have the promoters from albumin, prolactin, skeletal actin, transferrin, and phosphoenolpyruvate carboxykinase (PEPCK) genes. All but two of 21 growth constructs were tested in pigs, and the most striking phenotypes resulted from the use of MT-GH fusion genes [5].

Seven transgenes designed to enhance disease resistance and to produce immunologically related molecules have been introduced into pigs and sheep [9–12]. Although desirable expression patterns have been reported in several of the projects, none of the studies has progressed to the point of demonstrating a beneficial effect of transgene products.

Very recently it has been reported that transgenic sheep with enhanced wool production characteristics have been produced [13]. The results are quite promising; if no unforeseen anomalies occur, transgenically produced wool may be the first marketed livestock product.

C. Biomedical Transgenic Projects

Other proposed transgenic farm animal applications are decidedly nonagricultural. One of the first transgenic animal companies demonstrated the feasibility of producing new animal products by manufacturing human hemoglobin in pigs, to serve as a principal component of a human blood substitute [14]. Human antibodies have also been produced in transgenic

mice [15]. Another area in which transgenic animals will have a significant influence on society will be in the development of human genetic disease models. Genetic disease models have now been generated in mice for atherosclerosis [16], sickle cell anemia [17], Alzheimer's disease [18], autoimmune diseases [19], lymphopoiesis [20], dermatitis [21], and prostate cancer [22]. These models for the most part require "knocking out" the function of a gene or replacing an existing gene with a mutant form. Many of these models will be more useful if they can be replicated in farm animals because the physiology of some farm animals more closely resembles that of humans. Unfortunately, the stem cell technology required to generate most of the disease models is still in development for livestock (discussed later).

Commercial interests have focused primarily on modifying the genetic control of mammary glands to produce pharmaceutical proteins. However, transgenic technology also offers the opportunity to reformulate endogenous protein production of the mammary gland in a variety of other ways. Several excellent reviews have been written on the topic [23–27]. These reviewers and others have suggested strategies for changing milk composition to provide a source of milk for lactose-intolerant individuals, to provide milk that is more efficiently processed into cheese, and to provide "natural" low-fat milk. One of the most ambitious schemes proposed is to "humanize" milk. Some bovine milk proteins would be eliminated and others would be replaced with coding sequences derived from human genes. The resulting milk would more closely resemble human breast milk and would be used to supplement or replace infant formula.

Finally, a new use not reported in the aforementioned reviews deserves note. The objective of this new endeavor is to genetically engineer animals, primarily pigs, so that their organs can be used as xenografts for humans. Preliminary studies to test the concept have been performed in mice [28,29], and transgenic pigs have now been produced [30,31]. Although several strategies are being explored, the general approach has been to block activation of complement, which is normally part of the acute transplant-rejection response. These organs are intended for temporary use, until an appropriate human organ becomes available. However, as the technology develops, the goal will be the design of transgenic organs for extended use or for permanent replacement.

III. PRODUCTION OF TRANSGENIC LIVESTOCK

The methods used in the production of transgenic livestock have been described elsewhere [32] and will not be repeated here in detail. Currently, the only proved method of producing transgenic livestock is by microinjection of DNA into the pronuclei of one-cell embryos or the nuclei of two-cell embryos. Each of the major steps in the production of transgenic animals by pronuclear injection will be discussed in the following, with an emphasis on how these methods differ for the major domestic species (pigs, sheep, goats, and cattle).

A. Collection of Embryos

Usually, embryos for microinjection are recovered from donor females following superovulation and breeding. Two exceptions to this are (1) in some instances, one-cell pig embryos are recovered from nonstimulated females and (2) transgenic cattle can be produced by microinjection of in vitro-matured and fertilized oocytes from slaughterhouse material. Detailed methods for synchronization of the estrous cycle and superovulation have been described for pigs [33–35], sheep [36,37], goats [4,38], and cattle [37,39]. Large numbers of ova can be generated for the production of transgenic cattle when an in vitro production system is employed [40,41]. If a select population of oocytes are used, fertilization rates in excess of 90% can be expected [42,43]. These fertilized ova are capable of developing to the blastocyst

stage at a high rate, with a variety of in vitro culture systems [44,45]. As is true with in vivo-produced embryos, the viability of these embryos is reduced dramatically following microinjection.

B. Visualization and Injection of Pronuclei

Progress in the production of transgenic livestock was hindered for some time because, unlike mouse embryos, nuclear structures are not readily visible in embryonic cells of domestic animals. An exception to this is the sheep and goat in which pronuclei are visible (but not sharply defined) with differential interference contrast (DIC) microscopy [6]. In the other species the problem of visualization of pronuclei has been overcome by centrifugation of the ova to stratify cytoplasmic contents [34]. Ova are typically placed in 1.5- to 2.0-mL microcentrifuge tubes and centrifuged at 14,000–15,000g for 4–8 min. Following centrifugation, pronuclei are visible with DIC or Hoffman contrast optics. Visualization of pronuclei following centrifugation is generally more successful for pig zygotes than for those of the cow. Pronuclei of the pig zygote have a prominent nucleolus that aids in their visualization, whereas those of the cow do not. The ability to visualize pronuclei of cattle zygotes following centrifugation also limits the time frame for which microinjection is successful. This has been well characterized for in vitro-produced embryos for which the time of fertilization can be closely controlled. The proportion of zygotes with visible pronuclei following centrifugation peaks at 22–24 h following insemination [43,46], which is 8–10 h later than pronuclei are visible by cytochemical-staining techniques [43]. The potential significance of this for the production of transgenic livestock, especially cattle, will be discussed later.

Injection needles and apparatus used for microinjection of zygotes of farm animals do not differ from those used for laboratory animals. The manufacture of injection needles and injection apparatuses have been described in detail [47]. In this laboratory the same injection needles and apparatus are used for the microinjection of zygotes from mice, pigs, sheep, and cattle. Pronuclei are successfully visualized and microinjected in approximately 75% of pig zygotes [34,35], 80% of sheep zygotes [6,48], and 60% of cow zygotes [39,41].

C. Culture and Transfer of Injected Zygotes

For most species of livestock, microinjected zygotes are transferred into the oviducts of recipients immediately following injection. The exception to this is the cow. Because surgical transfer to the oviduct of the cow is time-consuming and expensive, and nonsurgical uterine transfer of blastocysts is a well-established technique, microinjected cow zygotes are cultured for 6–7 days and transferred to recipients as morula or blastocysts. A number of successful systems have been developed for the culture of one-cell bovine embryos to the blastocyst stage [see Ref. 45 for a recent review of this subject].

Microinjected embryos of the pig and sheep are normally transferred into the oviduct of recipients. As many as 40 microinjected pig eggs will be transferred into an oviduct, but the optimum number is between 20 and 25 [49]. Because transuterine migration is common in the pig, there is no need to transfer into both oviducts [50; V.G. Pursel, personal communication]. Occasionally, uninjected embryos will be transferred along with the injected embryos to ensure a litter size of at least four pigs, which is required for maintenance of pregnancy [51]. Two to five microinjected sheep embryos will be transferred to each recipient, with most ewes receiving three embryos [37,52]. In most instances, blastocysts resulting from microinjected bovine embryos are transferred singly into synchronous recipients. Increased calf loss owing to twinning and the risk of a transgenic female born twin to a male calf being sterile, makes multiple embryo transfer unadvisable in most transgenic cattle projects.

IV. EFFICIENCY OF PRODUCING TRANSGENIC LIVESTOCK

Efficiencies observed in the major steps of producing transgenic animals are given in Table 1 for the livestock species and for mice. The efficiency of producing transgenic animals is always low, ranging from about one transgenic animal per 40 injected eggs (mice) to one in 1600 injected eggs (cattle). The three major parameters resulting in this low efficiency—embryo survival, integration rate, and transgene expression—are discussed in the following three sections.

A. Embryo Survival

The data presented in Table 1 for survival of microinjected mouse embryos are somewhat biased. When mouse eggs are injected, a significant proportion will lyse immediately. These eggs are discarded and the number of discarded embryos is seldom documented. In this laboratory a 20–40% lysis rate is routine. Microinjected embryos of the pig or sheep seldom lyse immediately after injection, and few, if any, embryos are discarded before transfer. If a 25% lysis rate is assumed for the mouse data in Table 1, the percentage of injected embryos surviving to term is 11.3%, which is comparable with that observed for pig and sheep embryos. The situation for microinjected cattle embryos appears to be quite different. However, it must be recalled that this system includes *in vitro* culture from the one-cell to the blastocyst stage. The data for cattle embryos in Table 1 represent a mixture of *in vivo*- and *in vitro*-matured and fertilized ova. All of the embryos were cultured *in vitro* following microinjection. Of the *in vivo*-matured and fertilized oocytes, 2.8% of injected embryos developed to term, compared with 1.2% for the *in vitro*-matured and fertilized embryos. When *in vivo*-matured and fertilized cow zygotes were recovered from superovulated cows and cultured to the blastocyst stage in the oviduct of a ewe [53], 10% of the injected embryos developed to 60-day fetuses on transfer to synchronous cows. These observations taken together indicate a significant reduction in embryo survival after microinjection, but all species are affected similarly. Control studies in which buffer without DNA was injected indicate that this loss of viability is due to the physical trauma associated with microinjection and not the introduction of exogenous DNA. This is further supported by the work of Bondioli et al. [39] in which DNA concentrations ranging from 1 to 100 ng/ μ L were injected into bovine zygotes. A reduction in survival to 60-day fetuses was not observed until the DNA concentration was increased to 50 or 100 ng/ μ L. Embryo survival for all species can be highly dependent on the size of the injection needle, the methods used to prepare injected DNA solutions, and the experience of the individual performing the microinjection.

Table 1 Efficiency of Producing Transgenic Animals

Species			Fetuses or born		Transgenic animals produced			Ref.
	No. injected	No. transferred	No.	% of injected	No.	% of born	% of injected	
Mice	12,314	12,314	1,847	15.0	321	17.3	2.61	83
Pigs	19,397	19,397	1,920	9.9	177	9.2	0.91	83
Sheep	5,242	5,242	556	10.6	46	8.3	0.88	83
Goats	1,058	782 ^a	173	16.4	12	6.9	1.11	4
Cattle	11,206	1,018	193	1.7	7	3.6	0.06	41

^aSome embryos were cultured 72 h before transfer to the uterus of recipients.

B. Rate of Transgene Integration

Genetic diversity in livestock species may play a role in the low integration frequency observed. Laboratory animals employed to produce transgenic animals are derived from highly inbred lines, and investigators often choose to use specific strains, the embryos of which "culture easily." Scientists who work with livestock embryos do not have the same inbred resources. However, examples can be found in the livestock industry in which selection of animals is based partly on gamete quality. In the artificial insemination industry, selection is based not only on a bull's genetic merit, but also on the "freezability" of his sperm. However, very few in the research community have the resources to perform the same kind of selection on embryo donors. Even though breeds of livestock are genetically more diverse than inbred strains of mice, there may be some value in comparing transgene integration efficiency among livestock breeds.

Another cause of low transgene integration rate may be related to procedural differences between microinjection of livestock and laboratory animal zygotes. Livestock eggs are more challenging to microinject than eggs from laboratory species, in part because livestock pronuclei are more difficult to visualize. Cow and sow eggs must be centrifuged before microinjection, although there is little evidence that embryo survival is compromised by centrifugation [34]; however, the procedure may influence integration rate. Within livestock species, the lower integration rate observed in cattle embryos may also be attributable to the mobility of their pronuclei. Pronuclei tend to move away when touched by an injection needle, but that is not likely to account for the 50% or greater reduction in integration efficiency between livestock and laboratory animals.

It is also possible that transgenes become integrated at similar rates in all species, but the development of injected embryos is disproportionately compromised in livestock. However, the similar poor survival rate of injected embryos of both laboratory and livestock embryos does not support that hypothesis.

A more compelling argument could be made for an association of integration failure with inappropriate timing of microinjection. Cell transfection is most efficient in dividing populations. It has been inferred from this observation that DNA replication is required for integration of foreign genes into the genome [54]. If that is true, then the timing of pronuclear microinjection should be synchronized with onset of the DNA synthetic phase (S phase) of the first cell cycle to ensure the maximum likelihood of an integration event. As depicted in the stylized graph of [Figure 1](#), mouse eggs are microinjected about 8 h after mating. This results in DNA being introduced into zygotes during the beginning of S phase [55]. Sow and cow eggs are injected toward the end of the S phase [43, 56–60], possibly reducing the probability of an integration event. Characterization of pronuclear development in Figure 1 was determined by nonvital-staining techniques. Pronuclei in pig and cattle zygotes form well before the usual timing of microinjected [58–60]. However, the time of microinjection in those species was established to coincide with the time pronuclei can be visualized in a nondestructive manner (differential interference contrast microscopy). At earlier times pronuclei cannot be seen, even though they are present. Therefore, it does not seem possible to microinject transgenes into pronuclei until DNA synthesis is nearly completed in cow and sow eggs. Experimental evidence on the influence of time of microinjection on transgene integration frequency is lacking and may be difficult to achieve. Efforts to inject in vitro-fertilized bovine zygotes earlier than 13 h after insemination have failed because of difficulties in visualizing pronuclei [K.R.Bondioli, unpublished data].

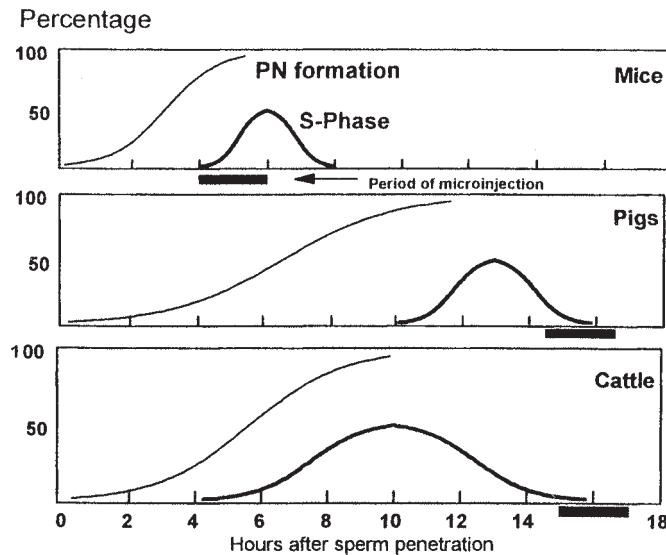


Fig. 1 Timing of S-phase relative to pronuclear (PN) formation in mice, pigs, and cattle: Data from several sources (see text) were combined to generate this stylized graph showing the relative relation of pronuclear development, DNA synthetic phase (S phase) of the first cell cycle, and typical timing of pronuclear microinjection. Pronuclear formation was characterized using nonvital DNA-staining techniques. Timing of pronuclear microinjection is based on the ability to visualize pronuclei with differential interference microscopy.

C. Expression of Transgenes

Aside from the differences in efficiency of producing transgenic animals, the effects of a specific transgene on phenotypic characteristics appear to be similar for transgenic goats, mice, pigs, rabbits, rats, and sheep. There are insufficient data to make the same claim for transgenic cattle, but there is no reason to expect that transgenes will behave differently in that species.

It is commonly thought that transgenes integrate at random locations within the genome. Unfortunately, that assumption is based on very limited direct data. Foreign gene integration in somatic cells does appear to seek specific sites. Analysis of almost 500 cell transfection studies demonstrated that a high proportion (over 90%) of integrations occurred in the vicinity of topoisomerase I cleavage sites [61].

Approximately half of transgenic animal lines express their transgenes, although some specific transgenes are expressed in a higher proportion of transgenic animals [62–65]. Adding matrix attachment region sequences to transgene constructs may “rescue” expression in lines that otherwise might carry nonfunctional transgenes [66,67]. About 70% of transgenic founder mice transmit the transgene to offspring [68]. Although few transgenic livestock experiments have produced enough offspring to precisely estimate transmission rates, it would appear that transgenes will be inherited in livestock as they are in mice [69]. Significant exceptions to predicted transmission frequency have been observed, but those exceptions were a consequence of specific transgene products [62,70–72].

V. INVESTMENTS REQUIRED TO PRODUCE TRANSGENIC LIVESTOCK

The production of transgenic animals of any species will require large intellectual and technical investment. These investments include determining which gene to express, in which tissue to express the transgene, how to build a construct to achieve these goals, and how to efficiently produce the transgenic animals. Although these investments are large, they will be essentially the same for all species. Two forms of investment will vary considerably for different species: animal resources and time.

A. Animal Resources

A high probability of success in a transgenic project requires the generation of several transgenic lines. In this laboratory transgenic projects are designed to generate a minimum of ten founder animals. From this, we expect to obtain five lines that will express the transgene. The levels of expression will be highly variable between these lines and we hope will include both male and female lines. Table 2 illustrates the major animal and labor resources required to complete a transgenic project for various species. The animal resources required to complete these projects are very substantial for sheep and cows. In addition to the cost associated with acquiring and maintaining many animals, the labor costs associated with estrus synchronization, superovulation, embryo collection, and transfer are also very substantial. The animal costs associated with transgenic cattle have been greatly reduced by the use of IVM or IVF zygotes produced from slaughterhouse ovaries. With the *in vitro* systems presently available, there is a significant loss in efficiency owing to low embryo survival, as discussed earlier. There has been recent progress in the development of IVM and IVF systems for pigs, but the development competence of these embryos is too low for practical use in transgenic projects [73]. These *in vitro* systems will have to become quite efficient before they are routinely used because the current approach requires relatively few animals. Technically, it is probably quite feasible to develop IVM and IVF systems for sheep or goat embryos, but little research has been done in this area. This is not a particularly practical approach in the United States because of the small number of adult sheep or goats slaughtered for meat. At least one group is developing techniques for ultrasound-guided aspiration of immature goat oocytes for subsequent IVM or IVF [74].

Even with IVM or IVF zygotes, there is a substantial animal resource requirement in the form of recipients for transgenic cattle projects. Large numbers of recipients are required

Table 2 Animal and Labor Requirements for a Transgenic Project Intended to Yield Ten Transgenic Animals, Five of Which Would Be Expected to Express the Transgene

Species	Recipients required	Donors required	Embryos injected	Embryos transferred	Animals born
Mice	15	33	525	400	60
Pigs	55	63	1,100	1,100	110
Sheep	375	300	1,125	1,125	120
Cows <i>in vivo</i> ^a	1,040	900	5,000	1,040	313
Cows <i>in vitro</i> ^b	1,500	—	20,000	1,500	313

^aIn *vivo*-matured and fertilized zygotes collected from superovulated cows. Cultured to the blastocyst stage in ligated oviducts of sheep.

^bIn *vitro*-matured and fertilized zygotes from slaughterhouse ovaries. Cultured to the blastocyst stage *in vitro*.

because of the low integration rates. If transgenic embryos could be identified before transfer, recipient costs would become almost insignificant. Attempts have been made to achieve this goal by analyzing a few cells taken from the embryo (embryo biopsies), by polymerase chain reaction (PCR) amplification or fluorescent in situ hybridization (FISH) for the presence of the transgene [75–79]. The results of these studies have not been very encouraging because the transgenic rates observed after transfer of only “positive” embryos have not yielded higher rates of transgenics. The persistence of nonintegrated transgenes through several cell divisions (leading to false-positive results) and the high incidence of transgene mosaic embryos (leading to both false-positive and false-negative results) [80,81] are likely explanations for these results. Our laboratory [82] has taken a slightly different approach to this objective. We have attempted to take advantage of the in vitro culture period normally employed in bovine transgenic projects by establishing a positive selection for transgenic embryos during this culture. We have injected a gene construct into pronuclei of bovine embryos that contains the *neo* antibiotic-resistance gene under the control of the chicken β-actin promoter, which is known to be expressed in early bovine embryos. This construct also includes the *lacZ* gene as a marker for expression in the blastocyst. After culturing injected embryos in the presence of the neomycin analogue G418, there is approximately a fourfold increase in blastocysts expressing the marker gene in most or all of their cells. It is yet to be determined if this approach will result in an increased proportion of transgenic calves following transfer to recipient cows. This approach could significantly reduce the recipient requirements for cattle transgenic projects. A similar approach would be feasible for sheep or goat embryos, as these embryos are readily cultured in vitro from the one-cell to the blastocyst stage [37].

B. Time Required to Complete Transgenic Projects

By far the greatest resource expended in livestock transgenic projects is time. The expenditure of time is particularly great for projects in which the transgene product is to be expressed in milk. It is this type of project that has received the most attention recently. Table 3 shows the time required to achieve significant milestones for livestock transgenic projects requiring lactation. The time required to produce a small group of lactating females from a founder line known to express the transgene is substantial for all livestock species, and especially so for

Table 3 Time Required (Months from Microinjection) for a Transgenic Cattle Mammary Gland Bioreactor Project

Event	Pigs		Sheep/goats		Cattle	
	Female founder	Male founder	Female founder	Male founder	Female founder	Male founder
Founders born	4	4	5	5	9	9
First lactation ^a	17	28	18	31	33	54
AI transgenic semen ^b	24	28	32	31	57	54
Production in milk ^c	41	45	50	49	90	87

^aFirst lactation in a female founder or daughter of a male founder.

^bFrom line known to express the transgene. For pigs it is assumed a transgenic male will be born in the first litter of a female founder. For sheep, goats, and cattle time is allowed to produce a son from a female founder known to express the transgene.

^cProduction of protein in the milk of a group females from a single line known to express the transgene.

cattle. The following discussion concerning techniques that might be employed to reduce this time requirement will center on transgenic cattle projects.

The small group of lactating females illustrated in Table 3 could be the animals required to produce enough of a pharmaceutical protein to enter clinical trials or enough animals for a pilot project to test the suitability of an agricultural product. Additional time would be required for herd expansion if these trials proved positive. Even some bioreactor projects would require significant time to generate a group of lactating females to satisfy the product demand. For example, it has been estimated that as many as 35,000 cows would be required to produce enough human serum albumin (HSA) to satisfy the market [83]. With present techniques, the only feasible way of producing an adequate number of cows for an HSA project would be by artificial insemination with semen of a transgenic bull. As discussed later, it would be feasible to generate small groups of transgenic females (eight to ten) for pilot studies or production of clinical trial material by embryo transfer from female founders.

Much of the delay in reaching the major milestones listed in Table 3 results from the need to breed a transgenic female and allow her to go through a lactation to determine which lines will express the transgene in adequate quantities. If expressing lines could be identified early, then herd expansion could proceed without delay. One alternative would be to expand all lines immediately and determine the expressing lines after expansion. This approach would be inefficient because only a few lines will have desirable transgene expression patterns. Unpredictable behavior of transgenes is a critical problem that remains to be resolved. A more practical approach would be to identify expressing lines early by induction of lactation in young females or even males. Expressing transgenic animals have been successfully identified by induction of lactation in female and male goats [84]. Regimens for induction of lactation without pregnancy have also been described for the pig [85] and the cow [86]. Lactation induction experiments have generally been carried out in young females approaching sexual maturity or parous females. It is not known how successful these procedures will be in younger animals, but lactation could possibly be induced as early as 6 months of age in cattle. It is also possible that transgene expression in mammary tissue could be determined in the neonate. It has been observed that some human neonates secrete a milk-like substance during the first 48 h after birth in response to the maternal hormones of pregnancy. Given that observation, transgenic rats carrying a fusion gene composed of the bovine αS_1 casein gene promoter and human growth hormone gene were subjected to mammary gland biopsies 24 h after birth [87]. Human growth hormone was detected in the biopsied tissue in both males and females. It has not been determined if this approach will be successful in livestock species or how reliable it is in predicting levels of expression in lactating females. An alternative to analyzing for the transgene product would be to test for the transgene mRNA in neonatal tissue by Northern blot analysis. A comparison between the levels of mRNA for the transgene and

Table 4 Time Required (Months from Microinjection) for a Transgenic Cattle Mammary Gland Bioreactor Project with the Aid of Follicular Aspiration or Semen Collection

Event	Female founder	Male founder
Birth of founders	9	9
Follicular aspiration or semen collection	15–24	21
Birth of F_1 females	24–33	30
Breed F_1 females	39–48	45
Lactation in F_1 females ^a	48–57	54

^aEight to ten F_1 females could be produced from a single female founder. Any number of animals could be produced from a single male founder.

levels of mRNA for an endogenous milk protein might be a more reliable predictor of expression levels at lactation. If either technique were verified to accurately predict high-expression lines from young founders, then herd expansion from the appropriate lines could proceed much earlier. One of the risks associated with identification of expressing lines in founders is the possibility of a founder being a somatic cell mosaic for the transgene. If this is so, then expression levels will be underestimated or a high-expressing line might be missed.

The development of ultrasound-guided follicular aspiration techniques combined with in vitro embryo production techniques has made limited herd expansion from a female founder more practical. Not only is the potential for producing embryos from a single female enhanced by these techniques, but embryo production can begin at a much earlier age [88,89]. The birth of live calves have been reported from oocytes aspirated from 5- to 6-week-old calves and subsequently matured, fertilized, and cultured to the blastocyst stage *in vitro* [90]. Others have found that fertilized oocytes lack developmental competence until the donor females reach 6–8 months of age [91]. It is not known why results from these laboratories differ, but taken together, they clearly indicate that the potential to produce embryos from 6-month-old calves exists. If follicular aspiration of a founder calf were started at 6 months of age and continued through breeding age (15 months) and through the first 3 months of pregnancy, it would be theoretically possible to produce 100–150 transferrable blastocysts from a single female founder. If germline transmission of the transgene was normal, 50–75 of these would be transgenic and 25–37 of these would be female. If one assumes a 30% pregnancy rate on transfer, these blastocysts would yield eight to ten transgenic females for a pilot study or production of a protein to be used in clinical trials. Again, if we assume the transgene was expressed at 1 g/L of milk, eight cows would produce 72 kg of unpurified protein in a 305-day lactation [83]. If the *in vitro*-produced blastocysts were sexed [92] and screened for the presence of the transgene by PCR before transfer, the number of recipients required to produce this sized group of females from several founder lines would not be extreme.

If transgene expression levels could be accurately predicted in male founders, the task would be much simpler. Semen could be collected from the male founder at about 12 months of age and used to inseminate cows. Any number of transgenic females could be produced in this manner from a single founder. [Table 4](#) summarizes the time required to reach these milestones for male and female transgenic founder cattle. By comparing the time required to produce lactating females presented in [Tables 3](#) and 4, it becomes clear that intervention either by follicular aspiration or semen collection can reduce this time by up to 1 year.

Other reproductive strategies that could significantly reduce the time required to produce a number of lactating transgenic animals, include growth and maturation of fetal oocytes [93] and nuclear transfer with fetal gonial cells [94]. Live young have been produced by fertilization of oocytes collected from the ovaries of newborn mice [95]. Experiments with domestic animals (pigs and cattle) have been restricted to developing procedures for collection of primordial follicles from fetal ovaries and definition of culture conditions for the growth of these follicles *in vitro* [96]. Developmental competency of fetal oocytes has not been achieved for any species except the mouse. Primordial germ cells of the mouse have been transformed into stem cells *in vitro* and used for germline transmission of exogenous genes by the generation of chimeric animals [97]. Attempts to produce young from cultured bovine primordial germ cells by fusion with enucleated oocytes (nuclear transfer) have thus far been unsuccessful [98,99]. In one preliminary experiment [94], bovine fetal gonial cells were recovered from 50-to 70-day-old female fetuses and fused to enucleated oocytes. Blastocysts developed from these reconstituted embryos and on transfer the reproductive cycles of recipients were extended and placental material was recovered, but no fetus. With either of these techniques the full gestation time is not required, and the time required for founders to reach sexual maturity is completely eliminated.

Thus, the time required to produce offspring from transgenic founders is greatly reduced. Although transgenic fetuses can be readily identified by amniocentesis [K.R.Bondioli, unpublished results], the identification of expressing lines before the investment of considerable resources for propagation would not be possible. Not only do these techniques require further development in livestock species, but their application to transgenic projects will require further understanding of the factors determining expression of transgenes.

VI. OTHER METHODS OF PRODUCING TRANSGENIC LIVESTOCK

The foregoing discussions have dealt exclusively with the production of transgenic livestock by pronuclear injection. This is because it is the only technique that has been demonstrated to be successful and repeatable. Other methods of gene transfer that have been suggested include sperm-mediated gene transfer, retroviral-mediated gene transfer, and transformation of embryonic stem (ES) cells.

A. Sperm-Mediated Gene Transfer

One way to ensure that the transgene is in place before the first mitotic S phase is to introduce the transgene at fertilization. That could be achieved by sperm-mediated gene transfer [100,101]. Notwithstanding the controversy this approach has generated [102], it clearly represents an intriguing method that shows some promise [103]. Accumulating evidence suggests that sperm of several species can bind transgenes [104–107] and carry the genes into oocytes in which the gene sometimes persists [100,108,109]. However, it appears that almost always, the transgene DNA becomes rearranged or otherwise mutated by the process (C. Spadafora, personal communication).

Another potential sperm-based delivery approach has been foretold by a pioneering study conducted by Brinster [110]. In that study, transplanted spermatogonial cells generated sperm capable of fertilizing oocytes and offspring were produced. If a means is found to culture, transfet, and select spermatogonia with transgenes, Brinster's transplantation scheme could be used to produce males capable of siring transgenic animals. Others have proposed directly transfecting testes as a means of transforming sperm [111].

B. Retroviral-Mediated Gene Transfer

Retroviral-mediated gene transfer is also a potential alternative approach for introducing transgenes into embryos with high efficiency [112,113]. Although the technique solves the lowintegration frequency problem, it creates other inefficiencies by generating mosaic founders that may not transmit their transgene. Furthermore, retroviruses can carry only a limited amount of exogenous DNA; therefore, the technique limits the size of transgenes. If cDNA-based transgenes, which are relatively short, were efficiently expressed, the transgene size restriction would not be a significant problem. However, many cDNA-based gene constructs are poorly expressed in transgenic animals [144].

C. Transformation of Embryonic Stem Cells

The only proved method for the production of transgenic animals other than pronuclear injection is the transformation of ES cells and "capture" of the transformed genotype through germline chimeric animals. Germline chimeras are formed by injecting ES cells into normal blastocysts or by aggregation with morulae. The only species for which germline transmission of ES cells has been demonstrated is the mouse, and most of these cell lines have been derived

from a single inbred strain of mice (129/SvJ). The ES cells that colonize the germline of chimeric offspring have been described for other strains of mice [115,116], but the efficiency of establishing these lines has been an order of magnitude less than for the 129 strain. This observation is not encouraging for the prospects of routinely establishing ES cells of domestic species. Embryo-derived pluripotent cell lines have been reported for the pig [117] and cow [98,99], but germline colonizations in chimeric offspring have not been reported.

If ES cells of domestic species are developed, it is not entirely clear what their real value would be. The ES cells of mice have been used extensively to study gene function. The most common experiment is to generate a "knockout" by specific mutation of a gene and to study the resulting phenotype. It is unlikely that domestic livestock species would be used extensively for such experiments. Opportunities do exist for the development of human disease models in livestock species, and these opportunities would clearly be enhanced by the availability of ES cells. The primary advantage offered by ES cells is the ability to efficiently perform site-specific genetic mutations by homologous recombination. It is possible that insertion of a transgene into a specific site within the genome would provide enhanced expression or better control of expression, but this has yet to be demonstrated. The primary disadvantage to ES cells is the requirement to produce chimeric animals as an intermediate step in the production of a fully transgenic animal. This is not a major constraint with mice, but as indicated earlier, the additional generational interval could be highly critical for transgenic livestock projects. If offspring could be produced by nuclear transplantation of genetically modified ES cell nuclei, this disadvantage would be overcome and ES cells would have a major effect on transgenic livestock projects. Attempts to produce offspring by nuclear transplantation with mouse ES cells [118] and bovine embryo-derived pluripotent cell lines [98,99] have thus far been unsuccessful. Calves have been produced by nuclear transplantation of "short-term" cultured inner cell mass cells [119]. Live young have been produced by fusion of cultured sheep embryo-derived cells into enucleated oocytes [120]. The production of live young from these cells has not been very efficient, but if they can be genetically altered and modified cells selected in culture, high levels of efficiency would not be required for transgenic projects. If these results can be duplicated and extended to other livestock species, they will have a tremendous influence on future transgenic livestock projects.

VII. CONCLUSIONS

In light of the overall low efficiencies of producing transgenic livestock, that these projects are being pursued is testimony to the perceived importance of this technology. Even with the present low efficiencies, transgenic livestock are being commercially developed for production of valuable proteins and xenographic organ transplant donors. Several companies have made significant capital investments in this technology, and several transgenic animal-produced products are in the commercial "pipeline" [121]. The low efficiencies have severely restricted the use of transgenic technology or the enhancement of livestock animal production. It has become clear that the economically important traits for livestock production are under complex genetic control, and it is difficult to predict how altering the expression of a single gene will affect these traits. The low efficiencies and resultant high costs have made empirical experimentation impractical for most laboratories and, for the most part, prevented commercial investment in these projects. Therefore, it is crucial that research in transgenic technology continue to increase these efficiencies and make this type of experimentation possible. It is fortuitous that commercial applications of transgenic livestock for nonagricultural products have arisen, for it is these applications that will provide the impetus for this research in the short term.

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Transgenic Dairy Animals for Production of Novel Proteins in Milk

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I. INTRODUCTION

Transgenic animals were first produced by Gordon and Ruddle, who produced live mice from microinjection of an exogenous DNA into pronuclei of mouse eggs. The resulting offspring were termed transgenic [Gordon et al., 1980; Gordon and Ruddle, 1981]. It was only in their second paper [Gordon and Ruddle, 1981] that the full definition of transgenic was met by the birth of offspring from transgenic parents who expressed the transgene. So, a *transgenic animal* is one in which new DNA sequences have been inserted, are expressed, and become a permanent part of the genome, transmitted by sexual reproduction.

Since then, transgenic mice have become a common research tool for studying genetic control of physiological function, control of gene expression, genetic disease, models for human viral diseases, and as models for genetic modification of domestic animals [First and Haseltine, 1991]. It has been estimated that more than 10,000 different strains of transgenic mice have been produced [J. Gordon, personal communication].

The efficiency ranges from 1 to 4% of the DNA microinjections resulting in transgenic offspring [Brinster et al., 1985]. Similar DNA microinjection procedures have been used to produce transgenic cattle, sheep, swine, and goats [Rexroad, 1992; Ebert and Schindler, 1993; Ebert and Selgrath, 1991; Eyestone, 1994], and the efficiency is 1% or less. For cattle, this efficiency is lower than other species or about 0.2%. The low efficiency makes each transgenic animal produced extremely expensive.

The usefulness of transgenic mice as a research tool has been expanded by development of systems for production of chimeric mice containing a transgene from cultured transgenic embryonic stem (ES) cells [Evans and Kaufman, 1981; Stewart, 1991]. The ability to transfet, microinject, or infect DNA with sequences homologous with known chromatic sequences has allowed use of homologous DNA recombination to accomplish site-specific gene transfer or deletion [Koller et al., 1989; Capecchi, 1989]. Hence, the use of ES cells has enabled us to study lost function after gene deletion, study of gene positional effects, and study of cell

lineage and differentiation [Joyner, 1991]. In domestic animals, offspring have only recently been produced from cultured embryonic cells of swine [Wheeler, 1994], cattle [Sims and First, 1994], and sheep [Campbell, et al. 1996]. In cattle, germline transmission was tested and shown positive [M. M. Sims and N.L. First, unpublished data].

Private industry, government, and university research laboratories are attempting to produce transgenic cattle, goats, sheep, and swine, even though the cost is high when this is done by conventional microinjection of DNA into pronuclei. This means initial efforts are with genes of an expected high-economic return. These areas, in order of economic interest are, (1) the production of pharmaceutical proteins by targeted expression in the mammary gland, (2) genetic alteration of milk proteins to produce humanized infant formula, (3) the genetic modification of milk proteins to increase the yield, quality, or kind of milk products produced, and (4) modification of the cow or other animals to increase the efficiency of milk production, meat production, or environmental adaptation. Genes can be targeted to be expressed only in the mammary gland by inclusion in the transgene construct of a promoter sequence for a milk protein. Promoters commonly used are casein lactoglobulin and α -lactalbumin promoters.

More than 37 gene constructs have been tested in livestock [Wall, 1996]. Over half of these constructs have been directed to the promotion of animal growth. The common genes introduced have been growth hormone, growth hormone-releasing factor (IGF-1), and the *C-ski* oncogene. Thus far, none has resulted in a transgenic animal of accepted commercial use or a genetically engineered product of wide-scale use or commercial value. Nearly one-quarter of these constructs have contained genes to influence the immune system, either by enhancing disease resistance or to allow organs of the transgenic pig to be used as human organ transplants. At least three commercial companies are doing and supporting research to develop human-compatible xenotransplant animals as human organ and tissue donors.

At least four publications have concerned transgenic attempts to target genes to the wool follicle [Damak et al., 1996a] or to improve the growth or composition of wool. One approach has been to transfer into sheep genes that encode enzymes for the biosynthesis of cystine, a major component of wool. The expectation is that wool growth would increase [Ward and Nancarrow, 1991]. Unfortunately, only one animal transgenic for increased cystine has now been produced, and it failed to show consistent gene expression [Powell et al., 1994]. Another approach has been to target genes to the wool follicle by building follicle-targeting sequences into the construct. Four transgenic sheep and germline progeny expressing a marker gene in the wool follicle have been produced [Damak et al., 1996a]. The ultrahigh sulfur keratin promoter used by Damak et al. [1996a] was used to target an ovine insulin-like growth factor gene to wool follicles of sheep produced by pronuclear injection of the foregoing construct. Five transgenic sheep and 43 transgenic progeny were produced. When shorn at 14 months of age, their clean fleece weight was 6.2% higher than that of comparable nontransgenic controls [Damak et al. 1996b]. These results suggest that strains of sheep with improved wool yield or wool characteristics may soon be developed. Additionally, the use of genetic engineering to make new fibers or protein products from harvested wool of appropriate transgenic sheep becomes a real possibility.

Several companies are attempting to produce novel valuable proteins in milk by transfer of genes for proteins of mammary-specific expression along with DNA sequences coding for a protein of interest. Some of these companies and the protein they are attempting to produce are shown in [Table 1](#). The ability to produce products in milk has been seriously impeded by the low efficiency of gene transfer by pronuclear injection of DNA. Because of this—the common method for gene transfer in mammals has been so inefficient. This and alternative methods will be discussed later.

Table 1 Summary of Companies Attempting Production of Milk Proteins

Company	Programs
American Red Cross	Human protein C in milk of pigs
Cell Genesys	Human monoclonal antibodies in mice
DNX	Blood substitute human hemoglobin in blood of pigs
GenPharm International	Nutritional supplements: human milk specifically human lactoferrin proteins
Pharmaceutical Proteins Limited	Human α 1-antitrypsin (AAT) in sheep milk
Genzyme	Antithrombin-III (AT-III) Cystic fibrosis transmembrane conductance regulator Human serum albumin (HSA) Oral tolerizers Monoclonal antibodies Long-acting tissue plasminogen activator (LA-tPA)

II. GENE TRANSFER

A. Microinjection of DNA into Pronuclei

Methods for producing transgenic cattle, goats, sheep, and swine are broadly similar to methods used for producing transgenic mice [Hogan et al., 1986], except that cattle zygotes for pronuclear injection are usually produced from in vitro fertilization, rather than by superovulation. The cytoplasm of cattle and pig eggs is dense and requires displacement by centrifugation to allow visualization of the pronuclei.

The process is very inefficient, with approximately 50–70% of the eggs suitable for microinjection and approximately 3–10% of injected bovine eggs yielding calves, of which 0.16–4% will be transgenic [Eyestone, 1994; Rexroad, 1992]. In two studies, over 600 zygotes were microinjected to produce a single transgenic calf [Eyestone, 1994; Bowen et al., 1994]. In spite of this, a few transgenic calves have been produced by this method [Krimpenfort et al., 1991; Hill et al., 1992; Bowen et al., 1994]. The efficiencies are somewhat better in swine, sheep, and goats in which numerous transgenic animals have been produced [Wall, 1996].

The variables affecting this low efficiency can be grouped into two categories: reproduction and molecular genetics. On the reproductive side, the zygotes can be produced by either in vitro fertilization or in vivo surgical recovery. In one study, 1.4% of in vitro-produced zygotes yielded live calves, compared with 3% for in vivo-recovered zygotes [Eyestone, 1994]. In vivo recovery is seldom used because the superovulation of nearly 120 cows would be required to produce one transgenic calf. Oviduct recovery of in vivo-produced embryos is the common method of producing embryos for gene transfer in goats, sheep, and pigs.

The in vitro technique for cattle involves retrieval of oocytes with either unknown and potentially low genetic value at the abattoir, or of oocytes with known parental background and potentially of high genetic value by transvaginal ultrasound-guided retrieval. The efficiencies of fertilization and embryo development are not greatly different between these two sources of oocytes. Without DNA microinjection, in excess of 30% of the in vitro-matured and fertilized oocytes become blastocysts capable of high rates of pregnancy initiation [Dominko and First, 1992; Monson et al., 1992] and 50–60% [D.Northey, personal communication] of the blastocysts become calves. With the addition of oocyte centrifugation and DNA microinjection for transgenic production, only about 12% become blastocysts and only 20% of these yield live calves [Eyestone, 1994]. The embryonic and fetal survival of in

vitro-produced transgenics is 16 and 20%, respectively [Eyestone, 1994]. Clearly, improvements in reproductive efficiency could improve the efficiency of bovine gene transfer and gene transfer in other domestic animals.

The efficiencies of the molecular genetic steps are also low. Gene integration frequencies at birth of 4–5% [Rexroad, 1992; Eyestone, 1994] have been reported for cattle, compared with approximately 8–10% for swine and sheep, or 30–35% in species such as mice [Brinster et al., 1985; Wall and Seidel, 1992].

The causes for low integration and expression in domestic animals are not understood. As in mice, the variables may include the construct microinjected, purity of DNA, timing of the microinjection, strain, or breed [Brinster et al., 1985]. The causes may also be random insertion, poor nuclear retention, degradation of the DNA, or availability of integration sites. Constructive approaches to improving gene integration frequency have been slow in development [Wall and Seidel, 1992]. It may be that the pronuclear stage is not the best stage for maximum DNA integration and expression. It may also be that integration and expression rates would be higher in domestic animals and more like mice if the male pronucleus was the injected pronucleus. The source of pronuclei cannot be easily distinguished in domestic animals. In support of this, there is evidence in mice that condensed chromatin is repressive to DNA expression. Whereas chromatin undergoing decondensation and especially sperm-derived chromatin undergoing histone remodeling and acetylation is extremely permissive to binding and enhanced activity of transcription factors [reviewed by Schultz et al., 1995].

One approach has been to screen embryos to select only those bearing the transgene before transfer to cattle, using PCR-based genetic screening or *in situ* hybridization on cells of embryos. If effective, this would require higher production of microinjected embryos, but reduce the costly component, the recipient herd. These attempts so far have resulted in little success. By using PCR-based methods, as many as 60 [Horvat et al., 1993] to 85% of the screened embryos [Krisher et al., 1995] have been positive at the morula to blastocyst stage, and a high level of PCR-positive results continue through at least 2 weeks of gestation [Roschlau et al., 1989], even though few born offspring are transgenic. It is likely that the sensitive PCR technique is detecting nonintegrated DNA, and even noninjected cells mixed with injected have been positive [Horvat et al., 1993; Eyestone 1994]. These false-positives appear to bear little relation to the low frequency of integration (4–5%) found in the offspring born [Eyestone, 1994]. *In situ* hybridization methods for screening have not yet allowed every embryo to be evaluated owing to problems of the procedures and low number of cells that can be biopsied from the embryo. These assays offer promise, and better transgenic assays are likely to be developed. One example is the green fluorescent protein expression assay (Chan et al. 1997).

Transgenic cattle have been produced by DNA microinjection [Roschlau et al., 1989; Krimpenfort et al., 1991; Hill et al., 1992; Bowen et al., 1994], and one transgenic bull has produced germline transgenic descendants [Eyestone, 1994]. However, the efficiency is so low that present efforts are directed almost exclusively to high-value transgenic products, such as targeted expression in milk for production of pharmaceutical proteins, or humanized infant formula. Clearly, more efficient and more versatile methods are needed.

B. Viral Vectors

The viral vectors most commonly used to introduce DNA into embryonic cells of mice or other species are replication-defective retroviral vectors [Miller, 1992; Kim et al., 1993a,b; Haskel and Bowen, 1995]. The retroviruses integrate the gene of interest as a single copy, whereas a microinjected transgene usually integrates as a tandem array. The microinjection

of viral vector containing a DNA of interest has resulted in a few transgenic embryos in cattle [Kim et al., 1993a,b; Haskel and Bowen, 1995]. Subzona injection of the viral vector at the one- to four-cell stage embryo, as done by Haskel and Bowen [1995], has resulted in 7% transgenic fetuses. Because some blastomeres are infected and integrate DNA, whereas others do not, transgene mosaicism results. Early embryos of mice are known to repress transcription of retroviral DNA unless it is driven by a powerful exogenous promoter [Soriano et al., 1986; Stewart, 1987]. This situation may be the same as reviewed by Schultz et al. [1995], wherein gene-microinjected early embryos of mice contain repressors of transcription that are not expressed until after the one-cell stage and that can be overcome by powerful exogenous promoter or enhancer sequences. This problem could be prevented by infection or injection of the DNA into embryos before the first cell division, as has been done in fish and insects [Burns et al., 1993; Yee et al., 1994]. The efficiency of production of transgenics by retroviral vectors should also be improved by use of higher-titer retroviral vectors. The PG13 LN vectors, as cited earlier, typically produce virion titers of 10^4 – 10^5 . Whereas the newer pseudotyped retroviral vectors, as reported for fish and insects [Burns et al., 1993; Yee et al., 1994], allow virion titers of 10^7 – 10^{10} and are highly infectious to cells of many species.

C. Sperm-Mediated DNA Transfer

Sperm of mice and cattle have been shown to bind and internalize exogenous DNA: mouse [Bachiller et al., 1991], and cattle [Atkinson et al., 1991; Schellander et al. 1995]. Sperm-mediated DNA has been transferred to the site of fertilization where some has integrated into the genome and has resulted in a few transgenic mice [Lavitrano et al., 1989] and one transgenic calf [Schellander et al., 1995]. In spite of these limited successes, the use of sperm to transfer exogenous DNA is, at present, poorly efficient, and is highly unreliable across laboratories [Brinster et al., 1989], and in some cases has been associated with genomic rearrangements [Schellander et al., 1995]. Additionally, the mechanisms of sperm-mediated DNA transfer are poorly understood. In spite of this the method presents intriguing possibilities for gene transfer in the normal practice of in vitro fertilization or artificial insemination. The transgenic calf reported by Schellander [1995] was born from artificial insemination of sperm containing exogenous DNA.

III. THE DEVELOPMENT OF EMBRYONIC STEM CELL SYSTEMS FOR BOVINE TRANSGENESIS

The production of offspring from transgenic bovine embryonic cells is potentially a more efficient and more versatile system for transgenesis than microinjection of DNA into pronuclei. The ability to produce transgenic cattle from embryonic stem cells could (1) provide an efficient system for gene transfer, (2) provide a means for site-specific gene transfer or deletion, and (3) provide a large number of identical cells for production of clonal offspring by nuclear transfer or other means.

Embryonic stem (ES) cells have been isolated from the late-stage inner cell mass (ICM) of mouse blastocysts [Evans and Kaufman, 1981] or morulae [Eistetter, 1989] and cultured on differentiation-inhibiting mouse fibroblast feeder layers to large cell numbers. These ES cells exhibit pluripotency. When chimerized with normal embryonic cells, they result in ES cell chimeric offspring and pure ES cell germline descendants [Evans and Kaufman, 1981; Stewart, 1991]. The ES cells of mice have not produced offspring when used in nuclear transfers. Pluripotent ES cells have been isolated and cultured from mice [Evans and Kaufman, 1981], hamsters [Duetchman et al., 1988], pigs [Notarianni et al., 1991; Wheeler et al., 1995], rabbits [Graves and Mareadith, 1993; Giles et al., 1993], mink [Sukoyan et al., 1993], rats [Iannaccone

et al., 1994], monkeys [Thomson et al., 1995], cattle [Strelchenko, 1996], and sheep [Campbell et al., 1996]. Totipotency, as evidenced by offspring from ES cells, has been documented for mice [Robertson, 1987], cattle [Sims and First, 1994], pigs [Wheeler, 1995], and sheep [Campbell et al., 1996].

Several variables affect the isolation, derivation, culture, pluripotency, and totipotency of putative ES cells. These variables include (1) methods of ES cell isolation, (2) culture methods and differentiation prevention, (3) stage of the embryo supplying putative ES cells, and (4) ES cell parentage and normalcy of karyotypes.

Most attempts to isolate and culture inner cell mass cells have been based on or adapted from the original methods of Evans and co-workers for mice [Evans and Kaufman, 1981]. These methods have involved placing either the entire day 4-hatched mouse blastocyst or its ICM after immunosurgical removal of the trophoblast cells onto a murine mitomycin C- and (leukemia inhibitory/factor (LIF)-treated fibroblast feeder layer. The ICM is allowed to outgrow for 3–4 days and is then picked off and briefly incubated in trypsin to disaggregate the cells.

Success in producing offspring from ES cells of species other than mouse have in some species required deviation from the original mouse methods [Sims and First, 1994; Campbell et al., 1996]. Some of these differences include stage of cells used to make a cell line, method or species specificity of materials used to prevent differentiation, method of culture, feeder layer, and the use of nuclear transfer to produce offspring. Data pertaining to production of offspring from cattle blastocyst inner cell mass cells are shown in Table 2.

We attempted to establish cell lines from 16- to 20-cell embryos because (1) absence of commitment to differentiation is critical for totipotency of an ES cell line, (2) differentiation begins with cell polarization and compaction—16- to 32-cell stage in bovine—and (3) in cleavage-stage embryos, cells can be immediately disassociated. These 16- to 20-cell embryos were pooled to provide a critical mass of 80–100 cells for establishment of cultures. The cultures were a microdrop containing the media CR1aa [Rosenkrans and First, 1993] plus insulin, selenium, and transferrin, with 15% fetal calf serum and a layer of bovine 30-day fetal fibroblasts. Bovine, rather than mouse, fetal fibroblasts were used to provide species specificity in the differentiation-inhibiting activity of the feeder layer. When the feeder layer was removed or overgrown, the cultured ICM cells differentiated to become embryoid bodies. The short-term growth of cultured 16- to 20-cell-staged cells and the effects of pooling embryos on establishment and proliferation of embryonic cell cultures is shown in Table 3.

If we assume that cultured embryonic cells of bovine or other domestic species can integrate and express DNA after its injection or infection into the cells, as mouse ES cells do, and

Table 2 Use of Loose Suspension Cultured Inner Cell Mass Cells as Donors of Nuclei in Nuclear Transfer to Produce Blastocysts and Calves

Cell lines	5	
Culture duration (days)	34	
Nuclear transfers	239	
Blastocysts	42	(14%)
Blastocysts ($n = 34$) transferred into cows ($n = 27$)		
Pregnant at 42 days	13	(49%)
Fetuses at 56 days	10	(37%)
Calves born	4	(12%)

Source: Adapted from Sims and First, 1994; First et al., 1994.

Table 3 Effect of Number of 16- to 20-Cell Precompaction Morulae Starting a Culture on ES Cell Survival and Proliferation

No. embryos in cell Line	Initial		Cultured 10 days		
	No. Lines	No. Cells	No. Lines	No. Cells	No. Cells/Embryo
1	10	21	4	2,788	2,788
3	16	44	16	25,246	8,415
5	10	96	10	93,334	18,667

^aDisaggregated embryos were cultured on a bovine 30-day fetal fibroblast feeder layer in a microdrop of the medium. CR1aa+selenium, insulin, transferrin, and 15% fetal calf serum.

Source: First et al., 1994.

assume that the transgenic cells can be identified and separated without damage, then reconstitution of embryos from cultured cells, either by nuclear transfer or other means, should provide a more efficient system for gene transfer than pronuclear microinjection. This is achieved by cell multiplication in culture and use of known transgenic cells in nuclear transfer. Although this saves recipient cow or animal costs, the data (see Table 2) suggests that with present nuclear transfer inefficiencies the efficiency would still only be about 12%. Greater efficiency would be achieved by chimerizing the ES cells with normal cells and transferring blastocysts derived from the aggregated cells into cows. In cattle, cells from normal day 5–6.5 embryos chimerized and transferred into cows, resulted in seven calves from 18 transfers, of which two exhibited a chimera marker [Brem et al., 1986]. The major problem with use of chimeras is that two generations would be required to produce germline offspring from the ES cells. Clearly, if perfect ES cell systems were available for cattle, sheep, swine, or goats today, their efficient use would be limited by inefficiencies in present methods for producing offspring from ES cells.

IV. TRANSOMATIC GENE TRANSFER

An alternative approach to testing expression of gene constructs in a species, such as cattle or other domestic animals, is the introduction of DNA into and production of protein from somatic cells. This has been done by bombardment of DNA into cells. This might be useful for testing gene expression *in vivo* or for DNA-based immunization of the mammary glands of sheep [Furth et al., 1995; Kerr et al., 1995].

Another approach receiving considerable interest for commercial production of proteins in milk is the infection of DNA into mammary glands by viral vectors. This approach produced small amounts of human growth hormone in the mammary glands of goats [Archer et al., 1994].

As further research is completed, it is anticipated that both the transgenic and transomatic approaches to gene transfer will become more efficient, and that each will be used for specific product production through milk, as reviewed by Bremel [1995] and Wall [1996]. It is also likely that transgenic animals will eventually be made that have value in improved milk composition [Bremel, 1996], wool production [Bullock et al., 1995], or meat composition and production efficiency. It is also expected that transgenic domestic animals will benefit human health and welfare through avenues such as production of xenotransplant organs [Fodor et al., 1994; Rosengard, 1995], through products from transgenic or transomatic mammary glands, such as pharmaceuticals, nutraceuticals, antigens for vaccines, and new food products, as well as becoming models for human disease.

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Somatotropin in Domestic Animals

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I. INTRODUCTION

Somatotropin (ST) belongs to a superfamily of hormones composed of ST, prolactin (PRL), and placental lactogen (PL). These three hormones are postulated to have originated by duplication of an ancestral gene [1]. Although PRL and ST bind to only their respective receptors, PL binds to both the PRL and ST receptor [1]. It is presently postulated that the putative PL receptor is a modified ST receptor [1].

The definition of ST's biological role has changed over time. The growth-promoting activity of ST was first demonstrated in 1921 by Evans and Long [2] using an extract of ox pituitary. This discovery led to its misnomer as primarily a "growth hormone." The galactopoietic (lactation-enhancing) role of ST was first demonstrated by Azimov and Krouze in 1937 [3], and the metabolic effects of ST were first demonstrated by a series of investigators beginning in the 1940s [4,5]. These studies led to categorizing ST as "lipolytic" or "diabetogenic," or both, and "nitrogen-sparing." In addition to these effects, STs are also involved in regulation of some aspects of immune function [6] as well as reproduction [7,8]. Thus, the term ST does not nearly describe the pleiotropic actions of this molecule.

Bauman and Currie [9], first described the multiple actions of ST as *homeorhetic* "the coordinated control of metabolism in support of a dominant developmental or physiological process." The concept of ST as a homeorhetic molecule involved in coordination of metabolism to support the processes of growth and lactation is much more encompassing. Possibly, with further discoveries, the other roles of ST in reproduction and immune function can be placed in this context as well. The advent of recombinant DNA technology has subsequently made available large quantities of human and animal STs, their specific receptors, as well as their mediators. This has resulted in rapid increase of knowledge about the regulation and metabolic effects of STs. The objective of this chapter will be to outline our present knowledge of the systemic and cellular ST cascade in domestic animals.

II. SYSTEMIC SOMATOTROPIN CASCADE

The systemic ST cascade begins with the neurohormonal system in the brain that regulates secretion of ST. Somatotropin is secreted from the anterior pituitary in a pulsatile pattern that is sexually dimorphic, nutritionally influenced, and ontogenetic [10]. Somatotropin secretion is low at birth, increases to peak levels at puberty, and declines with advancing age [11].

Secretion of ST in males is characterized by discrete peaks and prolonged troughs, whereas females tend to have peak of a lower amplitude and a more continuous secretion [10]. The pattern of secretion is important to the biological responses involved. In particular, the pattern of ST secretion influences absolute growth rate. Studies in the rat [12] demonstrated that growth was most sensitive to pulsatile exposure and peak amplitude, whereas the baseline ST concentration influenced the ST-binding protein (STBP) and hepatic ST receptor expression. Likewise, pulsatile exogenous porcine ST (pST) is more effective in stimulating growth in pigs than is continuous pST delivery [13].

The pulsatile pattern of ST secretion results from two interacting neurohormones: growth hormone-releasing factor (GRF) and ST release-inhibiting hormone (SRIH), also called somatostatin [14]. These two neurohormones are under the regulation of adrenergic and cholinergic neurotransmitters and neuropeptides (opioids, galanin, vasoactive intestinal peptide, and others [14]. Somatotropin secretion is also stimulated through a non-GRF-dependent mechanism, mediated through the recently characterized growth hormone-releasing peptide (GHRP) receptor [15]. The endogenous ligand for this receptor has not yet been discovered, although it is mimicked by synthetic hexapeptides [11]. In addition, the regulation of ST secretion also occurs at the transcriptional level through regulation of the promoter region of the ST gene [11].

Somatotropin travels in the circulation as either a free hormone or bound to a circulating BP [16]. For ST to affect a target cell, it must first bind to its target receptor as a free molecule. Thus, the BP-ST complex must dissociate for the ST to bind to a target cell receptor; hence, the total concentration of ST does not necessarily reflect the free or effective concentration. Formation of the circulating BP in humans is directly related to the secretion rate of ST [16]. Following binding and activation of the full-length receptor, the hormone-receptor complex dissociates, and the extracellular cytoplasmic domain or binding region of the receptor is cleaved and enters the circulation as a STBP. Somatotropin BP arises by a different mechanism in rodents in which the circulating STBP is the product of alternative splicing of the ST receptor primary transcript [17]. Circulating STBPs have been identified in cattle, sheep, humans, rodents, swine, and a putative BP has been identified in poultry [17]. The endocrine role of STBPs appears to be primarily protection of circulating ST from degradation [16]. Mathematical analyses and computer simulations indicate that STBP binds from 10 to 80% of circulating ST, depending on the concentration of the ST in circulation [16]. Somatotropin BPs have also been detected in cytosolic fractions of several cells [18,19] and have been demonstrated to affect adipocyte glucose metabolic responses to ST. Thus, considerable research will be required to fully elucidate the functions and role of STBPs in the systemic and cellular ST cascade.

There are many cell types that have been identified as targets of ST, some of which include hepatocytes, ovarian luteal cells, cells of the small intestine, bone, adipocytes, muscle, immune system, and cardiac cells [6–8,20,21]. The liver appears to be a key target for ST owing to its central role in regulating metabolism and its secretion of the ST mediators, insulin-like growth factors (IGF) and BPs, in response to ST binding. Thus, for many cell types ST has both direct effects by binding to its target receptor and indirect effects through mediator molecules. The mediator molecules produced by the liver, which are secreted into the circulation in response

to ST, include insulin-like growth factor-I (IGF-I) and insulin-like growth factor II (IGF-II) [22], six IGF-binding proteins (IGFBPs) [23], and serpins (serine protease inhibitors) [24]. In addition to endocrine regulation of ST mediators, there is paracrine regulation, for many cell types secrete their own IGFBPs and proteases that inactivate IGFBPs or enhance IGF-I action by preventing binding of IGF by its BPs [25]. Because of the complex nature of the systemic ST cascade, it is more useful to discuss these in relation to a specific metabolic process, such as growth, lactation, or metabolic homeostasis. This is even more apparent when considering the effect of physiological state on the ST axis.

III. CELLULAR SOMATOTROPIN CASCADE

The receptors for ST and PRL belong to the PRL-ST-cytokine receptor superfamily and are considered single-pass receptors, meaning they are not reutilized [26,27]. Several factors are known to influence the expression of the ST receptor, such as puberty, gender, and energy state [13,28,29]. Expression of receptor is minimal in the fetus and the neonate, but increases dramatically postnatally to reach maximal levels during the peripubertal period in liver, and to plateau thereafter. The peripubertal period also corresponds to the period of maximal growth in mammals. In addition to ontogenetic regulation, there is substantial evidence of nutritional regulation at all stages of life [10,30,31]. Baumbach and Bingham [10] have hypothesized that regulation of the ST receptor and BP is "hierarchical," with major regulators, such as nutrition, affecting overall transcriptional activity, whereas secondary regulators, such as ST pulsatility, affect the transcriptional or splicing activity among alternative 5' exons at the locus. The ST receptor gene has been assigned to chromosome 20 in cattle [32] and to chromosome 16 in swine [33]. Regulation of this gene is still poorly understood, although the promoter region has recently been sequenced [34].

The metabolic effects of ST on a single cell begin with the binding and dimerization of a single molecule of ST to two receptors. Each receptor molecule is a membrane-spanning protein composed of approximately 620 amino acids, with a single-binding site or region [35]. The ST molecule, on the other hand, has two separate-binding sites or regions. The initial event in the ST-receptor interaction always involves the binding of the extracellular domain of the ST receptor to site 1 of ST [36–38] (Fig. 1). This reaction involves primarily 11 hydrophobic contact residues on the BP and 8 hydrophobic residues on ST and accounts for approximately 85% of the binding free energy [36].

According to Clackson and Wells [36] the 1–1 hormone-receptor complex "hot spot" is an interaction between complementary functional epitopes that form a compact hydrophobic core, surrounded by five intermolecular salt bridges and hydrogen bonds. This 1–1 hormone-receptor complex then binds to another ST receptor at site 2 of the ST molecule and the same region of the extracellular domain that bound site 1 of ST, which requires lateral movement of the receptor or 1–1 hormone-receptor complex through the plasma membrane [38–41]. The lateral movement of the receptor also brings the transmembrane, box 1 and box 2 regions of the receptor (see Fig. 1) into contact with regions of the receptor that interact with Jak-2 and MAP kinases [42].

Binding of the second receptor to site 2 of ST then initiates activation of these kinases and subsequent phosphorylation of the receptor, tyrosine and MAP kinases [27]. Extensive studies have shown that the location of the amino acid residues involved in activation of these kinases are the transmembrane region and box 1 and 2 of the cytoplasmic domain adjacent to the transmembrane region [27,43–45] (see Fig. 1). Specific cellular events that follow phosphorylation of these kinases are primarily translational and include increases in protein and lipid synthesis [27].

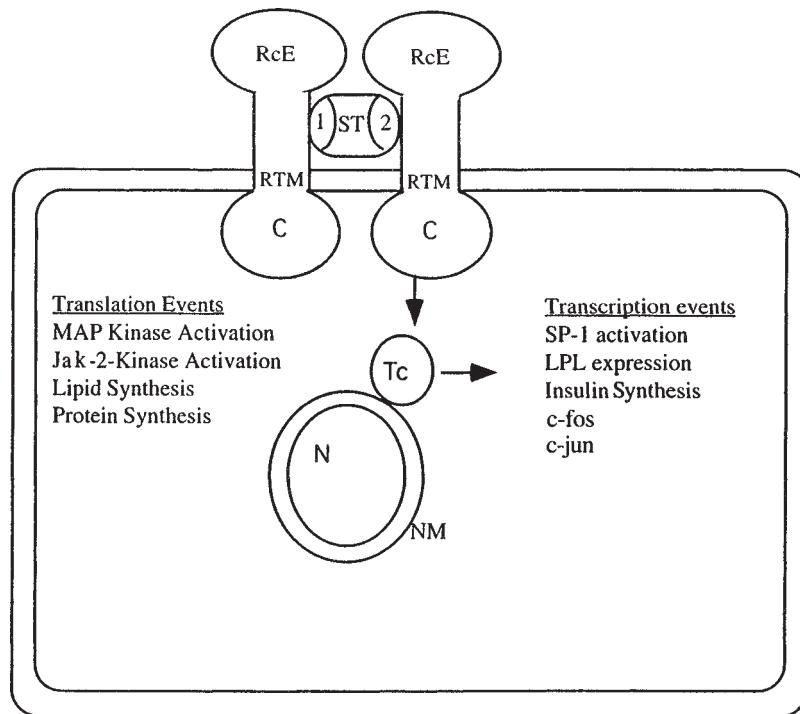


Fig. 1 Translation and transcription events initiated by formation of the somatotropin receptor-hormone complex. ST, somatotropin; RcE, receptor extracellular domain; ST₁, binding site 1; ST₂, binding site 2; RTM, receptor transmembrane region; C, cytoplasmic domain; Tc, translocated cytoplasmic domain; N, nucleus; NM, nuclear membrane. (Adapted from Refs. 35, 37–39, 41, 44.)

Subsequently, activation of the cytoplasmic domain of the receptor, and its internalization and translocation to the nucleus, are associated with an increase in transcriptional events [26,46]. These include activation of *c-fos*, *c-jun*, and STAT-factors [47]. Studies by Lobie et al. [39] have shown that prevention of internalization of the receptor does not abolish the increase in transcription, indicating that nuclear translocation is not required; however, translocation and nuclear binding likely have a function that is as yet undocumented.

Obviously, the type of responses initiated by the cellular ST cascade are directly related to the cell type involved. As with the systemic ST cascade, the cellular ST cascade is best discussed in context of a specific physiological process.

IV. ROLE OF SOMATOTROPIN IN LACTATION

Although PRL is required for the initiation of milk synthesis and secretion at parturition in all mammals examined in a subset of these species, it is ST that is required for maintenance of established lactation. This is well documented in ruminants, for which the PRL requirement in established lactation is minimal, and the ST requirement is nearly essential [48]. Bovine placental lactogen (bPL), which binds to both ST and PRL receptors, is a weak agonist of ST in enhancing established lactation in cattle, but it is much more potent as a mammary growth hormone than either ST or PRL [49,50]. The low galactopoietic potency of bPL is probably because it forms only a 1–1 hormone-receptor complex with the ST receptor, rather than a 1–2 complex [51]. Bovine PL also reduces ST secretion from the pituitary, likely by negative

feedback, and may ultimately reduce milk yield in concert with the steroids of pregnancy [52]. The enhancement of established lactation by ST in ruminants appears to be partly mediated by the somatomedin family in these species [53,54]. The members of this cascade include IGF-I and IGF-II, six different circulating IGFBPs, and the cellular IGF receptors. In addition, there are specific proteases that can modify the activity of the BPs and the IGF molecules. The complexity of this system has prevented its complete elucidation, but a few facts are known.

There are abundant type I and type II somatomedin receptors in mammary tissue [25]. In contrast, only a low level of expression of ST receptor message could be detected in bovine mammary tissue [55] and close-arterial infusion of the mammary gland with ST has not increased milk yield [56]. Close-arterial infusion of either IGF-I or IGF-II stimulates milk production, but to a smaller degree than ST [57,58]. IGF-I dramatically increases mammary blood flow, and this effect appears to be mediated by local production of nitric oxide [59,60]. Also, IGF-I in cell culture systems increases casein synthesis [25]. However, the specific roles of the IGFBPs and their proteases in regulating mammary gland synthetic activity remains to be established. Thus, considerable work remains in establishing the mechanism of action of ST in increasing milk synthesis and secretion. This includes determining if part of the mechanism of action of ST in lactating ruminants is either to bring resting cells back into production or to prevent apoptosis of cells already secreting.

Increasing mammary milk output can be sustained only by an increase in nutrient supply at the mammary gland. The diversion of cardiac output to the mammary gland induced by elevated IGF-I ensures delivery of substrate to the secretory cells. However, in addition, there must be increased mobilization of nutrients from body stores or increased flow of nutrients from the digestive tract [61,62].

In the initial response to ST, there is no change in feed intake necessitating an increase in body mobilization of milk precursors from body stores, which are subsequently replaced as feed intake rises to match nutrient outflow [63].

V. ROLE OF SOMATOTROPIN IN GROWTH

Issues relative to the influence of STs on growth and development of domestic animals varies with the species involved. As in lactation, the process of growth requires elaborate and exquisite coordination of all physiological and endocrine systems within the animal. The systemic and cellular ST cascades play a major role in accelerating growth to maturity while maintaining metabolic homeostasis. This permits the growth process to achieve priority over other physiological processes while preventing metabolic disorders. A major component of this coordination is the metabolic adaptations that occur in growing animals. These include enhanced ST secretion from the pituitary, increased expression of the hepatic ST receptor and, therefore, increased IGF-I and IGFBP production by the liver in response to ST. Also, fat accretion is reduced and muscle accretion increased as STs partition energy and protein into growth and away from adipose stores, with enhanced output of adrenal and gonadal steroids at puberty, which drive secondary sexual characteristics, and increased appetite to provide the nutrients to drive accelerated growth.

A. Ruminant Species

Somatotropins improve average daily gain and feed conversion, increase lean tissue deposition and nitrogen retention, while decreasing plasma urea nitrogen and urinary nitrogen loss in growing ruminants. Initially, growth responses to ST in ruminants was variable and difficult to repeat [13]. This was partly due to underestimation of the net carbohydrate and protein

Table 1 Effect of Somatotropins on Growth in Cattle

Sex	Gain	Intake (%)	Feed/Gain (%)	Dose -(mg/d)	Ref.
Bulls	+12	-10	-18	36	76
Steers	—	—	-13	36	
Steers	+15	—	NR	25	70
Steers	+3	-4	-7	11.4	71
Steers	+5	-5	-9	22.8	
Heifers	+19	—	-13	NR	72
Bulls	—	NR	NR	36	
Steers	—	—	—	13	73
Steers	+29	NR	NR	18	74
Heifers	8	—	11	11.4	75
Heifers	11	—	9	22.8	
Average		+12.8	-2.1	-11.4	

requirements of growing ruminants and lack of knowledge for how to calculate the increase in these requirements with increased growth rate [64]. In addition, there is a period in the young ruminant that is associated with poor growth responses to ST owing to a low ST receptor population in the liver [28]. Recent studies have demonstrated good growth and carcass composition responses in beef cattle supplemented with exogenous bST [65–70].

Typical responses to ST in cattle are shown in Table 1. The growth response to ST in cattle is additive to the growth response caused by anabolic steroids [65–67]. Hancock and Preston [65] have titrated the dose of ST required for maximal anabolic response in feedlot steers and have reported it to be between 41 and 64 µg/kg per day. Preston et al. [71] reported that the growth and carcass composition response to ST and anabolic steroids were additive and possibly independent.

Beef cattle are not handled by producers on a daily basis as are dairy cattle. Any attempt to use ST in the feedlot industry will require a long-term implant that is presently not commercially available.

B. Poultry

The role of ST in regulating growth in poultry has been difficult to elucidate owing to conflicting results and experimental paradigms. In general, mammalian STs have not increased growth rate or feed efficiency in intact poultry, but will increase both in hypophysectomized birds [77,78]. There are conflicting reports on the effects of poultry-derived STs on growth rate of intact domestic fowl [79,80]; however, a somewhat consistent trend relative to pattern of delivery is emerging.

Pituitary ST mRNA levels in chickens progressively increase from 18 days of embryonic development to a maximum at 4 weeks of life (posthatch); thereafter, they decline steadily [81]. Plasma ST concentrations in growing chickens parallel the same pattern [81]. Likewise, hepatic ST receptor levels and mRNA for IGF-I follow an identical pattern, peaking at 4 weeks of age [82,83].

Vanderpooten et al. [83] evaluated hepatic ST receptor levels in broiler chicks selected for improved feed conversion efficiency versus improved growth rate. At 4 weeks of age, there was a two- to threefold higher percentage of hepatic receptor binding in the fast-growth line compared with the improved feed conversion line.

Similar to mammalian species, the pattern of ST treatment is critical to the biological

responses evoked. Abdominal fat pad size was decreased by pulsatile, but not by continuous, exogenous ST treatment in broiler pullets [84]. In the same study, pulsatile, but not continuous, ST increased plasma triiodothyronine and ST, while decreasing plasma concentrations of thyroxine, corticosterone, and triglycerides. In another study, continuous intravenous infusion of chicken ST (cST) increased body fat content of young broiler chicks (4–7 weeks of age) [85]. Likewise, exogenous cST treatment stimulates hepatic IGF-I transcription and translation only when the pattern of infusion is pulsatile [86]. In two studies by Vasilatos-Younken et al. [80], it was demonstrated that pulsatile (16 pulses a day), but not continuous, cST increased feed efficiency, longitudinal body growth and body mass, and reduced abdominal fat pad size in broiler-strain pullets. The number and amplitude of pulses may also be critical. Burke et al. [78] could not demonstrate growth responses in broiler chickens using thrice daily injections. Pulsatile infusion of growth hormone-releasing factor (GRF) (8 pulses per day) depressed growth of young broiler chicks (4–7 weeks of age) [87].

Bacon et al. [88] demonstrated that pulsatile (12 pulses per day) infusion of turkey ST (tST) did not affect growth, feed conversion, final body weight, or muscle mass. However, the abdominal fat pad was decreased by 70% in 8-week-old female turkeys. Finally, twice-daily injections of cST failed to elicit body weight gain or growth in random-bred meat-type chickens [89].

C. Swine

The ontogeny of the systemic ST cascade in swine is similar to other mammalian domestic animals [90]. However, use of ST to increase growth rate and improve carcass composition in swine also leads to development of esophageal and gastric ulcers in some treated animals [91].

Growth responses to porcine ST (pST) in swine were first recorded [92] using crude pituitary extracts. Today, there is abundant literature on growth responses and carcass composition of recombinant, highly purified pST-treated swine [90]. All breeds respond to some degree to exogenous pST administration with the Chinese slow-growing and obese Meishan pigs demonstrating some of the most impressive growth and fat reduction responses [90].

As stated by Bonneau [90], “Overall, it can be considered that the relative improvement in performance due to pST treatment is in inverse proportion to the animal’s potential for lean meat growth.” Growth responses are more pronounced with pulsatile than with continuous delivery [13], and pST has more pronounced effects in castrated males than females, whereas females are more responsive than intact males [93]. Thus, demonstrating that pST induces clear growth and feed efficiency responses in swine.

During the finishing phase of swine development, pigs eat in great excess of their nutrient requirements [13]. Treatment with ST during this period is most attractive owing to high feed costs and enhanced opportunity to increase efficiency. In fact, ST treatment greatly reduces feed intake, while stimulating increased growth rate [13], but the two responses have different dose-response curves. The inhibition of feed intake and reduced carcass fat has a linear dose-response, whereas feed efficiency, growth rate, and muscle accretion is maximal at about 60–80 µg/day [90] and declines as doses increase.

This is more easily understood when considering the metabolic effects of exogenous ST. Glucose production and fatty acid release from adipose stores will increase in parallel with the dose of ST [94]. Increases in the circulating concentrations of these nutrients causes feedback on the brain to reduce feed intake. As feed intake is reduced the protein intake required to sustain increased growth rate falls below required amounts and growth rate slows down, even though fat mobilization and glucose production continue to rise. In lactating dairy cows, the mammary gland provides a sink for these nutrients by uptake for increased milk synthesis,

preventing large increases in circulating concentrations of these substrates and, thereby, maintains and eventually increases feed intake. In addition, in growing animals as muscle accretion increases, the protein requirement to sustain growth increases. Thus, the pST-treated pig has an increased dietary protein requirement and a decreased energy requirement. Diets for growing and finishing pigs treated with pST would need to be reformulated to meet the increased amino acid requirements.

D. Aquatic Species

There is growing interest in the possible use of ST to improve productivity of finfish aquaculture. Numerous studies using both recombinant fish and mammalian STs have demonstrated that growth rate can be increased in a wide variety of species [95]. Generally, increased growth rate stimulated by exogenous ST is associated with an improvement in feed conversion efficiency. Feed efficiency for rainbow trout and coho salmon was increased by 10–20% in several different studies [96–98]. However, reports of effects on feed intake have been variable. Agellon et al. [99] reported that trout treated with salmon ST consumed more feed than control fish, whereas Market et al. [100] observed no treatment effect in yearling coho salmon treated with bST. By contrast, Garber et al. [98] measured a decrease in average daily feed intake in rainbow trout treated with a prolonged-release formulation of bST. Increased growth rate stimulated by ST usually increases both body length and body weight, and an analysis of the carcass indicates that nonvisceral tissue growth is supported over organ growth [98–102]. In addition, ST treatment tends to increase the percentage of carcass protein [98,102] and reduce carcass lipid content [95,98].

However, the classic nutrient repartitioning and growth effects of exogenous ST are not always observed. One of the most striking examples was the study of Wilson et al. [103] in which channel catfish were treated for 8 weeks with bST. Although treated fish gained weight at a greater rate, there was very little increase in length, most of the weight gain was due to increased fat deposition. This appeared to be largely due to increased feed consumption. The differential effect of bST in this report may be due to species differences. However, there are many other factors that also influence response to ST, such as source of ST, dose, route of administration, maturity of the fish, season and photoperiod, water temperature, and composition and availability of the diet.

Intuitively, it would seem that the homologous fish ST should be more active than a heterologous mammalian ST because there is only 20–40% similarity between teleost and mammalian STs [104,105]. However, key constituents governing secondary and tertiary structure have been conserved and as a result mammalian, nonprimate, STs appear to have a biological activity and potency similar to homologous teleost STs [106,107]. Variability in response owing to source of ST, therefore, may not be as important an issue as might first appear.

The route and manner of administration of ST probably account for much of the variability of response seen with studies carried out with fish. Unlike mammals, for which ST must be administered by parenteral injection, oral and immersion routes of delivery are also effective in fish [95]. Potential differences in response to these different routes of delivery have not been investigated. Similarly, it is unknown whether fish respond differently to pulsatile versus continuous delivery, as has been shown for many species [13], or if prolonged administration of a heterologous ST will lead to immune attenuation of response.

Some studies also suggest that responsiveness to exogenous ST may be modulated by water temperature [107]. When food is not limiting, growth rates of salmonids are directly proportional to environmental temperature, up to the thermal optimum for the species [108].

As water temperature increases up to the thermal optimum, it appears that ST-stimulated growth may diminish, or that increasing doses of ST may be required to maintain the same growth advantage. Another related factor that affects growth rate is season (photoperiod). Most species of salmonid display seasonal fluctuations in growth, and there is evidence that endogenous secretion of ST is regulated by photoperiod [109], with reduced circulating levels present during winter months. Thus, dramatic increases in growth rate can be obtained in fish treated during the winter, owing to the relatively slow growth rate in the untreated controls [110].

Fish move through their environment with less expenditure of energy and require less energy than do land animals. However, their requirement for protein as a component of their diet is quite high. The percentage of crude protein in the diet may also modulate effectiveness of ST treatment. Previously, Campbell et al. [111] demonstrated that the dietary protein requirement of finishing hogs is increased by treatment with pST. This increase in protein requirement may be offset by the nitrogen-sparing activity of ST. For instance, ST-treated carp displayed a greater increase in growth rate when fed a low (23%) versus a high (38%) protein diet [112]. Obviously much needs to be learned about factors affecting teleost responsiveness to ST before recombinant ST, or modulators of ST secretion can be practically applied to aquaculture of finfish.

VI. CONCLUSIONS

Recombinant bST is a reality in the dairy industry for increasing milk yield. Studies in growing ruminants indicate it has potential for increasing growth rates in feedlot steers and growing dairy heifers. In addition, several aquaculture species are responsive to the growth-promoting properties of ST. Additional work is needed for use of ST in growing swine and poultry. However, additional members of this family, such as PLs and PRL, also hold promise for increasing productivity of domestic animals, and members of the ST cascade, such as the IGFs, their BPs, and receptors, also have potential uses in animal agriculture.

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Avian Transgenics

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I. AVIAN REPRODUCTION: PROBLEMS, LIMITATIONS, AND OPPORTUNITIES FOR TRANSGENESIS

Transgenic animals are those that have a stable modification to their DNA either because of the insertion of exogenous genes or because of manipulation of endogenous sequences. For these modifications to be inherited by the offspring, they must clearly be introduced at some stage into the germline. In practice, this means that the manipulation must be done (1) into the fertilized egg, (2) into embryonic tissues before the germ cells are segregated during embryogenesis, or (3) into the germ cells themselves. All three of these methods have been attempted in birds, and it will be clear that all of them depend on particular features of the bird's method of reproduction and development.

The system of avian reproduction differs from that of the mammal in several ways:

1. In most birds there is only one functional ovary.
2. Fertilization occurs at the top of the oviduct, in the infundibular region, but the mammalian phenomenon of capacitation of the sperm in the female reproductive tract [1] does not appear to be necessary in birds [2].
3. Polyspermy is common in birds. In the domestic fowl about ten sperm enter the oocyte, lose their cytoplasm, and swell up to about 50 times their volume to form the male pronuclei. There is no way of predicting which of these pronuclei will fuse with the female pronucleus [3].
4. Immediately after fertilization the zygote passes down the magnum region of the oviduct where, in the domestic fowl, about 15–20 g of albumen are secreted around it in about 2½ h. This secretion has a Na/K ratio of about 10 [4].
5. As the egg leaves the magnum and enters the isthmus region of the oviduct it is surrounded by the shell membranes on which the eggshell is subsequently secreted. It then enters the shell gland and remains in this region of the oviduct for about 18 h. During this period, cell division proceeds so that the new-laid egg contains an embryo of about 60,000 cells [5]. Simultaneously, the egg is “plumped” by the secretion of about 15 g of fluid into the

albumen, which decreases its Na content and increases its K so that the Na/K ratio of the albumen is about 2 at the time of lay [6].

These aspects of avian reproduction have influenced the approaches to transgenesis in three ways. First, injection of DNA into the male pronucleus is hampered by a large opaque oocyte in which it is not possible to identify the pronuclei. Furthermore, the presence of a large number of supernumerary male pronuclei reduce the chances that the one that might be microinjected with the vector DNA will actually be involved in the fertilization process. Second, to obtain a fresh zygote, it is necessary to remove the egg from the top of the oviduct, which usually means (1) killing the bird, (2) inventing a surrogate egg culture system [7], or (3) returning the zygote to the top of the oviduct. Third, if the egg is allowed to be laid normally, the embryo will have developed too far for microinjection to do anything but produce a mosaic embryo.

Consequently, several workers have attempted to manipulate the genome of the bird by exploiting the extensive knowledge of the embryology of the fowl. This has been described for several stages (I–XIV) covering the period from cleavage to the primitive streak stage [8], and from thereon for the period up to hatching (stages 1 to 46) [9]. The important features of avian embryology, as far as transgenesis goes, are as follows:

1. At the time of laying (stage X) the embryo is present as a blastodisk. In a series of experimental studies [8a], it was shown that these cells were pluripotent (i.e., capable of developing by different developmental routes). If these routes included the subsequent formation of the primordial germ cells (PGCs), it would then clearly be possible to enter the germline through these pluripotential blastoderm cells.
2. However, other workers [9] have suggested that the formation of the primordial germ cells is already determined by stage X, so that they already exist in the blastoderm at the time of oviposition.
3. Despite this confusion, it is generally agreed that the PGCs are present in the epiblast layer at the center of the blastoderm of the developing embryos and that they migrate from there onto the hypoblast at stage XII of development [10,11].
4. The PGCs are carried anteriorly on the developing hypoblast and come to lie in the germinal crescent in an anterior and lateral position, relative to the embryo's head. Here they accumulate in considerable numbers at this extraembryonic site in the 2-day-old embryo (stages 8–10) [12].
5. The PGCs migrate from the germinal crescent to the germinal ridge of the embryo where they form the definitive gonad. The transfer from the extraembryonic to the intraembryonic site occurs through the bloodstream [13] and about 30–40 PGCs per 10 µL of blood occur at about stage 16 of development when this migration is at its peak [12].

These features of avian embryology differ considerably from those of a mammal, and they are relevant to the production of transgenic animals for the following reasons: First, it is probable that cells can be isolated from the blastoderm which will subsequently form the germline. If these cells are similar to some of the embryonic stem cells of the mouse embryo, it may be possible to culture them *in vitro* and then use them to form germline chimeras by injecting them into recipient embryos. Such a cell line could be used for genetic manipulation and screening before integrating them into recipient blastodisks. Second, because the primordial germ cells of the bird arise extraembryonically and are easily transferred between the bloodstreams of embryos, this provides an alternative cell line for gaining access to the germline.

II. SPECIFIC APPROACHES

A. Manipulation of the Gametes

About 2 h before ovulation, the egg divides to form a secondary oocyte and a polar body. Immediately after this oocyte is ovulated it is available for fertilization, after which the second maturation division occurs to form the female pronucleus [14]. At this time, the entry of the sperm occurs followed by the loss of its cytoplasm and the formation of the male pronucleus. Nuclear fusion occurs shortly afterward.

Clearly one direct way of introducing foreign DNA into the zygote would be to use the gametes as vehicles. Toward this end Shuman [15] injected replication-competent reticuloendotheliosis virus (REV) directly into the developing follicle and recovered it in 26% of the embryos derived from these birds. She was also able to obtain 8% transfection with a replication-deficient retrovirus. Subsequently, Kopchick et al. [16] injected 1–25 µg of a plasmid containing the gene for bovine growth hormone (bGH) into follicles of the fowl ovary. Of the 400 offspring that were eventually produced, none possessed the foreign DNA, and it appeared to have been rapidly degraded in the follicle.

An alternative approach advocated by Lavitrano et al. [17] was to coat the sperm surface with plasmid DNA, on the basis that this would then be carried into the egg to produce a transgenic embryo. It was claimed that between 30 and 60% of offspring from DNA-coated rooster sperm contained the foreign DNA and that it was transmitted to the F₁ generation [18], but doubt has been cast on this approach [19] and attempts to improve the technique by using liposomes to introduce the DNA into the sperm [20] have similarly failed to produce convincing evidence of transgenesis [21].

Therefore, it now appears that it is possible to infect the gonads with retroviruses, but it has not been possible to introduce plasmid DNA into the avian genome by means of the gametes.

B. Microinjection into the Fertilized Egg

In an extensive series of experiments, several groups [22–24] have injected cloned DNA into the cytoplasm of the fertilized ovum close to the site of the female pronucleus (25 µm below the plasma membrane); these eggs were subsequently cultured in vitro. As might be expected, there was a progressive dilution and loss of the DNA from the developing embryos, although some of it formed large concatemeric molecules and replicated roughly 20 times during the first day. Tissue expression of the reporter gene was found in 40% of the embryos by day 4 [24], but only 8% of the surviving embryos were positive by day 7 [23]. However, there were groups of cells that continued to express the foreign DNA, suggesting the possibility that the plasmid had integrated into the chromosomes to give mosaic embryos. Two linearized reporter gene constructs (pRSVneo LTR and pDiMZlys 1) were used to pursue this possibility. A total of 1238 zygotes were microinjected with these constructs and seven healthy chicks were raised to sexual maturity. One was a cock with cells that were roughly 10% transgenic. The plasmid had clearly integrated during development to produce a mosaic bird. Fortunately, some of the germ cells were also mosaic so that of the 412 chicks bred from this cockerel 14 (3.4%) were transgenic and showed normal Mendelian transmission of this plasmid DNA [25].

The success of this approach has produced considerable interest, although similar results have been achieved by cytoplasmic microinjection into mice [26], *Xenopus* [27], and zebra fish eggs [28]. The main constraints on its application as a routine approach for the bird are (1) the need to kill the bird to obtain a freshly fertilized ovum and (2) the complexity of the culture system needed to incubate the ensuing zygote. This uses a three-stage technique [29]

that appears to be an empirical solution to the absence of the electrolyte changes that occur in the albumen during normal egg formation [4]. This culture system approach is continually being improved [24], but represents a major investment in effort.

C. Mosaic Blastoderms

So far the most successful way of producing transgenic birds is by infecting the blastoderm with either competent or replication-defective retroviruses. In most attempts the hatchling that is produced from these manipulations is a mosaic bird (i.e., it is composed of two genetically different populations of cells). One of these represents the original genome of the zygote, whereas the other consists of those cells that have been genetically modified by the vector. Frequently, the two genomes will be represented in the same tissue so that, for example, some of the spermatocytes will produce spermatozoa from the original cell line, but others will contain vector sequences. The first transgenic birds that used this approach were produced almost 10 years ago by injecting avian leukosis virus (ALV) into the blastoderm of new-laid White Leghorn fowl. Twenty-one cocks were raised to sexual maturity from these eggs, and the proviral inserts were stably inherited for two generations [30,31]. Most of the birds showed the complete ALV construct, but two were incomplete: one coded only for the envelope protein, whereas the other included a group-specific antigen that induced resistance to the subgroup A virus [32]. The complete retroviruses were able to replicate and, thus, transfected other cells, but the two inserts that were incomplete (*alv6* and *alv13*) were incapacitated and thus could be transmitted only as part of the bird's genome. Processes of this type presumably occur naturally and account for the endogenous viral regions that are found in the normal genome.

As an extension of this experimental approach [33] a replication-competent strain of the Rous sarcoma virus (RSV) was used that contained the gene for bovine growth hormone. Viremic chicks were produced by injecting 10^5 viroids into the blastoderm. Roughly 50% of the hens produced in this way laid infected eggs [16], and between zero and 4% of the first generation cocks transmitted these viral sequences to their offspring. Because male birds do not tend to transmit free viroids in their sperm, this was taken as showing that the foreign DNA had integrated at a variety of different sites. With a second construct, the bGH gene was linked to a metallothionein promoter. Infected blastoderms produced hatchlings that were shown by Southern blot analyses to contain the construct. However, there was a poor correlation between the presence of the construct and serum bGH, and no clear increase in growth rate. It appears that many of the cells that were secreting hormone were in a variety of somatic tissues, and the hormone, which is normally secreted in a pulsatile way, was ineffective in producing a normal physiological response.

Replication-defective retroviruses are so-named because they have been constructed to be deficient in those regions of their genome that make them capable of completing more than one round of their life cycle. Therefore, they are much safer to use as long as they do not revert to replication-competent forms by interacting with infectious forms. A replication-defective spleen necrosis virus (SNV) was used [34,35] to introduce foreign genes into fowl blastoderms. Out of 760 potentially mosaic offspring, these investigators obtained 23% showing evidence of vector-derived DNA. Four cocks, containing these vectors in their sperm DNA, were used to produce transgenic offspring showing 34 different sites of integration. In a subsequent experiment, the chick growth hormone (cGH) gene was inserted into the retroviral sequences and used to transfet blastodermal tissue. None of these embryos survived to hatch, but those that were sampled at 15-days incubation showed growth hormone levels that were up to ten times higher than normal [36].

D. Chimeric Blastoderms

Chimeric are produced by mixing cells from two different organisms, usually at an animal's early stage of development. The cells tend to mix freely and the technique has made important contributions to an understanding of the fate of particular cell lineages in the processes of embryologic development [37]. The cells that are mixed can be from different individuals of the same species, or even from different species, as in the quail-chick chimera.

The original zygote is totipotent in that it is capable of producing all the cells of the offspring. As development progresses this ability is constrained, and particular cell lineages become pluripotent (i.e., capable of developing into a certain number of tissues, but not all types). The production of chimeric embryos clearly necessitates mixing cell types at the appropriate developmental stages for them to integrate into particular organs. Therefore, there was considerable excitement when Evans and Kaufman [38] devised a way of obtaining and maintaining pluripotent cells from the mouse embryo. The technique involved inducing delayed implantation in these embryos so that they showed retarded development. Permanent cell lines of these so-called embryonic stem (ES) cells could then be maintained in culture before introducing them into recipient embryos to form chimeras. The opportunity to insert vector DNA into these cell lines, to select and maintain them in culture, and then to gain access to the mouse germline by using them to form chimeras was subsequently exploited [39]. The method clearly contains all the components necessary for a successful exploitation of the potential for transgenesis.

The application of this technology to birds clearly depends on two features: First is to show that cells from a pluripotent embryonic stage can be used to form a germline chimera; second, is isolation of these cells to demonstrate that they can be maintained in culture as embryonic stem cells.

In her detailed studies of the early stages of chick embryology Eyal-Giladi [8] concluded that the stage X blastodermal cells of the new-laid egg were pluripotent. On the basis of this Petitte et al. [40] injected the blastoderms of 53 Dwarf Leghorn eggs with blastodermal cells from Barred Plymouth Rock birds. Six of the hatchlings were chimeric for feather color and one male survived for breeding. It produced 719 chicks, of which 2, as shown by DNA fingerprinting were descended from Barred Plymouth Rock cells. This possibility of being able to populate the germline with blastodermal cells was extended by Naito et al. [24], who made chimeric embryos by transferring quail cells into the blastoderm of the fowl. A total of seven chimeric chicks were produced and further work showed that, by injecting about 10^3 quail cells into the center of a stage XI to XIII fowl blastoderm, it was possible to obtain identifiable quail cells in the gonads of fowl embryos in about 60% of cases.

These experiments demonstrate that precursors of germline cells exist in the early embryos, and that they can be transferred into recipient embryos. The efficiency of this process can be greatly facilitated by irradiating the recipient blastoderm. This apparently delays cell division and enhances the establishment of the transferred cells [41]. What remains to be established is whether the avian blastoderm contains embryonic stem cells (i.e., pluripotent cells that can be maintained in that state in culture). If such a population does exist, or if it can be artificially induced, then it should be easy to establish a whole transgenic technology for the bird. However, embryonic stem cells have not yet been produced in the bird.

E. Germline Chimeras

The cell line that will form the gametes of the bird consists of the primordial germ cells. In the early embryonic stages it is difficult to know how to characterize or define this population, but several groups have described these cells on morphological criteria in the stage X blastoderm

of eggs at the time of lay [9,42]. If this is true, then some of the results attributed to pluripotent blastoderm cells may be due to early primordial germ cells (PGCs).

As development continues, the primordial germ cell population becomes more clearly demarcated both histologically and anatomically. By stages 2–5 there are between 50 and 100 PGCs in the germinal crescent region of the embryo. They are large, roundish cells 14–18 µm in diameter, with a conspicuous spherical nucleus 8–12 µm across, and containing a granular fragmented nucleolus [43]. By stages 10–15 their numbers have increased to between 200 and 400, and they start to migrate through the bloodstream as a pulse of cells that settle in the germinal epithelium of the definitive gonad [12].

Because of this migration, it is relatively easy to isolate PGCs from the blood and transfer them to the vascular system of a recipient embryo, thereby creating a germline chimera [44]. This provides the opportunity for an alternative way to produce transgenic birds, as has been demonstrated [45]. In that experiment, PGCs were isolated from the germinal crescent of a White Leghorn embryo, transfected with a defective retrovirus containing the *lacZ* gene (NLB) and injected into the blood of a Rhode Island Red embryo. On hatching, the bird was raised to sexual maturity and was a germline chimera in that its sperm were mixtures of Rhode Island Red and White Leghorn cells. In the next generation, a cockerel, derived from the White Leghorn cells that were present in the sperm, contained the vector DNA in its blood cells (i.e., was transgenic).

The efficiency of producing germline chimeras by this route can be increased by partially sterilizing the recipient embryo so that the number of endogenous PGCs that are produced is reduced. This is most easily achieved by using the drug busulfan [46], introduced as an intravitelline injection to minimize teratological side effects. This drug is cytotoxic to migrating PGCs and when injected into embryos after a 50-h incubation, it induces sterility so that a subsequent injection of donor PGCs may repopulate the gonad. If used in this way, it is possible to obtain germline chimeras that produce almost equal numbers of offspring from each of the different cell lines of the PGCs [47]. By inserting foreign DNA into the donor PGCs, it can be shown that their survival is not affected by the earlier treatment with busulfan; since these cells are able to populate the partially sterilized gonad [48].

It will be apparent from this description that there is the basis for the production of transgenic birds by techniques that use the PGC system. Unfortunately, there is still one difficulty with this technique. Primordial germ cells are very difficult to maintain in cell culture, and this limits two aspects of the approach: First, if PGCs cannot be cultured *in vitro* they cannot be screened after exposure to vector DNA to select modified cells; seconds, because PGCs do not grow *in vitro* it is impossible to develop and keep stocks of these cells (i.e., they have to be continually produced from donor embryos).

F. Germline Mosaics

An entirely different approach to this problem was subsequently adopted [49]. There is now an extensive literature on the technique of introducing foreign genes into the tissues of animals and plants by using DNA-coated microprojectiles [50]. By firing 1 to 2 µm gold or tungsten particles at high velocity into cells, it is possible to carry the vector DNA into the nucleus of a wide variety of organisms [51] in which it can integrate to give a genetically modified cell. The technique has been used mainly to produce manipulated somatic cells, and it is the basis of the so-called gene therapy approach to metabolic diseases. It will be apparent, however, that the avian embryo provides a unique opportunity to apply this technology to germline studies. In the approach adopted by Li et al. [49], gold and tungsten microprojectiles were coated with vector DNA and fired at the germinal crescent of the 2-day-old embryo *in ovo*. A

small hole was made in the shell so that the germinal crescent was exposed, but the embryo was protected beneath the shell. Because the primordial germ cells are extraembryonic at this stage they can be bombarded with microprojectiles without any fear of damaging the embryo itself. The method is extremely simple, hence, embryos can be treated in less than 1 min before sealing the egg and returning it to the incubator. Embryos treated in this way continued to develop such that the PGCs migrated to the gonad and completed normal development. Hatchlings were raised to sexual maturity, and the sperm of cockerels was studied by Southern blot analyses for the vector DNA. This was present, suggesting that the bird was a germline mosaic particularly because the vector DNA was passed on to the next generation, in which it was identified in the blood cell DNA of the chick. However, it subsequently became apparent that the DNA had not integrated and, over the next 6 months, the signal decayed. This is an unusual result in that it implies the transmission of plasmid DNA in a nonintegrated way through specialized cells (such as the sperm). Other examples, however, are known for fish [52] and mice [53]. The experiment, which has been performed on only a few animals is very promising because it implies that the microprojectile approach could be an extremely simple way of introducing vector DNA into the germ cells. Getting the DNA to integrate should be no more difficult than with the microinjection technique [25] whereas this approach avoids all the problems of embryo culture.

III. FUTURE PROSPECTS: THE AVIAN WISH-LIST

The success of mammalian transgenesis has tended to obscure the obvious point that introducing foreign DNA into the germline of any particular species depends on a blend of relevant aspects of cell biology and embryology. Thus, those characteristics of avian reproduction that make the bird so commercially attractive (regular oviparity based on a large protein-rich egg) are the very features that make mammalian approaches to the problem difficult to apply. This is the essence of the problem of using pronuclear injection, which is a favored approach for mice, but which is an extremely difficult technique to use with large vitellogenic oocytes. The success [25] in obtaining integration from cytoplasmic DNA during the embryogenesis of the chick is encouraging, but it emphasizes the difficulty of applying this mammalian approach, especially when it is linked to the necessity for embryo culture.

The other mammalian approach to transgenesis that is highly attractive is the culturing of *embryonic stem cells*. These cells are defined by operational criteria (i.e., they are pluripotent cells that can be obtained from embryos and maintained in culture without differentiation, but they retain the ability to integrate into the germline when returned to an embryo). Whether such cells occur normally is unclear, but they can be encouraged to developed these properties by culturing them with differentiation-inhibiting factor (leukemia-inhibiting factor; LIF) or a variety of other growth factors [54]. Because the avian blastoderm is described as containing pluripotential cells, there has been a search for stem cells among these preparations. Thus, high on the avian wish-list for those who use this approach is the desire to isolate an avian stem cell [24,40]. The situation is complicated because mammalian LIF does not appear to influence avian cells, and the equivalent molecule does not appear to be present in birds, although there may be other growth factors with similar properties.

The whole question of the control of the differentiation of embryonic cells has recently been overturned by studies on primordial germ cells. These are extremely difficult to culture, but the mammalian forms can be induced to divide by using a cocktail of growth factors [55] including LIF, stem cell factor (SCF), and basic fibroblast growth factor (bFGF) [56]. Another candidate for the avian wish-list, therefore, would be to repeat these experiments with PGCs from the fowl. Several attempts have been made to culture these cells from various sources [57], but

without clear evidence of success. The phenomenon becomes more pressing with the discovery that the murine PGC can be induced to regress to form a stem cell [58]. This means that, under the influence of a particular mixture of growth factors, PGCs revert to stem cells (ES) that can be maintained in culture and, subsequently, reintroduced into embryos to form germline chimeras [59]. This ability to progress and regress the pluripotency of cells capable of populating the germline means that an understanding of the relation between growth factors and differentiation must also be added to any wish-list for workers on avian transgenesis. It also implies that the hunt for specific cell lines that will subsequently populate the germinal ridge of early embryos may be a technique-driven problem. The claims that the avian blastoderm contains cells that can populate recipient blastoderms and form germline chimeras [40], and the suggestion that presumptive PGCs exist in the blastoderm [59] or pregastrula stage [42], seem to suggest that the same cells are being identified by different approaches. Finally, it is clearly apparent that more attention needs to be given to the relation between the state of the DNA in various cell lines and the ability to integrate various vectors. Germline cells in a variety of organisms appear at various stages of embryology to be protected from cell-signaling systems directed at differentiation. This may be partly related to DNA methylation [60], and it would be interesting to have this studied that one can relate it to the factors influencing DNA integration in transgenics.

IV. CONCLUSIONS

An outline summary of the various approaches to avian transgenesis, indicating the types of vector, cell system and degree of success is given in Table 1. It is clear that oocyte microinjection, blastoderm transfection, and primordial germ cell manipulation, have all been successful in producing transgenic birds with germline transmission. However, there has never been a system produced that is free from technical problems. Thus, the production of transgenic poultry is possible, but not in a routine way. No doubt the next few years will show considerable advantages in perfecting these different approaches and, at that stage, the crucial question will be the use to which these methods are put. Potential applications include the following:

1. Manipulations of the bird's normal physiology: This could be directed at beneficial (health) effects, such as disease resistance, or at commercial production (egg, meat).

Table 1 Summary of Main Systems Used to Produce Avian Transgenics.

Stage	Vector type	Construct	Embryo expected	Result	Efficiency	Ref.
Gametes—male	Plasmid	pCK17	Transgenic	—	0	21
Gametes—female	Plasmid	pBGH	Transgenic	—	0	16
Retrovirus	REV	Transgenic	+	8%	15	
Zygote	Plasmid	pDiMZlys	Mosaic	+	3%	25
Blastoderm	Plasmid	pRSVneo	Chimera	—	0	64
	Retrovirus	ALV	Mosaic	+	4%	30,31
	Defective, retrovirus	SNV	Mosaic	+	23%	34,35
Primordial germ cell	Plasmid	pSV2neo+	Mosaic	+	20% (not integ.)	49
	Defective, retrovirus	NLB	Chimera	+	1–2%	45

2. Modifications to biosynthetic processes: Because of the relatively simple composition of some of the yolk (e.g., vitellin) or albumen (e.g., ovalbumin) proteins these could be modified to substitute pharmaceutical products (e.g., clotting factors) in the egg.
3. Novel changes to the cell biology of the genome: The production of immortalized cell lines could reduce the use of birds and replace them with in vitro systems for activities such as drug testing or vaccine production.
4. Manipulation of sex control: The prospect of being able to control the sex of offspring would be of enormous benefit to all branches of agriculture. The WZ sex chromosome system of birds has been compared with that of reptiles for which environmental sex control is common. The possibility that these control systems could be introduced into the bird is only one of various approaches that might be used in future molecular approaches [61].

To achieve these ends, it will be necessary to design suitable vectors. Retroviruses are widely considered with suspicion by the public, either for real or illusionary reasons. Plasmids are rather more difficult to insert, but they have the advantages of greater acceptability, larger size, and easier preparation. The number of avian genes that have now been cloned is relatively small, but the fowl genome is currently being mapped [63]. Attempts have been made to design vectors suitable for homologous recombination and gene targeting [62], but the number of insertions is generally very few, except for particular cell lines [63]. Again, the directions in which future developments will occur are apparent, but the amount of basic work that is being undertaken to achieve these ends is still very small when compared with mammalian species.

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Biotechnology and Veterinary Diagnosis: An Overview

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I. INTRODUCTION

In late September 1994, an outbreak of a severe respiratory disease occurred in a horse farm in Brisbane, Queensland, Australia, causing the death of 14 horses (out of 21 sick) and of a 49-year-old trainer. The cause of this new-emerging, fatal disease was unknown. Within less than 2 weeks, the agent was identified as a morbillivirus, closely related to dog distemper virus. A serosurveillance of horses and humans showed no more carriers, a vaccine and a diagnostic method were developed.

This successful operation, carried by the CSIRO, in the Australian Animal Health Laboratory, led by Dr. Keith Murray and her team, would not have succeeded without the skillful application of modern diagnostic technologies [1]. A full "arsenal" of serological, biochemical, and biotechnological diagnostic tools is essential to respond to the diversified needs of reliable and cost-effective methods to prevent, control, and eradicate enzootic and epizootic diseases and to give a rapid and accurate animal-side tools for the clinician.

II. BIOTECHNOLOGY IN DIAGNOSIS

A. Serological Methods

1. First Generation

Immunological techniques that detect the presence of disease agents have been in practice for many years in the diagnosis of human and animal diseases. The visualization of antigen-antibody complexes, using the reaction of complement and erythrocytes, such as the complement fixation test (CFT), indirect hemagglutination (IHA), hemagglutination inhibition (HI), and many similar versions, detects antibodies or antigens. Other methods, based on the neutralization of viruses by antibodies, are also used for quantitative measurement of both antibodies and viruses in the blood [2].

The low cost of reagents and the well-established procedures are the main reasons for the wide acceptability of these techniques. Moreover, automation realized by computed "robot" machines, permits a cost-effective screening of thousands of blood samples of cattle and sheep in control or eradication programs. It seems that the "traditional" serological methods will stay as important diagnostic tools in the veterinary laboratory practice in the future.

The direct labeling of antigens with fluorescent antibodies provided more accurate, but rather tedious and time-consuming practice. Similar but more quantitative direct antigen-antibody tests, such as the radial immunodiffusion (Coombs test), in which the radius of precipitation of the complex is directly proportional to the antibody concentration, are also used in laboratory diagnosis [2]. Nevertheless, some major disadvantages jeopardize the acceptability of these methods. First, the endpoint is subjectively determined. Secondly, discrepancies in the results are caused by differences in reagents, laboratory techniques, and sampling conditions. Third, most of these methods are time-consuming and difficult to implement in current veterinary practice, calling for more accurate and rapid diagnosis, and last, these methods do not differentiate between recent and latent infections and are prone to nonspecific cross-reactions.

2. Second Generation

The second generation of serological methods is based on the labeling of antibodies and antigens with radioactive atoms in the radioimmunoassay (RIA), or with enzymes in the enzyme immunoassay (EIA), which permit an objective and accurate reading of the results. The RIA, although most specific and accurate, shares the inconvenience of dealing with radioactive reagents.

In the enzyme-linked immunosorbant assay, (ELISA), antibodies are labeled with an enzyme by conjugation or by an avidin-biotin bond. The antigen-antibody reaction is visualized by the presence of a substrate that changes its color as the result of the enzymatic activity. Solid-phase ELISA is a rapid semi-quantitative method, in which reaction takes place on a plastic or nitrocellulose "dipstick, and a color dot that is formed demonstrates the presence of the antigen-antibody complex [3].

For the specific identification of many pathogenic agents, methods such as the rapid agglutination, competitive ELISA, and other enzyme immunoassays were developed [3]. Nevertheless, higher specificity than that of both RIA and EIA, was achieved with the introduction of the monoclonal antibodies (MAb).

B. Monoclonal Antibodies in Laboratory Diagnosis

Developed by Choler and Milstein in 1975 [4], this method is based on the mass production of epitope-specific antibodies that can virtually identify any molecule that can bind to an antibody. The use of MAbs in diagnosis permitted a wide use of RIA and EIA in human and veterinary diagnostic laboratories [5]. The ability to produce an antibody monospecific to a single virus or bacterium led to the development of highly specific diagnostic tools. Various methods to produce monoclonal antibodies in cell culture as well as in laboratory animals are described and documented elsewhere [6]. Efficiency, specificity, and sensitivity are now the "name of the game" in this highly competitive market, when diagnostic kits replace the methods previously used only in specialized laboratories.

C. Recombinant Proteins

The technique of recombinant proteins consists in expression of the selected genes by the protein synthesis apparatus of organisms such as *Escherichia coli* or *Canadian* spp. Once the

genetic code is inserted into the DNA strand, the desired polypeptides accumulate in the organism's cytoplasm, or in the medium [7].

The baculovirus expression system is yet another way to produce antigens for diagnosis and vaccination by DNA recombination technique. The lytic virus, known as *Autographa californica* is the prototype of the family *Baculoviridea*. It is a large, double-stranded DNA enveloped virus, that infects arthropods, preferably the larvae of the butterfly *Spodoptera exigua* [8]. A unique characteristic of this virus is the production of a specific polyhedral protein by the infected cells at the late-lytic phase of the virus life cycle. This protein serves as the major protein of the inclusion body and plays an important role in the natural defense of the virus in the dying larva, but is not essential for the multiplication of the virus in tissue culture cells. In an attempt to use this production mechanism as a "factory" for antigen production, the sequence of the DNA encoding for the antigen is cloned in a vector, usually a plasmid, and installed into the viral gene in a position adjacent to the polyhedrin promoter. The DNA sequence encoding the polyhedral protein is then eliminated, and intensive protein synthesis is expressed by the recombinant gene. The antigen produced accumulates in the culture medium [9].

This method is more suitable than the bacterial recombinant proteins for both diagnostic and immunization purposes. First, in many serological tests, cross-reactions between the serum tested and the antigens of the host cell proteins may cause false-positive readings. Such nonspecific impurities are always mixed with the antigen produced. This phenomenon is less likely to happen using the host cells of the *baculovirus*. Second, this method permits industrial production of both vaccines and immunodiagnostic antigens (the product constitutes up to 65% of the cells proteins), and third, it is possible to induce changes in the DNA code in a way that modifies the antigen according to the specific needs of the diagnostic test. This principle permits differentiation between the serological reactions to pathogens and to vaccines [10].

Another way to produce antigens for both immunization and differential diagnosis of highly virulent pathogenic viruses is the use of the *vaccinia* virus as a platform to introduce pathogenic antigens. The *poxvirus*, first used by Edward Jenner in 1792 to confer immunity against smallpox, was later named the *vaccinia* virus (VV). It is a large, double-stranded DNA virus, that contains a complete transcription system and is an excellent vector for different immunogenic antigens. When the DNA fraction of the necessary antigen is installed into the VV DNA strand, this usually harmless virus will induce high immunological response toward this antigen when introduced into a host animal. The use of this method to produce a vaccine against the rabies virus may serve as an example [11]. A chimeric gene, encoding for the rabies capsid protein G, was planted into a plasmid. This plasmid was incorporated into the VV genome by homologous recombination in tissue culture cells. A recombination with the wildtype, which possesses a thymidine kinase (TK) gene, or alternatively the β -galactosidase (*lac Z*) gene of *E. coli*, provides the marker known as "reporter gene." The change of the color generated by the enzyme identifies the recombinant plaques. In this way live nonpathogenic VV represents the antigen of rabies both for vaccination and for diagnosis. VV recombinant technology was also used to prepare diagnostic kits that permit differentiation between vaccinated and naturally infected animals. The advantage of this method is that, whereas natural infection elicits antibodies to many antigenic epitopes, the VV recombinant antigen evokes the production of antibodies specific to the encoded protein.

The safety, convenience, and controlled technology of production made VV recombinant technique a valuable tool for diagnosis and vaccine production against pathogens of humans and livestock [12].

III. MOLECULAR BIOLOGY IN VETERINARY DIAGNOSIS.

Immunological techniques although rapid and accurate, are incapable of identifying pathological entities that are not expressed by significant antigenic responses. The RNA and DNA hybridization and amplification methods fill the gap.

The basic principle of the molecular diagnosis is based on the reversible denaturation of the double-stranded helix of DNA, or the DNA-RNA complexes. When conditions for renaturation exist, each strand forms a hybrid that strictly depends on the matching of complementary base pairs: The extraordinary stability, or *stringency* of the base-pairing bonds is reduced sharply when the sequence of bases does not match perfectly. It is possible, then, to identify a sequence of nucleic acids that will hybridize perfectly with only one intended target, accurately recognizing one specific genomic unit. Such a set, called a *probe* [13] may be synthesized when the sequence of nucleotides is known.

A. Nucleic Acid Probes

Probes may be used as hooks to “fish” one intended target out of billions of different strands. The *capture probe* is fixed on a solid matrix, usually a membrane. Other probes, which are labeled with radioactive isotopes, biotin-avidin conjugates, chemiluminescent molecules, or enzymes, are used to signal the presence of the target strand and are referred to as the *labeling probes* [14].

This technique permits an accurate detection of viruses, bacteria, and parasites by *in situ* hybridization, in which the target strand is identified within the host cell, and it may also be used as a cytogenetic tool to identify chromosomal location or variations in a single copy of a gene [15].

In situ hybridization permits the definitive identification of parasite or virus-infected cells in biopsies or *postmortem* examination. Variations of the *in situ* techniques, enable the identification of free viruses on a membrane that selects the viruses contained in a sample of cell particles, and allows rapid, animal-side identification methods. Occasionally, when unbound probes may not be entirely washed out, it is necessary to translate the specific site of the DNA intended target to RNA by using the *nick translation* method to reduce the nonspecific “noise” of unbound probes here’s the labeled RNA molecule that was synthesized on the probe template is used as a signal [10].

For the characterization and typing of pathogens, it is necessary to analyze the differences in the genomic sequences of nucleic acids using blotting techniques.

B. Dot-Blot Hybridization

Dot-blotting tests are used for the simultaneous rapid screening of a target sequence in several samples. The whole DNA in the sample is immobilized as a dot on the membrane, and a labeled probe is than added. This method is used for rapid screening of viruses, bacteria, or protozoa in medical clinics or animal-side tests [16]. A similar method was applied in the test for feline peritonitis virus (FIP), a corona virus that causes a lethal disease and cannot be accurately identified by the immune response. The viral RNA was cloned and hybridized to a cDNA biotin-avidin-labeled probe. Due to the high specificity of this reaction, the virus could be identified in the body fluids sample [17].

More accurate and sensitive methods are the Southern and Northern blotting tests. In the Southern blot [18] method, the DNA content is digested by restriction endonucleases that specifically cleave the denatured DNA strands at their specific recognition sites to fragments that differ in length and weight. Electrophoresis is than applied to separate the fragments on

agarose gel. The shorter strands are transferred by blotting on the solid support and visualized by the introduction of labeled probes. Northern blotting [19] is a modification of this method, using DNA-RNA hybridization at the visualization step. The use of these methods for the diagnosis of parasitic diseases has been comprehensively reviewed [12]. Yet another application of these blotting techniques is to differentiate between nonpathogenic and pathogenic strongyles [20].

C. RNA and DNA Amplification

The hybridization techniques, although sensitive and accurate, depend mostly on the presence of minimal quantities of the specific target strands for visualization by nonradioactive methods. In many clinical situations, the pathogenic virus may hide itself within the cells, or present in very small quantities in the blood. It is then necessary to multiply the intended DNA or RNA

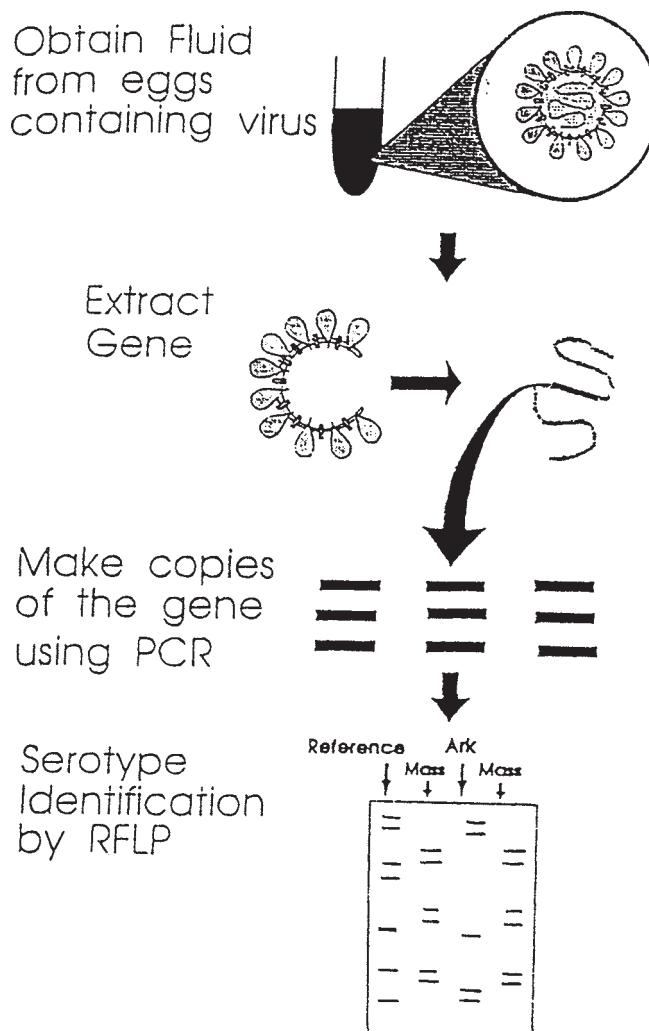


Fig. 1 Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) used to identify infectious bronchitis virus serotypes. (Ref 23.)

strands specifically and accurately to be eventually recovered. The polymerase chain reaction, known as PCR [21], is a DNA amplification method based on the reversible denaturation of the DNA double-helix at 90°C. This principle was described by Saiki and his colleagues to amplify the nucleic acid sequence of the mutated gene for human sickle cell anemia [22]. This powerful tool for DNA amplification consists of a cycle of three steps. The polynucleotide strand intended for multiplication is annealed between two primers. A thermostable DNA polymerase, produced by the thermophilic bacterium *Thermus aquaticus* catalyzes the polymerization of a homologous strand at 60–75°C. The new double-stranded DNA is then denatured to single strands at 90°C. The temperature cycle is repeated, with the multiplication of the existing DNA fragments in each cycle. A typical PCR amplification reaction consists of 20–40 cycles, which enables the visualization of one single preamplified DNA strand by hybridization with a labeled probe.

Use of PCR permits the identification of differences in viral genomes or serotypes. Such was the case with the typing of the infectious bronchitis virus (IBV) in chickens. By using the technique called restriction fragment length polymorphism (RFLP), Southern blotting was applied to determine the changes within a copy of amplified single viral gene isolated from an egg. The “fingerprint” of each DNA fragment and the accurate identification of each virus strain were obtained [23] as shown in [Figure 1](#). The complementary use of amplification and probing permitted fundamental research on the genetics and evolution of pathogens as well as an accurate evaluation of the findings in epidemiological studies.

New probes for almost any polynucleotide strands could now be produced. The amplified strand is analyzed by sequencing, and the complementary strand is synthesized by automatic DNA or RNA synthesizer. Nonspecific amplification should be avoided in the PCR by using highly purified reagents under noncontaminated laboratory conditions. Frequently, when attenuated viruses are used for vaccination, the PCR is not accepted as conclusive diagnosis of a field strain, as long as no specific genetic change in the DNA of the vaccine strain has been realized. New methods of enhanced nucleic acid amplification that are not dependent on the temperature cycle were also developed: The nucleic acid sequence base amplification (NASBA), which amplifies RNA target strands, is less sensitive to contamination than PCR and may better fit into both human and veterinary medicine.

IV. BIOTECHNOLOGY IN FIELD AND LABORATORY DIAGNOSIS

A. Differentiation Between Immune Responses to Vaccine and Pathogen Strains

Differentiation between vaccinated and naturally infected animals depends on a deeper understanding of the immunochemistry underlying the antigen-antibody reaction, as demonstrated by the *Brucella* case.

The *brucellae* are gram-negative, facultative, intracellular bacteria with four species that cause diseases in domestic ruminants, dogs and humans. Most important are *B. abortus*, which causes abortion and infertility in cattle, and *B. melitensis*, which causes abortion in small ruminants, as well as Malta fever in humans. Worldwide international cooperation has been established in an effort to control and eradicate these diseases [24].

The presence of antibodies against the bacteria in infected animals could be easily monitored with direct methods; such as the CF, plate agglutination, or direct antigen binding in milk (abortus band ring test). As a facultative intracellular pathogen, the *Brucella* spp. elicits a cellular immune response as well as a humoral antibody response. In the delayed-type

hypersensitivity test (DTH), sensitized animals respond with a swelling in the injection site of the *brucella* allergenic antigen [25].

As long as no vaccination is applied, the aforementioned tests may be efficiently used to screen sheep flocks and cattle herds for the presence of the pathogen. The vaccination of livestock in brucellosis endemic areas, calls for a diagnostic tool that can detect sporadically infected carriers in the vaccinated herds. For this purpose, an antigen intended for vaccination should elicit production of different antibodies compared with the antibodies produced in the natural immune responses against the pathogenic strains.

One approach was to identify a specific molecule, or epitope, on the bacterial lipopolysaccharide (LPS) envelope that may differ in the vaccine and the pathogen strains. Such antigen, an O-chain polysaccharide was purified from *B. abortus* LPS. Unfortunately, this antigen, a 4,6-dideoxy-4-formamido- α -d-mannopyranosyl, was also found in *Yersinia enterocolitica* and could not specifically identify the presence of brucellae. A monoclonal antibody that interacts with the pathogenic *B. abortus* LPS "O" side-chain but not with the nonpathogenic vaccine strain, was applied in a competitive ELISA [26,27].

Another approach for studying antigenic specificity was applied by Debbarh and colleagues [28]. They extracted proteins from the cytosoluble fraction of *B. melitensis* Rev-1 vaccine strain and from the pathogenic field strains. These proteins were separated by electrophoresis and some immunogenic proteins that reacted with antibodies from sheep infected with pathogenic field strains, but not with the antibodies produced in vaccinated sheep, were analyzed [28]. They suggested the use of these proteins as capture antigens for the differentiation between naturally infected and REV-1 vaccinated sheep. Bacharach and his colleagues [29] showed that a ribosomal subunit, the L7/L12 protein, is involved in the DTH reaction and is present only in intracellular multiplying bacteria. They identified and replicated the gene that codes for the protein, and ligated it into *E. coli* plasmid QE30. This plasmid transformed the *E. coli* and the recombinant protein was expressed, produced, and purified. The L7/L12 12kDa protein in the vaccine strain can be altered so that that its antigenicity will differ from that of the field strain, eliciting a DTH, positive response only in cases of disease.

A solid-state dot blotting ELISA that monitors both LPS antigen and a cytosoluble protein was also used to discriminate between the immune response of vaccinated and infected animals: It seems that the development of a feasible and efficient rapid selective test for immune response to *Brucella* spp. is foreseeable.

B. Biotechnology and the Control of Parasitic Diseases

Parasites cause major diseases in humans and livestock. More than 75% of the world population, and up to 70% of the world livestock are concentrated in developing countries where damage caused by parasitic diseases is enormous. It is estimated that a 6% reduction of their incidence could provide food for additional 250 millions persons [30]. In the developed countries, some parasitic diseases that were nearly eradicated recurred. The shipment of livestock from infected zones, invasion of vectors into disease-free areas, and the gradual establishment of resistance to drugs are identified as the main causes. Application of an effective diagnosis will reveal the pathogen and monitor the treatment, prevention, and eradication.

Tick-borne parasites, such as *Anaplasma marginale*, *A. centrale* and *Babesia* spp., have worldwide distribution and endanger some billion heads of cattle in the tropical and subtropical areas. Diagnostic methods, such as direct microscopic observation of blood smears, complement fixation, direct immunofluorescence, and ELISA [31] are used for the identification and surveys of the diseases in field stations and remote areas. More sophisticated methods, using monoclonal antibodies and DNA probes are used for species-specific diagnosis. Amplification

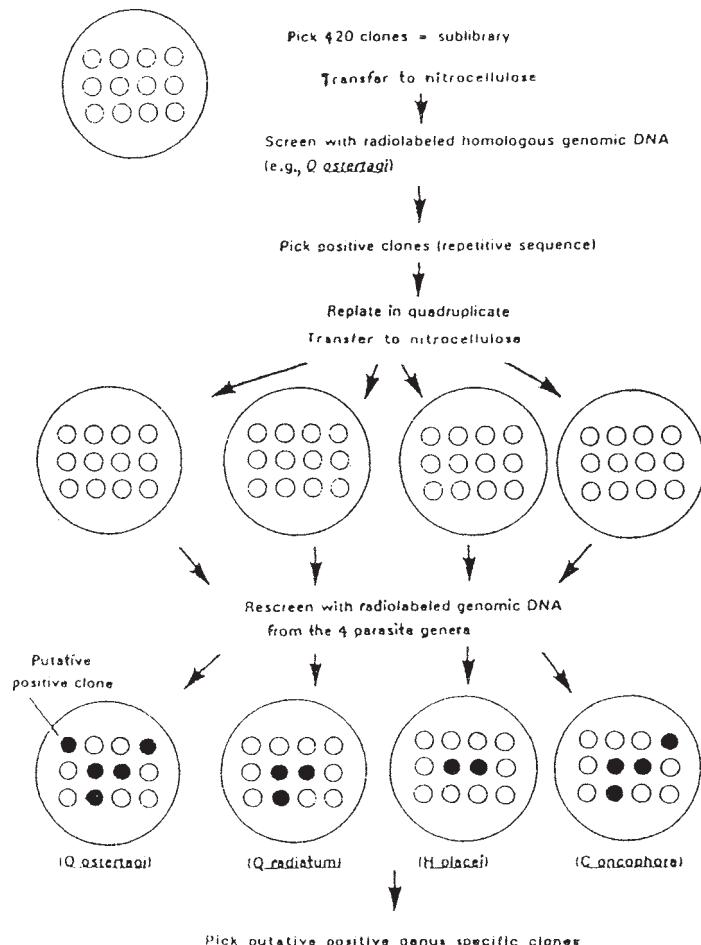


Fig. 2 Research protocol used to generate and screen an *Ostertagia ostertagi* partial genomic DNA library of genus-specific DNA probe. The same technique was used to identify probes specific to many other parasites. (Ref. 20.)

of the DNA content, followed by RFLP methods are applied for the differentiation and the identification of mutated field strains [16]. Southern and Northern blotting, DNA probes, and recombinant proteins with specific antigenic determinants are used in many cases of rickettsial, protozoan, and helminthic diseases for which the immune response may not always differentiate between an active or latent state of disease. For some gastrointestinal nematodes, differentiation between nonpathogenic and pathogenic strongyle strains cannot be obtained without highly specific DNA probes [20]. Figure 2 demonstrates the procedure used to screen partial genomic DNA library for genus-specific probes.

C. Diagnosis of Epizootic Diseases

Accurate and rapid testing methods are indispensable in cases of emergency, when despite the clinical symptoms, the precise identification of the pathogenic agent is needed for prophylactic measures and efficient treatment.

Many viral diseases, such as blue tongue, Eastern and Western equine encephalitis, and foot and mouth disease, rapidly spread in the herd after a short, acute phase. In these cases the IgM antibodies that appear during the viremic phase of the disease and decay soon after, are more significant than the IgG immunoglobulins that appear much later. In such diseases, the IgM-capture ELISA should be applied as a first-line, rapid test, and the competitive ELISA may serve later for confirmation [32].

The use of two different methods to ensure definitive diagnosis becomes a routine in current practice, such as for the bovine viral diarrhea virus (BVDV). This virus causes abortion, enteritis, and death in cows and their offspring, and sudden outbreaks may occur with significant damage. The noncytopathogenic virus is transferred from a nonsymptomatic dam to her offspring, which become immunotolerant toward the pathogenic virus. Usually, vaccination is applied to protect the uninfected cows. It is possible then, to identify the carriers that respond poorly to the vaccine using the indirect ELISA. The cows that lack immune response are then retested with the antigen-capture direct ELISA for the identification of the viral antigen [33].

A PCR technique may also be used to confirm the presence of the virus, although this method is still too complicated for screening herds in day-to-day laboratory practice [29]. Outbreaks of chlamydiosis, leptospirosis, or the syndrome of respiratory diseases caused by the infectious rhinotracheitis virus (IBR), parainfluenza-3 virus (PI-3), and the bovine respiratory syncytial virus (BRSV), call for rapid and conclusive diagnostic tests, substantiating prophylactic campaigns. "Animal-side" tests that identify the pathogen within a few minutes in one blood drop, such as the latex agglutination, or the dot ELISA, permit the screening of different antibodies or antigens by the clinician without the need of a laboratory.

A routine serological survey is sometimes recommended in nonvaccinated animals introduced to a high-risk area, or during vaccination and culling operations. Very fast immunoassay devices, based on chromatographic separation of the antibodies on a membrane, are applied. The adaptation of this technology in veterinary medicine, particularly of small animals, will be discussed at a later.

D. Diagnosis of Enzootic Diseases

Despite the low mortality, enzootic diseases, such as bovine leukemia (EBL) and paratuberculosis (Johne's disease) often cause heavy economic losses. The control and eradication of such diseases are essential for the milk and meat industries.

A multinational campaign to eradicate EBL has been successfully applied in the European Common Market, using antibody testing and culling of the positive reactors. John's disease, caused by *Mycobacterium paratuberculosis*, is a lethal intestinal infection characterized by a long incubation period. Calves may contract the disease within the first 6 months of life and develop signs that lead to death after 4–5 years. During this nonsymptomatic period the animal may shed the bacteria in the feces and contaminate the premises. The herd may be permanently infected with only a few acute cases and many carriers [34]. Direct identification depends on the isolation and definition of the pathogen from feces, which is laborious and time-consuming.

Nonpathogenic strains of saprophytic mycobacteria, such as *M. phlei* might elicit nonspecific reactions in the ELISA screening test [35]. Incubation of serum samples with *M. phlei* antigen, improves specificity, but reduces sensitivity. DNA probes and DNA amplification methods, such as the PCR [36], are both sensitive and specific, but are not economically feasible in large-scale screening of herds. Testing, vaccinating, and culling of the reactors are governed mainly by management and economic considerations [37].

E. Diagnosis of Zoonotic Diseases

Zoonotic diseases are characterized by the ability of the pathogens to be transferred from domestic and wild animals to humans. Some zoonotic pathogens, such as *B. melitensis*, *Toxoplasma gondii*, or rabies virus are well established in domestic and pet animals and thus are widespread in human populations. The eradication and control of these diseases depend on international cooperation.

In this international campaign, simple and nonexpensive diagnostic tests are used by the developing countries. Modern laboratories that use computer-assisted automatic robots also prefer to use the classic serological techniques, such as the CF, DTH, and ELISA. These most convenient measures permit international monitoring of the diseases and improve the standardization of techniques. However, more concern should be given to "emerging diseases" when a new pathogen invades susceptible livestock or humans. Without the natural balance, the rapidly spreading epidemic may cause severe damage [38].

Viruses, especially the RNA viruses, can rapidly change their genetic material. Mutations, recombination, or reassortments of the viral genome, can bring about adaptation to new hosts, modification of virulence, or stability in new environmental conditions. The risk is still amplified by the close proximity of humans, livestock, and wild animals. Human intrusion into new areas and clearance of forests may accelerate the transition of microorganisms from their well-established hosts to humans and livestock. Rift Valley fever (RVF) may serve as an example: RVF is a cattle disease caused by a *bunyavirus*, transmitted by mosquito of the genus *Aedes*. The disease is endemic in the African bush where seasonal floods determine the abundance of the vector. Under these conditions a balance was established between the vector, the grazing animals, and the virus. The construction of the Aswan dam in Egypt provided new irrigated breeding grounds and a longer reproduction season to the mosquitoes. Consequently, outbreaks of RVF occurred in Egypt and spread through western and southern Africa, killing hundreds of thousands heads of cattle as well as humans. Today RVF is one of the most dangerous cattle diseases, which worries the World Health Organization (WHO). Introduction of specific monoclonal antibodies in rapid laboratory tests, combined with vaccination and eradication, have already been applied in RVF control and call for multinational collaboration in the future.

Molecular genetic methods were successfully applied to study the source and evolution of the epidemic human influenza A disease. Influenza types A, B, and C evolved separately in horses and birds. The human virus appeared in the early 1900s, concurrently with the spreading of swine influenza virus. Since 1918, waves of acute human eastern virus types such as the Spanish (1918), the Asian (1957), Hong-Kong (1968), and Russian (1977), raged in the human population, leaving millions dead, and huge economic damage.

Influenza virus, an *orthomyxovirus*, has an RNA genome divided into eight segments, two of which are important determinants of the host range and virulence of the virus. One segment encodes for a hemagglutinin (HA), a membrane-bound protein that mediates receptor binding and fusion between the virus envelope and the cell membrane. The second determinant encodes for the neuraminidase enzyme (NA), that affects virulence. Different HA subtypes (H1-H14) have been detected in birds, two (H3 and H7) in horses, two (H1 and H3) in pigs, and three (H1, H2, and H3) in humans. Different mutations (N1,N2,N3) were also found on the NA cluster [39].

By using the RNA-DNA recombination, blotting, and sequencing techniques, it was shown that a change in the H1N1 and H3N2 combinations of heterosegments of DNA is needed to adapt avian virulent virus to humans. New avian influenza strains, originally borne by wild ducks or geese, are spread eventually to the human environment. It seems that pigs serve as a

pool, where recombination of two types of influenza virus, with an exchange of segments, generates the types adapted to humans [40].

New emerging diseases of both animals and humans are mostly caused by existing viruses shifted from their original wild host pool to domestic animals and man. The severe infection of farmers with pathogenic Sabia arenaviruses of wild rodents in Brazil (1990), the Sin-nombre virus in New Mexico, Colorado, and Nevada (1993), and the Ebola virus in Zaire (1995) are just a few examples.

V. DIAGNOSIS IN SMALL ANIMAL CLINICS

A. One-Step Rapid Single Testing

Very fast immunoassays, based on the techniques of chromatographic separation or membrane concentration in one-step, single tests were recently developed for human medicine, such as the pregnancy test or the sexually transmitted diseases tests. The adaptation of this technology in veterinary medicine, especially in pet animals, revolutionized the veterinary practice. Combined tests for antibodies to feline immunodeficiency virus (FIV) and antigens for feline leukemia virus (FeLV) are available, for which the concentration of the reagents as blotted dots on a membrane improves specificity and sensitivity of the reaction. Such a device is one example of the broad use of the over-the-counter diagnostic tests that may replace the laboratory services.

The adaptation of techniques to the practice should comply with some basic criteria:

1. Sensitivity and specificity: The sensitivity should allow diagnosis in the lower detectable concentration of the pathogen. The specificity should discriminate between the pathogen or the antibody and the presence of nonpathogenic cross-reacting antigens.
2. Turn-around time: The test should be rapid and free the clinician from time-consuming practices.
3. Ease of performance: A one-step test with clearcut results, and no need of preparation of solutions or reagents.
4. Stability and integrity: The device should withstand long storage and easily integrates in the day-to-day routine work in the veterinary clinic.
5. Cost-effective: A cost-benefit analysis of each test is essential when diagnosis involves economic decisions, such as in the dairy farm.

B. Diagnosis, Vaccination, and Immunity-Monitoring in the Small Practices

Over-the-counter diagnostic devices, such as the immunochromatographic tests for parvovirus antigen in dogs (CPV) and the need to monitor immunity demonstrate the dynamic changes in the pet clinics. In 1978, an acute epidemic of lethal diarrhea killed thousands of dogs and spread from North America to Europe, Japan, and Australia. The cause was a mutant of the feline panleukopenia virus (FPV) that was known to affect cats, now renamed as the CPV type 2 virus. DNA-sequencing analysis of the gene encoding the virus capsid proteins, revealed two different clusters of DNA. One gene cluster enabled the virus to interact with the dog's cells, whereas a change in another cluster enables the virus to multiply in the dog's gut cells. The recombination of the two clusters provides the virus with the ability to cause acute enteritis in dogs [41].

DNA sequencing, and virus recombination were employed to create the phylogenetic tree of the evolution of the FPV, and revealed that new mutations permanently change the virus type, and the new types rapidly replace the older ones. It is likely that intermediate hosts such as the raccoons, were involved in the first recombination steps that contributed to the rapid evolution of this virus [39].

C. Determination of Sex in Exotic Pet Birds

In many species of birds, including pet birds, there is no phenotypic difference between the female and the male. In these birds invasive methods that endanger the bird, such as endoscopy, are needed to identify the sex organs.

In birds the female carries the heterozygote ZW whereas the male carries the homozygote chromosome ZZ. The specific segment for the W chromosome found only in the female, is different in each species and sometimes in each variant of birds. Therefore, it is necessary to create a rich library of DNA segments that may be used in RFLP techniques, where the fingerprint of each bird is analyzed [42]. Such libraries are maintained only in specialized laboratories, and the use of this method is complicated and expensive. A new method was recently developed by Poltsky et al. [43]. In this method, a large quantity of male DNA (ZZ), is mixed with a small quantity of female DNA (ZW). Under conditions of competitive reassociation and denaturation, each chromosome type was processed by different nucleases, revealing different strand ends. Reassociation involves three ways of DNA binding: M-M, M-F, and F-F. Because the quantity of the male DNA is far greater than that of the female, reassociation of the F-F type will occur only when the W homologue is present. In this way, clones that contain free ends that can bind only to the W chromosome are identified. The DNA strand is cloned in a plasmid and inserted into a bacteria. In this procedure, large quantities of the DNA strands are produced from the bacterial colony, and the identification of the segments determining the female sex permitted the preparation of primers of the W segments by PCR. In this procedure, common, pooled primers and probes are used, enabling the development of more convenient techniques.

VI. BIOTECHNOLOGY IN MODERN FARM MANAGEMENT

A. Diagnosis of Mastitis in the Dairy Herd

Mastitis is an infection of the mammary gland; chronic and subclinical infections are the main causes for decrease in milk yield and losses because of culling, drugs, and labor. Over 100 different microorganisms have been reported to cause intramammary infection. However, the most significant economical damage is caused by species of streptococci, staphylococci, and a few gram-negative bacteria [44]. According to the mode of transmission, pathogens are classified as contagious, environmental bacteria, and the coagulase-negative staphylococci.

Diagnostic techniques assist in the detection of carriers, dictating treatments, and control. Mastitis control programs reduce the incidence of the infection within economical acceptable limits. Diagnostic tests should be rapid, accurate, and adaptable to the practice of automatic milking and programmed management. One of the classic tests is the California mastitis test (CMT), based on a color marker that interacts with free somatic cells detached from infected mammary tube epithelium. A similar, more quantitative method, is the somatic cell count in milk (SCC) [43]. A very sensitive test is the *Limulus* amoebocyte test. This method is based on the coagulation of the hemocoel fluid of the horse-shoe crab *Limulus polyphemus* in the presence of a lipopolysaccharide (LPS) of the bacterial cell wall. The presence of LPS triggers the reaction, which is visualized by a color signal.

Dry chemistry methods, which can measure free cell markers such as ATP, lacticdehydrogenase, or free ammonia ions, are also available and are currently used for screening [45]. Cytometric methods, such as chemiluminescence count of white blood cells, are also used. All these methods require sampling during milking, which interferes with the milking practice and demands extra skilled labor.

One of the earlier symptoms of subclinical mastitis, is a change in the electric conductivity of the milk owing to enhanced electrolytes transfer from blood to tissues [46]. The electric conductivity of the milk is monitored during milking. Information on behavior, milk conductivity, and production rate, is assessed and diagnosed, resulting in an on-line processing of the cow's health. The chronic carrier can be detected automatically during milking by on-line testing. Modern milking machines are equipped with monitors that gather information from both the milking machine and the cow, identified by a transmitter connected to its leg. Furthermore, the monitor registers through pedometry the number of steps realized by the cow per hour. The actual activity is compared with the mean activity of 7–10 previous days. Reduced activity may indicate stress, whereas hyperactivity signals the onset of ovulation, and calls for insemination. The monitor dictates appropriate treatment, while information containing the history, productivity, and economical value of the cow is assessed by a central computer. In this way, the manager can choose the most effective protocol suitable for each cow [47]. Although not defined as biotechnology *per se*, this new approach of an automatic computed diagnostic technique indicates the direction of modern diagnosis gradually replacing the traditional sampling and testing methods.

B. Monitoring Immunity in Vaccination Programs

Modern intensive livestock raising depends on strict management of animal health. The economic basis of any animal health program depends on accurate and efficient use of vaccines and medications. Intensively managed poultry flocks risk outbreaks when invaded by a pathogen, whereas unnecessary vaccinations are expensive and useless, and should be avoided. Efficient health management can be contemplated by monitoring the immunity status of the flock, resulting in an optimal vaccination program. The avian immunoglobulin Y (or G) level against pathogens, such as the Newcastle virus, infectious bronchitis virus, and bursal disease virus, should be maintained high throughout the life of the chicken. Maternal antibodies, transferred through the egg to the chick confer protection for the first few weeks after hatching, but the immunity decreases gradually with time. Once the level of antibodies is not sufficient to inactivate wild pathogenic strains of viruses, the young chick becomes susceptible. However, a vaccine given before the decline of the maternal antibodies, may be rapidly inactivated without conferring immunity.

For the screening of the flock's immunity, serum samples are checked automatically by computed indirect ELISA systems for the quantitative monitoring of the immunoglobulins. In small farms, where the expensive computed readers are not feasible, rapid self-contained kits using the solid-state dot ELISA technique, or the standard HI tests, are used routinely to determine the time and type of vaccination.

VII. SUMMARY

Disease control programs in livestock depend on reliable, cost-effective, and rapid diagnostic methods. Classic diagnostic methods were based on isolation of the pathogen, essentially using laboratory animals. Serology was mainly used for epidemiological surveillance and disease eradication campaigns. Methods, such as the serum agglutination, complement fixation,

immunofluorescence, and enzyme-linked immunosorbant assay, are still widely applied and have their place in modern preventive medicine. However, these laboratory methods do not respond to the ever-increasing needs of speed, specificity, and sensitivity. Furthermore, distinction between naturally infected and vaccinated animals is not possible, often jeopardizing disease control campaigns, such as for brucellosis, leptospirosis, and some viral diseases.

Biotechnology revolutionized diagnostic techniques, introducing higher sensitivity and specificity. Monoclonal antibodies, DNA probes, Southern and Western blotting, DNA and RNA amplification through PCR, nowadays constitute an integral part of the diagnostic arsenal at the disposal of the advanced laboratories. Given these techniques, rapid animal-side field tests used by the clinicians furnish results at real-time, permitting immediate decisions on the proper treatment, vaccination, or stamping-out policy. High-technology-based diagnosis will become a routine in every diagnostic laboratory, forming a sound basis for control and treatment of animal diseases. Molecular biology contributes to other livestock activities, such as sexing of birds as well as the study and prediction of new pathogenic mutants. Finally, industrial livestock management, particularly dairy and poultry farming, will depend more and more on higher biotechnological techniques.

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Transgenic Fish Technology and Its Application in Fish Production

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I. INTRODUCTION

Organisms into which heterologous DNA (transgene) has been artificially introduced and integrated in their genomes are called *transgenics*. Since the early 1980s, transgenic plants [1], nematodes [2], fruit flies [3], sea urchins [4,5], frogs [6], laboratory mice [7,8], and farm animals, such as cows, pigs, and sheep [9], have been successfully produced. In plants, transgenes are introduced into cells by infection with *Agrobacterium tumefaciens* or by physical means, such as ballistic bombardment. In animals, transgenes are introduced into the pronuclei of fertilized eggs by injection, and the injected embryos are incubated in vitro or implanted into the uterus of a pseudopregnant female for subsequent development. In these studies, multiple copies of transgenes are integrated at random locations in the genome of the transgenic individuals. If the transgenes are linked with functional promoters, expression of transgenes as well as display of change in phenotype is expected in some of the transgenic individuals. Furthermore, the transgenes in many transgenic individuals are also transmitted through the germline to subsequent generations. These transgenic animals play important roles in basic research as well as applied biotechnology. In basic research, transgenic animals provide excellent models for studying molecular genetics of early vertebrate development, actions of oncogenes, and the biological functions of hormones at different stages of development. In applied biotechnology, transgenic animals offer unique opportunities for producing animal models for biomedical research, improving the genetic background of broodstock for animal husbandry or aquaculture, and designing bioreactors for producing valuable proteins for pharmaceutical or industrial purposes.

Since 1985, a wide of transgenic fish species have been produced [10–12] by microinjecting or electroporating homologous or heterologous transgenes into newly fertilized or unfertilized eggs. Several important steps are routinely taken to produce a desired transgenic fish. First,

an appropriate fish species must be chosen, depending on the nature of the studies and the availability of the fish-holding facility. Second, a specific gene construct must be prepared. The gene construct contains the structural gene encoding a gene product of interest and the regulatory elements that regulate the expression of the gene in a temporal, spatial, and developmental manner. Third, the gene construct has to be introduced into the developing embryos for the transgene to be integrated stably onto the genome of every cell. Fourth, because not all instances of gene transfer are efficient, a screening method must be adopted for identifying transgenic individuals.

Although remarkable progress has been made in producing transgenic fish by gene transfer technology, a critical review of the published results has shown that most of the research effort has been devoted to confirming the phenomenon of foreign gene transfer into various fish species. Very few attempts have been made to explore the application of transgenic fish technology in basic as well as applied research. Recently, we have devoted a substantial amount of our research effort to this problem with promising results.

The worldwide harvest of fishery products traditionally depends on natural populations of finfish, shellfish, and crustaceans in fresh and marine water. In recent years, however, the total annual worldwide harvest of fish products has approached, or even surpassed, the maximal potential level of about 150 million metric tons (as calculated by the US Department of Commerce and the US National Oceanic and Atmospheric Administration). To cope with the worldwide demand of fish products and the escalating increase in fish price, many countries have turned to aquaculture for increasing production of fish products. In 1985, the world production of finfish, shellfish, and macroalgae by aquaculture reached 10.6 million metric tons, or approximately 12.3% of the worldwide catch generated by international fishery efforts. Although aquaculture clearly has the potential for increasing worldwide fish production, innovative strategies are needed to improve efficiency. What can transgenic technology offer?

Success in aquaculture depends on six factors: (1) complete control of the reproductive cycle of the fish species in culture; (2) excellent genetic background of the broodstock; (3) efficient prevention and detection of disease infection; (4) thorough understanding of the optimal physiological, environmental, and nutritional conditions for growth and development; (5) sufficient supply of excellent quality water; and (6) application of innovative management techniques. By improving these factors, the aquaculture industry has developed to a remarkable extent during the last decade. To sustain this growth, however, newly developed technologies in molecular biology and transgenesis will have to be increasingly applied by the aquaculture industry. These technologies can be employed to enhance growth rates, control reproductive cycles, improve feed compositions, produce new vaccines, and develop disease-resistant and hardier genetic stocks. In the last several years, we have been searching for strategies to increase fish production by manipulating fish growth hormone and growth factor genes. In this chapter, we will review results from our laboratory and those from others to demonstrate this point.

II. METHODS OF TRANSGENIC FISH PRODUCTION

A. Transgene Constructs

A transgene used in producing transgenic fish for basic research or application should be a recombinant gene construct that produces a gene product at an appropriate level in the desired tissue(s) at the desired time(s). Therefore, the prototype of a transgene is usually constructed in a plasmid to contain an appropriate promoter-enhancer element and a structural gene sequence.

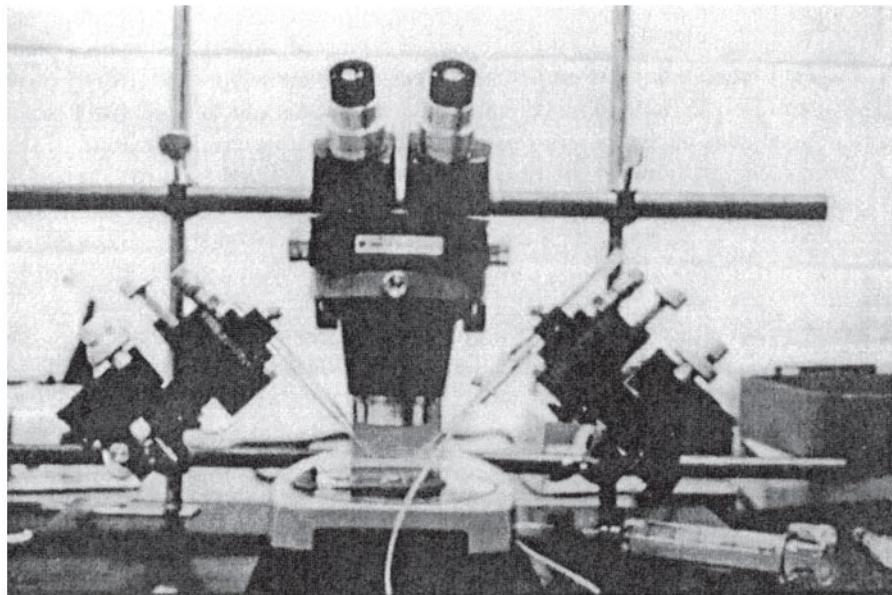
Depending on the purpose of gene transfer studies, transgenes are grouped into three main types: (1) *gain-of-function*, (2) *reporter function*, and (3) *loss-of-function*. The *gain-of-function* transgenes are designed to add new functions to the transgenic individuals or to facilitate the identification of the transgenic individuals if the genes are expressed properly in the transgenic individuals. Transgenes containing the structural genes of mammalian and fish growth hormones (GH, or their cDNAs) fused to functional promoters, such as chicken and fish β -actin gene promoters, are examples of the gain-of-function transgene constructs. Expression of the GH transgenes in transgenic individuals will result in increased production of growth hormone and ultimate growth enhancement [13–16]. Bacterial chloramphenicol acetyl transferase (CAT), β -galactosidase, or luciferase genes fused to functional promoters are examples of transgenes with *reporter function*. These *reporter function* transgenes are commonly used to identify the success of gene transfer effort. A more important function of a reporter gene is used to identify and measure the strength of a promoter-enhancer element. In this case, the structural gene of the CAT, β -galactosidase, or luciferase gene is fused to a promoter-enhancer element in question. Following gene transfer, the expression of the reporter gene activity is used to determine the transcriptional regulatory sequence of a gene or the strength of a promoter [17].

The *loss-of-function* transgenes are constructed for interfering with the expression of host genes. These genes might encode an antisense RNA to interfere with the posttranscriptional process or translation of endogenous mRNAs. Alternatively, these genes might encode a catalytic RNA (a ribozyme) that can cleave specific mRNAs and, thereby, cancel the production of the normal gene product [18]. Although these genes have not yet been introduced into a fish model, they could be potentially employed to produce disease-resistant transgenic brood-stocks for aquaculture or transgenic model fish defective in a particular gene product for basic research.

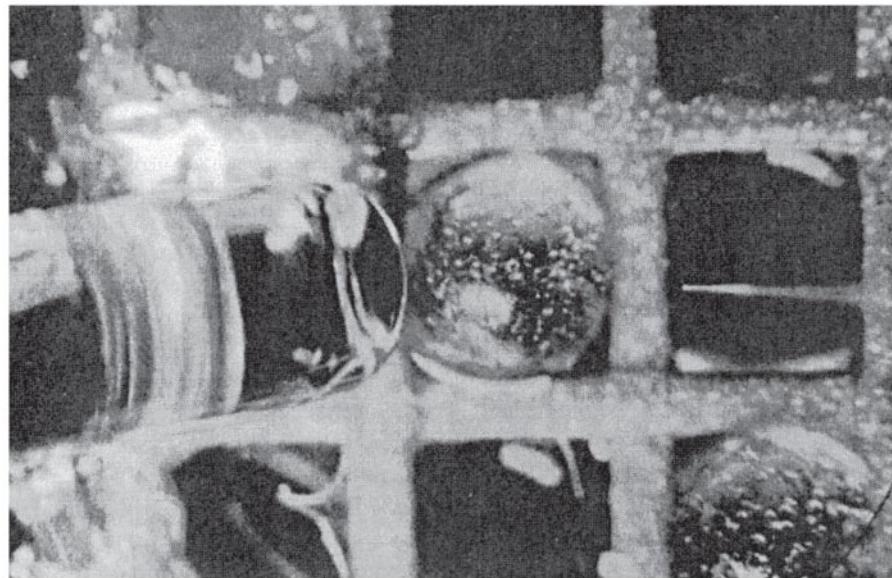
B. Selection of Fish Species

Gene-transfer studies have been conducted in several different fish species, including channel catfish, common carp, goldfish, Japanese medaka, loach, northern pike, rainbow trout, salmon, tilapia, walleye, and zebrafish [for review: 10,12]. Depending on the purpose of the transgenic fish studies, the embryos of some fish species are more suited for gene transfer studies than the others. For example, Japanese medaka (*Oryzias latipes*) and zebrafish (*Brachydanio rerio*) have short life cycles (3 months from hatching to mature adults), produce hundreds of eggs on a regular basis without exhibiting a seasonal breeding cycle, and can be maintained easily in the laboratory for 2–3 years. Eggs from these two fish species are relatively large (diameter, 0.7–1.5 mm) and possess very thin, semitransparent chorions, a feature that permits easy microinjection of DNA into fertilized eggs. Furthermore, inbred lines and various morphological mutants of both fish species are available. Therefore, these fish species are suitable candidates for conducting gene transfer experiments for (1) studying developmental regulation of gene expression and gene action; (2) identifying regulatory elements that regulate the expression of a gene; (3) measuring the activities of promoters; and (4) producing transgenic models for environmental toxicology. However, a major drawback of these two fish species is their small body size that makes them unsuitable for some endocrinological or biochemical analyses.

Channel catfish, common carp, rainbow trout and salmon, are commonly used large-body-sized model fish species in transgenic fish studies. Because the endocrinology, reproductive biology, and basic physiology of these fish species have been well worked out, they are well suited for conducting studies on comparative endocrinology and aquaculture applications. However, the long maturation time of these fish species and a single spawning cycle per year will limit research progress in the field.



(A)

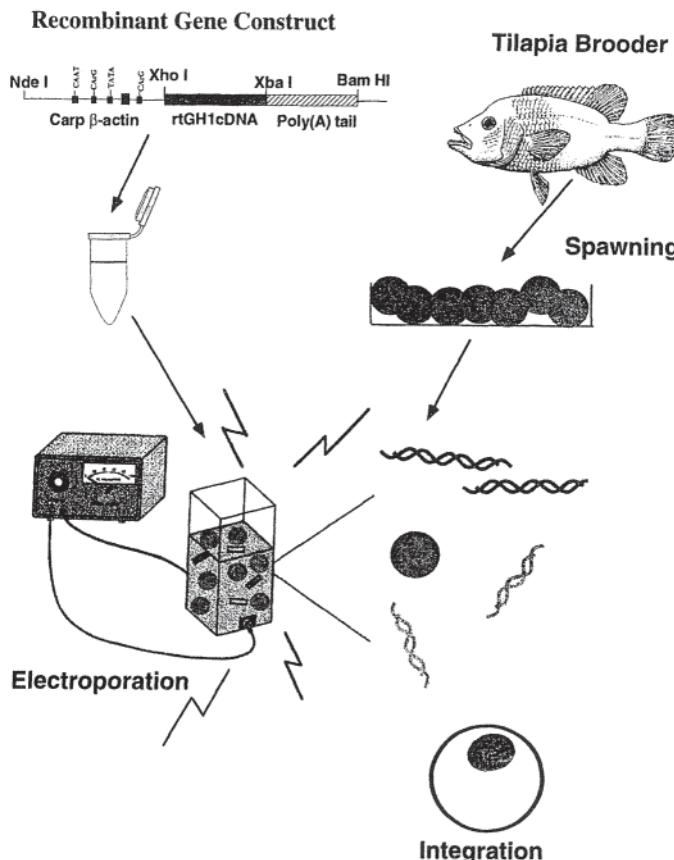


(B)

Fig. 1 Gene transfer apparatus: (A) Microinjection apparatus; (B) a close up view of medaka egg under the microinjection apparatus; (C) scheme of gene transfer by electroporation.

Loach, killifish, goldfish, and tilapia are the third group of model fish species suitable for conducting gene transfer studies because their body sizes are large enough for most biochemical and endocrinological studies. Furthermore, shorter maturation times, as compared with catfish, rainbow trout, or salmon, allow easier manipulation of transgenic progeny. Unfortunately,

(C)



the lack of a well-defined genetic background and asynchronous reproductive behavior of these fish species render them less amenable to gene transfer studies.

C. Methods of Gene Transfer

Techniques, such as calcium phosphate precipitation, direct microinjection, lipofection, retroviral infection, electroporation, and particle gun bombardment have been used to introduce foreign DNA into animal cells, plant cells, and germ-lines of mammals and other vertebrates. Among these methods, direct microinjection and electroporation of DNA into newly fertilized eggs have proved to be the most reliable methods of gene transfer in fish systems.

1. Microinjection of Eggs or Embryos

Microinjection of foreign DNA into newly fertilized eggs was first developed for the production of transgenic mice in the early 1980s. Since 1985, this technique has been adopted for introducing transgenes into Atlantic salmon, common carp, catfish, goldfish, loach, medaka, rainbow trout, tilapia, and zebrafish [10,11 for review]. The gene constructs that were used in these studies include human or rat growth hormone (GH) gene, rainbow trout or salmon GH cDNA, chicken δ -crystallin protein gene, winter flounder antifreeze protein gene, *Escherichia coli* β -galactosidase gene, and *E. coli* hygromycin-resistance gene [10,11]. In

general, transfer of foreign DNA into fish by direct microinjection is conducted as follows. Eggs and sperm are collected in separate, dry containers. Fertilization is initiated by adding water and sperm to the eggs, with gentle stirring to enhance fertilization. Fertilized eggs are then microinjected within the first few hours after fertilization. The injection apparatus consists of a dissecting stereomicroscope and two micromanipulators, one with a glass microneedle for delivering transgenes and the other with a micropipette for holding fish embryos in place (Fig. 1a). Routinely, about 10^6 – 10^8 molecules of a linearized transgene (with or without plasmid DNA) in about 20 nL is injected into the egg cytoplasm. Following injection, the embryos are incubated in water until hatching. Because natural spawning in zebrafish or medaka can be induced by adjusting the photoperiod and water temperature, precisely staged newly fertilized eggs can be collected from the aquaria for microinjection. If the medaka eggs are maintained at 4°C immediately after fertilization, the micropyle on the fertilized eggs will remain visible for at least 2 hs. The DNA solution can be easily delivered into the embryos by injection through this opening.

Depending on the fish species, the survival rate of injected fish embryos ranges from 35 to 80% while the rate of DNA integration ranges from 10 to 70% in the survivors (Table 1) [10,11]. The tough chorions of the fertilized eggs in some fish species (e.g., rainbow trout and Atlantic salmon) can frequently make insertion of glass needles difficult. This difficulty can be overcome by any one of the following methods: (1) inserting the injection needles through the micropyle, (2) making an opening on the egg chorions by microsurgery, (3) removing the chorion by mechanical or enzymatic means, (4) reducing chorion hardening by initiating fertilization in a solution containing 1 mM glutathione, or (5) injecting the unfertilized eggs directly.

2. Electroporation

Electroporation is a successful method for transferring foreign DNA into bacteria, yeast, and plant and animal cells in culture. This method has become popular for transferring transgenes into fish embryos in the past 3 years [15,63]. Electroporation uses a series of short electrical pulses to permeate cell membranes, thereby permitting the entry of DNA molecules into embryos. The patterns of electrical pulses can be emitted in a single pulse of exponential decay form (i.e., exponential decay generator) or high-frequencies multiple peaks of square

Table 1 Transfer of Foreign DNA into Medaka Embryos by Different Gene Transfer Methods

Microinjection ^a	Electroporation		Pantropic retroviral vector	
	I ^b	II ^c	Electroporation ^d	Incubation ^e
Viability (%) (at hatching)	50	70	90	50
Integration ^f rate (%)	20	15	25	50
Transgene expression	Yes	Yes	Yes	Yes
Efficiency (eggs per minute)	1–2	200	200	200

^aInjecting is carried out by micropyle before blastodisk formation.

^bExponential decay impulse mode.

^cSquare wave impulse mode.

^dElectroporation with square wave mode at 3.5 Kv.

^eFertilized eggs are exposed to a mixture of medaka-hatching enzyme and pancreatin for 2 h. The dechorionated embryos are incubated with the pantropic pseudotyped retrovirus overnight at room temperature.

^fIntegration rate is calculated from the surviving embryos after gene transfer.

Source: Ref. 63.

waves (i.e., square-wave generator, see Fig. 1b). Studies conducted in our laboratory [15,63] and those of others [19] have shown that the rate of DNA integration in electroporated embryos is one the order of 20% or higher in the survivors (see Table 1). Although the overall rate of DNA integration in transgenic fish produced by electroporation may be equal to or slightly higher than that of microinjection, the actual amount of time required for handling a large number of embryos by electroporation is orders of magnitude less than the time required for microinjection. Recently, several research groups have also reported successful transfer of foreign DNA into fish by electroporating sperm instead of embryos [20,21]. Electroporation, therefore, is considered as an efficient and versatile massive gene transfer technology.

3. Transfer of Transgenes by Infection with Pantropic Retroviral Vectors

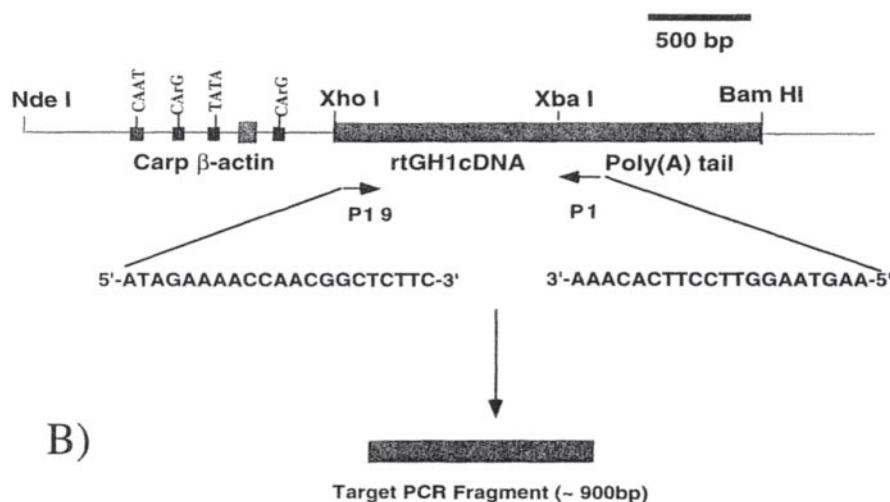
Although transgenes can be reproducibly introduced into various fish species by microinjection of electroporation, the resulting P₁ transgenic individuals possess mosaic germlines as a result of delayed transgene integration. Furthermore, these two gene transfer methods are not effective or successful in producing transgenics in marine fish and invertebrates. Recently, a new gene transfer vector, a defective pantropic retroviral vector, has been developed [22]. This vector contains the long terminal repeat (LTR) sequence of Moloney murine leukemia virus (MoMLV) and transgenes packaged in a viral envelop with the G protein of vesicular stomatitis virus (VSV). Because the entry of VSV into cells is mediated by interaction of the VSV G protein with a phospholipid component of the cell, this pseudotyped retroviral vector has a very broad host range and is able to transfer transgenes into many different cell types. Using the pantropic pseudotyped defective retrovirus as a gene transfer vector, transgene containing neo^R or β-galactosidase has been introduced into zebrafish [23] and medaka [24] (see Table 1). Recently, the feasibility of using a pantropic pseudotyped retroviral vector for introducing genes into marine invertebrates has been tested in dwarf surf clams and the results have shown that transgenes can be readily transferred into clams at high efficiency [61].

III. CHARACTERIZATION OF TRANSGENIC FISH

A. Identification of Transgenic Fish

The most time-consuming step in producing transgenic fish is the identification of transgenic individuals. Traditionally, the presence of transgene in presumptive transgenic individuals is determined by dot-blot or Southern-blot hybridization of genomic DNA isolated from the test individuals. These methods involve isolation of genomic DNA from tissues of presumptive transgenic individuals, digestion of DNA with restriction enzymes, and dot-blot. Southern-blot hybridization of the digested DNA products. Although this method is expensive, laborious, and insensitive, it offers a definitive answer whether a transgene has been integrated into the host genome. Furthermore, it also reveals the pattern of transgene integration if appropriate restriction enzymes are employed in the Southera-blot analysis. To handle a large number of samples efficiently and economically, a polymerase chain reaction (PCR)-based assay has been adopted [15,16]. The strategy of the assay is outlined in Figure 2. It involves isolation of genomic DNA from a very small piece of fin tissue, PCR amplification of the transgene sequence, and Southern-blot analysis of the amplified products. Although this method does not differentiate whether the transgene is integrated in the host genome or remains as an extrachromosomal unit, it serves as a rapid and sensitive screening method for identifying individuals that contain the transgene at the time of analysis. In our laboratory, we use this method as a preliminary screen for transgenic individuals from thousands of presumptive transgenic fish.

A)



B)

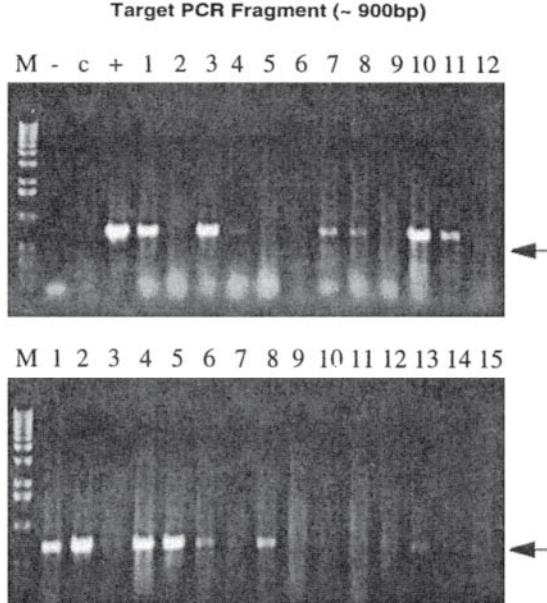


Fig. 2 Strategy for identifying the presence of transgenes in the presumptive transgenic fish by PCR and Southern blot hybridization. DNA samples were isolated from pectoral fin tissues of presumptive transgenic fish and subjected to PCR amplification. The amplified products were analyzed by electrophoresis on agarose gels and Southern blot hybridization. (A) Strategy of PCR amplification; (B) Southern-blot analysis of PCR-amplified products: lane M, molecular weight marker; lane -, PCR reaction without template; lane C, DNA sample from a nontransgenic fish; lane +, transgene construct; lanes 1–12 (upper panel) and 1–15 (lower panel), DNA samples from presumptive transgenic fish. Arrows indicate the size of amplified products.

B. Expression of Transgenes

An important aspect of gene transfer studies is the detection of transgene expression. Depending on the levels of transgene products in the transgenic individuals, the following methods are

commonly employed: (1) RNA Northern or dot-blot hybridization; (2) RNase protection assay; (3) reverse transcription-polymerase chain reaction (RT-PCR); (4) immunoblotting assay; and (5) other biochemical assays for determining the presence of the transgene protein products. Among these assays, RT-PCR is the most sensitive method and only requires a small amount of sample. The strategy of this assay is summarized in Figure 3 [16]. Briefly, it involves the isolation of total RNA from a small piece of tissue, synthesis of single-stranded cDNA by reverse transcription, and PCR amplification of the transgene cDNA by employing a pair of oligonucleotide primers specific to the transgene product. The resulting products are resolved on agarose gels and analyzed by Southern blot hybridization using a radiolabeled transgene as a hybridization probe. Transgene expression can also be quantified by a quantitative RT-PCR method [25]. Although this method is rapid and sensitive, it can be easily confused by transgene contamination in the reaction unless extra precaution is taken in setting up the reactions.

C. Patterns of Transgene Integration

Studies conducted in many fish species have shown that following injection of linear or circular transgene constructs into fish embryos, the transgenes are maintained as extrachromosomal units through many rounds of DNA replication in the early phase of the embryonic

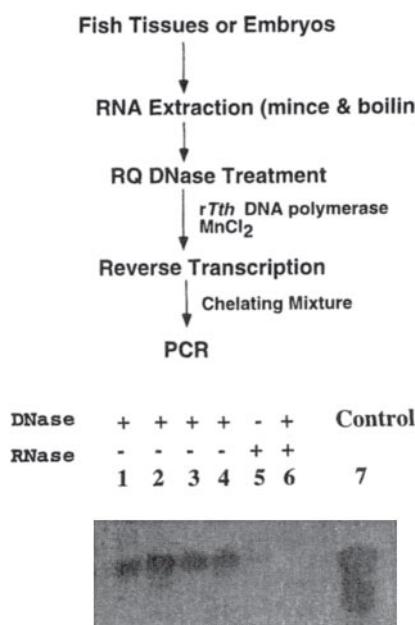


Fig. 3 Strategy of detecting rtGH transgene expression by reverse transcription (RT)-PCR assay: (A) Strategy of RT-PCR; (B) Detection of rtGH transgene expression in transgenic medaka by RT-PCR. Total RNA was isolated from whole fish of F₁ transgenic and controls fish following the acid guanidinium thiocyanate-phenol-chloroform method. Single-stranded cDNA was prepared by reverse transcription from each total RNA that has been pretreated with RNase-free DNase to remove any contaminating genomic DNA, and used as a template for PCR amplification of rtGH with synthetic oligonucleotides as amplification primers. The resulting products were analyzed by Southern blot analysis, using radiolabeled rtGH cDNA as a hybridization probe. Lanes 1–4, different F₁ transgenic fish; lane 5, PCR of RNA from lane 1 without pretreatment with RNase-free DNase and reverse transcription; lane 6, PCR of RNA from lane 1 with DNase and RNase treatment followed by reverse transcription.

Table 2 Effect of GH Treatment on the Growth of Rainbow Trout Fry

Treatment	Weight (g)		
	Initial	Final	% Gain
Saline control	1.33 ± 0.6**	3.94 ± 1.8*	196
GH (50 µg/L)	1.29 ± 0.7**	5.51 ± 1.6***	327
GH (500 µg/L)	1.35 ± 0.7**	5.30 ± 1.3***	293

Values presented as mean±SD. Groups of rainbow trout fry ($n=15$) were subjected to osmotic shock in the presence of absence of GH. Weight was measured before and 5 weeks post-treatment. Differences between mean weights of GH-treated and control groups were evaluated using Student's *t*-test ($\alpha=0.01$).

*Significantly different from the GH-treated groups ($P<0.01$).

**No significant difference between these groups.

***No significant difference between these two treatments.

Source: From Ref. 45.

development. At later stages of embryonic development, some of the transgenes are randomly integrated into the host genome, whereas others are degraded, resulting in the production of mosaic transgenic fish [for review, see Ref. 12]. In many fish species studied to date, multiple copies of transgenes were integrated in a head-to-head, head-to-tail, or tail-to-tail form, except in transgenic common carp and channel catfish where single copies of transgenes were integrated at multiple sites on the host chromosomes [13].

D. Inheritance of Transgenes

Stable integration of the transgenes is an absolute requirement for continuous vertical transmission to subsequent generations and establishment of a transgenic fish line. To determine whether the transgene is transmitted to the subsequent generation, P_1 transgenic individuals are mated to non-transgenic individuals and the progeny are assayed for the presence of transgenes by the PCR assay method described earlier [15,16]. Although the transgene may persist into the F_1 generation of transgenic zebrafish as extrachromosomal DNA [26], detailed analysis of the rate of transmission of transgenes to the F_1 and F_2 generations in many transgenic fish species indicates true and stable incorporation of the constructs into the host genome [for review see refs. 10,12]. If the entire germline of the P_1 transgenic fish is transformed with at least one copy of the transgene per haploid genome, at least 50% of the F_1 transgenic progeny will be expected in a backcross involving a P_1 transgenic with a nontransgenic control. In many of such crosses, only about 20% of the progeny are transgenic [13,15,16,26–28]. When the F_1 transgenic is backcrossed with a nontransgenic control, however, at least 50% of the F_2 progeny are transgenics (Table 2). These results clearly suggest that the germlines of the P_1 transgenic fish are mosaic as a result of delayed transgene integration during embryonic development.

IV. APPLICATION OF TRANSGENIC FISH IN BIOTECHNOLOGY

A. Biosynthetic Growth Hormone and Growth Enhancement

In recent years, growth hormone (GH) cDNAs and genomic DNAs have been isolated and characterized for several fish species [for review see Ref. 44]. Expression of rainbow trout or striped bass GH cDNA in *E. coli* cells results in production of large quantity of recombinant

GH polypeptide [45,46]. Because the GH polypeptide is highly hydrophobic and contains four cysteine residues, the newly synthesized recombinant GH polypeptide forms insoluble inclusion bodies in *E. coli* cells, rendering the hormone inactive. In an attempt to regain the biological activity of the recombinant hormone, Cheng et al. [46] developed a procedure for renaturing the protein. It involves dissolving the insoluble recombinant hormone in a buffer containing 8-M urea and renaturing the polypeptide by slowly removing the urea from the protein solution. The biological activity of the renatured protein was then assessed by an in vitro sulfation assay [47].

In a series of in vitro studies, Agellon et al. [45] showed that application of the recombinant trout GH to yearling rainbow trout resulted in a significant growth enhancement. After treatment of yearling rainbow trout with the recombinant GH for 4 weeks at a dose of 1 $\mu\text{g}/\text{g}$ body weight per week, the weight gain among the individuals of the hormone-treated group was two times greater than that of the controls (Fig. 4). Significant length gain was also evident in hormone-treated animals. When the same recombinant hormone was administered rainbow trout fry (see Table 2) or small juveniles by immersing the fish in a GH-containing solution, the same growth-promoting effect was also observed [45; Leong and Chen, unpublished results]. These results are in agreement with those reported by others [48–52]. However, importantly the growth enhancement effect of the biosynthetic hormone was markedly reduced when more than 2 $\mu\text{g}/\text{g}$ body weight of the hormone was applied to the test animals [45]. Recently, Paynter and Chen [53] have observed that administration of recombinant trout GH to spats of juvenile oysters (*Crassostrea virginica*), by the “dipping method”, referred to earlier also resulted in significant increases in shell height, shell weight, wet weight, and dry weight (Table 3). Furthermore, they also showed that oysters treated with recombinant trout GH, native bovine GH, or bovine insulin consumed more oxygen per unit time than controls.

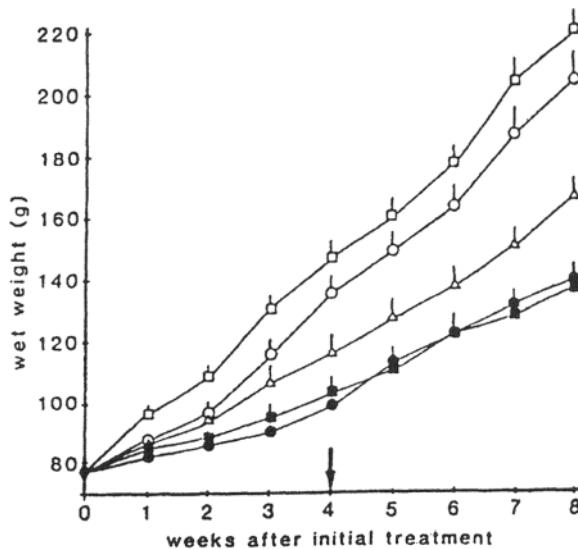


Fig. 4 Effect of recombinant trout GH on growth of yearling rainbow trout: Groups of yearling rainbow trout received intraperitoneal injection of recombinant GH or control extract for 5 weeks. Wet weights of GH-treated and control fish are shown (mean \pm SE). Open symbols, GH-treated fish: ○, 0.2 $\mu\text{g}/\text{g}$ body weight; □, 1.0 $\mu\text{g}/\text{g}$ body weight; Δ, 2 $\mu\text{g}/\text{g}$ body weight. Closed symbols, control fish: ●, mock-treated fish; ■, untreated fish. The arrow indicates the time of the last hormone treatment. (From Ref. 45.)

Table 3 Effect of Exogenously Applied recombinant Rainbow Trout Growth Hormone on Oyster Growth

Treatment	Initial ht (mm)	Final ht (mm)	Total wt (mg)	Shell wt (mg)	Dry wt (mg)
Control	8.14 (0.25)	11.68 (0.27)	206 (11)	136 (8)	6.10 (0.66)
10 ⁻⁹ M	8.04 (0.27)	11.74 (0.23)	199 (9)	131 (6)	6.87 (0.66)
10 ⁻⁸ M	8.72 (0.18)	12.79 (0.27) ^{ab}	244 (20)	171 (11) ^b	9.42 (0.41) ^{ab}
10 ⁻⁷ M	8.65 (0.32)	13.00 (0.36) ^{ab}	252 (13) ^b	189 (13) ^{ab}	9.41 (0.74) ^{ab}

^aSignificantly larger than the control group (*t*-test; *P*<0.05).

^bSignificantly larger than 10⁻⁹ M treatment group (*t*-test; *P*<0.05).

Initial ht represents mean size at the beginning of the experiment and final ht, total wt, shell wt, and dry wt are mean values determined after the 5-week treatment cycle was concluded. Height (ht) was measured in millimeters from the umbo to the ventral shell margin; weight (wt) was measured in milligrams. Standard errors of the mean (SEM) are in parentheses.

Source: Ref. 53.

The results summarized in the foregoing clearly suggest that exogenous application of recombinant fish growth hormone can enhance the somatic growth of finfish and shellfish.

B. GH and IGF-I Transgenic Fish

Although exogenous application of biosynthetic GH results in a significant growth enhancement in fish, it may not be cost-effective because of the following reasons: (1) high cost in producing large-scale purified biosynthetic GH; (2) treating individual fish with the hormone is labor-intensive; (3) the optimal hormone dosage for each fish species is difficult to determine; and (4) GH uptake into fish from an exogenous source is inefficient. If new strains of fish producing elevated, but optimal, levels of GH can be produced, it would bypass all of the problems associated with exogenous GH treatment. Moreover, once these fish strains have been generated, they would be far more cost-effective than their ordinary counterparts because these fish would have their own means of producing and delivering the hormone, and they could transmit their enhanced growth characteristics to their offspring.

Three aspects of fish growth characteristics that could be improved for aquaculture are (1) initial growth rate so that they reach maturation earlier; (2) enhanced somatic growth rate as adults to provide larger body size for market; and (3) fish with improved feed conversion efficiencies. Among these three, enhanced somatic growth rates by manipulation of GH or insulinlike growth factor gene show considerable promise. Zhu et al. [60] reported the first successful transfer of a human GH gene fused to a mouse metallothionein (MT) gene promoter into goldfish and loach. Although Zhu and his colleagues failed to present compelling evidence for integration and expression of the foreign genes in their transgenic fish studies, recent studies reported by many laboratories have successfully confirmed Zhu's work by demonstrating that human or fish GH gene can be readily transferred into embryos of many fish species and integrated into the host genome. Although a few groups have demonstrated expression of foreign genes in transgenic fish, Zhang et al. [13], Du et al. [14] Lu et al. [15], and Martinez et al. [62] have documented that a foreign GH gene could be (1) transferred to the target fish species; [2] integrated into the fish genome; and [3] genetically transmitted to the subsequent generations. Furthermore, the expression of the foreign GH gene may result in enhancement of growth rates of both P₁ and F₁ generations of transgenic fish [13–15,62].

In gene transfer studies conducted in common carp and channel catfish [13,16,54,55], about 10⁶ molecules of a linearized recombinant plasmid containing the long terminal repeat

(LTR) sequence of avian Rous sarcoma virus (RSV) and the rainbow trout GH cDNA were injected into the cytoplasm of one-cell, two-cell, and four-cell embryos. Genomic DNA samples extracted from the pectoral fins of presumptive transgenic fish were analyzed for the presence of RSVLTR-rtGH1-cDNA by PCR amplification and Southern-blot hybridization of the amplified DNA products using radiolabeled LTR of RSV or trout GH1 cDNA as hybridization probes. In the transgenic carp studies [13,16], about 35% of the injected embryos survived at hatching, about 10% of which had stably integrated the RSVLTR-rtGH1-cDNA sequence. A similar percentage of transgenic fish was also obtained when the RSVLTR-csGH-cDNA construct was injected into catfish embryos [55,56]. Southern-blot analysis of genomic DNA samples of several transgenic carp and catfish revealed that a single copy of the RSVLTR-rtGH1-cDNA sequence was integrated at multiple chromosomal sites [13].

The patterns of inheritance of RSVLTR-rtGH1 cDNA in transgenic common carp were studied by fertilizing eggs collected from nontransgenic females or P_1 transgenic females with sperm samples collected from several sexually mature P_1 male transgenic fish. DNA samples extracted from the resulting F_1 progeny were assayed for the presence of RSVLTR-rtGH1-cDNA sequence by PCR amplification and dot-blot hybridization [16]. The percentage of the transgenic progeny resulting from nine matings were: 0, 32, 26, 100 (four progeny only), 25, 17, 31, 30 and 23%, respectively (Table 4). If each of the transgenic parents in these nine matings carries at least one copy of the transgene in the gonad cell, about 50–75% transgenic progeny would have been expected in each pairing. Out of these nine matings, two siblots, both control $\times P_1$, transgenic progeny numbers as larger or larger than expected ($P < 0.05$) and the remaining had lower than expected numbers of transgenic progeny. These results indicate that, although most of these P_1 transgenic fish had RSVLTR-rtGH1 cDNA in their germline, they might be mosaics. Similar patterns of mosaicism in the germline of P_1 transgenic fish have been observed in many fish species studied to date [13,15,26,55,57,59].

If the transgene carries a functional promoter, some of the transgenic individuals are expected to express the transgene activity. According to Zhang et al. [13] and Chen et al. [16], many of the P_1 and F_1 transgenic common carp produced rtGH and the levels of rtGH produced by the transgenic individuals varied about tenfold Chen et al. [16] recently confirmed these results by detecting the presence of rtGH mRNA in the F_1 transgenic carp using an assay involving reverse transcription (RT)-PCR amplification. They found that

Table 4 Percentage of F_1 progeny inheriting PRSVLTR-rtGH1 cDNA

Family	Mating	N	Observed % inheritance	Expected % inheritance ^a
1	$P_1 \times$ control	17	0	50
2	$P_1 \times$ control	96	32	50
3	$P_1 \times$ control	26	42 ^b	50
4	$P_1 \times$ control	4	100 ^c	50
5	$P_1 \times P_1$	28	21	75
6	$P_1 \times P_1$	99	21	75
7	$P_1 \times P_1$	312	31	75
8	$P_1 \times P_1$	93	30	75
9	$P_1 \times P_1$	65	23	75

^aAll observed values are less ($P > 0.05$) than except

^bwhich is not significantly different ($P > 0.05$) than the expected, and

^cwhich is greater ($P < 0.05$) than expected (X^2).

Source: Ref. 16.

different levels of rtGH mRNA were detected in liver, eyes, gonads, intestine, and muscle of the F₁ transgenic individuals.

Because the site of transgene integration differs among individuals in any population of P₁ transgenic fish, they should be considered as totally different transgenic individuals and consequently, inappropriate for direct comparison of the growth performance among these animals. Instead, the growth performance studies should be conducted in F₁ transgenic and nontransgenic siblings derived from the same family. Recently, Chen et al. [16] conducted studies to evaluate the growth performance of F₁ carp in seven families. In these experiments, transgenic and nontransgenic full-siblings were spawned, hatched, and reared communally under the same environment. Results of these studies showed that growth response by families of F₁ transgenic individuals carrying these rtGH1 cDNA varied widely. When compared with nontransgenic full-siblings, the results of four out of seven growth trials showed 20, 40, 59 and 22% increases in growth, respectively (Table 5 and Fig. 5). The same extent of growth enhancement was also observed in F₂ offspring derived from crossing the fast-growing F₁ transgenics with nontransgenic controls. Similar results were observed when RSVLTR-csGH-cDNA was transferred into channel catfish. Because the response of the transgenic fish to the insertion of the

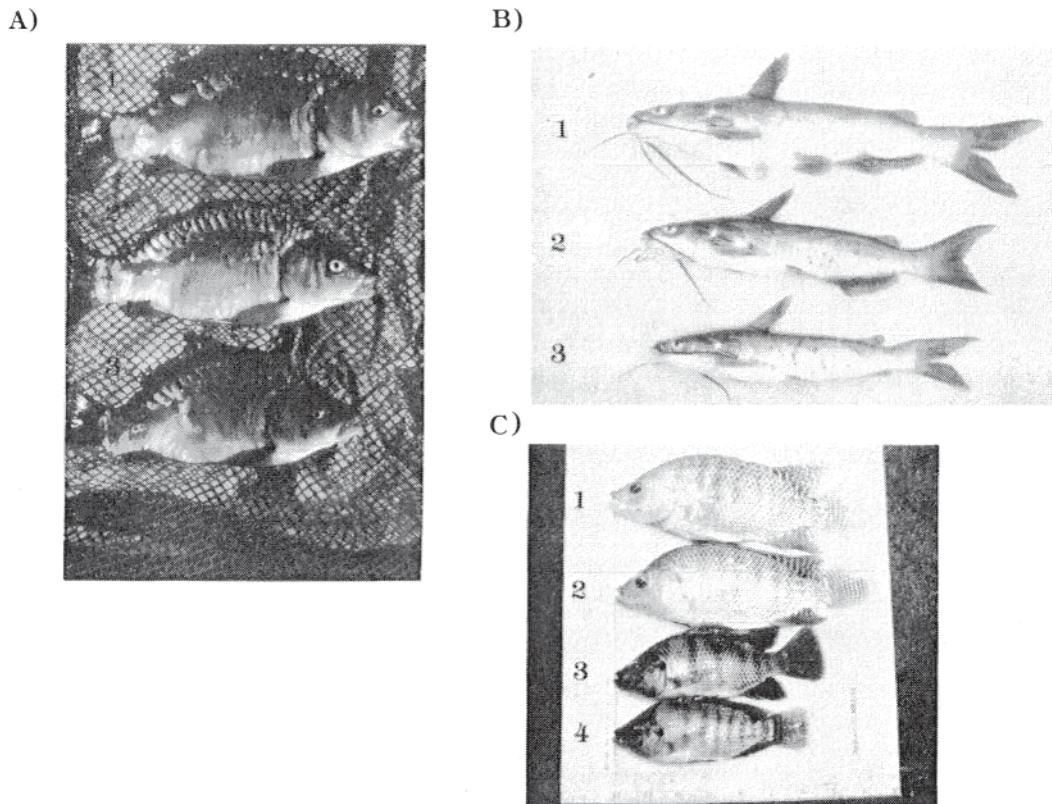


Fig. 5 Transgenic fish carrying rtGH transgene: (A) Transgenic common carp carrying RSV-LTR-rtGH1 cDNA (1, F₁ transgenic fish; 2, P₁ transgenic fish; 3, nontransgenic fish). (B) Transgenic channel catfish carrying RSV-LTR-csGH cDNA (1, F₁ transgenic fish; 2, P₁ transgenic fish; 3, nontransgenic fish). (C) Transgenic tilapia carrying carp β -actin promoter-rtGH1 cDNA (1 and 2, P₁ transgenic fish; 3 and 4, nontransgenic fish).

Table 5 Mean, Standard Deviation, Coefficient of Variation, and Percentage Difference in Body Weight of Transgenic Common Carp *Cyprinus carpio*, and their Nontransgenic Full-Siblings

Family	Mating	Genotype	N	Mean body weight (SD)	Coefficient of variation	% Difference	Range in body weight (g)
1	$P_1 \times$ control	T	31	120.6 (17.4)	14.4	20.8	95–173
		NT	65	99.3 (14.7)	14.8		65–129
2	$P_1 \times$ control	T	11	206.0 (45.2)	21.9	40.1	115–283
		NT	15	147.0 (48)	32.6		67–228
3	$P_1 \times P_1$	T	7	5.8 (3.4)	58.6	-26.6	1.8–11.3
		NT	21	7.9 (3.1)	39.2		3.3–17.9
4	$P_1 \times P_1$	T	28	66.1 (36.9)	55.8	58.5	18.5–338
		NT	65	41.7 (27.8)	66.6		8.3–141
5	$P_1 \times P_1$	T	17	14.7 (6.8)	46.3	21.5	6.5–30.4
		NT	82	12.1 (8.4)	69.4		3.9–56.1
6	$P_1 \times P_1$	T	97	114.2 (81.6)	71.5	-14.5	18.3–565.1
		NT	215	133.6 (83.6)	62.5		20.9–416.2
7	$P_1 \times P_1$	T	15	72.2 (58.0)	80.3	-1.5	7.1–214.4
		NT	48	73.3 (47.6)	64.5		8.7–203.3

T, transgenic; NT, nontransgenic; N, number of fish; SD, standard deviation.

Table 6 Growth Performance of Transgenic GH, IGF-I Fish and Control

Transgene	Regression line (y = a + bx)	Absolute growth rate (mg/d)	Feed conversion rate	K_n value
cBA-hGH	y = 4.27 + 1.52 ×	1.52	0.353	6.19 ± 0.43 ^{a*}
ccBA-rtGH1	y = 1.20 + 1.49 ×	1.49	0.327	5.98 ± 0.25 ^a
ccBA-rtIGF1	y = 0.37 + 1.32 ×	1.32	0.316	5.88 ± 0.39 ^a
Control	y = 0.06 + 1.04 ×	1.04	0.297	7.21 ± 0.56 ^b

*ANPOVA P<0.05

$$\text{Absolute growth rate} = \frac{\text{Final wt (mg)} - \text{initial wt(mg)}}{T - t}$$

$$\text{Condition factor } (K_n \text{ value}) = \frac{\text{Weight(mg)}}{\text{length}^{3.22} (\text{cm})} \times 1000$$

$$\text{Feed conversion rate} = \frac{\text{Wet weight (mg)}}{\text{dry food weight (mg)}}$$

RSVLTR-rtGH1-cDNA appears to be variable as a result of random integration of the transgene, the fastest growing genotype will likely be developed by using a combination of family selection and mass selection of transgenic individuals following the insertion of the foreign gene.

More dramatic growth enhancement in transgenic fish has been obtained by introducing Chinook salmon GH cDNA drive by the promoter of ocean pout antifreeze protein gene into Atlantic salmon embryos [14]. Some of these transgenic animals grew several times faster than their controls. A series of recent studies conducted by Lu et al. [manuscript in preparation] showed that both P₁ and F₁ transgenic medaka carrying a chicken β-actin gene promoter-human GH gene construct [15] or tilapia carrying carp β-actin promoter-rtGH1 cDNA exhibited a significant growth enhancement when compared with the nontransgenic siblings (see Fig. 5; Table 6). Some of the P₁ transgenic tilapia grow several times faster than their controls.

The effect of IGF-I transgene on somatic growth has also been tested in medaka and tilapia. IGF-I transgenic medaka and tilapia not only developed faster during embryonic development, they also exhibited a significant degree of growth enhancement (see Table 6).

V. GENERAL CONCLUSION AND FUTURE PROSPECTIVE

Transgenic fish technology has a great potential in revolutionizing the aquaculture industry. By introducing desirable genetic traits into finfish or shellfish, superior transgenic fish strains can be produced for aquaculture. These traits may include elevated growth enhancement, improved food conversion efficiency, resistance to some known diseases, tolerance to low oxygen concentrations, and tolerance to subzero temperatures. Recent progress in our laboratory and those of others has shown that transfer, expression, and inheritance of fish growth hormone and IGF-1 transgenes can be achieved in several finfish species, and the resulting animals grow substantially faster than their control siblings. This is a vivid example of the potential application of the gene transfer technology to aquaculture. However, to realize the full potential of the transgenic fish technology in aquaculture or other biotechnological applications, several important scientific breakthroughs are required. These are (1) identifying genes of desirable traits for aquaculture and other application; (2) developing targeted gene transfer technologies, such as embryonic stem cell gene transfer method or ribozyme gene

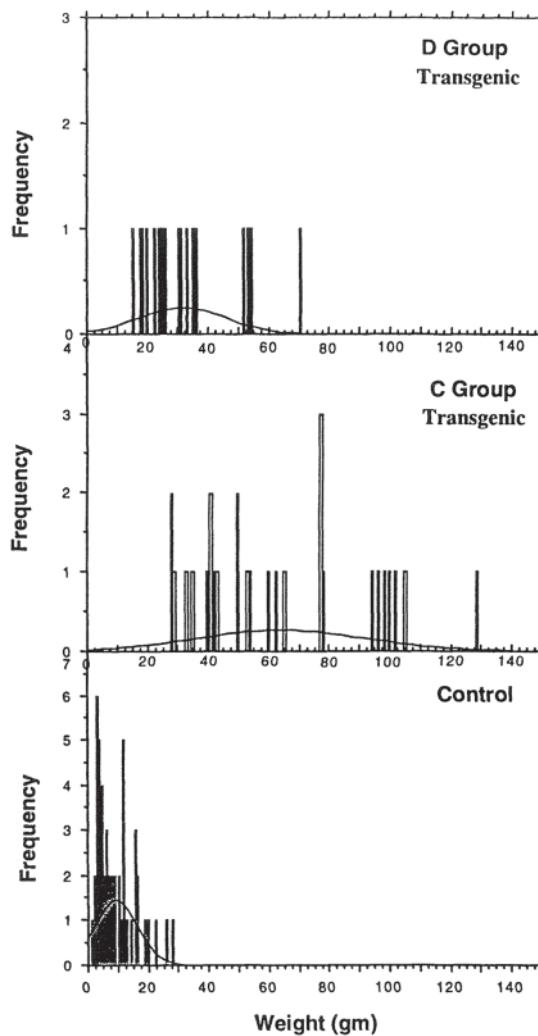


Fig. 6 Body weight distribution of P_1 transgenic and nontransgenic tilapia. D group, embryos electroporated about 2 h after fertilization; C group, embryos electroporated 30 min after fertilization. Frequency, number of animals in each weight group.

inactivation methods; (3) identifying suitable promoters to direct the expression of transgenes at optimal levels during the desired developmental stages; (4) determining physiological, nutritional, immunological, and environmental factors that will maximize the performance of the transgenic individuals; and (5) assessing safety and environmental effects of transgenic fish. Once these problems are resolved, the commercial application of the transgenic fish technology in aquaculture will be readily attained.

ACKNOWLEDGMENT

This work was in part supported by grants for NSF (DCB-91-05719, IBN-93-17132) to T.T. C. and USDA (93-37205-9073) and BARD (US-2305-93RC) to T.T. C. and Rex Dunham at Auburn University.

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Use of the Fish Antifreeze Protein Gene Promoter in the Production of Growth Hormone-Transgenic Salmon with Enhanced Growth Performance

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I. INTRODUCTION

The pioneering work of Gordon et al. [1], Brinster et al. [2], and others in creating transgenic mice by gene transfer technology has generated a new era in the application of different transgenic animals in basic and cancer research, animal husbandry, molecular farming, and many other biotechnology industries. In aquaculture, the production of transgenic fish is a powerful means to improve the performance of many farmed fish [3–5]. Unlike the traditional genetic-breeding methods, this new transgenic technology offers a direct and selective method for introducing a well-defined DNA encoding a gene product for a specific biological function into the genome of the recipients. Among its many possible applications, these manipulations could strengthen the genetically modified fish to tolerate cold or freezing temperatures, improve their disease resistance, enhance their growth performance, and permit utilization of low-cost feeds to cut operating expenses. There are many other desirable, but presently less well-defined, genetic traits that can be similarly integrated once the genes are isolated.

For the past several years, our laboratories have been pursuing two separate research programs using the teleost as our experimental model. One program focuses on the

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biochemistry and molecular biology of fish antifreeze proteins, which include studies of their structure and function, protein engineering, gene structure and expression, and gene transfer for the production of freeze-resistant transgenic fish [6]. The other program deals with the molecular endocrinology of teleost pituitary polypeptide hormones, their gene structure and control mechanisms, using both the gain of function (transgenic fish overexpressing the hormone of interest) and the loss of function (cell ablation techniques to selectively destroy hormone-producing cells) approaches.

The present chapter highlights an integrated approach using our knowledge from these two programs in the development of faster-growing transgenic salmon. These transgenic fish, in addition to their direct application in aquaculture, also serve as unique and important models to study growth hormone (GH) action [7].

II. BIOCHEMISTRY AND MOLECULAR BIOLOGY OF FISH ANTIFREEZE PROTEINS

One of the most intriguing biological phenomena is the freezing adaptation observed in several coldwater marine fish inhabiting ice-laden environments. Most fish species, because of the presence of sodium chloride and other small molecular weight electrolytes, have a freezing temperature of approximately minus -0.6°C and, therefore, cannot tolerate any lower temperatures. Yet the seawater temperature in the polar regions during the winter can be as low as -1.9°C. To avoid freezing, several marine teleosts inhabiting these regions produce a unique family of proteins, the antifreeze proteins (AFPs) or the antifreeze glycoproteins (AFGP) in their blood, with a concentration of 10–25 mg/mL. These proteins inhibit ice crystal formation and effectively lower their freezing temperature, allowing these animals to survive in their habitat. Since their first observation [8], these proteins and their corresponding genes have been isolated and characterized [6,9, and refs. within]. Although these proteins perform identical functions, they are structurally diverse and can be presently classified into one type of AFGP and three types of AFPs (Table 1). The origin and evolution of these proteins have been discussed [10]. In addition to the coldwater marine fish, antifreeze proteins or antifreeze-like proteins have been demonstrated to be present in overwintering insects, plants, fungi, and other microorganisms. However, the biochemistry of the AFPs from these organisms is not as well characterized as it is for the AFPs of marine fish [9, and refs. within].

The fish AFPs belong to the multigene family, with gene copies varying from 12 to 15 copies in sea raven, 40 to 50 copies in winter flounder, and 150 copies for the ocean pout.

Table 1 General Characteristics of Fish Antifreeze Proteins

Characteristic	Type I AFP	Type II AFP	Type III AFP	AFGP
Molecular mass and structure	3–5 kDa α-Helical, alanine-rich	11–13 kDa β-Turn, cystine-rich	6 kDa β-Bundle	2–23 kDa Disaccharide (Ala-Ala-Thr) _n
Biosynthetic pathway	PreproAFP	PreproAFP	PreAFP	Polyprotein precursor
Gene copy	30–40	15	150	Unknown
Tissue-specific expression	Liver-predominant and in skin, gills, scale	Liver-specific	Liver-predominant and in skin, gill, etc.	Unknown
Representing fish species	Winter flounder shorthorn sculpin	Sea raven, smelt, herring	Ocean pout	Antarctic fish, Atlantic cod

Coincidentally, the sea raven AFP, which has the least number of genes, is the most restricted in terms of its tissue-specific expression, being expressed only in the liver. Both the AFPs from the winter flounder (type I) and ocean pout (type III) are expressed primarily in the liver with some expression detected in other peripheral tissues, such as skin, gills, and so forth. The ocean pout AFP, with the largest number of gene copies, is the most promiscuous, and is expressed in a wide variety of tissues [11]. Whether some of these AFP genes in the larger multigene family are selectively expressed in certain tissues remains to be investigated. The seasonal appearance of these proteins are influenced by both environmental and hormonal factors. Growth hormone, in particular, inhibits AFP gene expression in the winter flounder [12].

III. ANTIFREEZE PROTEIN GENE PROMOTER AS A VECTOR FOR TRANSGENIC STUDIES

Compared with the mammals, there are now only a very limited number of well-characterized promoters from fish genes. These include the trout and salmon metallothionein [13,14], carp β -actin [15], salmon histone [14], protamine [16], and AFP genes from several fish species [17], thus limiting the selection of promoters suitable for aquacultural purposes. Except for a few cases, most transgenic studies involve the ectopic expression of a known or common gene in a different tissue in the recipient animal by artificially changing the promoter specificity. This results in the production of proteins outside their normal environment and separated from their usual physiological control mechanism. The selection of an appropriate promoter should include several considerations: namely, its tissue-specificity, the strength and inducibility of the promoter, ease of detection, as well as the possibility of any interference from the host genome. Because of the lack of appropriate embryonic stem cells, gene targeting by homologous recombination is not yet available in transgenic fish studies. The integration of transgenes is likely to be random.

The AFP gene promoters, when compared with other fish promoters, have several merits worthy of discussion. First of all, the AFP gene encodes naturally occurring nontoxic fish proteins. It might be more readily accepted by the consumer as compared with the use of viral promoter sequences. In addition, a recent report showed that retroviral vector sequences inhibit transgene expression in transgenic mice [18]. The synthesis of AFP is liver-specific or liver-predominant. Liver, a major secretory organ with well-organized cisternal, is well suited for protein secretion. In contrast, most of the other fish promoters lack the tissue-specificity to target the transgene selectively for liver expression. The protamine promoter, on the other hand, is restricted in the testis and has limited or specialized application.

Unlike other genes from which promoters are available, the AFP gene is present in only a few fish species. It is absent in most commercially important fish, including salmon, carp, catfish, tilapia, and many others, thus making its detection by PCR simple and at the same time, providing a useful marker for stock identification. On a molecular level, it is easier to manipulate the AFP gene promoters without any interference with endogenous genes [19].

Transient transfection studies in fish cell lines and Japanese medaka embryos [20], as well as transgenic studies in goldfish [21], loach [22], Atlantic salmon [23], and Pacific salmon [24] have demonstrated that the AFP promoters are active in a wide variety of fish species. Thus, common transcription factors present in the transgenic host can actively transcribe the AFP gene promoter properly. Recent studies from our laboratories show that the flounder AFP transgene is expressed specifically in the liver of transgenic salmon [P.Davies et al., manuscript in preparation].

Lastly, there are at least three types of AFP promoters with different degrees of tissue specificity that are responsive to different physiological signals. The promoters also contain

both positive and negative regulatory sequences. Thus, the AFP promoters offer a large repertoire of candidates for transgenic studies.

IV. DESIGN OF THE AFP-GH CHIMERIC GENE CONSTRUCTS FOR TRANSGENIC STUDIES

The AFP promoter from the winter flounder, *Pleuronectes americanus*, is the most extensively studied in our laboratories. However, this AFP gene is known to be inhibited by the action of

Name	Schematic of Gene Construct and PCR Strategy
(A) opAFP-GHc	<p>opAFP promoter GH cDNA opAFP 3' ATG TAG TATA AATAA</p> <p>A B 855 bp</p> <p>A D 333 bp</p> <p>C D 199 bp</p> <p>E</p>
(B) opAFP-GHf	<p>opAFP promoter salmon GH gene</p> <p>ATG TAG</p> <p>W E probes</p> <p>M N 543 bp</p> <p>C D 335 bp</p> <p>A D 898 bp</p>

Fig. 1 Design of AFP-GH gene constructs for transgenic studies: (A) Structure of the opAFP-GHc: [] ocean pout AFP gene promoter and 3' region; [] salmon GH cDNA 5' and 3' untranslated regions; [] salmon GH cDNA-coding regions. Three sets of primers were used to detect the presence of transgene, primers A/D, primers C/D, and primers A/B, with the expected size of PCR indicated. Primer A and primer B are specific for opAFP, and primer C and primer D are from the chinook salmon GH cDNA. Probe E (5'-GAAAATGTCAATGACT-3') corresponds to nucleotide positions 132–148 relative to the initiation ATG codon of chinook salmon cDNA. (B) Structure of opAFP-GHf: [] opAFP 5' promoter; [] exons of GH; [] 5'-untranslated sequence of opAFP; [] GH introns. The position of the primers A, C, D, M, and N, are indicated. Probes used are W (ocean pout-specific) and G and E (GH-specific).

GH [12,25], and it is obviously inappropriate to use it for GH-transgenic studies. Instead, we have selected the AFP promoter from ocean pout *Macrozoarces americanus* for most of our transgenic studies. Unlike the winter flounder, the AFP level in the ocean pout is not affected by GH. Although levels vary seasonally, significantly high AFP levels occur in the summer [26], indicating the proteins might be more constitutively expressed all year round.

Two different AFP-GH gene constructs have been made. They are opAFP-GHc and opAFP-GHf, representing the AFP promoter linked to the chinook salmon GH cDNA and a chimeric cDNA-genomic GH gene respectively (Fig. 1). As expected, the precursor RNA for GH mRNA was apparently processed properly when opAFP-GHf was used. Our ongoing experiments indicate that there is no apparent difference in these two constructs. Both constructs are effective in producing faster-growing transgenic salmon, and these results have been reported [27,28].

V. DETECTION OF GH TRANSGENESIS BY PCR

A. Incorporation in P₁ Founders

To screen the large number of samples available (500 eggs injected for each construct), a convenient and sensitive polymerase chain reaction (PCR) method was used. The strategy and the design of the PCR primers are shown in Figure 1. The detection of P₁ founders from the 1989 opAFP-GHc injection is used as an example (Fig. 2). The DNA from red blood cells of eight presumptive transgenic fish were analyzed by PCR using the A/D primers. As seen in Figure 2, the expected 333-bp PCR products were generated in these positive transgenic fish as well as in the opAFP-GHc. In contrast, both the distilled water alone and DNA from a nontransgenic sibling were negative, indicating the specificity of the PCR. To further demonstrate the identity of the PCR fragment, a GH cDNA probe was used for hybridization (see Fig. 2B), which confirmed the PCR data. Similarly, the presence of the expected 855-bp and 199-bp products using the A/B pair and C/D pair was demonstrated (see Fig. 2C and 2D). In the C/D pair, an additional larger 344-bp PCR fragment was generated from the host chromosome owing to the presence of an intron in the endogenous GH gene. This is used as a positive control to monitor PCR reaction. A detailed description of the data has been published [27].

Similarly, the opAFP-GHf founders were detected using the PCR technology [28]. The integration frequency is generally 2–3%, consistent with our previous studies on AFP gene transfer [23]. The incorporation of the transgene into the Atlantic salmon genome was demonstrated by genomic Southern analysis in both opAFP-GHc and opAFP-GHf-injected transgenics.

B. Inheritance of the F₁ Offspring

Both the male and female transgenic founders from both constructs were fertile and sexually mature and were used to generate F₁ offspring. Although it is straightforward to detect whether the transgene is present in the germline in the founder male by the direct analysis of the milt using PCR, it is as yet, difficult, if not impossible to detect the transgene in the egg directly owing to the small amount of DNA in a single female germ cell. To overcome the difficulty, the yolk sac frys generated from the cross between a presumptive transgenic female (positive in the blood cells or scale samples) and a control male were used instead. Shown in Figure 3 is the positive yolk sac frys of several females (Eo1, opAFP-GHc injection; FSJ-2, F0281, opAFP-GHf injection) by PCR using the M/N pair of primers. However, the inheritance of the transgene varies from a low value of 0% to a high value of 18%, suggesting that most if not all of the founders are germline mosaic [M. Shears et al., manuscript in preparation].

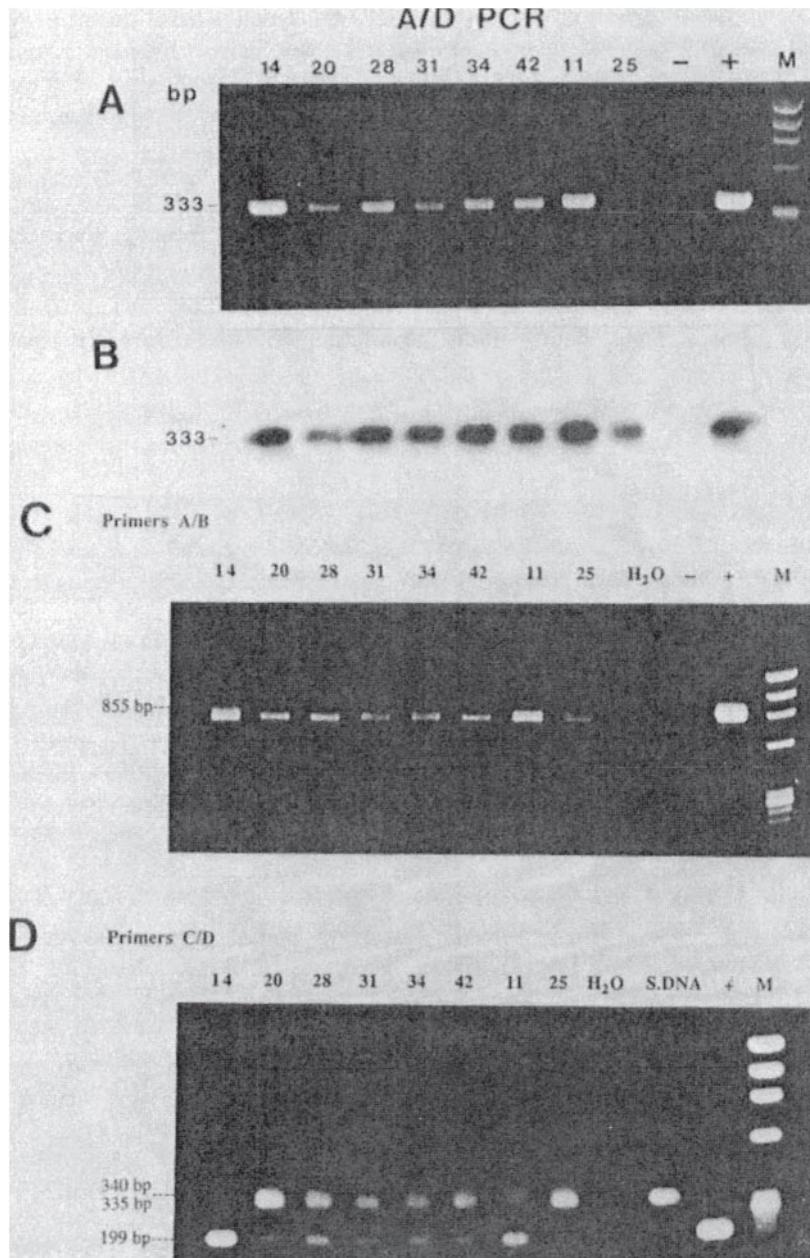


Fig. 2 Screening of Transgenic Salmon by PCR: Numbers 14, 20, 28, 31, 34, 42, 11, and 25 correspond to the positive transgenic fish. (S) DNA from noninjected salmon; (-) DNA from injected nontransgenic salmon; (+) opAFP-GHc; (M) molecular weight markers; λ -X-174RF DNA *Hae* III digest. (A) screening the transgenic salmon by PCR using primers A/D; (B) Southern-blot analysis of the PCR product from (A) using GH-specific probe E; (C) study of the integrity of the transgene by PCR using primers A/B; (D) confirming the presence of GH transgene by PCR using primers C/D. (From Ref. 27.)

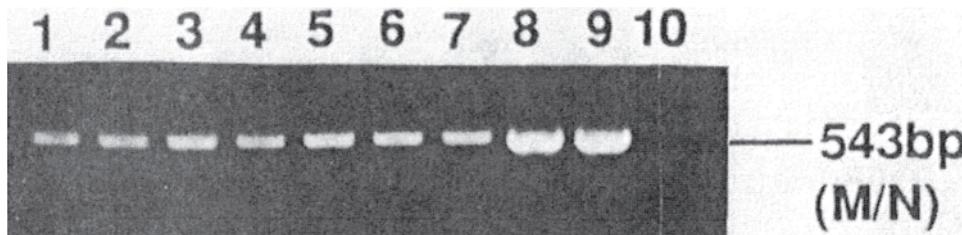


Fig. 3 PCR screening of yolk sac fry from crosses of transgenic females with wild-type males using primers M/N: Lanes 1, 2, and 3, Eo-1, (opAFP-GHc)×wild male; lanes 4, 5, and 6, FSJ-2 (opAFP-GHf) × wild male; lane 7, FO-281 (opAFP-GHf)×wild male; lane 8, 30 ng of plasmid opAFP-GHc; lane 9, 30 ng of plasmid opAFP-GHf; lane 10, 30 ng of genomic DNA from nontransgenic salmon.

C. Tissue-Specific Mosaicism in GH Transgenic Founders

In addition to the germline mosaicism, the variability of transgene incorporation in different tissues was similarly observed. As compared with transgenic mice, the mosaicism is generally more pronounced in fish, presumably a reflection in the late integration of the injected DNA in the latter species [3].

Figure 4 shows the results for the presence of GH transgene in gill, liver, and spleen for two opAFP-GHc-injected, large transgenic founders, Eo1 and Eo4. These two founders were positive when their red blood cells were analyzed. Although the GH transgene was present in all tissues examined for Eo1, only the spleen was positive for Eo4. The absence of the GH

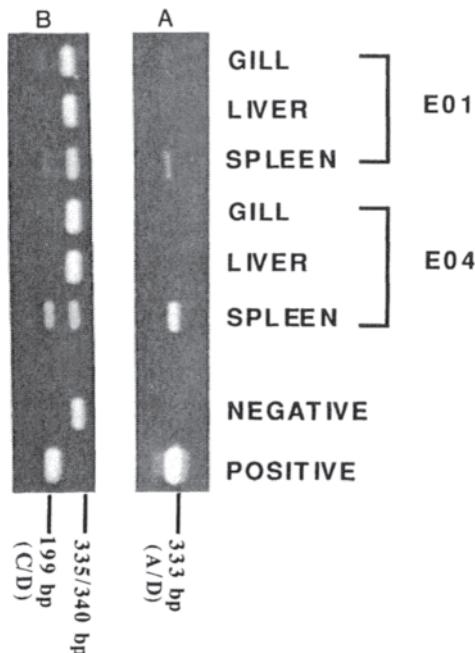


Fig. 4 Study of the integration of opAFP-GHc transgene in different tissues of transgenic fish Eo-1 and Eo-4: Eo-4 was a mosaic that contained the transgene in spleen cells, but not in the gill or liver cells.

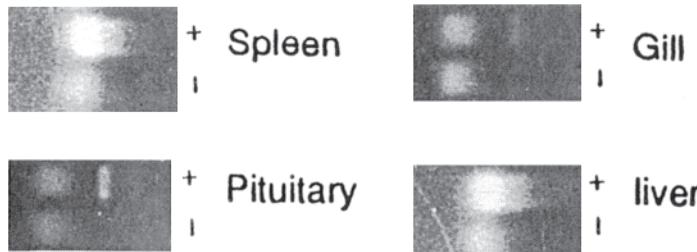


Fig. 5 Reverse PCR analysis of the expression of opAFP-GHc transgene: Poly(A)+mRNA was isolated from different tissues of transgenic fish Eo-1. The pituitary mRNA sample was extracted from nontransgenic salmon. (+), with M-MLV reverse transcriptase in the RT-PCR; (-), without M-MLV reverse transcriptase in the RT-PCR.

transgene in the liver has raised an interesting question about the mechanism of enhanced growth observed in this fish, which will be discussed in a later section.

VI. THE ACCUMULATION OF GH TRANSGENE mRNA

The ocean pout AFP mRNA is expressed in liver, skin, and gills, with liver being the predominant tissue [11]. One would anticipate, therefore, significant transcription of the GH transgene in the liver and other peripheral tissues. Total RNA was extracted from several tissues of Eo1 and Eo4, as well as from liver and pituitary from a nontransgenic control and analyzed by Northern blot analysis, using GH cDNA as a probe. Except for the pituitary of the control, the GH mRNA was not detectable in any nonpituitary tissues, indicating that either there was no transcription or the GH mRNA was below the limit of detection by Northern blot analysis. In a separate experiment, pituitary RNAs from the transgenic Eo1 and Eo4 were shown to contain levels of GH mRNA similar to the nontransgenic control (data not shown).

A more sensitive technique, reverse transcription PCR (RT-PCR) was used to examine the GH mRNA in these nonpituitary tissues from Eo1. As can be seen in Figure 5, GH mRNA was detected in liver, gill, spleen, as well as the pituitary.

These experiments, taken together, suggest that the GH transgene is weakly transcribed in the liver and several other peripheral tissues, which is consistent with the tissue-specificity of the ocean pout AFP promoter. In addition, the pituitary continues to synthesize endogenous GH mRNA without any significant feedback inhibition which has been observed in GH transgenic mice [29].

VII. GROWTH DYNAMICS OF THE GH TRANSGENIC SALMON

A. Growth Performance of the Founders

Since 1988, we have injected 500–1000 fertilized eggs for each construct during the spawning season of the Atlantic salmon. The hatched fry were screened as described in the foregoing. As a result, we have obtained transgenic salmon at different stages of development up to the F₂ generation. Invariably, the largest and faster-growing are the transgenic salmons, with an average four- to sixfold increase over the nontransgenic siblings. The results of the opAFP-GHc 1989 injection series is used as an example (Table 2). Although there is only a 1.4-fold difference in the GH level, which might be statistically insignificant, there is four- to fivefold

Table 2 Growth Rate and Plasma GH Levels in GH Transgenic (opAFP-GHc) and Nontransgenic Atlantic Salmon

	GH level (ng/mL)	Weight		Growth rate (% day)
		Oct 4/90	Jan 12/91	
Transgenic	39.9 + 14.8 (5)	20.9 + 5.13 (6)	47.3 + 9.5 (6)	0.836 + 0.186 (6)
Sibling	28.2 + 8.8 (7)	7.4 + 0.26 (43)	9.48 + 0.6 (43)	0.213 + 0.023 (43)
Fold difference	1.4	2.8	4.9	4.0

Source: Ref. 27.

difference in the body weight, length, and growth rate between these two groups. Our investigation was the first to demonstrate the dramatic increase in the growth rate of transgenic fish using the gene promoter and growth hormone gene derived from fish species. Similar results are obtained for all subsequent injection series for both constructs in Atlantic salmon [28] and the opAFP-GHc construct in Pacific salmon [24].

B. Growth Performance of the F1 Offspring

In addition to the inheritance of the genotype, the growth performance of the transgenic descendants (i.e., the expected large phenotype of the transgenic offspring) is vital to the successful development of superior transgenic broodstock. Our investigation demonstrates that the large phenotype is inheritable. Table 3 shows the results of a study using a transgenic male (Fo-1, opAFP-GHf) crossed with a control female. Although the inheritance is low (less than 3%), all the largest F₁ offspring (5 out of 200) are transgenic.

C. Growth Abnormality

Some of the largest transgenic salmon exhibit growth abnormality similar to the acromegaly observed in mammals. Similar characteristics have been observed in the transgenic Pacific salmon generated using the opAFP and MT promoters [R.H.Devlin, personal communication.] Whether these are the consequences of ectopic expression of the GH transgene, inadequate nutritional requirements, or other factors is at present difficult to assess. With the production of a large number of F₁ and F₂ offspring, we intend to examine these issues, as well as a means to minimize growth abnormality.

VIII. GENERAL DISCUSSION AND MECHANISMS OF ACTION

Our results with the growth hormone gene transfer using the antifreeze protein promoter can be summarized as follows:

Table 3 Body Weight of Transgenic Salmon Produced from the opAFP-GHf Construct

	Transgenic average weight (g)	Nontransgenic average weight (g)	Average fold increase
Founder P ₁	30 (22)	8 (964)	3.8 ×
F ₁ (Fo-1 × female)	23.8 (5)	4.5 (200)	5.2 ×

1. Both opAFP-GHc and opAFP-GHf are incorporated, integrated, expressed, and inherited in the transgenic salmon.
2. Both the opAFP-GHc and opAFP-GHf constructs are effective in stimulating growth in transgenic salmon, suggesting that there is little, if any, variation between the GH cDNA and GH cDNA-genomic chimeric gene.
3. The enhancement of growth is dramatic, an average of four- to sixfold increase, with several individuals as much as 10–30 times larger than the control.
4. Both the phenotype and genotype are inheritable.
5. There is no obvious impairment of reproductive ability in the transgenic founders and their F₁ offspring. Both fertile male and female transgenic founders and F₁ offspring were obtained. Viable offspring have been generated using both the eggs and milts of transgenic salmon.
6. The GH transgene is transcribed in the liver, gill, and some other tissues, in addition to the pituitary. However, compared with levels in the pituitary, the GH mRNA in these nonpituitary tissues is low.
7. Surprisingly, there is no direct correlation between the enhanced growth rate of the transgenic salmon versus its circulating GH level, indicating a paracrine or autocrine function, instead of the endocrine function.

In this study, we have tested two GH chimeric genes constructed with chinook salmon GH cDNA or a chimeric cDNA-genomic GH gene. This study showed no significant difference between these two constructs. Transgenic Atlantic salmon with either one of the GH gene constructs grew considerably more rapidly than nontransgenic siblings, suggesting that both constructs are functional. This indicates that the intron sequences in the transcripts of the opAFP-GHf transgene have been properly removed. This is further confirmed by RT-PCR analysis. This result differs from the study of the human GH gene (hGH) in fish for which the hGH transcripts could not be spliced efficiently [30]. It is, therefore, our opinion that the GH genes from closely related fish species may function better in transgenic fish, compared with those from mammals.

The results showed that there was no strict correlation between blood plasma GH levels and growth rates. The largest transgenic salmon with the fastest growth rate had the lowest GH levels (9 ng/mL), whereas one of the smaller, slower-growing transgenic fish had the highest GH level (91 ng/mL). Both rapidly growing nontransgenic fish had low GH levels (<12 ng/mL). The smaller nontransgenic salmon had GH levels ranging from less than 4 to 61 ng/mL. This result suggested that the level of circulating GH may not be a determining factor in growth-promoting activity. This also suggests that a high level of GH gene expression is not necessary for producing transgenic animals with an enhanced growth rate. In contrast, the lower expression of GH by a moderate gene promoter may be more suitable. Overexpression of GH has caused several notable pathological changes in transgenic mice, such as hepatomegaly and glomerular sclerosis, which shorten the life span [31,32]. In addition, female transgenic mice expressing hGH gene are commonly infertile owing to an impaired release of prolactin [29,33]. However, in our studies, both male and female transgenic salmon mature normally, produce fertile sperm and eggs, and moreover transcribe their endogenous GH genes normally. These findings suggest that the expression of the opAFP-GH transgene does not affect normal function of the pituitary in the transgenic fish. This result may be due to either low-level expression of the GH transgene, or to the lack of lactogenic activity of the fish GH, or both.

It is generally thought that production of a fast-growing transgenic animal depends on an increased level of GH in the circulation. Our data does not support this idea, because the

larger transgenic salmon have a low level of circulation GH. The dramatic growth observed in the transgenic salmon is probably due to locally produced GH. Because the opAFP promoter is active in many tissues (e.g., liver, gill, kidney, and skin [11]), it is likely that the opAFP promoter can direct the expression of the GH transgene in many tissues in the transgenic salmon. Our RT-PCR data demonstrated that at least two tissues, liver and gill, express the GH transgene. The locally produced GH may be more effective compared with the GH secreted from the pituitary, because the locally secreted GH can bind to its receptor immediately after its secretion. In contrast, GH secreted from the pituitary usually binds to the GH-binding protein [34] that transports it to its target tissues. The GH receptor [35] has to compete with the GH-binding protein for GH. Therefore, GH may be more effective in an autocrine and paracrine fashion.

The traditional somatomedin theory suggested that growth-promoting activity of GH is mediated by stimulating the production of IGF-I in liver [36]. This theory has been challenged recently by the identification of GH receptors in several different tissues, suggesting that GH may also function directly at its target cells. Results from our laboratories fully support this notion. One of the large transgenic salmon (Eo-4) found to be mosaic by blood and scale PCR died recently. DNA from different tissues was analyzed for the presence of the GH transgene. It was found that Eo-4 did not contain the GH transgene in its liver cells (see Fig. 4), suggesting that the action of GH on liver may not be absolutely necessary for regulating body growth. This implies that direct local effects of GH on other tissues may also be important in promoting body growth. The local effects of GH on peripheral tissues are not yet well understood. It has been suggested that GH may have a direct mitogenic effect. On the other hand, GH may act indirectly, possibly by stimulating local IGF-I production, which, in turn, could locally stimulate tissue growth.

IX. FUTURE DIRECTION

Many laboratories, including our own, have attempted to develop faster-growing transgenic fish with different degrees of success. However, several obstacles need to be overcome before the commercialization of these transgenic fish can be realized. On a molecular biological level, it might be necessary to regulate GH transgene expression levels, either by modulating the strength of the promoter, the time of expression, or the number of integration sites, as well as protecting the transgene from transcriptional inactivation. On a physiological level, the action of GH requires further examination to avoid growth abnormality caused by the overexpression of the hormone. Nutritional requirements of the transgenics is another area worthy of investigation. Lastly, there should be complementary techniques, such as the improvement of chromosome set manipulation or other novel transgenic technology, to ensure the sterility of the transgenic fish when farmed in open spaces. Considering the progress that has been made in the past several years, these new and exciting challenges will be fully explored to accelerate the adoption of this new technology.

ACKNOWLEDGMENTS

We thank Linda Mark for the preparation of the manuscript. This work is supported by a Natural Sciences and Engineering Research Council of Canada Strategic Grant to C.L.Hew, G.I.Fletcher, P.Davies, and D.Saunders, and a DFO Studentship to S.J.Du.

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Biotechnology of Marine Invertebrates: Current Approaches and Future Directions

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I. INTRODUCTION

Since ancient times, man has harvested marine invertebrates both as food and as a source of useful materials. In the past two or three decades, however, three major areas of emphasis can be clearly identified: (1) The rational exploitation and management of the natural shellfish resources, (2) the transition from harvesting the marine environment to farming those useful species through aquaculture technology; and (3) The increased interest in screening marine invertebrates as potential sources of bioactive compounds of potential medical and agricultural interest. However, despite the rapid advances in biotechnology over the last decade, the use of new molecular-based techniques in the aforementioned areas of interest has lagged far behind other agricultural applications. This is due to the relatively small body of knowledge that exists, possibly except for sea urchin embryos, on the molecular biology of marine invertebrates as compared with the large amount of published information on insects, vertebrate animals, and terrestrial plants. In comparison, there is just not enough currently known about gene expression events during reproduction, growth regulation, defense against disease, and symbiotic interactions in marine invertebrates to allow the establishment of successful strategies for biotechnological interventions. In this chapter, we will summarize selected approaches and current problems in the exploitation of marine invertebrates and the development of shellfish mariculture, and we will discuss the gaps in our knowledge that must be addressed for us to fully develop biotechnology-enhanced applications in these areas.

II. MARINE INVERTEBRATES AS SOURCES OF NATURAL PRODUCTS

A. Useful Materials from Marine Invertebrates

Sponges, oysters, clams, snails, corals, and other marine invertebrates have been collected for centuries for the extraction of natural dyes, nacre, pearls, coral, and a variety of useful materials.

More recent applications of materials from marine invertebrates, including the development of novel glues from mussels [1] and the use of seafood byproducts, such as the exoskeleton of crustacea for chitin production [2], are very promising indeed. Chitin is a homopolysaccharide of N-acetylglucosamine, and a major constituent of the shell of crabs, lobsters, and shrimp. After cellulose it is the most abundant polysaccharide in the biosphere, and because of its unique properties, together with its by-products, chitosan, chitotriose, and chitobiose, it has found applications in industries, medicine, and agriculture [2,3]. Application of a biotechnological approach to both prokaryotic and eukaryotic organisms that use and degrade chitin by enzymatic means (i.e., chitinases) may, in the near future, provide the molecular tools for a more efficient extraction and processing of this polysaccharide [2,3].

One of the most successful application of biological substances from marine invertebrates to medicine is the use horseshoe crab amoebocyte lysates for endotoxin detection in pharmaceuticals and medical devices [4]. The observation that fatal intravascular coagulation occurred in the American horseshoe crab *Limulus polyphemus* following injection with live or heat-killed gram-negative bacteria [5] and the in vitro gelation reaction between endotoxin and a lysate made from its amoebocytes [6] led to the development of the *limulus* amoebocyte lysate (LAL) test as a useful test in pharmacology and clinical medicine. This gelation reaction can also be observed in the other three species: the Indian horseshoe crab *Carcinoscorpius rotundicauda* and the Japanese horseshoe crabs *Tachypleus tridentatus* and *T. gigas*, although the coagulogens isolated from the four species are somewhat different [7]. For the preparation of *limulus* lysate the horseshoe crabs are bled under aseptic conditions and immediately returned to the sea. The amoebocytes are separated by centrifugation, lysed, and processed according to the various manufacturer's specifications. Following preparation of the lysate pool, cations are added and the sensitivity of the lysate is adjusted. The liquid lysate is dispensed into either multitest vials or single-test vials and lyophilized. Each lot of *limulus* lysate produced by a licensed manufacturer is tested by the Center for Evaluation and Research, US Food and Drug Administration against a standard endotoxin [8], and the sensitivity is expressed in endotoxin units (EU) per milliliter. Several modifications and improvements of the original gel endpoint method LAL test have been developed and include turbidimetric and chromogenic substrate methods, automated systems, or combinations with enzyme-linked immunosorbent assay (ELISA) [9,10]. The original LAL test was used as an alternative to the rabbit fever test to detect contaminating "pyrogens" in radiopharmaceuticals [11]. Nonisotopic pharmaceuticals and invasive medical devices were eventually included for testing as well, and the application of the LAL test as a possible method to detect bacterial endotoxins in various clinical specimens was evaluated. It has already been used for the detection of endotoxemia [11] and bacteriuria [12] and for the diagnosis of gram-negative spinal meningitis [8]. Additional applications of the LAL test are foreseeable, such as the detection of gram-negative bacterial contamination in waters and food products. It has already been used to assess the microbial quality and condition of several species of raw fish and to measure the horizontal and vertical distribution of bacteria in oceanic waters [13].

During the past few decades the LAL test has performed remarkably well and, although today, the LAL test is the most sensitive and specific procedure available for the detection of bacterial endotoxin because it can detect as little as 1 pg [8], considerable effort has been invested in the reproducibility of the test results. Significant variations in the activity of the *limulus* lysate pools have been reported and attributed to the seasonal influences on the crabs [14]. Sullivan and Watson [11] improved the sensitivity and eliminated much of the seasonal variability of the lysate by extracting it with solvents, such as chloroform. However, preparation methods may affect the sensitivity of different lysates to native endotoxin [15], and sensitivities may vary among lysates produced by different manufacturers [16]. Although much has been

improved in the standardization of the test by the removal of inhibitors, addition of divalent cations, and lyophilization, because the variability between stocks is not well understood and horseshoe crab populations are declining dramatically worldwide, there is a clear need for alternative solutions. Certainly, the application of molecular techniques to the production of recombinant components of the horseshoe crab clotting cascade would likely alleviate the problems just indicated. Several components of the clotting cascade of *L. polyphemus*, the endotoxin-binding protein [17], and the factor C [18] have already been purified, cloned, and fully characterized, and it is likely that in the future a recombinant LAL test will become available.

B. Marine Invertebrates as Sources of Bioactive Compounds

In addition to the useful materials and biologicals illustrated in the foregoing, many marine invertebrates produce natural compounds that affect the growth, metabolism, reproduction, and survival of other types of organisms, and, hence, are referred to as "bioactive." The marine environment represents an enormously diverse ecosystem harboring organisms that have shown great potential as sources of bioactive molecules. These include effective diagnostic tools and therapeutic drugs with antiviral, bacterial, fungal, parasitic, immunostimulatory, immunosuppressive, and cytotoxic properties. The targets of the studies have included marine invertebrates, and their mutualistic associations with marine microbial communities that inhabit environments subject to various conditions of UV radiation, temperature, hydrostatic pressure, or nutrient concentration, such as the tropical and subtropical sea, deep sea, thermal vents, and polar environments. So far, limited screening of this rich natural resource has provided a variety of unique biologically active compounds isolated from invertebrate and microbial taxa. Those include potentially effective therapeutic agents with antiviral, antibacterial, and antitumor properties produced by invertebrates from the classes Porifera, Cnidaria, Mollusca, Echinodermata, Bryozoa, and Urochordata. The production of bioactive substances in sessile or slow-moving benthic invertebrates has been hypothesized to have evolved as part of an overall antipredatory strategy [19]. So far, a large number of bioactive chemicals have been described and have been the subject of comprehensive reviews [20]. Examples of these bioactive substances are peptides (didemmins, eudistomins, ecteinascidins, ulicyclamides, clavepictins), toxins (holotoxins, holoturins, mucotoxins), pigments (tunichromes), and alkaloids [19–23]. Structural studies on the identified bioactive compounds have provided information on the molecular basis of their biological activity, contributed to drug design through molecular modeling, and will facilitate the efficient large-scale manufacturing of such therapeutic agents through organic synthesis or expression in prokaryotic or eukaryotic cell culture systems. Moreover, the deep understanding of the modes of action of the bioactive compounds within the source organism, between community components, or between these organisms and the environment will provide insight on novel mechanisms of molecular adaptation to function under extreme environmental conditions.

Another group of bioactive molecules from marine invertebrates are carbohydrate-binding proteins (lectins). The biological activities of lectins are diverse and include cell activation, insulin-like, cytokine-like, and cytotoxic activities. Lectins have been useful tools for cell and glycoconjugate separation, histochemistry, tumor diagnosis, and cell-specific gene targeting [24]. They have been identified in taxa from viruses and bacteria to vertebrates [25]. Their potential ligands, simple or complex carbohydrates, occur in all cells and biological fluids, suggesting that protein-carbohydrate interactions constitute a basic phenomena common to all organisms, most likely within an extensive functional diversity. The potential therapeutic value of lectin ligands with antiviral properties, such as sulfated polysaccharides from marine

invertebrates, has been determined [26]. Recent interest on lectins in marine invertebrates species stems from the fact that they may represent self-nonself recognition and defense molecules that constitute precursors of specific and nonspecific recognition, opsonic and complement-activating factors such as the acute-phase reactants, and humoral or cellular bactericidal lectinlike molecules related to inflammatory processes in homeotherm vertebrates [27]. Furthermore, a large number of colonization factors of those microbial communities associated with invertebrate and vertebrate species, such as bacterial adhesins and protozoan cell adhesion molecules, are actually lectins [28].

Marine microorganisms become equally rich sources of compounds with novel properties, including toxins produced by bacteria and dinoflagellates, bacterial polysaccharides, and enzymes [29–31]. Close relations between marine invertebrate species and microorganisms, including symbiotic associations and interactions during larval settlement, have been characterized and provide insights to the regulation of host-symbiont-microbial community molecular interactions. Many of the compounds isolated from marine organisms, such as sponges, may be produced by associated bacteria. For example, several diketopiperazines previously ascribed to the sponge *Tedania ignis*, are produced by a marine *Micrococcus* sp. associated with this sponge [32]. The halichondrins, complex polyether macrolides from the marine sponge *Halichondria okadai* [33], clearly illustrate this interesting interaction. The isolation of these compounds from a taxonomically unrelated sponge species in the genus *Axinella*, suggested that these compounds were probably originated in microbial flora components common to both genera [34]. Halichondrin B, an extremely potent antimitotic agent, inhibits tubulin polymerization and microtubule assembly [34] and has been recently selected as representative of a new chemotype for anticancer drug development programs by the NCI Decision Network Committee. This represents only one example of the wide range of bioactive compounds produced by marine organisms and emphasizes their great potential for biomedical applications, which has encouraged large-scale screening by the National Cancer Institute, US Public Health Service. A standard screening system consisting of 60 *in vitro* cell lines, representing various tissues, has been established, together with human immunodeficiency virus (HIV)-infected human lymphoblastic cell lines, for systematically testing extracts from organisms for cytotoxic and antiviral activity.

III. FISHERIES AND AQUACULTURE OF MARINE INVERTEBRATES

As the seafood demand has been projected to increase substantially worldwide during the next century, it has also become evident that most of the major natural fisheries are being exploited beyond sustainable limits. *Aquaculture* (the farming of aquatic organisms under controlled conditions), which is still a relatively new industry, will undoubtedly be the major contributor to satisfying such demand. Globally, marine invertebrate aquaculture initiatives and most significantly mariculture, have made impressive progress during the past two decades, especially for crustacean (shrimp, prawn, and lobster) and mollusk (oysters and clams) species. However, despite considerable improvements concerning the design and implementation of more efficient growing and harvesting techniques, there is a clear need in the shellfisheries and shellfish aquaculture industries to address a variety of problems related to reproduction, larval recruitment and settlement, growth regulation, disease diagnosis, prevention and remediation, as well as those associated with shellfish as vectors of disease to the consumer. Innovative approaches are needed to address the aforementioned issues, and recombinant DNA techniques are likely to play a major role in the process.

A. Biotechnological Intervention in Reproduction, Larval Recruitment and Settlement, and Growth Regulation of Marine Invertebrates

1. *Shellfish Reproduction*

It is widely accepted that efficient cultivation of a species requires that its life cycle be completed in captivity, and one critical aspect in the commercial cultivation of some crustacean species is the lack of a reliable seed supply. In Ecuador and other South and Central American countries, where the penaeid shrimp species farmed do not spawn naturally in captivity, larvae are collected in the natural environment and, although it is claimed that these are of higher quality compared with those hatched in captivity [35], serious problems develop from this practice. First of all, as the larvae are continuously harvested in the wild to meet the growing demands of the shrimp aquaculture industry, a critical reduction of the natural stocks is to be expected in the near future and, thus, increasing shortages in the seed supply are foreseeable. Second, the continuous introduction of environmental material to the shrimp-rearing facilities is associated with a significant risk of disease by viral, bacterial, and fungal pathogens. Current empirical methods for the induction of gonad maturation, such as the unilateral eyestalk ablation [35,36], yield low-quality larvae and are not conducive to the establishment of the much needed selected broodstock. Therefore, studies on the reproductive cycles of the shrimp species of interest, and the development of technology for the manipulation of gonadal development and maturation and spawning constitute relevant needs for this industry. As in the finfish aquaculture industry, a detailed knowledge of the physiological and molecular aspects of crustacean endocrinology are required for accomplishing any substantial progress in this area. A considerable body of information about the endocrinological control of gonadal maturation by juvenile hormones in insects already exists [37,38], and is currently being used as a source of possible approaches to gain a better understanding of equivalent phenomena operative in the crustacea. The synthesis rates of methyl farnesoate, a juvenile hormone-like compound, in mandibular organs, appear to correlate with ovarian developmental stages [39] and induce noticeable increases in blood vitellogenin levels when injected into crab females [40]. Considerable progress has been made in the past few years in the understanding of regulation of gonadal maturation and activity by juvenile hormones, ecdysteroids, and heterologous factors [41,42]. However, more research in the molecular aspects of crustacean endocrinology is needed to efficiently apply biotechnological approaches to control and manipulate the spawning of commercial species in captivity. Such efforts should allow the establishment of genetically improved shrimp broodstock and, by doing so, the risks of disease in shrimp farming and collateral depletion of the natural resources would be avoided.

2. *Larval Settlement*

Another sensitive step in the aquaculture of most marine invertebrates is ensuring that larval metamorphosis into juveniles occurs with a minimum mortality loss. Understanding the biochemical and molecular basis of the biological processes involved in metamorphosis has been a key area of research for the aquaculture of mollusks [43]. Many sessile invertebrates, such as the oysters and mussels, have free-swimming larvae which, on completion of development, will become competent and respond to environmental cues that indicate an appropriate substrate for settlement and metamorphosis [44–46]. Therefore, the predictable and efficient settlement of larvae on appropriate substrates for their further distribution on growout areas in ponds or open waters, is a crucial step in the farming of shellfish such as oysters and mussels. Several environmental factors, including light, temperature, salinity, water

flow, surface texture, and composition, affect larval settlement [47]. In addition, exogenous chemical factors that affect the larval nervous system appear to be responsible for inducing the behavioral changes that result in setting. Membrane receptors have been proposed to mediate the perception of those environmental cues [47–49] and may be present at early stages, even before the effector systems or morphogenetic pathways are functional [46,52,53]. Bacterial films or their soluble products are effective inducers of larval settlement and metamorphosis in many sessile marine invertebrate species, including oysters, polychaetes, and coelenterates [52–56]. Among the soluble cues of bacterial origin, l-dopa (l-3,4-dihydroxyphenylalanine) [46,56] and ammonia [57] induce larval settlement in oysters. Morse et al. [58] were the first authors to describe a potential neurotransmitter regulation of metamorphosis in abalone larvae by demonstrating that these larvae could be induced to settle and metamorphose in the presence of γ -aminobutyric acid (GABA). Morse and Morse [59] were able to show that natural inducers of abalone metamorphosis do exist in nature demonstrating that such bioactive compounds were present in the environment on the surfaces of red algae. Although the specific endogenous metamorphic inducer still remains undescribed in *Haliotis*, subsequent work has slowly dissected the molecular components to demonstrate the importance of calcium ion flux [60] and G protein-gated signal transduction pathways [61,62]. Once specific metamorphic regulatory mechanisms are described for a species, effective strategies for manipulating larval metamorphosis can be implemented to control the timing, survivorship, and uniformity of metamorphosis in an aquaculture setting.

3. Growth Regulation

Marine invertebrates present an interesting challenge because there is now no known gene that is specifically responsible for growth regulation. There is significant experimental evidence suggesting that the growth of marine invertebrates may be controlled by factors similar to those of vertebrates, such as growth hormone and insulin-like growth factors. Treatment of oysters or abalone with fish growth hormone and insulin results in significant growth enhancement [63]. Therefore, the identification and characterization of putative invertebrate growth hormone (GH) and insulin-like growth factors (IGFs) becomes a clear priority. The cloning of the GH and IGF genes from the species of interest will allow the production of recombinant hormones for growth enhancement in aquaculture and, in the long-term, develop fast-growing transgenic oysters through gene transfer technology.

It is likely that vertebrate GH has evolved from an ancestral invertebrate gene and thus trying to decipher the evolution of GH may shed light on how to identify an invertebrate homologue. In considering a physiological function for the vertebrate GH family ancestral gene, there are three critical points: (1) we know that no homologues of GH family genes have yet been identified in any invertebrates, suggesting that the common ancestral forerunner of these hormones may have appeared after the chordate ancestors diverged from the mainstem of deuterostome invertebrates; (2) then, in looking for an ancestral physiological function, we are restricted to considering the oldest extant group of chordates in which GHS are found, which are fish; and (3) when looking at what is currently known about the physiological functions of GH members in fish, one common function does emerge: GH, prolactin, and somatolactin, all are known to be actively involved in the osmoregulatory responses of teleosts [64]. These observations suggest that the GH family ancestral gene may have been involved in the osmoregulation response of the deuterostome invertebrate ancestors of the chordates. Thus, a detailed consideration of osmoregulation is warranted in establishing a functional model of GH family gene evolution [63].

In focusing on osmoregulation as a potential area of physiological activity for the GH ancestral gene, it is important to note that modern marine invertebrates generally exhibit an isosmotic cellular response to changes in environmental salinity. Following hyperosmotic exposures (i.e., a sudden increase in external salinity), cellular metabolism is shifted toward the catabolism of large molecules to increase the number of intracellular osmolytes, thereby increasing the osmotic pressure of the cytoplasm. Following hypoosmotic exposures, cellular metabolism is shifted toward the anabolism of macromolecules to reduce the number of intracellular osmolytes. The metabolites used for this metabolic shuffling are generally in flux between the cytoplasm and short-term nutritional reserves, with the net mass balance shifted one way or the other, as appropriate, to maintain cell volume. Animals also have structural deposits that serve as long-term sources or sinks of metabolites, but these are generally not accessed for short-term, immediate metabolic responses. The flux of metabolites to structural components are reserved for long-term allocations, such as growth and development, or dire metabolic needs, such as starvation. It is possible that the vertebrate GH family ancestral gene produced a protein product that regulated the intracellular flux of metabolites to and from the pool of short-term nutritional reserves in response to sudden changes in environmental salinities. In looking for invertebrate GH homologues, we should be focusing our attention on trying to identify genes that regulate the translocation of small macromolecules. Interestingly, two recent investigations of cerebral ganglia-derived proteins in the mussel *Mytilus edulis* have demonstrated a potential role in regulating amino acid uptake into proteins [65,66], which strongly supports this idea of an ancestral-like invertebrate GH being involved in amino acid transport.

B. Molecular and Serological Approaches to Disease Diagnosis and Control in Fisheries and Aquaculture of Marine Invertebrates

Viral, bacterial, fungal, and protozoan epizootic diseases are recognized as significant detrimental factors for the successful exploitation of natural and cultivated stocks of marine shellfish, such as crabs, shrimps, oysters, and clams, from coastal areas around the world. For example, mass mortalities caused by protozoan infections such as "dermo" (*Perkinsus marinus*) and MSX (*Haplosporidium nelsoni* and *H. costalis*) have caused a critical reduction of existing populations and impaired production of oysters in the Chesapeake Bay [67]. Other microorganisms, such as *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, are facultative bacterial pathogens of marine shellfish that may be latent in natural populations and become patent (and cause mass mortalities) under circumstances of stress caused by pollution or temperature changes of the natural environment or marginal aquaculture conditions. One of the major problems to address resides in that the molecular mechanisms for defense against disease in marine invertebrates are largely unknown. Therefore, the elucidation of these mechanisms is critical for understanding two important aspects: (1) the pathobiology of invertebrate species important from the ecological and economic standpoints, in particular, molluskan and crustacean marine species that are harvested commercially or considered with potential for aquaculture, and (2) the host-pathogen interactions in shellfish species that constitute vectors of disease to humans.

1. Disease in Fisheries and Aquaculture of Marine Invertebrates

As the invertebrate mariculture industry has undergone explosive growth in the past few decades, it has become increasingly apparent that the prevention and control or remediation of disease

remains as the one essential unsolved problem. In particular, under high-density rearing, animals may become stressed, and thus more susceptible to viral, bacterial, fungal, and protozoan infections. An additional problem that has faced the shrimp culture industry as it has developed over the past two decades, has been the dependence on wild stocks for larvae from several penaeid species that do not breed regularly in captivity. Moreover, the problem has been compounded by the common practice by the shrimp farmers of transferring and testing local and exotic penaeid shrimp species that may be more profitable, based on desirable characteristics, such as larger harvest size, faster growth rates, disease resistance, easier reproduction, and larval rearing and growth at colder water temperatures [68,69]. Hence, larvae and broodstock from shrimp farms or from wild stocks have been transferred between different areas, together with the pathogens that have caused mass mortalities of native stocks. Although shrimp aquaculture is a relatively recent activity in several countries of Asia and the Americas, it has been a fast-growing industry and presently constitutes a significant source of employment and export revenue. However, the acquisition of knowledge and technical ability to prevent the frequent mass mortalities that occur from viral and bacterial epizootics has not made comparable progress, and catastrophic losses in productivity are becoming increasingly frequent. Therefore, the effect of infectious disease in shrimp farming, as for most shellfish aquaculture initiatives, is severe and the serious economic consequences for this industry clearly reveal the need for intense research in this area. The development of specific and sensitive diagnostic methods is needed for epizootiological surveys that would allow the certification of disease-free stocks. In the long term, the development of disease-resistant shellfish varieties through transgenic technologies will provide the appropriate broodstock for continued growth of this industry.

Several viral, bacterial, and parasitic diseases have significantly affected the development of commercial mariculture. For example, at least six distinct viral diseases of cultivated penaeid shrimp can now be identified [68–70], and new viruses are reported in the current literature each year. One of the great difficulties in addressing the problem of viral diseases is the lack of invertebrate cell lines. Therefore, the isolation and genetic characterization of viruses to identify those genes that could constitute targets for inhibition strategies through antisense mRNA approaches is severely hampered.

Bacterial infections, in particular by *Vibrionaceae*, are relevant both as shellfish diseases for mollusks and crustacea, as well as for the seafood consumer. The massive use of antibiotics in the aquaculture setting is a common, but high-risk, practice because, in the long-term, selection of resistant strains may occur. Vibrios are facultative bacterial pathogens of marine shellfish. From the human health standpoint, the genus *Vibrio* includes a very significant group of pathogens, among which *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* are probably the most important species. *Vibrio parahaemolyticus* is an important species because it is a major cause for a dangerous acute gastroenteritis [71] and a considerable number of fatalities occur yearly in the United States as a result of consumption of improperly prepared seafood, very commonly raw oysters, or contact with contaminated seawater. In Japan *V. parahaemolyticus* is the leading cause for a common infectious food poisoning syndrome [72,73]. Hemolytic strains (Kanagawa-positive: K⁺) are usually recovered from patient stool specimens while K⁻ strains are more commonly isolated from environmental sources and are assumed to be nonpathogenic, but inconsistencies abound and frequently, K⁻ strains are the only ones isolated from patients. Strains vary in their hemagglutinating activity, usually mannose-sensitive, and adherence to epithelial cells [72] that would be mediated by the bacterial pili [73]. Although vibrios are part of the normal flora and do not produce disease when ingested by shellfish, these bacteria exhibit remarkable proteolytic and chitinolytic properties that may be the cause for their pathogenicity and probably enter the invertebrate body through

a cuticular injury or during ecdysis. Among the *Vibrio* the species most frequently associated with crustacean septicemia and mortality are *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum*. *V. parahaemolyticus* is present in wild stocks of blue crabs from the Chesapeake Bay and constitutes the most common *Vibrio* spp. isolated from the diseased crabs [74]. In addition, vibrioses frequent in oyster larval and adult populations as well as other mollusks of commercial importance, such as the abalone. *V. vulnificus* has been proposed to serve as a symbiont that contributes to the homeostasis of its host, the eastern oyster *Crassostrea virginica*, by mediating osmoregulation. However, in the cultured juvenile oyster *C. virginica* and other bivalves, such as the clam *Mercenaria mercenaria* and the European oyster *Ostrea edulis*, vibrios infect the conchiolin-containing ligament and the periostracum, preventing normal ligament function and shell deposition [75]. We have already documented [76] that lectins from the blue crab *Callinectes sapidus* interact with selected serotypes of its pathogen *V. parahaemolyticus*, a facultative pathogen of this species [77]. Multiple lectins present in serum of *C. sapidus* recognized 10 of 11 different isolates of *V. parahemolyticus*. Interestingly, the only *V. parahemolyticus* strain that did not interact with the crude serum or the pure lectins had been isolated from *C. sapidus* hemolymph [77]. This observation suggests that different lectins from crab serum recognize distinct *V. parahemolyticus* strains and probably mediate their clearance; the only strain that could be isolated from the crab hemolymph would represent those that, because of their surface properties, escape recognition by serum and hemocyte lectins.

Little information is available, however, on the relations of the "normal" shellfish microbial flora, which may contain latent pathogenic vibrios, and the effect of acute or chronic pollutant exposure and development of protozoan parasitic infections. Because oysters are filter-feeding organisms, there is an intimate relation in gut tissues between nutrition and internal defense because phagocytosis by granular hemocytes appears to be the basic mechanism that mediates both functions. If compromised by stress, for example, owing to exposure to pollutants or the presence of intracellular hemocytic protozoan parasites, such as *Perkinsus marinus*, it is very likely that components of the "normal" microbial flora, including latent pathogens or nonpathogenic opportunistic bacteria, may become patent. Parasitic diseases of shellfish, such as those caused by *Haplosporidium*, *Perkinsus*, *Bonamia*, and *Marteilia* spp. are a global problem [78], and the effect of these pathogens on mollusk populations has been quite rapid, unexpected and in many cases catastrophic [67,69,78]. Following a major breakthrough recently developed in our laboratory, the establishment of in vitro continuous cultures of the parasite *P. marinus* [79], we have now optimized the culture conditions [80] and developed a fully defined culture medium that promotes very high multiplication rates [81]. This has provided not only the first reliable and abundant source of parasites for developing molecular probes for diagnostic purposes [82] and evaluating antiparasitic drugs, but also a unique opportunity to obtain a clear understanding of the organism's physiology, in particular the factors that promote its virulence and regulate its differentiation stages [81,83]. It should be realized that the adverse effect of environmental pollution and other consequences of human activity, particularly the increased transfer of shellfish species with potential for aquaculture that may constitute new hosts for native pathogens, and likewise, the accidental transfer of pathogens between geographic locations, all are factors that will make disease outbreaks in farmed shellfish more likely in the future. Therefore, there is an urgent need to focus on the development of specific and sensitive diagnostic technologies that will aid in the rapid detection and identification of shellfish pathogens.

2. Molecular and Serological Approaches to Disease Diagnosis in Marine Invertebrates

a. Nucleic Acid Targets. The use of nucleic acid probes to detect the presence of specific target sequences in complex sample mixtures is an established and rapidly expanding technique. Especially in combination with the exponential target amplification of the polymerase chain reaction (PCR), these probes are becoming invaluable tools for the rapid detection and identification of an increasing number of pathogens, both in routine and research applications. Nucleic acid probe-based methods of parasite detection compare favorably in sensitivity with routine microscopy and can be used in a batchwise fashion on large numbers of samples. There are several approaches for applying nucleic acid probes to detect specific DNA or RNA sequences. As a general rule, it is important to select a nucleic acid target of the pathogen that has the highest possible degree of sequence dissimilarity from its host and other potential pathogens to minimize the degree of cross-reactivity or false-positives. Although rRNA sequences can be good targets for diagnostic assays because of their high-cell-copy number, the degree of evolutionary sequence conservation makes rRNA probes highly cross-reactive with species other than the pathogen of interest. Thus, rRNA-based method usually involve microscopic validation as with *in situ* hybridization applications where visual examination of tissue sections is used to identify pathogen localization within a host. In contrast, the high degree of unique species-specific sequences that can be found in DNA provides diagnostic assays with greater fidelity, allowing single-step method such as Southern blotting or PCR amplifications, without necessitating routine secondary validations.

The sensitivity of PCR for trace quantities of foreign DNAs in heterogenous samples has made this technology an ideal choice for identifying infectious agents and has been used with great success to detect protozoan pathogens in humans [84,85]. Any PCR assay requires a specific DNA sequence to be targeted for amplification; thus, in diagnostic applications, it is advantageous to use a DNA target sequence that has a high-copy number to increase the likelihood of detection at low levels of infection. Mitochondrial DNA (mtDNA) markers are an appropriate target for such an assay over genomic sequences because of the high number of mitochondria in a cell. Overall the use of PCR to amplify a mtDNA target provides the most practical and economical approach for extensive field-sampling programs of pathogens within a host population. The amplified mtDNA target also provides some sequence information on the mitochondrial genome and, therefore, could be secondarily employed to distinguish between related genetic strains of a pathogen if the mtDNA target region is carefully selected. The ability of a PCR assay targeting mtDNA to distinguish between genetically related species and subspecies has been documented in marine invertebrates. Recent studies with the pink barnacle [86], blue mussel [87] and calanoid copepods [88] all provide evidence for genetic drift among closely related populations based on small differences in nucleotide sequence of the mtDNA genome.

A PCR-based diagnostic method for detecting infections of the oyster protozoan pathogen *P. marinus* in tissues of its host *C. virginica* has been described [82]. The success of this particular assay was based on the fact that a noncoding domain present in a 32-kb DNA fragment present in high-copy numbers *P. marinus* selected as the target, is very specific for the parasite [89]. Thus, by identifying an intergenic domain between two rRNA genes, a highly sensitive and specific PCR amplification under stringent conditions can be performed to detect as little as 10 fg of total Pm DNA in a 1- μ g sample of total oyster tissue DNA (eight orders of magnitude difference in DNA levels) [82].

b. Protein Targets. The sensitive detection of specific pathogen proteins by monoclonal antibodies is an attractive alternative to nucleic acid-based assays. Although the development of monoclonal antibody probes is more labor intensive than for nucleic acid probes, they offer detection assays that are just as sensitive as those for nucleic acids. However, the use of monoclonal antibodies for detecting pathogens has one fundamentally different characteristic that nucleic acid method do not have to consider: the presence of a protein epitope or carbohydrate moiety within a pathogen may be temporal. Monoclonal antibody-based assays for diagnosing infections must take into account that the epitope the antibodies recognize may not (1) be present in all life-stages, (2) be expressed under all environmental conditions in which the infected hosts are found, or (3) be constitutively expressed among different environmental strains or geographic races of the pathogen. These three factors make monoclonal antibodies a double-edged sword. On the one hand, they potentially offer a greater degree of target specificity than nucleic acid method because it is possible for them to distinguish between different pathogen life-stages, physiological states, or regional populations. On the other hand, this level of specificity requires a much greater level of effort to properly validate with controls to determine the full range of expression variability exhibited by an epitope. For the oyster pathogen *P. marinus*, antibody techniques have been used with some success for detecting infections in *C. virginica* [90,91].

IV. MARINE INVERTEBRATE CELL AND TISSUE CULTURE

The use of animal cell, tissue, and organ culture has evolved from constituting a set of basic experimental procedures to become, in recent years, a powerful biotechnological tool, with wide applications to various aspects of biological research and commercial exploitation. Among those, continuous animal cell lines have proved invaluable in the identification, isolation, and molecular characterization of pathogens and the elucidation of their pathogenic mechanisms (that mediate pathogenesis). Another fundamental feature has been the possibility to manipulate *in vitro* the continuous and reliable production of intermediate metabolites and final secretion and excretion products, such as immunoglobulins, hormones, and a variety of recombinant proteins. Many bioactive products from marine invertebrate sources, such as halichondrins, briostatins, and many of those discussed earlier can be isolated only in minute amounts from the organisms collected in the environment, and it becomes clear that the current availability and variability of the wild stocks pose a serious threat to the efficient screening, characterization, clinical evaluation and, eventually, the large-scale production of such useful agents. Therefore, there is an urgent need to develop methods for the establishment of invertebrate continuous cell lines from the organisms producing the bioactive compounds in question. Moreover, this need is buttressed by an attractive possible alternative in which established invertebrate cell lines from a different taxa could be transfected with the appropriate gene constructs and synthesize the factors of interest. However, despite numerous attempts to maintain and propagate marine invertebrate cells *in vitro*, success has been limited to primary cultures that remained viable for only relatively short periods and, in a few cases, to a few generations.

A. Tissue and Cell Primary Cultures from Marine Invertebrates

Primary cell cultures, obtained from enzymatically or mechanically dissociated tissue explants from marine invertebrates were developed many years ago [92,93]. In a few cases, limited proliferation extended the viability of the cultures from weeks to several months [94,95]. Although primary cultures of several marine invertebrate taxa have been reported throughout

the years, there is as yet no firm evidence that established cell lines from marine invertebrates are currently available.

In the past few years, however, considerable efforts appear to have met some measure of success with tissue explants from tunicates [94,95] and cnidarians [96]. Past approaches included developing original culture media that would mimic the marine organism internal milieu, both in composition and osmolarity, or by simply adapting standard mammalian culture media to the osmotic properties of the marine organism by adjusting the ionic strength. Thorough characterization of optimal surfaces temperatures and media compositions have been carried out, and the use of mitogens that have proved successful for vertebrate cells has been carefully explored [97]. A culture medium for blood cells and pharyngeal explants from the solitary tunicate *Styela clava* [94] was developed from a base of RPMI 1640 powder in artificial seawater, supplementing with antibiotics and by adjusting the salt concentration and composition to those determined for the tunicate plasma. Embryo-derived cell cultures from the colonial protostome *Botryllus schlosseri* and cell cultures from the hermatypic branching coral *Stylophora pistillata*, from the gorgonian *Plexaura* spp., and the hydrozoan *Millepora dichotoma* were successfully maintained in vitro in a variety of commercial media, such as L-15, M199, and BCCM, in some instances considerably dilute and supplemented with heat-inactivated fetal calf serum [95–98]. Despite the recent reports of limited success in producing stable cell cultures from marine invertebrates, however, the development of method for the establishment of continuous cell lines from those organisms is still in its primary stages, lagging behind those procedures for continuous cell lines from vertebrates, including finfish.

B. New Approaches for the Establishment of Continuous Cell Lines from Marine Invertebrates

Extensive work has shown that spontaneous or experimentally induced in vitro transformation is required for the immortalization of mammalian cells [99–101]. Because spontaneous transformation is a relatively infrequent event in normal mammalian cell populations, the use of chemicals or oncogenic viruses for this purpose is a routine practice for the establishment of cell lines in culture. It has been recently proposed that the application of similar principles to the development of equivalent continuous cell lines from marine invertebrates may be the approach of choice [102]. Because of the abundance of information concerning molecular genetics, the sea urchin *Strongylocentrotus purpuratus* is proposed as the model organism, and stable cell lines will be developed by transfecting gastrula cells with oncogenes, such as those from *myc*, *ras*, nonfunctional *PKR*, and *eIF-4E* through pseudotyped retroviral vectors, such as pLGRNL, that have been successfully used in finfish and the dwarf surf clam [103]. In this vector, the Moloney murine leukemia virus coat protein has been replaced by the VSV-G protein giving rise to the vector's characteristic wide host range, and the neomycin phosphotransferase gene has been incorporated to allow selection of stable transformants [104,105]. If successful, this novel and rational approach will provide a strategy for the development of continuous cell lines from other scientifically and economically important marine invertebrates.

V. APPLICATION OF TRANSGENIC APPROACHES TO AQUACULTURE OF MARINE INVERTEBRATES

There is an enormous potential for the use of transgenics to vastly improve aquaculture production of marine invertebrates. There are also significant obstacles to overcome before such applications can be used. The primary concern for the production of transgenic marine

invertebrates is in containment. It is very difficult to guarantee that any aquaculture facility that constantly draws fresh seawater from the environment and discharges used seawater back into that environment can assure that the returning water is 100% free of transgenic larvae or gametes. External fertilization and wide-dispersing larval stages are two common reproductive characteristics that make most marketable marine invertebrates poor candidates for transgenic manipulations because of the societal concerns over the accidental environmental release of transgenic invertebrates. No current national government openly supports the environmental release of genetically engineered animals (eukaryotic metazoans). Beyond this political reality, there are significant moral and ethical considerations to employing transgenics in a situation where the risk of release and subsequent interbreeding with natural populations is very high. Consequently, the present production of transgenic marine invertebrates is limited to research applications where animals can be confidently contained from being released.

Despite the large gap in our knowledge of gene expression events in marine invertebrates (excluding sea urchin embryos), there have been numerous attempts to introduce heterologous transgenes with various promoters and reporters derived from distantly related phyla, usually vertebrates. These studies have generally been unsuccessful at establishing an integrated transgene in a germline cell, possibly because the transgene constructs have involved pieces of DNA that are "too foreign" for incorporation into an invertebrates genome. Recently, the use of a VSV-G pantropic retroviral vector has been developed to specifically facilitate the direct genomic incorporation of transgenes into eukaryotic cells [104,105]. This method offers a great potential for the future of transgenic invertebrates and has been successfully used on the dwarf surf clam *Mulinia lateralis* (see Chap. 28). Thus, the techniques for manipulating transgene constructs within marine invertebrates are presently available with a retroviral vector, electroporation, and biolistics. The real challenge now facing us is identifying what genes are relevant for us to manipulate and what regulatory elements do we use to control the expression of these *de novo* genes?

A. Growth Regulation

Without a doubt, the advantages offered by transgenic manipulations of aquaculture stocks are great. The primary focus of most transgenic applications in agricultural stocks has been to increase production by unit growth per individual. As indicated earlier, there is now no known gene that is specifically responsible for growth regulation in marine invertebrates, and what we currently need is the identification of an invertebrate homologue of the vertebrate growth hormone (GH) gene. A successful transgenic invertebrate stock evidencing enhanced growth rates will probably involve a genetic manipulation to increase amino acid assimilation or transport, thereby shifting the general pattern of cellular metabolism to favor the deposition of structural proteins.

Once a suitable target gene has been identified, the only remaining decision for the construction of a transgene is to identify a suitable promoter. Because the metabolic activity of all organisms is finely tuned by a complex system of checks and balances, it is likely that a strong, constitutive promoter (such as for β -actin) would have as much of a negative effect on growth as it would a positive effect. In this case, swamping a metabolic pathway with a signal promoting growth would have the equally opposite effect of stimulating all the metabolic pathways that serve to limit or regulate growth. A more effective transgenic strategy may be implemented by using a less-responsive or less-constitutive promoter to gently increase the metabolic balance of an individual toward protein deposition, without conversely increasing the level of constraining regulatory elements. In truth, transgenics is not going to reliably produce stock where the organism size is dramatically enhanced. Because of all the regulatory

pathways that exist in an organism to tightly regulate growth, the transgenic strategy most likely to succeed is one for which growth efficiency is enhanced in terms of tissue deposition per unit food by increasing the rates at which nutrients are allocated to structural components such as muscle-protein mass.

B. Disease Resistance

In addition to the considerable technical difficulties in developing stable transgenic organisms, as explained in the foregoing, a major problem that can be envisioned when considering enhancing disease resistance in marine invertebrates through transgenic approaches, is the identification of the suitable target gene(s). Experimental evidence has shown that, below the level of the Agnatha (lampreys and hagfish) no *bona fide* immunoglobulins, T-cell receptors, or lymphoid cells have yet been identified, and which are there invertebrate precursors, if any, has remained an open question. However, it has become clear that in the vertebrates, major histocompatibility complex (MHC) components, T-cell receptors, and immunoglobulins, do not necessarily mediate all recognition-defense events. Several other specific and "nonspecific" recognition and defense mechanisms operative in vertebrates have been identified and well characterized. Those include the complement system, humoral bactericidal proteins, acute-phase reactants, such as opsonins and lectins, and other components related to inflammatory processes, such as lymph node-homing receptors and endothelial leukocyte adhesion molecules [27,106–112]. When considering the invertebrates, the questions about their immunoevolution may be formulated differently because we are confronted with an enormous diversity of taxa exhibiting a variety of specialized defense mechanisms. Although it seems almost certain that the invertebrates, as a broad group, lack the typical adaptive vertebrate immune response characterized by immunoglobulins and lymphocytes, invertebrates are able both to differentiate self from nonself and to respond against nonself substances [112].

A wide variety of defense effector molecules, constitutive or inducible, have been identified, namely lysozyme and other bacteriolytic enzymes, bactericidal and bacteriostatic peptides, the prophenoloxidase system, bactericidal toxins, and others [106,113,114]. Among these, antibacterial peptides from invertebrates, such as cecropins, attacins, magainins, and defensins, have generated strong interest and have been characterized in considerable detail [113]. Insect cecropins, for example, are small basic peptides of about 30–40 amino acids in length, first described in the *Cecropia* moths, that on binding to the bacterial membrane through strongly hydrophobic stretches of sequence produce pores that result in the leakage of the intracellular components, thus killing the microorganism [115].

In addition, there is a considerable body of evidence that recognition-opsonic molecules bind to the nonself substances, such as microbial pathogens, promote their phagocytosis and trigger the production or release of those effector molecules. Humoral lectins, which are ubiquitous among invertebrates, and are known to be present in most body fluids, are likely candidates for the role of specific recognition molecules. Because lectins bind specifically to polysaccharides and glycoconjugates, humoral lectins may attach to or agglutinate, and thus immobilize, microbial or metazoan parasites that come in contact with hemolymph and mediate their subsequent phagocytosis, encapsulation, and melanization. Some humoral lectins from invertebrates can function as opsonins [116], and their activation from a precursor can be induced by trauma or by injecting foreign particles into the body cavity. Although invertebrate lectins have not yet been shown to express a recombinatorial diversity similar to that of antibodies, multiplicity in carbohydrate discrimination is attained to a certain extent by the occurrence of multiple lectins with distinct specificities; the presence of more than one binding site, specific for different carbohydrates, in a single molecule, and by certain "flexibility" of

the binding sites that would allow the recognition of a range of structurally related carbohydrates. Despite their nominal specificity for sialic acids, many lectins from arthropods (horseshoe crabs, shrimp, crabs) also bind, although with lower affinity, other structurally related and imrelated molecules, such as 2-keto-3-deoxyoctonate, N-acetyl-d-glucosamine, N-acetyl-d-galactosamine, d-galactose, glucuronic acid, colominic acid, and phosphorylcholine, all present in the bacterial cell wall or products of bacterial metabolism [117]. As we showed elsewhere in limulin [118], unrelated structures, such as sialic acids and PC, are apparently recognized by different binding sites. This multiplicity in lectin specificity and the nature distribution of the molecules recognized suggest that serum lectins may contribute as a carbohydrate-based recognition system for potentially pathogenic microorganisms.

Therefore, one approach for the application of transgenic technology to disease resistance could be at the level of promoting the efficient recognition of the pathogen in the organism in question. Therefore, lectins may be considered as suitable target gene(s) for transgenesis because after recognition of the pathogen, the complex lectin-pathogen would be phagocytosed and would trigger the organism's natural defense mechanisms. In vitro manipulation of the lectin, aimed at the recognition specificity of the appropriate pathogen carbohydrate moiety, could be accomplished by site-directed mutagenesis and the modified gene incorporated into the germline of the invertebrate of interest. A second alternative to be considered is the choice of effector factors, such as wide-spectrum antibacterial peptides, as the suitable target gene(s) for transgenesis. The overall lack of specificity of cecropins, attacins, magainins, and defensins for bacteria becomes a considerable advantage and their potential negative effects for the transgenic invertebrate becomes the major criterion that would determine their selection as the transgene of choice.

VI. CONCLUSIONS

The explosive growth of the shellfish mariculture industry in the past decade, has revealed the need for the application of biotechnology approaches to the solution of numerous essential problems. Despite significant obstacles to overcome before such applications can be used, there is an enormous potential for the use of transgenics to vastly improve aquaculture production of marine invertebrates. In particular, the development of fast-growing and disease-resistant varieties is a long-term goal worth pursuing, and the development of technologies for the genetic transformation of cells and organisms, now foreseen as a feasible endeavor thanks to the aforementioned recent breakthroughs, is a critical step in this regard. The identification of genes relevant to reproduction and growth in marine invertebrates and the characterization of their regulatory regions will provide the base for the enhancement of shellfish productivity in aquaculture through the development of transgenics. Because shellfish do not synthesize immunoglobulin antibodies, established methods for control of disease in vertebrates, such as rational vaccination programs, cannot be applied to molluskan and crustacean species that are environmentally or economically relevant, such as oysters, mussels, clams, shrimp, lobsters, and crabs. Therefore, the elucidation of their internal defense mechanisms and the identification of the immunoglobulin molecular equivalents is of utmost importance. In the long-term, the characterization of structure and function of the recognition-effector gene products and the detailed understanding of how their expression is regulated, will enable us to enhance disease resistance through adequate stimulation and to further apply transgenic approaches to the development of disease-resistant shellfish species. In the meantime, specific and sensitive diagnostic molecular tools for early detection of pathogens in the environment, vectors, and intermediate hosts, and increased knowledge of shellfish immunology will aid in improving disease control and management in fisheries and aquaculture.

and, in reducing the transmission of shellfish microbes that are pathogenic to the seafood consumer.

ACKNOWLEDGMENTS

Part of the studies described here were supported by grant MCB-91-05875 from the National Science Foundation, grant F31 GM14903 from the National Institutes of Health, DOC Cooperative Agreements NA47FL-0163 and NA57FL0039 awarded by NOAA/NMFS, Oyster Disease Research Program and grant NA90AA-D-SG063 awarded by NOAA through the University of Maryland and Sea Grant College to GRV.

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Disease Diagnosis and Control of Marine Organisms

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I. INTRODUCTION

Before addressing the issues of disease diagnosis and control, it is pertinent to inquire about the meaning of the term, disease. Although most individuals will have an understanding of this term, definitions may be fairly unhelpful. A valiant attempt at a definition was published in the *British Medical Journal* during 1979. Thus, a *disease* is defined as “the sum of the abnormal phenomena displayed by a group of living organisms in association with a specified common characteristic or set of characteristics by which they differ from the norm of their species in such a way as to place them at a biological disadvantage” [1]. In short, a disease may result from biotic, namely bacteria, parasites, and viruses; and abiotic factors (e.g., pollution). In this chapter, emphasis will be placed on the diagnosis and control of bacterial fish and shellfish diseases, reflecting the many recent advances in this area.

II. DIAGNOSIS

What is the need for disease diagnosis? In fish and shellfish pathology, there is an obsessive desire to isolate and identify the offending pathogen or parasite. Thus, much time may elapse before results of the diagnosis are obtained, and the client notified. Often diagnosticians seem reticent to make speedy interim judgments. Yet, it is necessary to take corrective action as early in a disease cycle as possible to maximize the effectiveness of control strategies. Therefore, rapid diagnoses should be considered essential prerequisites for control measures. If the diagnosis cannot be achieved within a short time, then the value of the effort is questionable. The categories of information, that are currently used for diagnoses of fish and shellfish diseases are included in [Table 1](#).

The involvement of biotechnology in disease diagnoses largely centers on serology and molecular biology techniques. However, for completion, it is necessary to mention miniaturized biochemical systems, for example, API 20E and Biolog, which although developed initially for use with bacteria of medical importance, have been used extensively in fish pathology [2].

Table 1 Categories of Information Used for the Diagnosis of Disease

Behavioral changes (e.g., abnormal swimming, inappetance)
External signs (e.g., Hemorrhaging, fin/tail rot)
Internal signs (e.g., granuloma, ascites, liquefying tissues)
Histology ^a
Microbiology, namely recovery and identification of pathogens ^a
Determination of the presence of pathogens without recourse to isolation procedures, namely by serology and gene probe technology ^a

^aRelevance for biotechnology.

Indeed, such systems are routinely used for the identification of many gram-negative bacterial fish pathogens, particularly in the families *Aeromonadaceae*, *Enterobacteriaceae*, and *Vibrionaceae*. In addition, bacteriophage typing is extremely useful for purposes of epidemiology and epizootiology [3,4]. For the future, it may be anticipated that species-specific bacteriophage could be modified genetically, such as by inclusion of target (e.g., luciferase) genes, so that incorporation of the virus into the host bacterium could be measured by analysis for the specified gene product. This could then be used diagnostically to indicate the presence of the host cell (=pathogen).

With current interest in serology and molecular biology, increasingly rapid and sensitive methods have entered the armory of fish and shellfish disease diagnostics. Nevertheless, caution needs to be advocated insofar as the interpretation of results can be troublesome. For example, does a positive result for a named pathogen, in the absence of culturing techniques, indicate the presence of live, avirulent or virulent cells, or inactivated antigen, which could be present in and around recently vaccinated fish? Thus, it is apparent that a history of the animals is important when contemplating disease diagnoses by means of serology or molecular biology.

A. Serology

Most rapid diagnostic techniques currently used in fish and shellfish pathology center on serology, some systems of which have been commercialized. The ongoing dilemma, concerning the relative advantages and disadvantages of polyclonal versus monoclonal antibodies, remains to be resolved. Certainly, the extreme specificity and sensitivity of monoclonal antibodies has been considered advantageous for diagnostic systems. Yet, single monoclonal antibodies would be unlikely to recognize subtle changes in antigenic structure, such as occurs regularly with some bacterial and many viral pathogens. The compromise of using batteries of many monoclonal antibodies to compensate for possible changes in a few antigens can become costly and potentially unwieldy. Thus, interest in polyclonal antibodies has been rekindled, although careful consideration needs to be given to potential problems with cross-reactions. In some serological systems, that rely on stages with several antibodies (e.g., the enzyme-linked immunosorbent assay; ELISA), the antigens are captured by means of broad-spectrum polyclonal antibodies, whereas positivity or negativity in the secondary assay depends on reactions with specific monoclonal antibodies.

Descriptions of the serological techniques currently used in fish and shellfish pathology are detailed in the following. Additional methods (e.g., immunodiffusion and immunoelectrophoresis) have found limited use for the diagnosis of some diseases, notably bacterial kidney disease, although there is only negligible scope for biotechnology. Therefore, these techniques will not be considered further.

1. Whole-Cell Agglutination

The whole-cell agglutination (WCA) test is a quick and easy technique that provides much useful data. Essentially, a drop of dense bacterial cell suspension (*ca.* 10^8 c/mL, prepared in saline) is added to a microscope slide. This is followed by adding a drop of antiserum, with gentle mixing for 2 min. A positive response is indicated by clumping of the cells. The reaction may also be carried out in microtiter wells, using serial dilutions of antisera [5]. It must be emphasized that the reliability of WCA reflects the specificity of the antiserum. Nevertheless, WCA is used routinely in diagnostic laboratories for serotyping (e.g., of *Vibrio anguillarum*) and identification of pure cultures. The possible involvement of biotechnology essentially concerns the need for standardized monospecific antisera.

2. Indirect or Latex Agglutination

The so-called latex agglutination test has found widespread use for diagnosis of enteric redmouth, furunculosis, and vibriosis (including Hitra disease). The technique may be readily adapted for most bacterial fish pathogens. As originally described [6], the test involved the use of globulins from hyperimmune serum (titer=>1:5000) and sensitized latex. Now, systems use whole polyclonal antisera. In the original study, the globulins were precipitated by the addition of saturated ammonium sulfate to the antiserum, and the precipitated proteins removed and redissolved in saline, dialyzed overnight at 4°C against three changes of saline, and, after centrifugation, the supernatant, which contains the globulins, stored at -20°C until required. The latex particles (0.81- μm diameter) are sensitized in globulin solution at 37°C for 2 h. For the test, a few drops (200 μL) of the antigen (bacterial suspension in glycine-buffered saline supplemented with Tween 80) are mixed for 2 min with an equal volume of sensitized latex on a clean glass plate. A positive result is indicated by clumping of the latex (Fig. 1). As before, positive and negative controls are necessary. The system has been commercialized, and forms a reliable means of diagnosing disease caused by a range of bacterial pathogens, including *Aeromonas salmonicida*, *V. anguillarum*, *V. ordalii*, *V. salmonicida*, and *Yersinia ruckeri*. There is scope for more products to identify the ever-increasing range of serious fish or shellfish pathogens of importance to aquaculture.

3. Coagglutination with Antibody-Sensitized Staphylococci

As reported for *A. salmonicida* and *R. salmoninarum*, this technique is similar to the latex agglutination test [7]. Essentially, *Staphylococcus aureus* is suspended in 0.5%

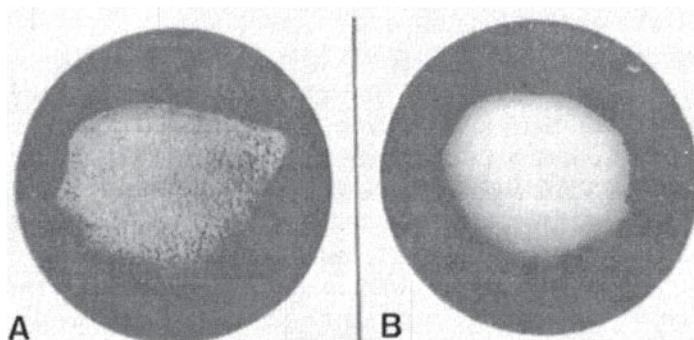


Fig. 1 The latex agglutination system for *V. anguillarum*. (A) and (B) correspond to positive and negative reactions, respectively.

formalinphosphate-buffered saline (PBS) for 3 h at 25°C to inactivate the cells, and washed three times in fresh PBS, before mixing with antiserum in the ratio of 10:1 and incubated at 25°C for 3 h. An equal volume of a boiled bacterial suspension and the sensitized staphylococci are mixed on a glass slide. Following incubation in a moist chamber at room temperature for up to 2 h, a positive response is indicated by clumping of the cells. Although the technique is adequate for the recognition of target antigens, it is unlikely to replace the latex agglutination systems as a commercial product.

4. Immuno-India Ink Technique (Geck)

A microscopic method involving the use of India ink and serum mixtures, this technique has been described only for use with *A. salmonicida* [8]. A drop of bacterial suspension is smeared onto a clean (defatted) microscope slide, air-dried, and heat-fixed. The smear is covered with a 1:1 mixture of India ink and antiserum, before incubation in a moist chamber for 10 min at room temperature. Subsequently, the mixture is removed by washing with ferric chloride, and the slide air-dried before microscopic examination. A positive result is indicated by the presence of cells, clearly outlined with India ink. Whereas positive reactions are readily visualized, it is often difficult to discern cells in negative reactions. Interest in this technique has decreased substantially over the last decade, and it seems unlikely to be developed further as a commercial product.

5. Fluorescent Antibody Technique

There are two variations to this test; namely, the direct and indirect methods. The fluorescent antibody test (FAT) has found use for the diagnosis of many fish diseases, particularly bacterial kidney disease, for which it is considered a highly effective procedure [9].

For the direct method [10,11], fluorescein isothiocyanate is conjugated with whole or with the IgG fraction of the antiserum. Twofold dilutions (i.e., 1:5 and 1:8) are prepared in PBS and used to standardize the "conjugate". Then, a dense bacterial suspension or tissue (namely kidney) smear or section is applied to grease-free microscope slides, air-dried and fixed at 60°C for 2 min (or fixed in 95% ethanol for 1 min, and then air-dried). The conjugate is pipetted onto the slide and left in a moist chamber for 5 min to react at room temperature (i.e., 15–20°C). Subsequently, the excess antiserum is removed by draining, before the slide is thoroughly rinsed for 10 min in PBS. The slide is air-dried, and the smear covered with a drop of buffered glycerol (Difco; at pH 9.0) before covering with a coverslip. This should be quickly examined with a fluorescence microscope. However, it is always important to include positive and negative controls. Increased sensitivity in the detection of *R. salmoninarum* has been reported to occur when kidney smears are pretreated with organic solvents; namely, acetone, methanol, or xylene, for 2 min [12].

For the indirect fluorescent antibody test (IFAT) [13], bacterial smears are prepared and fixed (as before). Doubling dilutions of rabbit antiserum are prepared, and 20-µL volumes added to the bacterial smears. These are placed in a moist chamber, left for 30 min to react, and washed for 30 min in two changes of PBS. After air-drying, the smears are covered with a suitable dilution of fluorescein-labeled sheep antirabbit globulin, incubated for 30 min in the moist chamber, rinsed thoroughly in PBS, air-dried and mounted in buffered glycerol. Examination, with a fluorescence microscope, should proceed as quickly as possible. Clearly, positive results, involving masses of fluorescing cells, are readily discerned. However, some inanimate particles may autofluoresce. This may be problematic in samples containing only a comparatively few cells. Here, the interpretation of positivity versus negativity need not be clearcut. Certainly, FAT has been established as invaluable for fish and shellfish pathology.

Further developments, from the commercial viewpoint, would be the provision of antisera, standardized reagents, and membrane filters, which capture and thereby concentrate the antigen, and appear to dramatically enhance the sensitivity of IFAT.

6. Sandwich Techniques

Sandwich techniques, involving antigen capture or separation and subsequent assay to determine its (their) presence, have evolved into the most sensitive of the serological procedures [14,15]. Moreover, systems have been developed into successful commercial products, some of which are suited for field use. It is considered that such techniques will continue to be developed further, offering reliable diagnostic systems for the foreseeable future. Nevertheless, numerous pitfalls occur in development programs, and it is worthwhile to consider the essential requirements [16]. In brief, it is essential to

1. Verify the specificity of all antisera
2. Standardize all steps
3. Optimize the dilution of all reagents

Sandwich techniques include the enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and immunoblotting or Western-blots.

a. *Antigen Capture and Separation.* It is personal experience that many problems with sandwich techniques reflect the initial capture or separation of the antigen. With ELISA, cells are captured on an antibody-coated surface. There is a practical problem of ensuring that sufficient cells are trapped to react in the secondary assay stage. This has led to some fascinating capture procedures, most of which involve samples containing low numbers of cells, such as might occur with asymptotically diseased fish. Fortunately, this is not a problem with overtly clinically diseased fish. A recent refinement concerns the magnetophoretic separation of cells by use of magnetic microspheres of magnetite (Fe_3O_4). Indeed, there is one commercial product that uses this system to separate and concentrate cells of *R. salmoninarum* from pathological material. With immunohistochemistry, histological preparations form the basis of the subsequent assay. For Western-blot analysis, there is initial electrophoretic separation of proteins. Here, the main problem centers on ensuring an adequate concentration of material for subsequent recognition by the blotting assay.

b. *Assay System.* A common link with sandwich systems concerns the detection of the specific antigen. The most common methods have involved antibody-conjugated enzymes, notably alkaline phosphatase or horseradish peroxidase, and their respective enzyme substrate. Alternative approaches have involved use of anionic dyes (e.g., coomassie brilliant blue) for the detection of proteins, radiolabeling, biotin-streptavidin, chemiluminescence with luminol, and colloidal gold.

With antibody-based assay systems, it is essential to block all unbound sites on the antibody-coated capture device. Experience suggests that suitable blocking agents include bovine serum albumin, casein, gelatin, or Tween 20.

c. *ELISA.* This is a technique that is becoming widely adopted for the detection and diagnosis of bacterial fish pathogens, with some commercial kits having been developed (Fig. 2). ELISA is a useful technique that has already gained widespread use in human and veterinary medicine. Essentially, there is a requirement for a specific antiserum (polyclonal or monoclonal), an enzyme (e.g., alkaline phosphatase or horseradish peroxidase), and a substrate (e.g., o-phenylenediamine [for use with alkaline phosphatase]) [17]. A positive result is indicated by a color change, which may be recorded quantitatively with a specially designed reader.

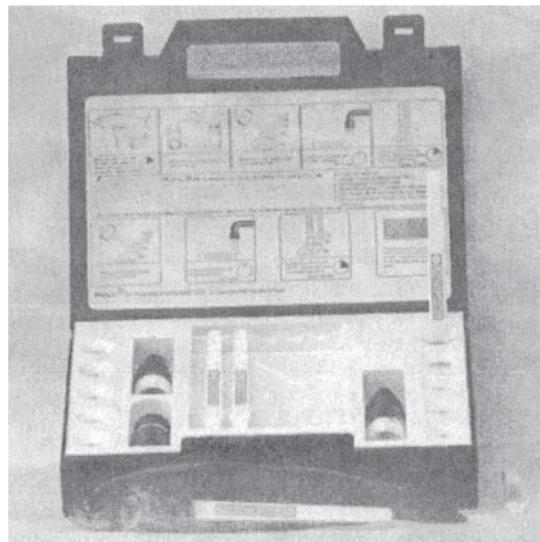


Fig. 2 An ELISA system for the rapid diagnosis of furunculosis. The antigen is captured on the monoclonal antibody-coated stick (probe), and the subsequent assay is carried out in the wells. Initially, the antigen is transferred to a antibody-alkaline phosphatase conjugate, and then reacted with the enzyme substrate. A color change indicates a positive reaction. This system, designed for field use, is capable of detecting 10^3 – 10^4 cells within 1 h.

In a detailed comparison, ELISA was more sensitive than FAT in the detection of *R. salmoninarum* [14,15]. Thus, ELISA was capable of detecting 20 ng of *R. salmoninarum* antigen per milliliter of kidney homogenate. Moreover, FAT did not detect 80% of samples containing renibacterium, which were positive by ELISA [14]. A recent development involves an NADP-based cycling assay, which appears to offer enhanced sensitivity.

d. Immunohistochemistry. Immunohistochemistry has been used especially for the detection of *R. salmoninarum* in histological preparations. Thus, after the addition of an antibody-enzyme-based assay, positivity is again measured by a color change. Fortunately, the system is fairly sensitive, being capable of detecting approximately 10–16 µg of antigen [16]. Moreover, an advantage of the system is that a permanent record results. This may be invaluable if the results of the diagnosis are challenged.

e. Immunoblotting: Western-BLOTS. Labeled antibodies have been increasingly used to identify antigens that have been previously separated by gel electrophoresis and then transferred to membranes for assay. In particular, Western-blotting techniques have fast become established for the diagnosis of *R. salmoninarum*, particularly the 57-kDa protein (Fig. 3) [18]. However, the technique, whereas commendable for the detection of overtly clinically diseased fish, is less useful for the recognition of asymptomatic animals [19]. Tissues are homogenized, centrifuged, and the proteins separated, such as by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), before transfer to nitrocellulose membranes. Unoccupied sites are blocked to prevent the nonspecific binding of antibody. The detection stage involves the use of fluorochromes (chemiluminescence; these emit light, which is captured on film), radio-labeling, colloidal gold (this is bound to antibody), or a sandwich assay with antibody conjugated to alkaline phosphatase or horseradish peroxidase. Thus, in one working system

after electrophoresis, the membrane or gel is blocked (with bovine serum albumen or gelatin and Tris, or Tween 20 in PBS), and washed thoroughly for at least 15 min before the addition of primary (rabbit) antibody with incubation at room temperature for 1–2 h. Following further washing, either horseradish peroxidase (HRP)-labeled antibody (e.g., goat antirabbit IgG) or amplification with biotinylated, conjugated antibody followed by application of streptavidin-HRP. Finally, the enzyme substrate, namely a tetrazolium compound, will lead to a color change. The system is capable of detecting 10^4 cells per milliliter [20]. A development, that has been successful with the detection of about 10^3 cells and 1–10 µg of the 57-kDa protein of *R. salmoninarum*, involves chemiluminescence with luminol. Here, the hydrogen peroxide oxidation of luminol in alkaline conditions may be detected with blue-light-sensitive autoradiography film.

B. Gene Probe Technology

The most recent developments in diagnosis of fish and shellfish diseases have centered around molecular biology and, in particular, the use of the polymerase chain reaction (PCR), which has been developed for the detection of several pathogens, including *A. salmonicida* and *R. salmoninarum*. With *A. salmonicida*, a detection limit of only two cells has been reported [21]. With *R. salmoninarum*, the technique offers important opportunities for improvement in diagnosis. Several systems have been described. Thus, two 24-base oligonucleotide primers have been used to amplify a 50-bp region of the gene encoding the 57-kDa soluble protein of *R. salmoninarum* [22]. The technique was specific and sensitive enough to detect two renibacterium cells per egg. Thus, DNA was extracted, and the primer synthesized using an automatic synthesizer. Each primer contained 24 nucleotides, which were chosen to amplify a 50-bp segment (codons 28–194 inclusive) of the gene encoding the 57-kDa protein. In a separate study, 30-base-long oligonucleotides were used as a specific probe for the detection of renibacterium, particularly in infected tissues, by filter hybridization [23]. The success was illustrated by the lack of hybridization with 22 other bacterial species. Moreover, the detection limit was reported as 2.5×10^4 cells. There is scope for other systems. Moreover, there is commercial application. However, gene probe technology seems likely to remain laboratory-based for the foreseeable future. Developments, suitable for use in field conditions, are sorely needed.

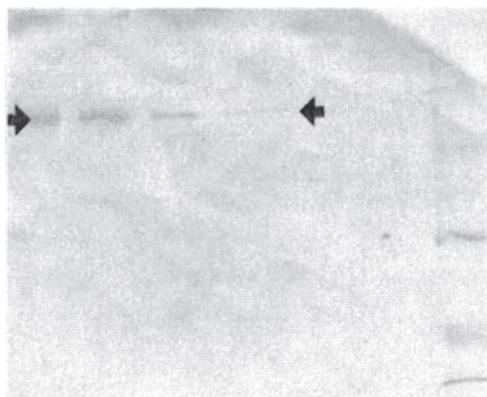


Fig. 3 A Western-blot for the 57-kDa protein (arrowed) of *R. salmoninarum*.

III. CONTROL

It is pertinent to recall the age-old adage that prevention is better than cure. However, experience suggests that despite the various disease control strategies available, emphasis is still placed on the use of antimicrobial compounds. Whereas antimicrobial compounds have a valued place as part of an overall disease control strategy, indiscriminate use can lead to environmental problems, as illustrated by a build up of antibiotic resistance in members of the aquatic microflora. Moreover, there is good evidence that, in some countries, there is widespread use of medically important antibiotics, such as chloramphenicol.

Involvement of biotechnology includes the development of antimicrobial compounds, disease-resistant stock, dietary improvements, nonspecific immunostimulants, probiotics, and vaccines (Table 2). These will be considered separately.

A. Disease Resistance

Disease resistance is a topic worthy of greater attention, insofar as there are numerous observations that point to the value of genetically disease-resistant strains for reducing the problems of disease. As a word of caution, however, comparative studies need to be carefully controlled so that meaningful results are obtained. In any comparison, the age, size, and relative condition of the animals need to be standardized. Nevertheless, there has been prolonged interest in breeding disease-resistant fish. The starting point was the work of Embody and Hayford [24], who increased resistance in brook trout to furunculosis. Subsequently, attempts were made to develop ulcer disease and furunculosis-resistant strains of brook trout and brown trout [25]. Then, it was concluded that disease was, indeed, genetically determined [26–28]. Thereafter, it was reported that varying degrees of resistance to furunculosis, which was correlated with the serum neutralization titer, occurred among 11 different strains of rainbow trout [29], with the McConnaughy strain (neutralization titre=1:80) being the most susceptible. In contrast, the Wytheville strain, which demonstrated a serum neutralization titre of 1:2560, was the most resistant [29]. Resistance to bacterial kidney disease has also been documented [30–32]. Consequently, it is apparent that the breeding of disease-resistant fish is worthwhile and is worthy of greater effort for future disease control efforts.

B. Dietary Improvements

Dietary influence is an area of comparatively recent interest in fish health. Indeed, it is ironic that, despite widespread publicity to the contrary, the precise nutritional value of commercial feeds is largely unknown. It would be pertinent to inquire about whether or not essential

Table 2 Possible Disease Control Strategies Used in Aquaculture

Improved husbandry practices, such as reduction of stocking levels, attention to hygiene
Improved water quality
Restricted movement of infected stock
Disease-resistant stock ^a
Dietary improvements ^a
Nonspecific immunostimulants (e.g., glucans) ^a
Specific immunostimulants, namely vaccines ^a
Antimicrobial compounds ^a
Use of beneficial microorganisms (e.g., as probiotics)

^aRelevance for biotechnology.

nutrients were present in the required amounts, and if other, potentially harmful substances, were present in dangerous excess. Nevertheless, it has been established that some dietary supplements are certainly beneficial for maintaining the health of fish. For example, there is a requirement for arginine and lysine by rainbow trout fry [33], with fin erosion resulting from a deficiency of lysine. The level of bacterial kidney disease may be reduced by feeding with high levels of trace elements, notably cobalt, copper, iodine, iron, fluorine, and manganese, and reducing the quantity of calcium [34]. Vitamins (i.e., A, C, and E) in diets are of value for controlling infections by a range of bacterial pathogens [35,36], including *Edwardsiella tarda* [37] and *Yersinia ruckeri* [38]. For example, it was reported that 48,000 IU/kg, 8,650 mg/kg, and 500 mg/kg of vitamin A, C, and E, respectively, enhanced resistance to enteric redmouth [38]. Evidence points to the effectiveness of glucans at enhancing the nonspecific resistance to disease, including enteric redmouth, Hitra disease, and vibriosis, probably by immunostimulation [39]. Moreover, spray-dried, heterotrophically grown (but not phototrophically grown) preparations of the unicellular alga *Tetraselmis suecica* possess antimicrobial activity and possibly immunostimulatory activity when used as dietary supplements [40]. In contrast, the presence of heavy metals, principally copper, which may be exposed to fish in diets (such as fish meal) or by way of environmental pollution, have been implicated as an initiating factor of disease, such as vibriosis in eels [41]. Personal experience suggests that the emergence and spread of some diseases, such as rainbow trout fry syndrome, may be aided by—or result from—the use of inadequately prepared or stored diets. It is not difficult to see that a poorly nourished fish would be more prone to disease; accordingly, further research effort to improve diets is clearly justified.

C. Nonspecific Immunostimulants

Success has resulted from the use of immunostimulatory compounds in fish. Such compounds, which have inevitably been applied by intraperitoneal injection, include β -1,3-glucans, synthetic peptides, and killed cells of mycobacteria. Initially, it was observed that administration of killed cells of mycobacteria enhanced resistance in coho salmon to various bacteria [42]. Then, synthetic peptides were determined to enhance resistance of rainbow trout to *A. salmonicida* [43]. However, the greatest interest has been toward the potential for β -1,3-glucans, particularly for dietary supplements. Yet, it is worthy of emphasis that there is only a tenuous link between data obtained following application by intraperitoneal injection and their subsequent extensive use as food supplements, although there has been one claim of success following the use in diets [44]. Nevertheless, a rapidly increasing literature points to the success of glucans in preventing disease [39,45–49]. For example, β -1,3-glucans, when applied by intraperitoneal injection at 2–10 mg/kg of fish, enhanced resistance to infection by *E. tarda* [45]. Parallel success was reported with glucans for the prevention of infection by *R. salmoninarum* [49] and streptococci [48]. Certainly, this aspect of research looks promising, and it is envisaged that there is a role for biotechnology in developing other immunostimulatory compounds for the future.

D. Specific Immunostimulants (Vaccines)

The rationale for the development of fish vaccines is identical with that of veterinary and human medicine (i.e., a utopian desire to rid stocks of disease coupled with a healthy regard for profit). The first published account of the development of a bacterial fish vaccine involved chloroform-inactivated cells of *A. salmonicida*, which were used to protect cutthroat trout (*Salmo clarki*) against furunculosis [50]. Since then, vaccines have been formulated against approximately half of the total number of bacterial fish pathogens [2]. From these endeavors,

vaccines to protect against edwardsiellosis, enteric redmouth, furunculosis, Hitra disease, and vibriosis (in finfish and shellfish) have reached large-scale commercial production. It is noteworthy that the simplistic approach of using formalin-inactivated whole cells, which works well with edwardsiellosis, enteric redmouth, Hitra disease and vibriosis, has met with conflicting results with furunculosis. However, more sophisticated approaches, such as involving genetic-engineering techniques, have been used and may offer hope for the future. Certainly, there has been no lack of imagination in vaccine development programs. Chemically inactivated whole-cell preparations have included use of conventional broth cultures [2], cell wall-defective or deficient cells (L-forms) of *A. salmonicida* [51], and cultures (of *A. salmonicida*) grown under conditions of iron limitation. Research has also focused on the use of subcellular components, such as lipopolysaccharide and extracellular products, which have met with some success for the control of infection by *A. hydrophila* [52] and *A. salmonicida* [53], respectively. Avirulent cells of *A. salmonicida*, lacking the extracellular (A[®]) layer that is involved with pathogenicity, have also been proposed as vaccine candidates [54]. Arguably the most modern approach concerns the use of molecular biology [2]. Clearly, molecular techniques are being used to develop improved vaccines for the control of *A. salmonicida* and *R. salmoninarum* infections. With the latter, efforts are currently being directed at cloning genes for putative virulence factors (e.g., fragments of the 57-kDa protein) into *Escherichia coli*, with a view of overexpressing the gene product for use as a vaccine.

Various methods of administering vaccines to fish have been tried with varying degrees of success [55]. Injection techniques tend to result in the best protection. However, injection is slow and will inevitably require prior anesthesia of the animals. Fortunately, automated mass injection techniques are under development and should offer promise for the future. The comparative benefit of adjuvants, especially Freund's complete adjuvant, has been well established [42]. Consequently, efforts to find superior adjuvants are justified. Nevertheless, in terms of ease of application to fish, oral uptake with food, deserves attention, particularly for microencapsulation of the vaccine to prevent breakdown of the antigens in the stomach.

E. Antimicrobial Compounds

The use of antimicrobial compounds in aquatic animals is an emotive issue in which the possibility of tissue residues and the development of bacterial resistance feature prominently in any list of concerns. Nevertheless, it is astounding that a wide range of compounds have been used in aquatic animals [56–58]. Antibiotics, many of which are important in human medicine, appear with compounds used exclusively in fisheries [56]. The use of antimicrobial compounds in fish essentially started with the work of Gutsell [59], who demonstrated the use of sulfonamides for controlling furunculosis. Indeed, it may be argued that the effectiveness of sulfonamides led to a temporary decline of interest in vaccine development. In fact, the eventual emergence of antibiotic-resistant strains of fish pathogenic bacteria led to renewed interest in vaccines. However, immediately after World War II, sulfonamides were considered the savior of fish farming. Following the introduction of sulfonamides, the range of antimicrobial compounds used expanded to include chloramphenicol [60], oxytetracycline [61], nifurprazine [62], oxolinic acid [63], sodium nifurstyrenate [64], and flumequine [65]. A pattern has emerged that points to the benefits of quinolines for controlling diseases caused by a wide range of gram-negative bacteria. Subsequent work highlighted the value of 4-quinolones or fluoroquinolones for inhibiting *A. salmonicida* [66–72]. Enrofloxacin and sarofloxacin are more effective than oxolinic acid, in terms of the minimum inhibitory concentration, at inactivating *A. salmonicida*. The search for other related compounds would be appropriate. Here, the aquatic microflora have been demonstrated to be an ideal source of

potentially useful antibiotics. Thus, in the context of fish diseases, it has been demonstrated that aquatic bacteria produce compounds with marked inhibitory activity against pathogens. For example, antiviral substances have been recovered, and determined to be effective against infections hematopoietic necrosis virus [73–75].

Another aspect worthy of consideration is the palatability of fisheries antimicrobial compounds. Whereas it is accepted that little can be done to improve the palatability of the active ingredient, effort could be directed toward improving binders and bulking agents, which are commonly contained in proprietary mixes. Perhaps, consideration could be given to using chemical attractants.

A possible involvement for biotechnology concerns the development of rapid systems for determining antibiotic-resistance patterns of fish and shellfish pathogens. A method has been described whereby pathogens are captured with a broad-spectrum antibody-based system, exposed to solutions of antibiotics, and viability of the cells assessed with tetrazolium dyes (e.g., thiazolyl blue) [76]. A commercial system would be invaluable in determining appropriate chemotherapy for diseased animals.

F. Water Treatments

Apart from the use of antibiotics and other antimicrobial compounds, the application of chemicals to water as disinfectants is effective for disease control. Examples include benzalkonium chloride, chloramine B and T, chlorine, formalin, iodophors, malachite green, and methylene blue [2]. The search for alternative chemicals is ongoing.

G. Beneficial Microorganisms

1. Bacterial Antagonists

There is some evidence that members of the normal aquatic microflora are effective at inhibiting fish pathogens. For example, antibiotic-producing bacteria, from the marine environment, inhibit bacterial fish pathogens [77]. The inhibitory mechanism centered on the presence of low molecular weight (<10-kDa) anionic, thermolabile antibiotics [77]. Others have demonstrated microbial inhibitors of *A. salmonicida*, *Flexibacter columnaris*, *Serratia liquefaciens*, and *V. anguillarum* [78–81]. So, it is argued that either the antagonists are recovered and used (as probiotics) in disease control or measures are devised to promote the natural selection and development of potential inhibitors to pathogens in the vicinity of the animals. Here, appropriate strategies would likely center on the use of selective nutrients.

2. Probiotics

There is little published information concerning the potential of probiotics in aquaculture [82], despite observations of widespread use for primary disease control purposes in shrimp hatcheries within Ecuador. Here, the use of probiotics is largely artisanal, with bacteria cultured in open-batch fermentation conditions, and added directly to the water containing larval animals. Yet, there is some indication that this approach has led to a large-scale (estimated at 90%) reduction in the use of antibiotics in shrimp hatcheries.

An alleged probiont, which was obtained from a commercial shrimp hatchery in Ecuador during 1993, was identified as *V. alginolyticus* [83]. This is interesting because most of the published work about probiotics, albeit in agriculture, has centered on gram-positive bacteria, notably putative *Lactobacillus* [84]. Moreover, the culture of *V. alginolyticus* was inhibitory to fish pathogenic vibrios, and was capable of colonizing the intestine of salmonids. In

laboratory-based experiments, application of the probiont to Atlantic salmon led to a reduction in mortalities after challenge with *A. salmonicida* and to a lesser extent *V. anguillarum* and *V. ordalii*. Thus, it would appear that there is indeed a potential role for the use of such probiotics in aquaculture as part of a disease control strategy. Clearly, further work is justified to identify other probiotics.

IV. CONCLUSIONS

There is an increasing role for biotechnology in diagnosis and control of fish and shellfish diseases. For the future, emphasis needs to be placed on diagnostic systems suitable for use in field conditions. Serological techniques, particularly those based on sandwich systems (e.g., ELISA) are well suited for adaptation for field use. Unfortunately, it seems that molecular methods will remain restricted to laboratory use for the foreseeable future. Control measures continue to attract the attention of researchers. Likely future developments will center on disease-resistant stock; vaccines, particularly involving genetic engineering; nonspecific immunostimulants (e.g., glucans); probiotics, which seem especially well suited for use in underdeveloped countries; and antimicrobial compounds purposefully developed for use in fish and shellfish.

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Marine Algae as a Tool for Bioremediation of Marine Ecosystems

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I. INTRODUCTION

Anthropogenic pollution of marine ecosystems, including rivers, bays, estuaries, and other coastal areas, is a major global environmental issue requiring immediate attention. Ongoing efforts by researchers at research institutions and universities worldwide have indicated that use of large-scale algae populations could possibly provide new and cost-effective technologies for sequestering and preventing dissemination of waterborne pollutants. These contaminants may include heavy metals, high sewage-based nutrient concentrations, bacterial and viral pathogens, and radionuclides.

A coordinated international focus is needed to implement in situ demonstration projects in "real" polluted environments to show the feasibility of this concept. Potential demonstration sites may include U.S. areas, such as the Pacific Coast off San Diego and Tijuana, the Chesapeake Bay, the Florida Keys, and Guam; the Baltic and North Seas, as well as areas within Russia, such as its many rivers and the Black Sea; and areas in the Mediterranean, including the coasts at Marseille and Malta. Note that, although each location may have different physical and biological requirements and resources, they provide a unique opportunity to do comparative research and technology development studies never before possible.

II. RATIONALE FOR USE OF SEAWEEDS IN BIOREMEDIATION

A large number of approaches exist for bioremediation of freshwater lakes, rivers, and streams, as well as groundwater. However, there are far fewer biological approaches available for seawater.

Some approaches used for freshwater ecosystems are not appropriate for marine systems. The large water volumes of bays and estuaries, much less the seas themselves, preclude economically pumping water into landbased bioreactors that use microbial degradation for bioremediation. Use of constructed marshes is limited to a narrow fringe of coastal areas and will not result in significant detoxification of large bodies of seawater. For marine systems, therefore, it is necessary to take a more extensive approach, such as use of seaweed farms that can be located over areas of the sea surface. This requires adapting and integrating current marine plant cultivation systems into reasonable bioremediation approaches.

Before describing different approaches to using seaweed for bioremediation, this section reviews some of the characteristics of seaweeds that make them useful in bioremediation. Seaweeds can remove several types of pollutants from the environment through pollutant assimilation and detoxification. These include the possibility for removal of excessive nutrients, such as nitrates or phosphates, organic pollutants, heavy metals, and radioactive nucleotides through bioconcentration within the seaweeds and detoxification.

A. Types of Seaweeds

Three groups (divisions) of algae encompass the seaweeds (or macroalgae). The differences between these divisions are primarily due to their photosynthetic pigments, followed by life cycles [Dawes, 1981].

The brown algae (*Phaeophyta*) include the largest-sized, especially the kelps. The kelps are a particular group of brown algae belonging to the brown algal order *Laminariales*. This particular group of seaweeds also provides the largest tonnage of farmed and harvested marine plants, mainly for food and chemicals, such as polysaccharides. They grow mainly in cooler marine waters at temperatures below 24°C. Other important brown algae from a marine farming perspective (and possibly bioremediation) include species of *Sargassum* and *Cystoseira*. These genera are found worldwide. All brown algae grow poorly in low-salinity seawaters and are often absent in estuaries with significant freshwater flow.

The red algae (*Rhodophyta*) provide more species of macroalgae than any other algal group. The red algae provide the highest-value products of the macroalgae. Genera such as *Porphyra* (nori) are cultivated extensively as food, whereas other genera, such as *Gracilaria*, *Eucheuma*, and *Kappaphycus*, are cultivated for their commercially useful polysaccharides. Red algae can grow over wide ranges of salinity and temperature, although there are only a few species of strictly freshwater red algae.

The green algae include only a few genera that have commercial importance such as *Monostroma* and *Caulerpa*, both eaten as food. Although green algae have polysaccharides as well, none of them are used by industry on a large scale. However, this group may offer tremendous potential for bioremediation purposes. Green algae such as *Ulva* spp. grow over extremely wide ranges of salinity and temperature. Their growth rates are fast, and they have very high rates of nutrient uptake and assimilation. In addition, they tend to be less fastidious in their environmental requirements, often growing in more polluted waters than either brown or red algae.

B. Removal of Nutrients

Seaweeds require inorganic nutrients for growth, as do other plants. The fast-growth rates of some seaweed species can account for rapid nutrient removal from marine waters. In fact, several aquaculture facilities now use "seaweed scrubbers" for removal of inorganic nitrogen sources from aquaculture effluents.

The primary nutrient that seaweeds are effective in removing is nitrogen in the form of ammonia or nitrate. Red and green algae actually require as much as 2% N of their dry weight for N-replete growth [Hanisak, 1979; Bird et al, 1982]. On the other hand, kelps such as *Macrocystis pyrifera* require less N, generally on the order of 1.5% N of dry weight. An important feature of seaweeds is that a number of species show luxury consumption of N. The red alga *Gracilaria tikvahiae* can assimilate up to 5% of its dried weight in N when the nutrient is provided in the form of ammonium [Bird et al., 1982]. The rates of N-nutrient removal can be very rapid, especially when the thalli are N-deficient. *Gracilaria tikvahiae* showed almost complete removal of millimolar levels of ammonium nitrate within 24 h, in conditions that were not light-dependent [Ryther et al., 1981]. Usually, the assimilated N is converted into an organic form, such as proteins, such as the red algal pigments [Bird et al., 1982], although some Arctic kelps store N primarily in an inorganic form such as nitrate [Chapman and Craigie, 1977].

C. Bioaccumulation of Heavy Metals

Most seaweed species show a high capacity for assimilation of heavy metals. Often, this assimilation includes significant bioaccumulation [Burdin, 1985]. There appears to be little difference as to whether or not these metals are radioactive, consequently, seaweeds can also act as sinks for these pollutants.

Seaweeds accumulate a wide range of heavy metals, such as lead, copper, nickel, zinc, cadmium, gold, silver, manganese, tin, chromium, aluminum, and cobalt. Rates of heavy-metal bioaccumulation are metal-specific, and the order of heavy metal removal varies from species to species. Amounts of bioaccumulation vary, but can be as high as 1000 mg per dry gram of tissue of seaweed sampled from highly polluted waters [Burdin, 1985]. More typical ranges are on the order of 5–100 mg/g in “normal” seawaters [Grüven et al., 1993; Sirota and Uthe, 1979]. This bioaccumulation acts to cause significant bioconcentration of heavy metals within the seaweeds, compared with the concentrations in seawater, sometimes on the order of 1000-fold [Ferreira, 1991]. The high rates of bioaccumulation appear to result partly from the cell wall components of algae. There are now engineering attempts to developed dried algal biomass as a biosorbant [Ramelow, 1993].

It is not yet certain about details of how this bioconcentration occurs. Weinberger et al. [1992] report that alginic acid is responsible for heavy-metal binding in kelps. Isolated carrageenan and dried cell wall material does cause the binding of lead and cadmium [Veroy, 1980]. However, Burdin and Bird [1994] could see no patterns of heavy-metal accumulation owing to different types of carrageenan and agar in the bioaccumulation of six heavy metals by red algal species.

D. Organic Pollutants

Although considerable research describes the effects of herbicides, pesticides, and other organic pollutants on microalgae and phytoplankton, far less work has been done on the seaweeds themselves.

Several higher plants produce a suite of enzymes that are useful in detoxification. Two key enzymes that appear to be universal are glutathione S-transferase and cytochrome P-450. Both of these enzymes act to detoxify a wide range of organic pollutants in organisms such as bacteria, flowering plants, invertebrates, and vertebrates [Jacoby, 1978; Edwards and Owens, 1986; Otto et al., 1994; Mauch and Dudler, 1993]. These ubiquitous enzymes have not been studied well in seaweeds, but it is highly likely that they exist and can provide a mechanism for detoxification of organic pollutants.

III. CURRENT RESEARCH PROGRAMS ON USE OF MACROALGAE IN BIOREMEDIALTION

Increasing awareness that certain seas, such as the Baltic Sea; major estuarine systems, such as Chesapeake Bay; and coastal regions, have high levels of pollution has encouraged the growth of bioremediation research programs. Some of the current research activity focuses on eliminating pollution at its source, one of the most effective methods of bioremediation. In some cases, such as Chesapeake Bay, water quality is already beginning to show some improvement [Swanson, 1994].

This approach cannot have universal application, because pollution does not always come from point sources, but instead may arise from seepage and groundwater transport. Multinational boundaries along the seas provide additional problems in marine bioremediation. As a consequence, various programs are being developed to investigate the use of seaweed farms as an in situ marine bioremediation system.

For example, within the United States, activities have been ongoing at specific universities investigating the processes and plant physiologies by which certain species of algae may sequester heavy metals and sewage-based nutrients. In addition, industry as well as the U.S. Navy are considering, if not already using, certain sea grasses as backup for tertiary remediation.

Demonstration projects are being proposed to test the feasibility of such technologies on a "pilot scale." The objectives of such projects are to

1. Implement an in situ demonstration project using a macroalgae moored grid farm system to sequester or act as a barrier to waterborne pollutants in an identified highly polluted marine environment
2. Obtain data and information on operational aspects of the farm system, such as
 - a. System efficiencies and processes
 - b. Additional marine ecosystem applications
 - c. Engineering design criteria and performance data
 - d. Interactions between engineering and biological mechanisms, structures, and components
 - e. System maintenance and support requirements
 - f. Farm operations and survivability in adverse weather and sea conditions
 - g. Costs, system economics, and manpower requirements
3. Conduct overall system life-cycle cost analysis and economic studies
4. Determine mechanisms for identifying and monitoring characteristics of different waterborne pollutants
5. Conduct comparative studies in different locations and ecosystems worldwide having different forms of pollutants
6. Explore optimum methods for harvesting and processing plants, followed by the appropriate disposition of the pollutants
7. Investigate additional biomass by-products applicable for sustainable development
8. Enhance international collaboration with sharing of expertise, resources, and facilities

In parallel with these efforts, Norsk Hydro (a major corporation in Norway) and the Norwegian academic community are developing a program entitled MARICULT. It addresses the cleanup and remediation of the Baltic Sea and the southern portions of the North Sea and will explore the potential use of marine biomass.

The University of Hamburg is actively exploring ways to cultivate certain species of algae in the North Sea, as well how different species sequester specific pollutants.

Because of these previous and ongoing efforts, both domestic and international, there is a strong and identified basis for using algae, a pool of available background and practical knowledge, and both scientific and operational experience supporting the importance of these demonstration projects.

IV. APPROACHES FOR USING SEAWEEDS IN BIOREMEDIATION

As with any engineering choice, the first requirement for using seaweeds in bioremediation is to clearly define the problem and what the operators expect from the system. Although this may sound obvious, it is usually ignored. Bioremediation approaches using macroalgae are also complicated by various choices that must be made. Systems using seaweeds can provide seaweed biomass that can be converted into a product. Alternatively, the desired approach may be to use the algae solely for bioremediation.

The first type of system will require more operational expenditures and possibly more capital. However, these costs can be offset by the revenue stream received from the seaweed products. An example of this is use of seaweeds as nutrient scrubbers for mariculture systems. This process (described later) results in production of algal biomass that can be harvested and processed.

The second type of system used strictly for bioremediation may not be able to produce products that can be sold. In this instance, the design should be less intensive and require lower operating costs to be cost-effective. An example of this kind of system is a proposal for a created *Macrocystis pyrifera* bed that would divert the flow of sewage nutrients from along the shore out into the sea.

Seaweeds can be cultivated on land using intensive tank systems or in ponds; in nearshore coastal areas on rope, net, or raft farms; or on offshore structures. These different systems indicate a large number of possible system designs in using seaweeds for bioremediation.

A. Land-Based Seaweed Cultivation Systems

Seaweeds are already used in some aquaculture systems as a method to remove nutrients from aquacultural effluents [Vandermeulen and Gordin, 1990; Ryther et al., 1979]. These systems generally use seaweeds growing in tanks receiving a steady stream of aeration and seawater. The aeration provides vigorous water movement in the tanks, sending the algal thalli up and down in the water column in a circular pattern. This vigorous aeration reduces diffusion boundary layers next to the seaweed thallus, and permits rapid uptake of dissolved carbon dioxide. The algae in these systems are characterized by high yields, on the order of 20–50 dry grams per square meter per day [DeBusk et al., 1986; LaPointe et al., 1978; Ryther et al., 1979; Hanisak, 1987; Vandermeulen, 1989].

Land-based tank systems are especially good for removal of nutrients. Cohen and Neori [1991] reported that *Ulva lactuca* biofilters, integrated into a fish culture system, removed 90% of the ammonia. This same macroalga also has excellent growth (and presumably nutrient removal) on municipal sewage effluents [Guist and Humm, 1976].

The algae in these kinds of systems have to be harvested for optimum nutrient removal. As a result, aquaculturists need some kind of plan for what to do with the algal biomass. In some instances, algal biomass can be fed to aquacultured herbivores, such as abalone, sea urchins, or seahares, all of which are significant consumers.

Macroalgae can also be grown in ponds. An advantage of this approach is the low-operating costs. Generally, water exchange in the ponds is accomplished by use of tide gates. Seaweed

yields are low because of the lack of water movement in the ponds and the low exchanges of water. They vary from 3 to 10 dry grams per square meter per day [Shang, 1976; Horstmann, 1983]. Depending on the design perspective, this can be an advantage or a disadvantage because there will be less algal biomass as an end product.

Pond systems are not maintenance-free. Tank systems are usually in smaller modules that allow a predictable maintenance schedule. Ponds are larger, and a significant outbreak of epiphytes or other weedy species may be much harder to bring under control. There are no known cases of seaweed culture ponds being used primarily for the purpose of heavy-metal removal or detoxification, although Haglund and Pedersén [1993] report on use of seaweed culture ponds for nutrient removal.

B. Seaweed Cultivation in the Sea

Aquaculturists grow most macroalgae in the sea as opposed to land. Cultivation occurs along coastal areas. To hold the seaweeds in place, a variety of farm structures are used. These include long lines to which ropes with seaweeds are attached, nets stretched out on frames, and ropes supported by poles. None of these systems are now being used on a large scale for bioremediation purposes. There are several experimental trials being conducted, most notably longline culture of the kelp *Laminaria* for removal of heavy metals from the Baltic Sea [Weinberger et al., 1992]. Details of these different systems are provided in the following sections. The capital costs associated with these systems vary depending on locale, water

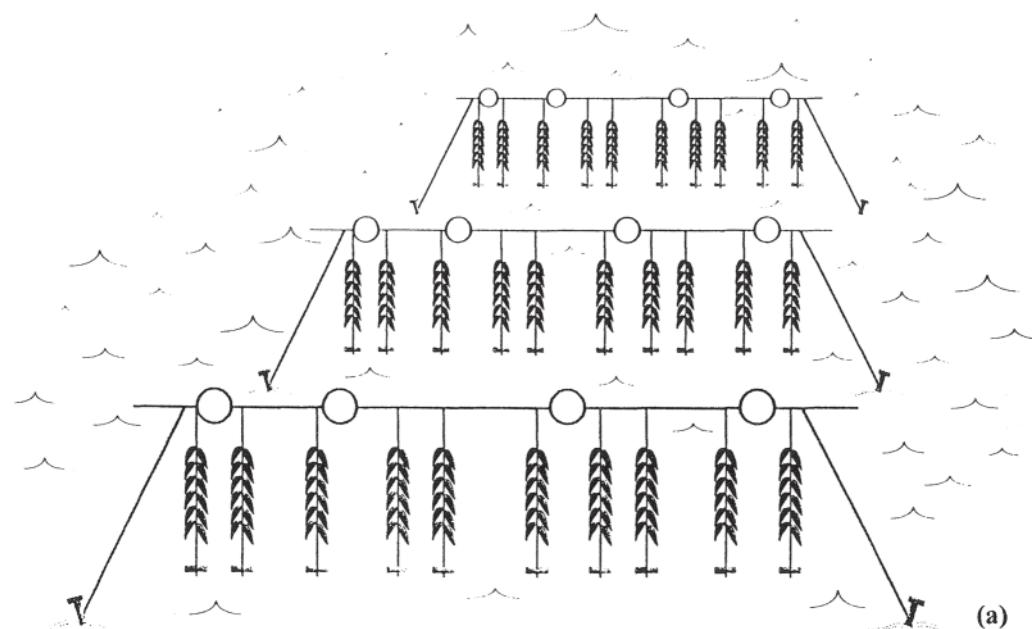
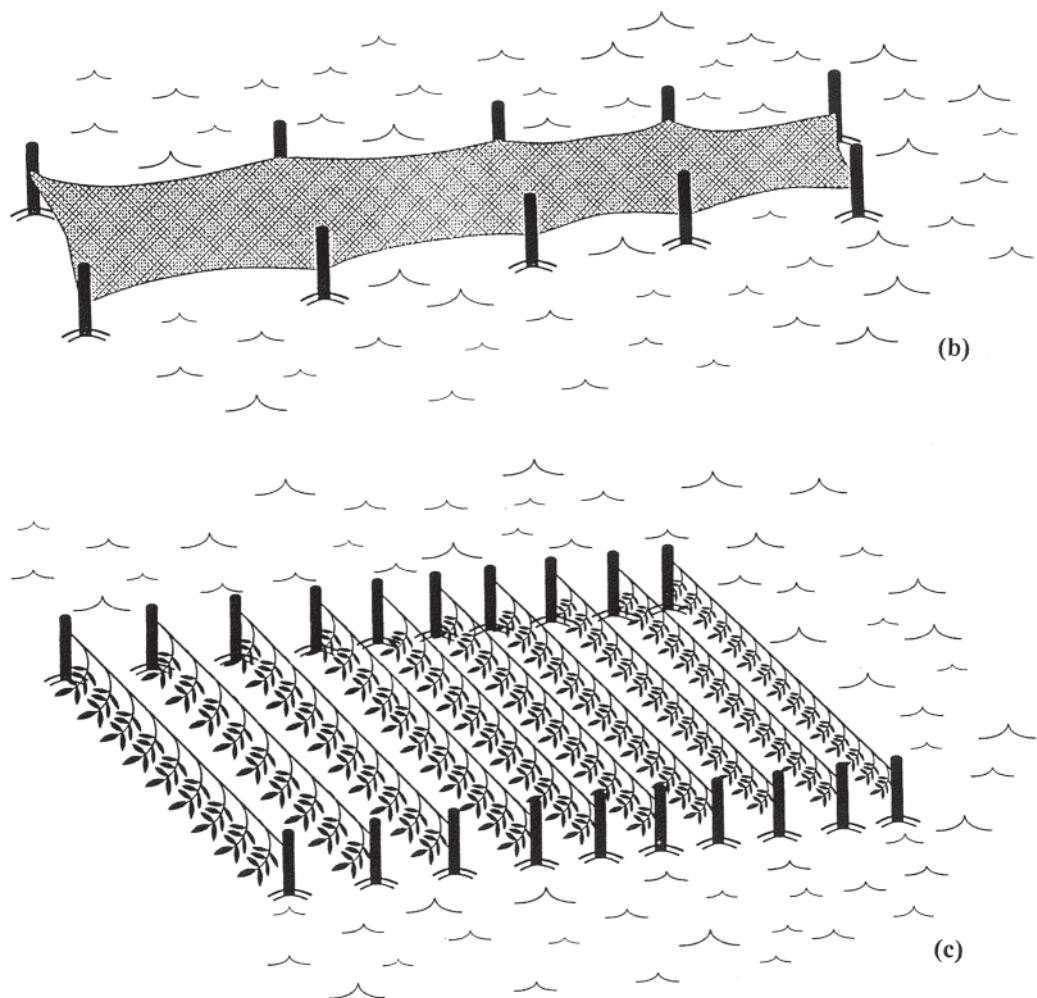


Fig. 1 (a) A Chinese long-line farm for cultivation of *Laminaria*: The long line is anchored by a rope at each end and held afloat by buoys. Culture lines with attached *Laminaria* include a spacing segment at the top and bottom of the 2- to 5-m-long rope and weighed. The anchor ropes are held by stakes or concrete weights. (b) A Japanese *Porphyra* culture net system: Poles hold the net in place so that it is exposed at low tides. The *Porphyra* grows attached to the nets. (c) A Philippine *Eucheuma* and *Kappaphycus* line (or rope) cultivation system: These seaweeds are tied to the lines that are held just above the sediment surface. The depth of the plants are adjusted so that they are not exposed at low tides.



depth, and local economies. The operating costs for these kinds of macroalgal culture systems vary as a function of harvest frequency and lifetime of materials in a site-specific environment.

1. Long-Line Cultivation Systems

Cultivation of kelps on long lines provides the largest bulk of farmed macroalgae (more than 250,000 dry metric tons per year). Species of the kelp *Laminaria* provide most of this harvest. In 1952, the Chinese tethered floating lines to the sea floor for *Laminaria* cultivation, and found this long-line method to be highly successful (Fig. 1a). The Chinese now cultivate over 15,000 hectares of *Laminaria* for food [Tseng, 1981]. *Laminaria* farms in Japan are similar, but produce a smaller crop (Ohno and Matsuoka, 1992; Brinkhuis et al., 1987; Torkko et al., 1987]. The Japanese also farm a similar kelp, *Undaria*, which is sold as the food "wakame."

The Oriental farms have been developed by trial and error. The early work of Matsumota [1959] in Japan clearly showed that by selecting an effective farm design and proper siting, one can greatly increase crop yields [Harger and Neushul, 1982]. The large increases of *Laminaria* cultivation are due to several factors. Chief among these are the long-line cultivation

technology, breeding *Laminaria* for superior growth and characteristics, developing fertilization techniques, and culturing summer sporelings. Summer sporelings are grown at low temperatures in greenhouses so that seeded lines can be placed in the field early in the fall [Tseng, 1981, 1987]. The Japanese use a forced cultivation technique to produce plants with 2-year-old characteristics in a single growing season.

2. Net-Style Farm Systems

Porphyra, or nori, is the world's most valuable marine crop. The crop is valued at more than 1 billion dollars worldwide and is used mainly for human food. These plants grow mainly in cooler waters. The algal spores are seeded onto nets that are then put in place on fixed support systems (see Fig 1b). The fixed support system exposes the nets to the atmosphere at low tides. This periodic exposure and drying acts to improve the growth of nori [Tseng, 1981]. Nori grows as a flat sheet and has a high surface area/volume ratio, a feature that aids nutrient uptake. There is a considerable amount of infrastructure and specialized marine-farming equipment available for this seaweed crop from various Japanese companies.

3. Line and Rope Farm Systems

Fishermen in the Philippines use old rope and lines to grow tropical seaweeds (see Fig. 1c). These seaweeds comprise species of genera *Eucheuma* and *Kappaphycus*. The seaweeds are tied to the lines, and the lines are then staked over the bottom. The water depths are on the order of 1–1.5 m [Trono, 1992].

Seaweed cultivation in the Philippines is quite successful, and some fishing families have seen incomes triple or quadruple. Seaweed yields on these farms vary, but 50 dry tons per hectare per year are now possible [Padilla and Lampe, 1989]. The crop is now the second largest aquaculture product of the Philippines, and one of its largest agricultural exports.

4. Offshore or Deep-Water Seaweed Cultivation

In the late 1970s and early 1980s, various attempts were made to cultivate the brown alga *Macrocystis pyrifera* on farm structures designed for use in deep water. The nutrients for these seaweeds were to come from cold, deep waters that would be pumped up into the growing kelps. The benefits of this approach is that growth of macroalgae would not be constrained by available shallow nearshore regions [Bird and Benson, 1987].

Despite several trials using different farm designs, operational problems prevented the production of actual kelp crops. However, the experiments did establish that *M. pyrifera* could grow in an open ocean environment, and that deep waters supplied ample nutrients for sustained growth without toxic side effects [North, 1987]. It is probable that these research results could be extended to other brown kelps, such as *Laminaria* spp. or *Undaria* spp. However, no reports are available that describe such offshore-farming approaches with green or red algae.

V. INTEGRATION OF SEAWEED FARMING WITH BIOREMEDIATION

Macroalgal farming should be able to provide the removal of nutrients and heavy metals from the environment. With the exception of nutrient removal by seaweeds cultivated in raceways, there have been few trials to use marine farms for these purposes. Fortunately, some of the operational features that lead to sustained, well-functioning macroalgal cultivation systems also contribute to their effective bioremediation potential.

The most obvious restriction is that seaweeds cannot be placed in an area so heavily polluted that mortality occurs. A first step in deciding on any seaweed species for use should include a marine floristics survey of the area. The use of seaweeds that already grow where the pollution occurs provides a natural selection process. In addition, aquaculturists need to look for seaweeds that can be cultured. Several seaweed species do not seem to do well when grown on different types of cultivation systems. Some seaweeds have very slow growth rates, which may be a problem or benefit, depending on the bioremediation strategy employed.

Macroalgae show a strong relation between growth and nutrient removal [DeBoer, 1981]. However, removal of organic pollutants through detoxification or heavy-metal sequestration may or may not require vigorous seaweed growth. In some instances, the accumulation of heavy metals requires the involvement of biological processes such as respiration [Klumpp, 1980] or protein synthesis [McLean and Williamson, 1977]. Markham et al. [1980] found that more cadmium accumulation occurred in slower-growing plants and parts of plants for the species *Laminaria saccharina*. Alternatively, even freeze-dried algal thalli that were rehydrated showed significant heavy metal accumulation in short-term experiments [Burdin and Bird, 1994].

Water motion is a significant factor affecting nutrient and dissolved inorganic carbon uptake by marine algae [Wheeler, 1980; Koch, 1993], but the effects of specific types of water flow (laminar and turbulent flow) on heavy-metal uptake have not been well documented. Nonetheless, there is some empirical evidence that increased water velocity leads to increased uptake of heavy metals [Burdin, 1985]. Therefore, the same principles that seaweed aquaculturists use to site farms in relation to currents for improved growth can be hypothesized as providing better heavy-metal assimilation.

These principles have varied from simply placing seaweed culture systems perpendicular to currents, to more sophisticated analyses of current flow within farms [Tseng, 1981, 1987; Wang and Ditmars, 1982; Jackson and Winant, 1983]. A necessary first step in using seaweeds for bioremediation should include a more detailed analysis of how different types of current flows affect heavy-metal uptake and bioaccumulation by seaweeds.

Both light and temperature are two other important physical factors that affect the success of any seaweed cultivation effort. Selection of seaweed candidates that are local to an area is one approach to using species that are adapted to local conditions. However, sites with strong seasonal changes in water temperatures may require the use of different seaweed species at different times of the year. For example, year-round marine biomass cultivation in Long Island Sound called for a strategy of growing *Laminaria* spp. in the colder winter months and *Gracilaria tikvahiae* in the warm summer months [Brinkhuis et al., 1987].

Before too many large-scale efforts go forward to use seaweed farming in bioremediation, a number of physiological experiments may help clarify some potential strategies. The use of seaweeds for nutrient removal will require cultivation systems that provide growth. Because these systems will be producing biomass, operators will find it necessary to harvest the seaweeds to maintain them at optimal-stocking densities for growth and nutrient uptake [Hanisak, 1987]. Therefore, operators will need to develop a plan for what to do with the harvested biomass.

Seaweeds have various uses and produce a number of products, which will be described later. Systems that do require significant amounts of seaweed harvesting and handling will incur higher operations costs. Such costs may be offset by revenues coming from sales of seaweeds or their products.

The role of seaweed growth in heavy-metal accumulation or pollutant detoxification is less clear. If some slow-growing species can actively remove heavy metals from water or show detoxification activities, such systems may not require significant operational costs other

than routine maintenance. Several physiological and cultural experiments that compare growth rates with bioaccumulation rates or detoxification activity may provide some evidence for strategies to use these species in systems with lower operating costs.

VI. DISPOSAL OF SEAWEEDS CONTAMINATED WITH POLLUTANTS

Any well-operated seaweed farm will require periodic removal of the crop and replanting the farm with fresh seaweed. If seaweeds have been used for heavy-metal accumulation or detoxification, other uses of the biomass may not be possible owing to high internal levels of pollutants. One approach may be to dry these contaminated seaweeds, and dispose of them or further concentrate the pollutants in them. A number of such strategies are described elsewhere in this book.

Another approach to treating toxin-contaminated biomass is the use of bioconversion to methane. An advantage of this approach is that seaweeds do not have to be dried before the bioconversion process. Bioconversion can result in as much as 80% transformation in the amount of organic matter being converted into methane and carbon dioxide [Chynoweth et al., 1987]. The bioconversion process produces three streams: a gaseous stream (mostly methane and carbon dioxide) that would contain no heavy metals, but possibly volatile toxins; a liquid effluent that would have significant concentrations of toxins; and solid residues containing toxins bound to the particulate carbon. Nonetheless, a bioconversion approach could represent an excellent method to further concentrate the amounts of accumulated pollutants for other methods of recovery or disposal.

VII. USES AND PRODUCTS FROM SEAWEEDS

There are various uses for seaweeds and products from seaweeds. Their integration into any kind of bioremediation approach for disposal of algal biomass will depend on the degree of pollutant accumulation and safety factors about the product's use. Seaweeds that are simply used for removal of inorganic nutrients provide the fewest problems for disposal in terms of safety issues.

Seaweed can be consumed directly as food, and this is still the largest use of seaweeds today [Jensen, 1993]. The major seaweed consumed is nori, followed by different kelps. Small amounts of the seaweeds *Caulerpa* spp., *Gracilaria* spp., and a few others are also eaten.

Macroalgae are also consumed by animals, and can serve a feed in aquaculture systems. Much of the *Gracilaria* cultivated in Taiwan has now been diverted from the hydrocolloid market and is sold as a feed to abalone farmers [Ajisaka and Chiang, 1993]. Abalone eat kelps as well. Other important herbivores grown in aquaculture include Caribbean queen conch, sea urchins, and certain herbivorous fish species.

Perhaps the best known approach for Westerners is the use of algae as a feedstock for polysaccharide extraction. The algae provide a variety of commercially important polysaccharides, such as agar, different types of carrageenan, and alginate [Waaland, 1981]. Estimates vary, but the total market for algal polysaccharides is in the range of 500 million dollars/yr [Jensen, 1993].

Smaller uses of seaweed include its conversion into fertilizers and meals. The use of such seaweed products is growing in Europe, especially where organic farming is popular.

Composting of seaweeds is also becoming more important. Large blooms of macroalgae can clog estuaries and foul recreational beaches. After harvesting, the biomass must be reduced in volume. A composted product offers some opportunity for creating additional revenue

streams. There are several different methods for composting seaweed, but particularly effective methods involve the addition of small amounts of lignocellulosic residues [Mazé et al., 1993].

VIII. ROLE OF BIOTECHNOLOGY

Although seaweeds already offer many advantages for bioremediation of marine waters, their usefulness can be improved. Conventional genetics and breeding may not suffice for the kinds of improvements needed to increase the effectiveness of macroalgae in bioremediation. Detoxification and accumulation can be more easily accomplished by the introduction of target genes into algal genomes. Among these genes are bacterial genes for the production of metallothioneins, which can both aid in accumulation as well as detoxification of heavy metals. Another approach would include gene amplification for increased production of glutathione-S-transferase and cytochrome P-450. Recently, O'Keefe et al. [1994] reported on the transformation of crop plants with a bacterial cytochrome P-450, with the objective of increasing detoxification of herbicides. There are recent reports on the introduction of single genes into macroalgae, but this field is still in its infancy [Cheney and Kurtzmann, 1992].

IX. SUMMARY AND RECOMMENDATIONS

The use of macroalgae in bioremediation of marine waters appears to have potential. The development of these systems will require some physiological studies as well as field trials. Among the major physiological studies needed is whether algal growth is required for heavy-metal accumulation and detoxification of other pollutants. These kinds of data can provide some significant input for the design of system approaches for marine bioremediation.

The major goal of bioremediation is both efficiency in pollution reduction and cost-effectiveness. Systems that have high operational requirements may not be feasible from an economic perspective. Slower-growing species of algae that show good bioremediation potential may provide a better overall choice than use of faster-growing algae for which intensive harvesting operations would have to be used. Studies that compare growth rates with heavy-metal accumulation or detoxification activity would not require elaborate field trials.

At some point, field trials and demonstration projects will be necessary to determine how well macroalgal cultivation systems do in bioremediation. Such trials are also necessary to begin optimization at a field level. The history of algal cultivation shows that strict physiological studies are insufficient to predict performance of seaweed farms. Other ecological variables such as farm hydrodynamics, plant-to-plant competition, competition with other species, epiphytes, and grazers have significant effects equal to strict physiological factors.

Environmental issues are also an important consideration. Many algal species produce significant amounts of dissolved organic carbon and particulate carbon. The interaction of this carbon with pollutants needs to be determined, particularly relative to the possibility that this carbon could cause precipitation of pollutants into sediments. This would only transfer the pollutants from one part of the ecosystem to another, without any long-term benefits.

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Environmental Applications of Marine Biotechnology

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I. INTRODUCTION

Marine biotechnology, which may be broadly defined as the application of scientific and engineering principles to the processing of materials by marine biological agents to provide goods and services [Zilinskas et al., 1995], involves a variety of industries, products, and production processes, and is now poised to enter a phase of rapid growth in commercial and environmental applications. Since 1983, more than 1000 publications have described the discovery of new compounds and natural products; the molecular genetics of fish and shellfish growth, metabolism, and reproduction; and new approaches to bioremediation, biofouling, and biocontrol. Promising new antibiotics, anticancer agents, adhesives, pharmaceuticals, enzymes, specialty chemicals, aquaculture stocks, marine polysaccharides, and energy sources are in various stages of research and development [Colwell, 1995; Zilinskas et al., 1995]. In the environmental arena, appropriate applications of marine biotechnology will help retain environmental productivity and restore environmental quality.

Most marine pollution problems occur in coastal areas, but originate in terrestrial inputs. Rivers carry pollutants to the sea from various inland sources. Agricultural practices—including the widespread use of fertilizers, pesticides, and herbicides—lead to nonpoint-source leaching of contaminants directly into the oceans from coastal regions and indirectly by rivers. Many shore-based operations, such as ship loading, result in point discharges of a variety of pollutants. In contrast, only a few problems arise offshore, such as those related to oil well blowouts and wrecks of oil tankers, and these problems have few measurable effects unless shorelines become contaminated. The vast dilution capacity of the sea and the degradative capacities of diverse marine microorganisms minimize the effect of contaminants.

Protecting and restoring the marine environment has become an increasingly high international priority, and marine biotechnology can play many roles. We have chosen to discuss six environmental application areas of marine biotechnology.

1. Waste processing and treatment
2. Monitoring of general ocean pollution
3. Bioremediation of spilled oil
5. Bioremediation of dredged sediments
6. Environmental restoration and preservation
7. Marine biotechnology and global change

II. WASTE PROCESSING AND TREATMENT

The treatment of raw sewage is perhaps the oldest of the environmental biotechnologies, and it is practiced on an enormous scale in the developed world [Organization for Economic Cooperation and Development, 1994]. Although advanced waste-treatment facilities are now a standard feature of many cities and the technology is mature, many coastal communities either do not treat their sewage or use only primary screening to remove solids before discharging it into the sea. Only some 10% of the total global production of waste is treated, and the remainder can place a severe burden on natural self-purification processes, particularly in the marine environment. Although small coastal villages often release their wastes directly into the sea with minimal effects, the output from large municipalities can cause substantial consequences. The sediments that receive such sewage often become anoxic, so there is little marine life, and biodegradation, which is often most rapid under aerobic conditions, is slowed. Natural cleansing processes are thus inhibited and, once problems occur, they tend to be self-amplifying so that the problem impinges on an ever greater area. Polychlorobiphenyls (PCBs) and other xenobiotic pollutants and heavy metals in sewage also contaminate sediments.

Modern biotechnology has much to offer the waste-treatment industry that produces effluents that enter marine ecosystems, although terrestrial and freshwater systems are receiving the most attention. Recent work has focused on better removal of nitrogenous and phosphorus nutrients, which stimulate algal growth in outfall areas; microbial nitrification is a promising approach [Fang et al., 1993], as is the construction of artificial food chains [Tredici et al., 1992]. Kortstee et al. [1994] have reviewed recent advances in the understanding of bacterial phosphorus accumulation and how it might be exploited more effectively in the future. Zelibor et al. [1988] proposed a method of using microbially mediated electrostatic fields to accelerate the formation of spherical vivianite $[Fe_3(PO_4)_2 \cdot 8H_2O]$ crystal aggregates in sediments; the method could be applied to aquatic systems that have received large amounts of phosphate from waste-treatment effluents.

Some waste-treatment systems that were designed to remove only organic compounds from wastewater aerobically have been modified to include anaerobic zones for removal of inorganic chemicals, such as nitrates. Under anaerobic conditions, microorganisms can transform nitrate to molecular nitrogen, thereby decreasing the nitrate in wastewater that might otherwise cause eutrophication of the receiving bodies of water. Microorganisms can also immobilize phosphates during wastewater treatment. Removing nitrates and phosphates prevents eutrophication in coastal waters that receive sewage effluent.

Waste-treatment systems can also be designed to maintain populations of specific genetically modified microorganisms that can biodegrade compounds found in industrial plant wastewater [Fujita and Ike, 1994], such as hydrocarbons and chlorinated solvents. These compounds sometimes escape biodegradation in traditional wastewater-treatment systems. Multistage treatments with individual bioreactors that contain microorganisms to remove individual contaminants are particularly useful in treating industrial wastes. One stage can reduce the overall biochemical oxygen demand through the general biodegradation of organic compounds,

whereas stages can be designed to biodegrade compounds in the waste stream that are not attacked in the initial stage. Textile-manufacturing plants in Asia use this approach to degrade azo dyes that are not biodegraded in traditional activated sludge or trickling-filter treatments. The bright yellow wastes of these plants are released as colorless effluents from the treatment facilities.

Another idea that has received increased attention is recent years is the discharge of partially treated waste into constructed wetlands where a final "polishing" can occur. Although this is principally a terrestrial concern [e.g., Knight et al., 1993; Baillie, 1995], there are marine applications. For example, Guida and Kugelman [1989] constructed marsh mesocosms for treating sludge effluents from a clam-processing plant and were able to meet direct-discharge standards; a similar approach can be used to remove metals from wastewater [Wildeman et al., 1994]. Constructed wetlands are not only effective water-treatment options, but also have substantial public appeal because they can have a dramatic influence on highly valued fauna, such as birds. Artificial wetlands constructed as part of the Des Plaines River Wetlands Demonstration Project resulted in a 400% increase in the number of species and a 4000% increase in the number of birds using the area [Hickman, 1994]. Similar increases, although perhaps less dramatic, can be expected for marine applications.

III. MONITORING OF GENERAL OCEAN POLLUTION

Modern biotechnology is providing numerous and powerful new ways to define the extent of marine pollution caused by coastal runoff and deliberate inputs. Since the 1970s, scientists of several countries have been using common bivalves, such as blue mussels and oysters, to monitor chemical contaminants in coastal waters [Farrington and Tripp, 1993; Regoli and Orlando, 1994]. Enzymatic techniques are widely used to monitor the exposure of marine species to pollutants [Livingstone, 1993]. Van Dover et al. [1992] have followed the incorporation of dumped sewage sludge into the food web by studying the abundance of stable isotopes, and this approach can be extended to other situations in which organic matter is dumped in the sea [Gearing, 1991; Shearer and Kohl, 1993]. Understanding the extent of the effect of ocean pollution is clearly an important determinant of whether remediation is needed, or will be needed in the future.

Microorganisms with reporter genes, such as the *lux* genes that code for bioluminescence, can be used to detect pollutants [Hill and Stewart 1994]. The *lux* genes can be fused with a promoter for stress-related genes, such as the SOS system of *rec* genes. The promoters are activated when bacteria encounter environmental stress factors, such as pollutants. The fusion of the *lux* genes to stress gene promoters permits the simple monitoring of their activation (stress detection) by the observation of bioluminescence. Samples are collected from a waste stream and added to contained cultures of the genetically engineered bacteria with the *lux* reporter genes. Biosensors are also being developed for environmental monitoring, particularly of pesticides [Dennison and Turner, 1995]. Developing such systems for marine bacteria will permit simple biomonitoring of pollutants in marine ecosystems.

IV. BIOREMEDIATION OF SPILLED OIL

The major role of bioremediation in the marine environment has been in speeding the natural biodegradation of spilled oil when spills have affected a shoreline [Atlas and Atlas, 1991; Prince, 1993; Hoff, 1993; Swannell and Head, 1994, Lethbridge et al., 1994]. Crude oil has been a part of the natural marine environment for millennia; oil seeps add an estimated 90×10^6 gal/year [National Research Council, 1985]. It is not surprising that hydrocarbon-degrading

bacteria are found in all environments where they have been looked for [Leahy and Colwell, 1990; Atlas and Bartha, 1992; Prince, 1993]. Today, total input of oil into the sea is perhaps ten times greater, with most of the additional oil coming from municipal runoff and shipping. Although major tanker spills have serious local effects, their total contribution is actually on the same scale as that from natural seeps [National Research Council, 1985]. To date, 1991 has been the worst year for catastrophic oil releases; the Persian Gulf spill of about 240×10^6 gal accounted for 86% of the total [Welch and Yando, 1993]. The second-worst year was 1979, when the IXTOC 1 well in Ciudad del Carmen, Mexico, released some 140×10^6 gal [Ross et al., 1980]. Those spills dwarf those from tankers; for example, the largest tanker spill was probably that from the *Amoco Cadiz* off Brittany, France, in 1978, which amounted to about 67×10^6 gal [Bellier and Massart, 1979]. Indeed, the U.S. Coast Guard estimates that 98% of spills from vessels are of less than 0.1×10^6 gal [U.S. Department of Transportation, 1992].

Microbial degradation consumes most of the molecules in crude oils, regardless of whether the oil is from a natural seep or from a damaged vessel, pipeline, or production facility. Unlike many other carbon sources, oil contains little nitrogen or other essential nutrients. In aerobic environments, the degradation of substantial amounts of oil, therefore, is typically limited by the supply of nitrogen and phosphorus. Hence, adding such nutrients is a promising approach to stimulating degradation. It is important to remember, however, that the growth of marine algae is also typically limited by the availability of nitrogenous nutrients, so care needs to be taken to stimulate the growth of oil-degrading microorganisms without stimulating algal growth. One elegant solution is the use of oleophilic nutrients [Atlas and Bartha, 1973]; another is the use of slow-release fertilizer [Townsley, 1975]. Rosenberg et al. [1992] have reported the use of a urea-formaldehyde polymer fertilizer and an inoculum of oil-degrading bacteria that use the polymer as a nitrogen source. Many proposals have suggested more sophisticated approaches [see Prince, 1993], and the first patent awarded for a genetically engineered organism was for an organism designed to degrade hydrocarbons [Chakrabarty, 1981], but few of these approaches have been used in response to real spills.

The most successful application of bioremediation to a marine oil spill has been that in Prince William Sound and the Gulf of Alaska after the spill from the *Exxon Valdez*. Adding a combination of oleophilic and slow-release fertilizers to stimulate biodegradation by the indigenous microflora sped degradation substantially and had no adverse ecological effects, and the process took several years less than unassisted natural cleansing would have taken [Pritchard and Costa, 1991; Prince et al., 1994; Bragg et al., 1994]. That success can probably be extrapolated to other well-aerated shorelines, and more than 40 companies offer similar bioremediation products and services in the 13th edition of the *International Oil Spill Control Directory* [1993].

Spilled oil bioremediation with the addition of exogenous bacteria has been less successful. It was attempted at the spills from the *Mega Borg* in the Gulf of Mexico [Leville, 1991] and the *Apex* barges in Galveston Bay [Mearns et al., 1993], but with no apparent success. Rosenberg et al. [1992] might have been more successful in treating a spill that affected a beach in Israel with their combined polymer-fertilizer and bacteria application described in the foregoing, but their analytical methods may have overestimated the extent to biodegradation [see Prince, 1993]. There has been little success with the addition of exogenous bacteria in terrestrial applications [Pritchard, 1992]. Whether engineered bacteria with specific degradation capabilities will achieve their much-touted potential to remediate spilled oil is an open question that hinges not only on the capabilities of the bacteria, but also, at least in the United States, on regulations governing their release into the environment [de Lorenzo, 1992; Wilson and Lindow, 1993; Day, 1993; Miller, 1994].

Given the ubiquity of hydrocarbon-degrading microorganisms in marine ecosystems and the natural increase in such microbial populations in oil-polluted waters and sediments [Atlas, 1981; Leahy and Colwell, 1990; Prince, 1993], it is not surprising that seeding with microorganism has not been demonstrated to be efficacious for spilled oil bioremediation. Most bioremediation applications follow physical cleanup activities, when the numbers of indigenous hydrocarbon-degrading microorganisms have increased owing to the presence of the spilled oil. This increase, often by several orders of magnitude, then limits the usefulness of seeding with exogenous hydrocarbon-degrading microorganisms. An increase in the number of hydrocarbon-degrading bacteria by more than a factor of 1000 was observed along the coast of Brittany within 24 h of the arrival of oil spilled from the tanker *Tanio*. Adding more microorganisms with similar hydrocarbon-degrading enzymatic capacities would not have been of great benefit in that or similar cases. Competition between the enriched indigenous microbial populations and exogenous cultures would further have decreased the likelihood of successful application of microbial seeding for spilled oil bioremediation, even if the seed cultures had a greater range of hydrocarbon-metabolizing capacities for degradation than the indigenous microbial populations.

The cleanup of spill-affected marshes and wetlands, including mangroves, presents a promising opportunity for bioremediation. There seem to be no well-characterized and environmentally benign treatments for such ecosystems, which tend to be anaerobic and fragile [Baker, 1983], although bioremediation is being considered [Scherrer and Mille, 1989; Oudot and Dutrieux, 1989; Nadeau et al., 1993]. Anaerobic hydrocarbon degradation is well documented [see Prince, 1993]; nitrate, sulfate, and metal ions are all capable of functioning as terminal electron acceptors in place of oxygen, but there have been no reports of successful stimulation of such activity after a spill. Coral reefs are also fragile environments [Jackson et al., 1989] for which there are no generally accepted approaches to cleaning spilled oil and for which biotechnology might have much to offer.

The bioremediation of floating oil slicks so that they are degraded before they hit a shoreline has great public appeal. No successes have yet been reported, despite some efforts by the oil industry [see Prince, 1993], and the industry's preferred response to spills is collection of the oil and use of dispersants [National Research Council, 1989]. Although dispersants received a bad reputation after their use in connection with the *Torrey Canyon* spill in 1967 [Southward and Southward, 1978], dispersion is the natural fate of most spilled oil even without the use of dispersants; the January 1993 spill from the *Braer* off Shetland is a recent example [Thomas and Lunel, 1993]. There are good theoretical reasons to believe that dispersion with nontoxic dispersants will increase the rate of natural degradation, and inoculation with oil-degrading organisms might also have a role in such situations.

V. BIOREMEDIALION OF DREDGED SEDIMENTS

Most harbors in the United States require periodic dredging to maintain safe water depths, but sediments in many harbors have been contaminated over the last 300 years with a variety of pollutants that makes disposal of dredged material problematic. A major problem for a bioremediation approach is that the varied contaminants require different approaches for safe and effective degradation, and appropriate strategies for some contaminants might increase the potential hazard from others. For example, dechlorination of the most-chlorinated polychlorinated biphenyls requires anaerobic conditions, but complete removal from the environment requires aerobic degradation [Abramowicz, 1990, Harkness et al., 1993]. Current hydrocarbon bioremediation technologies rely on aerobic degradation, but immobilization of potentially harmful metal ions requires anaerobic conditions [e.g., Fude et. al., 1994].

Nevertheless, bioremediation technologies potentially have much to offer in treating dredged material; perhaps there will be a role for engineered organisms in the treatment of some of the halogenated pollutants [Wackett et al., 1994; Furukawa et al., 1994; Timmis, 1995].

VI. ENVIRONMENTAL RESTORATION AND PRESERVATION

Modern biology is providing important insights into ways of improving, maintaining, or restoring the marine environment. One well-known example is the growing of various seagrasses and halophytic plants to protect sand dunes and marshes against erosion. Sylvia and Will [1988] studied the colonization of such plants by vesicular-arbuscular mycorrhizal (VAM) fungi and other soil microorganisms, and it was rather slow. The possibilities of stimulating such colonization is an appealing prospect. Another example is the cultivation of macroalgae in the Venice Lagoon to prevent eutrophication [Cuomo et al., 1993].

Promising advances that might lead to valuable environmental tools include the recent isolation of a virus that is apparently capable of controlling the brown-tide microalga *Aureococcus* [Milligan and Cosper, 1994]. Perhaps the virus can be used to keep these potentially toxic organisms under control, and it is likely that viruses can control blooms of other nuisance organisms. The recent discovery of the "phantom" dinoflagellate as the causative agent of fish kills in estuaries [Burkholder et al., 1992] tells us that there is much to be done in characterizing and controlling these probably ubiquitous organisms.

A quite different use of modern biotechnology is the forensic use of biomarkers to monitor compliance with international and state laws [e.g., Stone, 1995]. Baker and Palumbi [1994] have used a molecular-genetic approach to determine which whales are being sold in Japan, and Jahncke et al. [1991] have reported techniques for differentiating wild-caught fish from farmed fish by analyzing their fatty acid composition; at least for striped bass (*Morone saxatilis*), there are clear fatty acid markers in fish fed on vegetables. Fatty acid profiles also allow the detection of marine turtle oils in cosmetics and other preparations [Joseph et al., 1985], and it is not unreasonable to expect that they will be able to distinguish fish species and populations, which is vital for sound stock management.

A rather different aspect of the use of biotechnology in the pursuit of a cleaner environment is in pollution prevention. Examples of problems in the marine environment include oil sheens from motor vessels, plastic trash along the world's shorelines, and contamination by antifouling paints. All are amenable to at least some amelioration with biotechnology. Fully biodegradable lubricating oils are now available for marine lubrication [Wilson, 1991]; fully biodegradable plastics for the marine market, made from bacterial polymers, are available to replace nonbiodegradable petrochemical plastics [Doi, 1990]; and progress is being made to develop biomimetic antifouling paints [Todd et al., 1993].

VII. MARINE BIOTECHNOLOGY AND GLOBAL CHANGE

The tremendous consumption of fossil fuels since the dawn of the industrial revolution has undoubtedly resulted in increasing concentrations of CO₂ in the atmosphere. The influence of this increase, however, is debatable [e.g., Flannery, 1991]. A major uncertainty in predicting future trends is the role of the ocean in absorbing and transforming atmospheric CO₂, and it seems unlikely that we will be in a position to predict trends [Siegenthaler and Sarmiento, 1993] without at least some understanding of the fundamental marine biological phenomena.

The last few years have seen a reappraisal of marine biomass in the global carbon cycle. For example, Parkes et al. [1994] have provided evidence of a previously neglected biological

community that might add 10% to estimates of the amount of living biomass on our planet (the microbial communities in deep-ocean sediments), and others have shown that primary productivity at the surface is much greater in southern oceans than previously thought [Arrigo and McClain, 1994; Sullivan et al., 1994]. Marine viruses are being recognized as an important constituent of marine ecosystems, but their role in determining plankton primary productivity [Suttle et al., 1990; Hennes and Simon, 1995], bacterial numbers [Proctor and Fuhrman, 1992], and pelagic food webs [Thingstad et al., 1993] is only beginning to be understood. Molecular– biological approaches to elucidating the magnitude of the oceans' contribution to the global carbon cycle will undoubtedly play an important role in extending our understanding of the fate and effect of atmospheric CO₂ levels.

Even in the absence of a clear understanding of the effects of globally increased atmospheric CO₂, there is political pressure to at least slow the trend. Obvious approaches include reducing the use of fossil fuels by improving energy efficiency or by using renewable energy sources, although some believe that there will be a role for growing biomass to sequester atmospheric CO₂ out of the biosphere. It is widely viewed, especially in Japan, that marine biotechnology has the potential to lower global atmospheric CO₂, both by sequestering carbon and by producing CO₂-neutral fuels.

Carbon sequestration is a potential approach to reducing CO₂ accumulation in the atmosphere, although biological debate usually focuses on terrestrial forests [Hall and House, 1994]. Ocean disposal of the biomass is one potential option, and it is not impossible to envision using marine organisms instead of trees to harvest the CO₂. Kodama et al. [1993] recently found marine microalgae that grow rapidly on gas streams that contain over 70% CO₂, and Fukuzawa et al. [1992] have shown that carbonic anhydrase, the zinc enzyme that interconverts soluble CO₂ and bicarbonate, is essential for photosynthetic growth. The problem of how to store the biomass for long periods without anaerobic fermentation has not yet been addressed, but deep-sea disposal is sometimes suggested.

An alternative approach that has been discussed would be to stimulate marine photosynthesis in situ by adding some limiting nutrient, so that CO₂ would be taken from the atmosphere. Perhaps the best-known suggestion is what is sometimes referred to as the “ocean Geritol” effect: the addition of iron to the southern oceans. This was tested recently on a large scale; although primary productivity was stimulated, it is hard to envision that such an approach will have much influence on the global CO₂ budget [Martin et al., 1994]. Zinc supplements have also been suggested to stimulate photosynthesis [Morel et al., 1994], but again it is hard to imagine this as useful on a global scale. Both approaches, however, provide important and fundamental information on the global carbon cycle that is essential if we are to understand the fate of anthropogenic CO₂.

Most discussion of renewable fuels focuses on land plants [Hall et al., 1993], but marine algae—both macroalgae (e.g., kelp) and microalgae (microscopic)—have also been considered. They could be grown in the sea in enclosures, or on land with otherwise-unexploited saline water in arid regions [Chelf et al., 1993].

Cultivated macroalgae are reported to have yields of up to 150 tonnes dry weight per hectare per year [Gao and McKinley, 1994], although more realistic estimates are probably near 34 tonnes dry weight per hectare per year [Bird, 1987]. Even the latter is higher than average yields of sugarcane, the most productive land plant in widespread cultivation. The history of various attempts to use macroalgae as a source of renewable fuels is recounted by Neushul [1987]; no demonstrations are under way in the United States, although there are reports of large kelp harvests in China [Tseng, 1987].

Microalgae are receiving rather more attention, both in the United States and in Japan. Current estimates of annual productivity are up to 30 tons/acre (81 tonnes/ha) if saturating

concentrations of CO₂ were provided, perhaps from power-station flue gases [Nishikawa et al., 1992; Negoro et al., 1993]. Cell walls of microalgae from both freshwater and marine environments contain highly cross-linked, macromolecular, paraffinic hydrocarbons that resist biological and chemical degradation [Zelibor et al., 1988; Derenne et al., 1992a]. The macromolecular paraffinic hydrocarbons are similar to those found in the humin fraction of aquatically derived humic substances and are believed to be the precursors of marine kerogens [Hatcher, 1983; Derenne et al., 1992b]. It is reasonable to view the macromolecular paraffinic hydrocarbons in algae as a carbon sink because they can be buried, thereby removing CO₂ from the carbon cycle [P.G.Hatcher, personal communication]. Chelf et al. [1993] estimate that all the CO₂ emitted from fossil fuel power plants in Arizona and New Mexico could be captured by microalgae farms that occupy only 0.25% of the states' area. However, current cost estimates are rather high, and the only commercial large-scale exploitation of microalgae today is for food additives [Becker, 1994].

The simplest way to use algal biomass would be to harvest it, dry it, and use it as a CO₂-neutral fuel. Jenkins [1993] claims that such a process could provide electricity at half the cost of using coal and marine disposal of the CO₂, but this work is still in the pilot stage of development. Catalytic gasification is another alternative [Arauzo et al., 1994]. Converting algal biomass to methane by anaerobic digestion would be only slightly more complicated [Gerard, 1987]. Anaerobic degradation is an important part of modern waste treatment, although usually in agricultural situations for which gas production is secondary to waste disposal [Colleran, 1992; Rajabapaiah et al., 1993]. The process can be modulated so that most of the biomass is converted to CO₂ and CH₄, typically with a large excess of methane [Lettinga and van Haandel, 1993]. The CO₂ could be recycled back to algae [Conde et al., 1993], leaving a clean methane fuel. Marine algae are a particularly good feed for anaerobic digestion because they contain no lignin; land plants typically contain some 20% lignin (dry weight), which is quite resistant to anaerobic degradation. Kelp is perhaps the best source of biogenic methane yet found [Neushul, 1987]. An important caveat is that if anaerobic gasification were to be used on a large scale in a "greenhouse-limited" scenario, any leakage of methane would have to be minimized, because methane is a substantially more potent greenhouse gas than CO₂ [Rodhe, 1990]. It should also be remembered that there are tremendous reserves of natural gas in both the United States and the rest of the world, and biogenic methane is unlikely to compete unless there were a substantial incentive for a CO₂-neutral fuel.

Another simple approach would be to ferment the algal biomass to produce acetone, butanol, and ethanol, the ABE fermentation [Jones and Woods, 1986]. A variant of this was practiced on an enormous scale in Chula Vista, near San Diego, California, during World War I. The Hercules Powder Company built a plant that worked 24 h/day, 365 days/yr, with 800 workers designed to handle 1500 tonnes of kelp per day to produce acetone for cordite production; it was discontinued as soon as the war ended [Neushul, 1987]. ABE fermentation remained important in industrial processing (using corn and other terrestrial biomass) into the 1960s, but it cannot compete with petrochemical processes in today's market.

An approach that conceivably would yield a more valuable product would be to grow microalgae that produce oils; the National Renewable Energy Laboratory in Golden, Colorado, has had a small research program for several years [Wyman, 1994], and work is progressing in Japan [Dote et al., 1994; Kishimoto et al., 1994]. It is estimated that total microalgal productivity could be as high as 30 ton/acre (81 tonne/ha) if saturating concentrations of CO₂ were provided, perhaps from power station flue gases. If 50% of the product were oil, a reasonable goal, this would be the equivalent of more than 100 barrels of petroleum per acre per year. Current estimates are that liquid fuel could be produced from microalgae at a cost of

177 dollars per petroleum barrel equivalent [Wyman, 1994], with realistic hopes to reduce it to 50 dollars. That is very high for a transportation fuel, but some specialty oils are as valuable, and these would probably be the first target for commercialization. About one-third of the estimated current cost is for the CO₂, and it is conceivable that this would change dramatically if there were pressure to limit CO₂ release. In Japan, Dote et al. [1994] and Kishimoto et al. [1994] have described the recovery of liquid fuel from hydrocarbon-rich microalgae with thermochemical liquefaction.

VIII. CONCLUDING REMARKS

Marine biotechnology has progressed during the last decade to a point where it is generally accepted by many countries that it will contribute to the sustainable development of their marine resources. Environmental applications of marine biotechnology can help retain environmental productivity and restore environmental quality in areas adversely affected by industrial, municipal, agricultural, and aquacultural activities. It has clear roles in waste processing, environmental monitoring, bioremediation of spilled oil, bioremediation of dredged spoil, prevention of pollution, and environmental restoration.

Many of the scientific, economic, regulatory, and perceptual limitations that were viewed as barriers a decade ago still exist, and it is unlikely that recombinant microorganisms and plants will be deliberately released into the marine environment in the near future. There remain concerns over the uncertainties associated with recombinant organisms: the potential risk of harm to the environment or any of its inhabitants, their dispersal and persistence, and the possible integration of their genes into the genomes of nontarget organisms.

Miller and Gunary [1993] discussed the appropriateness of a vertical approach to biotechnology risk assessment and Zilinskas and Lundin [1993] argued that this approach is suitable for evaluating the risk associated with the introduction of recombinant organisms into the marine environment. Understanding and assessing risk associated with the release of recombinant organisms into the marine environment requires genuine risk assessment experiments. When risk is not readily demonstrable, the most rational approach to risk assessment is to use established scientific principles [National Research Council, 1989] and to identify important gaps in our understanding that can be filled by properly designed experiments.

Because commercial use of recombinant microorganisms and plants for environmental applications in the marine environment is not likely in the near future, a greater emphasis will be placed on the use of these and naturally occurring microorganisms in contained systems, such as bioreactors. Bioreactors create optimal environmental conditions for microbial-degrading metabolic activities and allow containment and monitoring. New bioreactors create zones, sometimes in sequential bioreactor chambers, where specific metabolic activities are favored; for example, creating anaerobic zones favors particular processes, such as denitrification and anaerobic dehalogenation. Many technological advances remain to be made through environmental engineering, but future environmental applications of biotechnology might also take advantage of genetic engineering. We are on the threshold of a biotechnological revolution that will have an influence on all our lives.

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Production of Valuable Products from Microalgae: An Emerging Agroindustry

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I. INTRODUCTION

Although the contribution of agriculture to the overall economies and life styles of different countries tends to wax and wane, the importance of agriculture as a provider of food and fibers cannot be underestimated. If we look at agriculture as a whole, with all its associated upstream and downstream agroindustries, we see that it plays an important role in the global economy.

Recent developments in the areas of information biotechnology and molecular genetics are paving the way for the development of new agroindustries, which will reach well beyond the food and fibers that have formed the basis of traditional agriculture for the last 10 millennia. The new industries will harness biological systems, just as traditional agriculture does, but the products will be aimed at different markets. Unlike the bulky perishable traditional agricultural produce, the products of the new agroindustries will be nonperishable and of high value per weight unit, and this will facilitate their entry into distant markets. Most of the products of these industries will be used not as foods or fibers, but as essential ingredients in variety of industries, from pharmaceuticals to oil drilling.

In this chapter, we set out to assess the potential of candidate agroindustries by comparing them with traditional agriculture. A typology of agroindustries is used to assess the relative suitability of particular agroindustries in a specific environment. This typology describes an industry in terms of its demands on the physical environment and its pattern of usage of production factors, as well as in terms of the organizational features of the production process; by the effect of the logistic barriers on the ability of the products to reach the market; and the markets at which the products are aimed. The environment is described by production factors endowment, by the distance from the market, by the markets themselves, and by the physical environment. An example of such a new agroindustry is the production of valuable products from microalgae. By means of our analysis, we were able to show that a candidate industry based on the technology of growing

the green microalga *Dunaliella* in vertical sleeves for production of β-carotene, would have major advantages over traditional agriculture under desert conditions.

II. AGRICULTURE IN HISTORICAL PERSPECTIVE

The prevailing notion of agriculture—as well as the definition used in national statistics—has its roots in the Industrial Revolution. Before that time, agriculture was virtually the only industry known to humans. In the course of the Industrial Revolution, some components of the agricultural production process have been differentiated to become industries in their own right; for example, downstream industries, such as bakeries and dairies, and upstream industries, such as fertilizers and seeds. Today, the output of these industries is much larger than what was left within the traditional definition of agriculture (see [Table 2](#)).

In the 1950s, the technology of producing food and fibers changed the organizational structure of agriculture once again, this time into vertically integrated agroindustries. In the poultry industry, for example, this integration includes hatcheries, feed mills, chicken husbandry, chicken abattoirs, and distribution chains. Similar integration has become common in pork production and, to a lesser extent, in other branches of food and fiber production. Thus in real life—although not yet in the official statistics—agriculture has gained back much of the ground lost. The existing official definition of *agriculture* has nothing to do with modern production technologies for feed and fibers or with the prevailing organizational structure of this industry.

Recent scientific developments in the fields of information, genetics, and biotechnology will change agriculture yet again. A variety of new agricultural industries will emerge: some of them are already just around the corner. The new industries, as well as the traditional ones, have to conform to some of changing philosophical, social, economic, political, and technological environments as follows:

1. Population increases will change attitudes in society toward favoring large, open spaces in and around a megalopolis, toward an increased awareness of the expected hazards inherent in human-environment interactions, and toward an overall holistic view of nature.
2. Society will become more aware of the link between health and food.
3. Better technology in the areas of detection of dangerous materials will reinforce the aforementioned awareness.
4. Affluence will change consumer behavior, and this combined with recent developments in technology, will lead to a new wave of product differentiation.
5. The political barriers used to insulate domestic markets for agricultural produce will diminish, and products from all over the world will compete in every market.

III. REDEFINITION OF THE BUSINESS

It seems that profound changes are taking place in the environment in which the agricultural industry will have to function. From past experience, we have learned that in such situations, a new definition of agroindustry is needed. The definition proposed here is *harnessing biological systems for the sake of mankind*.

This definition does not include others, for example, health works in the area, but it defines agriculture far more comprehensive than the prevailing definition. [Tables 1](#) and 2 serve to illustrate the new definition by presenting the industries (see [Table 1](#)) and describing their sizes (see [Table 2](#)).

Table 1 Industries Associated with Agriculture

Potential partners for vertical integration		Potential partners for horizontal integration	
Upstream industries	Downstream industries	Side industries	New industries
Fertilizers	Packing	“Green lungs” in urban areas	Production of valuable materials through the use of animals, plants, and algae
Pesticides	Conserving, drying and freezing	Forests, groves, and pasture	
Reproduction material	Dairies and ice cream	Vegetation of road-sides and installations	
Irrigation	Abattoirs	Gardening	
Mechanization	Retail trade	Lake and sea	
Feeds, meals			

IV. TYPOLOGY OF AGRICULTURAL INDUSTRIES

In general terms, agricultural industries can be described by the degree of separation from the external environment and by the control of the internal environment; by their demands on the physical environment; by production factor usage; by the markets for their products; by the logistics and political barriers that stand between the producer and the markets; and by their organizational features. A typology for agricultural industries was developed by Spharim and Vaadia [1982]. According to this typology, an archetype is described by positioning an industry along a variety of dimensions. In the context of agriculture these could be as follows:

1. *Degree of Separation and Control:* A biological system is harnessed by separating it from the external environment and controlling its internal environment. Various degrees of separation and control can be used, and the degree of control will affect the seasonality and reliability of the production process as well as the quality of the produce.
2. *Demands of the Physical Environment:* As long as there is no complete separation of the internal environment from the external one, the local physical environment will continue to play a role. The physical environment is described in terms of climate, land quality, and pest and disease threats.
3. *Production Factor Usage:* The pattern of production factors usage of an industry determines the fitness of that industry in a region with a specific pattern of production factors availability. The specific pattern of production factors usage can be described by calculating the ratios between the production factors quantities and the value of the product (i.e., production factors intensity).

Table 2 Agriculture in the Context of the Value-Added Chain for 1993

	Upstream industries	Agriculture	Downstream industries
Output (million NIS)	7,445	8,387	9,871
Employees (workers)	21,210	68,800	19,596
Export (million \$)	816	547	322

Sources: *Statistical Abstract of Israel* (1994), The Central Bureau of Statistics; *Industry in Israel* (1992), The Ministry of Industry and Trade; and FAO *Fertilizer Yearbook*.

4. *Product Features:* The main range of possibilities includes perishable and non-perishable; valuable or cheap (bulky or nonbulky); basic food or luxury; new, improved, or existing product; commodity versus specialty; and raw material to another industry or for use by an individual consumer (intermediate or final product).
5. *Type of Market:* The market may be large or small; seasonal or all-year-round; market niche or whole market; local or foreign; other industries or the final consumer.
6. *Effect of Logistics and Political Barriers:* Logistic and political barriers that separate the market from the producer affect the profitability of most agricultural produce either by closing a large market to the producer or by adding costs. The cost effect of the barriers depends on the type of product; for example, it costs more for a perishable product to cross barriers, and valuable products are less affected by them. On the other hand, logistics and political barriers insulate a local market from foreign competition to a certain degree. Technology has helped to reduce the logistics barrier by providing the means to condense and preserve products and by improving transport efficiency. The General Agreement on Tariffs and Trade (GATT), signed recently, will reduce political barriers, but the logistics barrier still determines, to a certain degree, the suitability of perishable products for a particular region.
7. *Knowledge Barrier:* We do not have to move a product from one country to another to enable the product to compete in the second country—technology transfer can do just that. In the past, agricultural knowledge was dealt with as public property, and was transferred freely to farmers in the country of origin as well as in foreign lands. However, agricultural research institutes and governments are now seeking ways to confine some of the benefits of their research and development to themselves, and this invites knowledge barriers. Knowledge barriers may be both technical and legal, and their effectiveness depends on the type of innovation.
8. *Organizational Features:* The main organizational features that determine the organizational pattern are advantages to scale, generic or specific technology, suitability to vertical and horizontal linkages, and synergism.

The following examples of grains and strawberries will help demonstrate the explanatory and predictive values of this typology. Grain production requires much land and relatively little labor, and there is only marginal control of the environment around the plant. Grains are nonperishable, low-value agricultural commodities. The technology of bulk transport by sea that has developed over this century has reduced the logistic barriers for grains to the degree that grains can be shipped economically from anywhere around the world. As grains are a basic food, governments attempt to be self-sufficient in this product, and they do this by controlling imports. Because grains are grown in an environment in which control is minimal, it is essential to import grains in bad years, which helps overcome existing political barriers against import. The peculiarities of the grain industry, which are encapsulated in this archetype, help explain why many countries still produce their own grains, even when production of grains is inefficient, and why a large international market for grains exists in a world that, until recent years, tends to set up political barriers. This archetype also helps predict where grains will be grown if these barriers should be reduced.

Strawberries are produced by a technology that is more controlled and much more laborintensive than that of grains. The produce is a seasonal, perishable specialty product. The logistics barrier for strawberries is very high, but out-of-season strawberries can be sold profitability in a special market niche, in which the price is high enough to cover the cost of crossing the logistic barrier. Israel has thus developed an improved variety that is sold in Europe out-of-season.

The aforementioned typology may help explain why out-of-season strawberries are grown in the Gaza Strip, and grains in Iowa; and it may help predict the success of technology transfer from one region to another. In fact, a similar typology was used a few years ago to predict the transfer of strawberry technology from Israel to the Gaza Strip [Spharim, 1992].

Predicting the success of a new industry is a special case of fitting technology to the environment, a case in which the source of the technology is not another region, but a laboratory. In such a case, the laboratory with a proposed technology searches for an environment in which to fit the technology.

The environment can be described by its social, economic, physical, and political features. The social features may be the quality and quantity of labor, the size of farms, and the distribution of land ownership. The economic environment can be described by production factors, endowment, the prices of inputs and outputs, and the distance from the major markets. The physical environment can be described by the climate and land and pests and diseases, and the political environment by entry barriers, land acquisition rules, and the rules that govern intellectual property rights.

Fitting a new industry to the environment involves matching the type of industry to the type of environment. In mature industries, the production process comes with a prevailing technology. New industries that are still in the research and development stage may come in a variety of production technologies. Here, fitting the industry to the environment is a task of matching a variety of hypothetical technologies to a variety of candidate environments. Coping with this task should be part of the research and development process.

V. PRODUCTION BIOTECHNOLOGY

A. Microalgae Production Systems

Microalgae are the most primitive and the most simply organized members of the plant kingdom. The vast majority of microalgae exist in aqueous habitats as single, small cells of about 3–20 µm, although a few species are organized into simple colonies. Macroalgae—the seaweeds—have achieved a further level of organization, with cells that are grouped into structures that resemble the leaves, stems, and roots of higher plants.

Although the biochemical mechanism of photosynthesis in algae is similar to that found in all plants, microalgae can be particularly efficient converters of solar energy to biomass. Free of the need to generate elaborate support and reproductive structures, microalgae can devote most of the energy they trap to biomass production. Unlike higher plants, single cells suspended in an aqueous environment can directly acquire the water, carbon dioxide, and nutrients they require for growth. The energy efficiency of microalgae and their ability to produce large portions of their total biomass in the form of various biochemicals, together with the future possibilities of molecular biology, make them a promising source of valuable biochemicals.

The different species and varieties of microalgae constitute a virtually inexhaustible reserve of natural substances and other by-products of commercial potential [Aaronson et al., 1980; Dubinsky et al., 1978]. A number of large international companies have already begun to market products of algal origin, including food additives and fluorescent dyes for use in the pharmaceutical industry and as diagnostic markers. In addition, industry throughout the world is presently engaged in developing new products and testing a new generation of natural products derived from microalgae. These include natural colors, pharmaceuticals, health foods, unsaturated fatty acids, and polysaccharides [*Chem. Eng.* 1987; Klausner, 1986]. This picture is in keeping with the tendency of the market to switch from synthetic to natural products.

A major advantage of microalgal production technologies lies in the fact that they can be based on saline waters and land that is unsuitable for conventional agriculture or for the production of higher plants. The fundamental components of a microalgal mass production technology include water, nutrient, and carbon dioxide supply systems; culture systems; harvesters; and processing equipment.

The production and development of commercially viable products from microalgae require the elaboration of an agrobiotechnological system that must include the following stages: cultivation of microalgae, enrichment of the required product; harvesting the microalgae; and extraction of the chemicals. Each of these stages involves a specific technology designed to yield maximum output for minimum operation outlay. The stages of the process are interdependent. The biotechnological process is specific to the algal strain and to the desired product.

A major advantage of microalgal production technologies is that they can be based on saline waters and on marginal lands, which are not suitable for conventional agriculture, but in which temperatures and light availability are high. The main obstacles lie in problems pertaining to large-scale cultivation systems. Various experimental designs for large-scale algal cultivation systems—both open and closed—have been suggested. Previously, the most common systems were based on open designs, but currently there is a trend toward closed systems.

The standard method used for large-scale production is based on shallow ponds, 10- to 20-cm deep, that are open or covered. The ponds, whether raceways or circular, are stirred with paddle wheels to prevent settling of the algae and to produce a homogeneous suspension, in which the light allocation to an individual algal cell varies in a fixed cycle. The main structure used today in commercial systems is that of the open raceway, which usually consists of several units joined together ("back-to-back") and stirred with paddle wheels [Richmond, 1986].

The main problems in large-scale cultivation of microalgae outdoors in open ponds are low productivities and contamination [Richmond, 1986]. To overcome these problems various types of horizontal or vertical closed systems have been suggested, including horizontally arranged glass or polyethylene tubes [Chaumont et al., 1988; Dicorato, 1980; Richmond, et al., 1993; Torzillo et al., 1986] or covered convectors [Anderson and Eakin, 1985]. Vertically arranged bioreactors were first proposed by Trotta [1980], and further developed for large-scale cultivation in the form of a system of polyethylene sleeves. Tubular (alveolar) bioreactors made of Plexiglas alveolar sheets have also been suggested [Tredici et al., 1993],

Microalgal cultivation in vertical closed bioreactors is superior to that in open ponds, because they facilitate

1. Significant reduction of contamination (by microorganisms or chemicals) [Cohen and Arad, 1989]
2. Improved light availability to the cells, resulting from a better surface area/volume ratio [Cohen and Arad, 1989; Cohen et al., 1991]
3. Improved temperature control—in the summer elevated temperatures can be prevented by spraying the sleeves with water [Cohen et al., 1991]
4. Prevention of evaporation and, thereby, prevention of salination [Cohen and Arad, 1989]
5. Better turbulence, resulting in improved light-dark cycles

Thus, the microalgae can be grown by various technologies that differ in the degrees of separation from the external environment and control of the internal environment. In Australia, where land and water are abundant, they are grown in large pond farms with little separation from the external environment, and a low degree of control. In Eilat, Israel, they are grown in pond farms with a higher degree of control.

The technology of growing algae in vertical sleeves encompasses a high degree of separation from the external environment, which is matched by intensive control of the internal environment. Potentially, a variety of microalgae can be grown in this manner. According to this technology, the algae are grown outdoors in rows of vertical plastic sleeves. The sleeves separate the algae from the external environment, except from the sun. The environment within the sleeves is mainly controlled by the density of materials in the water. The water is transported by a system of tubes, which connects the sleeves to a control center. In this center the temperature and the densities of the materials (including algae) are controlled, and harvesting starts. Potentially, this technology can be used for a variety of algae-product combinations.

B. Processing

The metabolism of microalgal cultures can be shifted by nutrient deprivation from the production of cellular machinery to the generation of storage products. For example, lipid production can be induced by nitrogen deprivation in most algae. Likewise, polysaccharide production can be enhanced by nitrogen starvation in red microalgae [Adda et al., 1986; Thepenier et al., 1985; Wanner and Kost, 1984]. Through this mechanism, cells can be induced to accumulate storage products amounting to a large proportion of their cell mass. By controlling the internal environment, the yields and the content of the desired product can be increased.

The growth stage can be followed by an enrichment stage aimed at concentration of the desired product [Ben Amotz and Avron, 1983]. Before processing, the microalgae must be harvested from the culture medium, further concentrated, and dried. The optimal harvesting technique depends on the characteristics of the species. The microalgae are harvested by physical means (centrifugation and filtration), or physicochemical means (flocculation and settling or flotation) [Shelef et al., 1983]. Harvesting is followed by processing stages, the nature of which depends on the particular product involved. Processing may include removal of water and drying, extraction of the product, and various degrees of purification.

The growing and harvesting techniques are components of a production process, which includes concentration, separation, and processing. In this production process, the technique used for harnessing the biological system is generic to microalgae. The rest of the production process, which includes the functions of concentration of the algae biomass, separation of the valuable materials from it, and further processing, has components that are not specific to microalgae. For example, the technique used for algae drying is essentially the same as that which is generally used for drying coffee.

Acquiring the capability of harnessing algae will lead to the emergence of a new agricultural industry, that will afford a variety of different products.

VI. VALUABLE PRODUCTS FROM MICROALGAE

A large industry revolves around the polysaccharides extracted from giant ocean algae, such as agar, carrageenan, and alginic acid, which are complex polysaccharides used in the food industry and for biomedical purposes [Renn, 1987]. The extraction of chemicals from microalgae, in contrast, has started only recently.

A. Pigments

In the food industry, the growing tendency to use natural, rather than synthetic, dyes has encouraged the search for new natural dyes [Taylor, 1984]. In particular, carotenoids and phycobiliproteins extracted from microalgae are gaining increasing commercial acceptance as natural colorings.

1. β -Carotene and Other Carotenoids

All algae contain carotenoids (lipid-soluble pigments composed of isoprene units), of which β -carotene is commercially the most important. The best-known and most-advanced chemical process for obtaining β -carotene is that from the green microalga *Dunaliella*. When cultivated under appropriate conditions, *D. bardawil* and *D. salina* accumulate more than 10% of their dry weight as β -carotene [Ben Amotz et al., 1982]. The amount of β -carotene accumulated is a function of the integral amount of light adsorbed by the alga in one division of cycle [Ben Amotz and Avron, 1983]. It thus seems that β -carotene accumulation protects the cells against the deleterious effects of high light intensity [Ben Amotz et al., 1987]. The β -carotene produced by *D. bardawil* is composed of two isomers, all-*trans* and 9-*cis*, in approximately equal proportions [Ben Amotz et al., 1988], a ratio considered superior to that of the synthetic product. The biotechnology for growing *Dunaliella* will not be discussed here, because it has been reviewed in depth by Ben Amotz and Avron [1989].

β -Carotene is used primarily as a yellow food coloring and, being a source of vitamin A, as a component of health foods and animal feeds. Also, in humans and animals above-average ingestion of β -carotene predisposes to a lower incidence of certain types of cancer [Jensen et al., 1987; Suda et al., 1986].

The general tendency to substitute natural for synthetic dyes, the recent findings of anticarcinogenic effects of β -carotene, and the superior isomer ratio of the natural product are the reasons for the sharp rise in demand for the natural pigment and the high prices it commands. Various companies throughout the world are engaged in its commercial production from *Dunaliella*; these include: NBT (Israel), Microbio Resources Inc. (California), Cyanotech Corporation (Washington), Western Biotechnology Ltd. (Bayswater, Australia), and Betadene Ltd. (Victoria, Australia).

Other carotenoids of commercial importance are xanthophylls, which are yellow to orange pigments. Of these astaxanthin and canthaxanthin, which are extracted from certain species of green microalgae are used as food dyes, especially in the pigmentation of chicken skin, egg yolk, and fish (e.g., salmon). Canthaxanthin is also used as a tanning agent.

2. Phycobiliproteins

Red algae and blue-green algae are characterized by the presence of red and blue pigments (in addition to chlorophyll a), which are responsible for their red-brown and blue-green color, respectively. These pigments—phycobiliproteins—have promising potential as food dyes, especially because many red and blue pigments currently in use are thought to be carcinogens. Other advantages of the phycobiliproteins are their intense color, their high solubility in water, and their stability to changes in pH [Yaron et al., 1992]. Their application in cosmetics is also of great potential.

The red, highly fluorescent phycobiliprotein, phycoerythrin, which is extracted from microalgae, is used mainly in diagnostics in multiple color flow cytofluorimetric analysis. In this technique, conjugates of phycobiliproteins and molecules with specific biological activity bind specifically to their target molecules, making them highly fluorescent. The method is very sensitive because the specific absorbance of phycoerythrin is very high, even at very low concentrations. Glazer and Stryer (1984) developed a series of conjugates, designated phycoproteins, that are composed of fluorescent phycobiliproteins (mainly phycoerythrin) covalently bound to biologically active molecules. These fluorescent markers include phycoerythrin-immunoglobulins, phycoerythrin-protein, phycoerythrin-avidin, and phycoerythrin-biotin. Ryan et al. [1984] succeeded in increasing the sensitivity of the method to enable the detection of leukemia at early stages by means of antibodies conjugated to phycoerythrin. They were able to detect as few as

5×10^6 positive cells, compared with about 300×10^6 by former methods. The use of phycoerythrin together with a second fluorescent marker enables multiple cytotoxic examinations. The other advantages of phycoerythrin conjugates are that their fluorescence is not affected by most natural molecules; they are soluble in water; and they can be stored for long periods [Ol et al., 1982]. Today, conjugates with phycoerythrin sell for about 100 dollars/mg, and their diagnostic uses are constantly widening.

The blue pigment phycocyanin, which is obtained from blue-green algae, is used in the food industry. Dainippon Ink and Chemicals of Japan has commercialized it under the name of Linablue [Dainippon Ink and Chemicals Inc., 1980]. It is used for coloring candy, ice cream, dairy products, and soft drinks. There are, in fact, other Japanese patents describing the uses of the blue color of phycocyanin in various food products [Patent no. 79-95770].

Undoubtedly, the algal world still has a vast undiscovered potential of valuable pigments.

B. Unsaturated Fatty Acids

Another class of algal biochemicals with potential commercial value are unsaturated fatty acids [Borowitzka et al., 1986]. Unsaturated fatty acids are traditionally consumed in the human diet in the form of fish oil. They are, however, not synthesized by the fish themselves, but are acquired by the fish from the marine algae in the food chain [Klausner, 1986]. By extracting these unsaturated fatty acids directly from microalgae, the less desirable components of fish oil—related fatty acids and cholesterol—and the unpleasant fish odor can be eliminated.

In addition, relatively high quantities of essential fatty acids have also been demonstrated in the marine red microalga *Porphyridium* [Ahern et al., 1983; Nichols and Appleby, 1969]. The most important of these is arachidonic acid (5,8,11,14-eicosatetraenoic acid), a highly unsaturated fatty acid that is only rarely found in higher plants. In addition to being an essential fatty acid in the human diet, arachidonic acid is the natural precursor of a large family of structurally related C₂₀ compounds that include prostaglandins, thromboxanes, leukotrients, and prostacyclins, all of which are potent biological regulators. Arachidonic acid and its C₂₀ derivatives are usually obtained from animal tissue. The use of this family of compounds is becoming increasingly important in clinical research and pharmacy, since arachidonic acid derivatives are synthesized and released in sparingly low concentrations by many tissues and are not concentrated in any organs to a significant degree. To overcome this shortage, new sources must be found. In heterotrophic microorganisms fatty acids account for no more than 9% of the dry weight of the organisms and the C₂₀ fatty acids constitute less than 2.5% of total fatty acids. In *Porphyridium*, arachidonic acid alone constitutes as much as 36% of the total fatty acids [Nichols and Appleby, 1969; Ahern et al., 1983]. Thus, *Porphyridium* has been suggested as a new source of arachidonic acid [Ahern, 1984].

The red microalgae are also rich in highly unsaturated fatty acids, especially eicosapentaenoic acid (EPA), which are effective in reducing levels of plasma lipids and lipoproteins and in ameliorating coronary heart disease, arthritis, and inflammatory ailments. When red microalgal biomass containing EPA (up to 40% of the fatty acids) and over 50% polysaccharides was fed to rodents (at a dietary level of up to 10% dry algal biomass of their diet), liver EPA content increased from 0.1% in the control animals to 0.7% in the experimental group. There was a decrease of 25% in serum cholesterol and triglyceride levels, probably owing to the effect of the EPA present in the algae, or to the polysaccharides acting as dietary fiber, or to a unique combination of the two components [Dvir et al., 1994; Yaron et al., 1995].

C. Polysaccharides

Seaweeds have been used by humans as food since ancient times, as early as 600–800 bc in China. In times of famine because of failure or destruction of terrestrial food crops, seaweeds were often eaten by coastal inhabitants to ward off starvation [Newton, 1951]. Seaweeds, especially the red macroalgae, are the conventional source of sulfated polysaccharides, which are used commercially as gelling agents, stabilizers, thickeners, and emulsifiers, mainly in food-stuffs, but also in paints, photographic films, pharmaceuticals, and in tertiary oil recovery. The best known of these sulfated polysaccharides are agar-agar and carageenan. Polysaccharide-containing macrophytes are usually harvested from their natural habitats [Gellenbeck and Chapman, 1983]. In fact, the term "marine agronomy" [Doty, 1977] was coined to describe the large-scale commercial cultivation of macroscopic marine algae. However, this source is not constant because of intensive harvesting and ecological damage. The increasing demand for seaweeds, mainly for the colloids that can be extracted from them, can thus not be met by traditional seaweed sources. Therefore, industry has initiated a search for new sources of a more stable nature (e.g., xanthan gum, which is extracted from *Xanthomonas campestris* B-1459) [Baird et al., 1983]. Another potential source could be the red microalgae [Arad, 1987; Ramus, 1986], the cells of which are surrounded by gel polysaccharides, as are the cells of the red macroalgae. This unique group of polymers comprises several structurally different polysaccharides, most of which are heteropolysaccharides. The chemical composition, structure, and rheology is under comprehensive study in our laboratory [Geresh and Arad, 1991].

Red microalgal polysaccharides may be used in a wide field of activities, from engineering through cosmetics, to health foods. In tertiary oil recovery, the biopolymer of *P. aeruginosum* has been applied as thickening agent for aqueous driving fluids to enhance recovery of petroleum trapped in the pore space of reservoir rocks [Savins, 1978]. It was a superior product, showing a fourfold higher apparent low shear rate viscosity yield than an equivalent weight of Kelzan (from *Xanthomonas campestris*).

The polysaccharides may also find application in human and animal health as dietary fibers and antiviral agents. Serum cholesterol, triglyceride, and very low-density lipoprotein levels were considerably lower (about 25%) in rodents, receiving the biomass of *R. reticulata* or its polysaccharide alone in their diets, than in control animals; no toxic effects were found. In glucose-loading experiments, the levels of serum insulin and glucose were much lower in rats fed with algal biomass or polysaccharide than in the control animals. These findings suggest that the polysaccharide acts as a dietary fiber [Dvir et al., 1994; Yaron et al., 1995]. The sulfated polysaccharide of a red microalgae showed promising antiviral activity against *Herpes simplex* virus types 1 and 2 and *Varicella-zoster* virus, with no cytotoxic effects on Vero cells. It seems that the polysaccharide is able to inhibit viral infection either by preventing adsorption of virus into the host cells or by inhibiting the production of new viral particles inside the host cells [Huheihel et al., 1995].

A variety of other applications have been the subject preliminary investigations. For example, addition of red microalgal polysaccharide to aloe vera gel prevented its degradation, as was shown by rheological studies (e.g., increased apparent viscosities that did not deteriorate during storage, apparent yield points, and in some cases, hysteresis). Therefore, it was proposed that the algal polysaccharides could serve to stabilize the network structure of fresh aloe vera polysaccharides [Yaron et al., 1992]. Applications of the heteropolymers as natural soil-aggregating agents (biofertilizers), as matrixes for slow release [Kolani et al., 1995], and for cosmetics are also of potential interest.

D. Food Additives

Microalgae (including the blue-green algae) use photosynthesis to convert water and carbon dioxide into organic materials, including proteins. Many species, including those of *Spirulina*, *Chlorella*, *Dunaliella*, and *Scenedesmus*, are used as traditional sources of protein, with nutritional values similar to those conventional protein sources such as soybean. Under optimal conditions *Chlorella* contains 50% or more of its dry weight as protein. The total protein content of *Spirulina* grown under optimal conditions can amount to 70% of its dry weight. Despite these high protein contents, production costs are high, and human consumption of microalgae as a protein source is limited to expensive health foods.

E. Wastewater Treatment:

A symbiotic interaction of microalgae with bacteria is exploited in photosynthetic wastewater treatment in high-rate algal ponds (HRAP) [Soeder, 1986]. In this process, microalgae use the end products of bacterial metabolism (CO₂, ammonia, and so forth) and, in turn, supply bacteria with the oxygen required for total degradation of organics. The advantages of using algae for wastewater treatment include reoxidation, mineralization, and contribution to the food chain [Oswald, 1987a,b]. Promising results were also obtained in a study investigating the contribution of microalgae to enhancing sedimentation and removing heavy metals [Kaplan et al., 1987] and organic toxins. In addition, algal biomass produced in water reclamation can be used for animal food and for production of energy by fermentation. It seems that the combined use of algae for wastewater treatment and biomass production has potential as a commercial process [Shelef, 1982]. Possible disadvantages are the build up of biochemical oxygen demand (BOD) and the need for large areas for the HRAP.

VII. MICROALGAL BIOTECHNOLOGY: AGROINDUSTRY OR AGRICULTURE?

Existing industries have been fitted to their environment through a long process evolution. The “fitness” of an established industry to a new environment (technology transfer) is usually assessed by cash-flow analysis using the prices of inputs and outputs prevailing in the relevant environment. How can one assess the fitness of a candidate industry that does not exist anywhere? Such candidate industries have usually not yet reached the phase in which a reliable financial forecasts can be easily produced. When there are many candidates industries, detailed assessment of each will drain resources and, in such case, a method for preliminary screening is needed. A broad brush approach for assessment the fitness of candidates is thus proposed in the following:

1. Describe the established industries using the typology described in Section IV.
2. Describe the candidate industry in the same way.
3. Describe the relevant environment.
4. Assess the fitness of the candidate industries by matching each industry with the relevant environment.
5. Compare the candidate with established industries.

In **Table 3**, each column describes a type of agroindustry (or agricultural branch) in a multidimensional way. The dimensions cover the demands of the physical environment, the product and its market, the logistic barriers, and the organizational features of the production process.

Table 3 Algae in Comparison with Other Agroindustries

	Micro-algae	Strawberries	Wheat	Dairy cows	Avocado	Cherry tomatoes (sheltered)	Tomatoes (in open area)	Melons	Citrus
Separation and control	Very high	High	Low	Medium	Medium	High	Medium	High	
Physical environment	All year-round sun	Mediterranean	Large plains	Mild	Sub-tropical				
Production factors ^a									
Labor (h/1000 IS)	3	23	6	8	8	18	14	9	22
Land (m ² /1000 IS)	7	58	5400	460	354	28	119	224	936
Water (m ³ /1000 IS)	14 ^b	46	0	32	248	23	60	101	655
Capital (IS/IS)	1128	71	1110	989	1192	2234	161	2805	872
Picking and packing (IS/IS)		367	157		374	375	334	245	284
Materials and services (IS/IS)	675	294	737	902	152	187	279	260	430
Product features									
Seasonality	Low	High	High	Low	High	Medium	High	High	
Perishability (days)	Low	7	400	10	20	10	10	10	40
Value (\$/ton)	2.2 million	3750	Low	Medium	1140	2088	752	1331	471
Novelty	Improved	Improved	Existing	Improved	Existing	Improved	Existing	Improved	Existing
The market									
Type of market	Specialty	Niche	Commodity		Niche	Niche	Niche		
Market place	Whole world	Europe	Local		Europe	Europe	Europe		
Type of client	Industry	Consumer	Industry	Industry	Consumer	Consumer	Consumer		
Logistic barriers effect									
Product (%)	5	31	Medium		30	19	53	45	68
Bulky raw material	Salt			Forage					
Organizational features									
Advantage to scale	Medium	Low	Medium	Medium	Medium	Low	Low	Low	Medium
Linkages	Vertical		Horizontal	Vertical					

^aMeasured by "input unit per output value."

^bBrackish water.

The pattern of production factors usage was measured quantitatively. The data related to different agriculture branches were normalized per unit of output value to escape the trap of "comparing apples with oranges." The logistics barrier was measured as far as possible by the costs of transport and insurance as well as other costs that are involved in getting the product to the market. As for the other dimensions, a qualitative evaluation was performed using expert knowledge in the field. The relevant environment, in this case Israel, or the Arava valley, was described by its physical as well as the socioeconomic features.

Let us use the example of the assessment of the relative fitness to the Israeli environment of avocados and cherry tomatoes.

In a nutshell, the Israeli socioeconomic environment can be described as follows: labor is scarce and expensive, capital is available, but not cheap, fresh water and land are scarce, the local market is small, and large foreign markets are far away. We assume that industries that use less scarce or expensive resources will be better fitted to this environment. In our example, cherry tomatoes require more labor and capital than avocados, but less water and land. This is a classic example of a trade-off. Israeli farmers had managed to substitute water and land, which were scarce, against labor and capital, which were available. Today, the socioeconomic environment is changing and, in the future, the price of labor will be relatively more expensive and thus reduce the competitiveness of cherry tomatoes in foreign markets. The loss of the competitive advantage will, in turn, reduce the relative fitness of cherry tomatoes in the Israeli agricultural scene.

We have to ask ourselves how microalgae grown in vertical sleeves will fare in such a contest. In Table 3 the candidate industry aimed at production of valuable products from microalgae, was represented by β -carotene production from the *Dunaliella*. The production process of this industry uses a technology in which the algae are grown in vertical sleeves. This technology uses less labor, land, and water than any of the other agroindustrial types represented in the table. This technology uses half the capital needed by cherry tomatoes and melons, which represent here the technology of high separation and control. On the other hand, growing algae requires more materials than any of the other industries represented here. Of the materials required for growing algae, salt, which is the most bulky material, is readily available in the Arava valley, and the rest of the materials are available everywhere.

Unlike most of the Israeli agricultural produce, the perishability and the seasonality of algal products are low; the products are improved versions of existing synthetic products; and the improvement is directed toward the health consciousness of the consumers. The products will be marketed in specialty markets, and the clients will be other industries.

Most perishable agricultural products are aimed at the consumer. In Table 3 only two products are sold as raw materials for further processing by other industries, wheat and milk. Selling a raw material makes the task of attracting the clients and distribution of the product easier, but selling a raw material to one industry can put the sellers in the hands of a powerful buyer. The products that can be produced from microalgae will be used as raw materials by a variety of industries, and this make them less vulnerable. A project for production of valuable products by growing microalgae in vertical sleeves will fit desert areas where the sun shines all year around, and brackish water and salt are available. A project of an efficient scale need about 20,000 m² of land that is not suitable for conventional agriculture, with an investment of about 3 million dollars (which in manufacturing is considered relatively small).

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Agricultural Biotechnology and the Law: Patents, Plant Patents, Plant Variety Certificates, and the Rise of Intellectual Property Rights in Biological Subject Matter

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Man, as two handed manipulator...has projected himself outward upon his surroundings in a way impossible to other creatures...since the first...man-ape hefted a stone in his hand...[His] creations...ride in the skies and the sea's depths; he has hurled a great fragment of metal at the moon...he once feared. He holds the heat of suns within his hands....

"Natural" is a magician's word.... Perhaps there may come to us in some...moment, a ghostly sense that an invisible doorway has opened...which, widening out, will take man beyond the nature he knows.

Loren Eiseley, How natural is "natural," *The Star Thrower*, pp. 282–283, 296, 1977.

The atmosphere of the "Front": it was...from having plunged into that atmosphere...that I ceased to notice any break (if not any difference) between..."natural" and "artificial." ...It was not merely that I [saw] the organic unity of the living membrane [that] is stretched like a film over the...surface of the star which holds us...[A]n ultimate envelope was...becom[ing] apparent to me.... This envelope was not only conscious but thinking....

Pierre Teilhard de Chardin, The heart of matter, *The Heart of Matter*, pp. 31–32 (Rene Hague trans., 1978).

I. INTRODUCTION

When Teilhard and Loren Eiseley penned these words, humans stood at the threshold of a new era of biotechnology that has taken him or her "beyond the nature that he knows" in a way perhaps no other technology has. Indeed, this most recent voyage—into the vast inner space of the cell's depths—epitomizes the ironic conception of our essential nature as artifice: A power to reshape the "natural" world, which itself is the product of nature.

In a broader sense, biotechnology has been with us ever since farmers first began “artificially” selecting and breeding plants and animals, and bakers and brewers harnessed a microorganisms—yeast—in their craft [1]. Only in the last two decades, however, has it been possible to operate directly on the heart of biological matter, transcending the reproductive barrier between species, and creating entirely new life forms that either would be impossible to bring about through breeding or would take many generations, and much trial and error, to develop [2].

As the other chapters in this book vividly detail, this development has had particular significance for agriculture. Indeed, the advent of recombinant DNA technology heralded a second, more radical Green Revolution of plants, animals, and microorganisms engineered to defeat disease, pests, and harsh conditions, or to yield more and better produce with reduced inputs, without the adverse effect on health and the environment associated with the rise of mechanization in the 1930s and agricultural chemistry in the 1950s [3].

Because of the enormous effort so often required to advance “science and the useful arts,” it is essential, if such advances are to be made, that inventors have some way of preventing others from unjustly reaping the fruit of their labor. Intellectual property [4] law provides that mechanism by allowing those who can demonstrate true invention a temporary legal monopoly on its commercial exploitation (if not its very use) [5]. Not surprisingly, biotechnology companies, many of which have little capital beyond their know-how, consider intellectual property protection as the very “lifeblood” of their industry [6].

The patent law, however, was cast in the crucible of the Industrial Revolution. Biological matter did not fit comfortably into its categories. Substantial change was required to accommodate the major biotechnical advances that span this century—whether by legislative or judicial fiat or some combination of the two [7]. The recently completed process of adaptation was not easy. Legal change generally lagged years behind the major technical advances. It also was not without controversy. There has been considerable political opposition, for example, to the most recent move to allow patents on genetically engineered animals [8]. In addition, extending full patent protection to living things, by some lights, poses a danger of overprotection by denying subsequent innovators the use of genetic material to make further advances, thereby robbing the storehouse of knowledge of “some of the basic tools of scientific and technological work” [9]. This concern contributed to the development of two rival approaches to protecting new plant varieties internationally—patents and the somewhat less protective plant variety certificates [10].

The rest of this chapter describes in greater detail the evolution and current state of the three different forms of intellectual property rights that can be acquired in biological subject matter, with primary reference to U.S. law to illustrate the basic concepts. The United States is one of the only countries that offers all three, yet otherwise, it is fairly typical of the regimen that obtains in the 100-odd countries that have signed the long-awaited Uruguay Round Agreement on Trade-Related Aspects of Intellectual Property Rights (the TRIPS Agreement) [11].

II. THE U.S. LEGAL REGIMEN

Under current law, it is possible to obtain a U.S. Patent on any sort of “artificial” life—from cells and plant parts to microorganisms, plants, and animals (except human beings) [12]. Indeed, the developer of a new plant variety may obtain either a patent, or a specialized plant patent, or a patent-like, “plant variety certificate.” This scheme comports with the TRIPS Agreement, which allows, but does not require, member states to “exclude from patentability ...plants and animals other than micro-organisms,” as long as they “provide for the protection of plant varieties either by patents or by an effective *sui generis* system or by any combination thereof”

[13]. Before delving into the details of the three modes of protection, it is necessary to explain how this unusual complex of three overlapping statutes came to be. For to quote Oliver Wendell Holmes, “[u]pon this point, a page of history is worth a volume of logic” [14].

The basic patent statute is the Patent Act [15]. It was promptly enacted by the First Congress in 1790 pursuant to the provision of the U.S. Constitution that had included among the limited powers of the Federal Government the power “[t]o Promote the Progress of Science and the useful Arts, by securing for limited times to...Inventors the exclusive Right to their ...Discoveries” [16]. The first section of the Patent Act—virtually unchanged since then—currently provides: “Whoever invents or discovers *any new and useful process, machine, manufacture, or composition of matter*, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title [17].

To qualify as an “invention,” then, the subject matter claimed in a patent application must meet three basic requirements: Novelty (“new”), utility (“useful”), and subject matter eligibility (“process, machine, manufacture, or composition of matter”). For most applicants, the first requirement—establishing true invention—has always presented the biggest challenge in obtaining a patent. Applicants claiming biological subject matter, however, faced an insurmountable obstacle in establishing the very eligibility of their discoveries for patent protection. Until quite recently, the Patent and Trademark Office (PTO) [18] rejected out of hand any application that claimed a living thing—no matter how much human ingenuity might have been involved in causing it to occur.

This rule apparently originated in the 1889 decision of the Commissioner of Patents in the case of *Ex Parte Latimer* [19]. In that case, the patent applicant had claimed the fiber of the needle of the *Pinus australis* tree, whose uniquely desirable properties he claimed to have been first to “discover.” The Commissioner approved the rejection of the Claim on the ground that the fiber was a “product of nature,” not man, and thus was not a “discovery” in the patent law sense [20]. To hold otherwise, reasoned the Commissioner would mean “that patents might be obtained on the trees of the forest and the plants of the earth, which of course would be unreasonable and impossible” [21].

Because Mr. Latimer had claimed a naturally occurring plant part, the Commissioner in fact never addressed the difficult legal question whether “artificial” life might qualify as a “manufacture” or “composition of matter” and thus constitute eligible subject matter. Be that as it may, the *Latimer* decision came to stand for the broad proposition that one cannot patent a living thing.

The Supreme Court’s landmark decision in *Diamond v. Chakrabarty* [22] finally changed all that in 1980. *Chakrabarty* involved an application claiming a genetically engineered microorganism with a unique ability to completely break down crude oil into compounds edible to aquatic life [23]. Such an organism was thought to be quite useful in cleaning up oil spills.

The Supreme Court noted the *Latimer* decision and its association with “the belief that plants, even those artificially bred, were products of nature for purposes of the patent law” [24]. The Court concluded, however, that the proper distinction was not “between living and inanimate things, but between products of nature, whether living or not, and human-made inventions” [25].

Chakrabarty thus cleared the way for patenting biological matter under the Patent Act. In its wake, the PTO extended its rule first to plants in *Ex Parte Hibberd*, [26] a 1985 case involving claims to new variety of maize genetically engineered for elevated tryptophan levels, and then to animals 2 years later [27].

Some 80 years before *Chakrabarty*, however, horticulturalists armed with the newly uncovered work of Gregor Mendel [28] had refined the technique of cross-pollinating selected

plants to produce offspring with distinctive characteristics and multiplying them asexually (i.e., by growing genetically identical individuals from cuttings) [29]. To obtain protection for such creations seemed to be foreclosed not only by the “product of nature” doctrine [30], but also by the inherent difficulty of verbally describing a new plant and how to produce it with the precision required by the Patent Act [31].

Horticultural interests appealed to Congress as early as 1906 for legislative change [32], but their efforts did not succeed until a quarter century later with the enactment of the Plant Patent Act of 1930 (PPA) [33]. The object of the PPA was to place agriculture on an equal footing with industry by giving plant inventors the *full* measure of protection that industrial inventors had always enjoyed [34]. Accordingly, Congress did not enact a free-standing statute, but rather, “[e]ngrafted” three short sections “onto the basic patent law,” the provisions of which otherwise were to apply equally to plants [35]. The first section established the subject matter eligibility of “any distinct and new variety of plant” that was successfully reproduced asexually [36]. The second relaxed the “written description” requirement for such subject matter (and limited applicants to one claim per patent) [37]. The third defined infringement as the “asexual [] reproduc[tion]” of a patented plant or the “selling or using the plant so reproduced” [38]. Conspicuously absent was any modification of the scope of the patent monopoly to accommodate what we now call “breeders’ rights.” Again, the idea was to treat agricultural and industrial innovators equally.

The reason for the exclusion of sexually reproducible new plant varieties from the PPA’s coverage is historical. In 1930, it was not yet practicable to sexually reproduce a new and distinct plant derived by breeding without losing its distinctive characteristics in subsequent generations [39]. The limited protection afforded under the PPA became inadequate by the 1950s when it became practicable to “stabilize” new and distinct varieties and reproduce them sexually [40].

At about the same time, momentum was building in Europe to establish an international convention to govern plant variety protection [41]. States who joined the convention would modify their national law as necessary to conform to its rules, in exchange for the reciprocal benefit of guaranteeing to their nationals in other member states [42]. This movement culminated in the creation of the Union for the Protection of New Varieties of Plants (known by its French acronym “UPOV”) in 1957 [43], and the promulgation of the International Convention for the Protection of New Varieties of Plants (the UPOV Convention) in 1961 [44].

The authors of the UPOV Convention confronted an extremely uneven international landscape that proved quite difficult to smooth out politically [45]. In the end, it reflected a basic compromise between countries that had extended patent protection to plants [46] and those that had adopted so-called “breeders’ rights” statutes [47]. These statutes created an exclusive and entirely separate system of plant variety protection that was administered by agricultural ministries and, more importantly, permitted a range of activities that would be considered infringing under a patent. To compound the problem, some countries had adopted breeders’ rights statutes *and* allowed general, “utility” patents on plants [48].

In 1960, the Group of Legal Experts tasked with recommending a model convention reflected the prevailing European view, which favored the more limited degree of protection afforded by the breeders’ rights statutes. But faced with the political reality of competing systems, the experts fell short of insisting that patents no longer be available for plants. They allowed that the idea of patenting plants was “not absolutely impossible” because the patent laws could be revised to guarantee breeders’ rights [49].

The final wording of the UPOV Convention, however, did not clearly require such revisions. It allowed signatory states to offer both patents and variety certificates on plants, provided only that they did not allow both for the same botanical genus or species [50]. Nevertheless,

even this relatively modest intrusion into national patent systems proved too much for countries such as Japan and the United States. They would not join the UPOV until the convention was revised *mutatis mutandis* [51] in 1978 to allow their accession [52].

Although the United States did not join the UPOV Convention until 1981 [53], it did not wait until then to cover the gap in U.S. intellectual property law that had developed by the 1950s with the advent of sexually reproducible varieties. Indeed, as early as 1966, an independent commission convened by the president to consider possible reforms to the patent laws actually recommended repealing the PPA in favor of an exclusive plant variety protection system modeled on the UPOV breeders' rights paradigm [54]. The bill introduced in Congress the next year to implement these recommendations, however, dropped the idea of repealing the PPA in order to enhance its prospects for passage—and even that half-measure failed [55]. The American Seed Trade Association subsequently drafted a similar measure, which 3 years later was enacted as the Plant Variety Protection Act of 1970 (PVPA) [56].

The United States thus replicated internally the conflict between patent and breeders' rights that confounded the UPOV Conference. One could patent an asexually reproducible new variety under the PPA—and after 1985, any sort of variety under the Patent Act—and thus bar virtually any use of the variety or its genetic material even though the PVPA purported to provide breeders and farmers with a safe harbor to carry on their traditional practices [57]. As a practical matter, however, the conflict between patent and breeders' rights is not as extensive as it seems, for as the following discussion suggests, it is rather difficult to claim a new plant variety successfully under the Patent Act because of its "inventive step" and "written description" requirements [58]. It is to these requirements that this chapter now turns.

A. The Requirements of Novelty, Utility, and Disclosure Under the Patent Act

An applicant for utility patent protection must, above all, prove that the subject matter claimed is worthy of the name "inventive" or "discovery" [59]. In a word, that means that the thing or process claimed must be "new" [60]. Newness, however, has come to have two distinct aspects—novelty" and "nonobviousness" ("inventive step" in European parlance). These concepts, which are defined in the second and third sections of the Patent Act [61], respectively, are also reflected in the TRIPS Agreement [62].

Generally speaking, a claimed invention is said to lack novelty if the exact same thing or process already exists in the public domain (although if the inventor himself put it there, he has a year to file a patent application) [63]. This is determined by comparing the language of the claims by which the applicant has described his or her invention [64] with the "prior art" in the field, which includes everything from earlier patents to papers published in scientific or technical journals [65]. If the claim language more or less perfectly describes a single piece of prior art, the claimed invention is said to have been "anticipated" and thus lack novelty under the second section of the Patent Act [66].

The term *novelty* is somewhat misleading, however, because a claimed invention also must be new in the sense that it does not represent an "obvious" combination of elements from several prior inventions [67]. The test is whether all of the significant elements of the invention can be found in the prior art [68] and, if so, whether the hypothetical "person of ordinary skill in the art" would have some "reason, suggestion, or motivation" to combine them [69].

The "nonobviousness" requirement is probably the most common stumbling block for patent applicants [70], and it poses unique challenges to applicants claiming biotechnology [71]. Indeed, because the development of new plant varieties through conventional-breeding techniques does not really involve an inventive step, as a practical matter, protection for such

creations may be available only under the PVPA, which eliminate the nonobviousness concept and require only novelty for this reason.

The last of the three basic requirements of patentability—in addition to subject matter eligibility and novelty or nonobviousness—is that the claimed invention must be “useful” [72]. Similar to the eligible subject matter requirement, most inventors have little difficulty in satisfying the utility requirement. It is not particularly strict, mainly because applicants can be expected not to claim useless things. It is enough that a claimed invention has some apparent, lawful use—however slight—in the “useful arts” [73] (as opposed to the liberal arts, fine arts, or pure science) [74].

Again, however, biotechnology has been somewhat of an exception to the rule. Applications that assert no use beyond laboratory research, for example, may be rejected on the ground that the invention falls within the ambit of pure science (or lacks “substantial” utility [75], whereas those who assert a utility in curing a putatively “incurable” disease (such as cancer or AIDS) may be deemed inherently incredible absent proof to the contrary. Recent judicial decisions finally have adopted a more relaxed stance, however [76].

In addition to the three requirements of patentability, the Patent Act also places on the applicant the burden of making a full disclosure of the claimed invention and how to make it in exchange for a legal monopoly on its exploitation [77]. This serves two purposes. First, it assures that the *de jure* monopoly will not be extended *de facto* beyond its term (which is now 20 years from the date the application is filed) [78] by assuring that commercial rivals will be “enabled” to enter the marketplace immediately and drive prices down to competitive levels. Second, it assures that the knowledge and insights embodied in the invention become part of the public domain as soon as the patent issues, thus facilitating further invention as well.

To these ends, the Patent Act provides that each application must “contain a *written description* of the invention, and the manner and process of making and using it, in such *clear and full, clear, concise, and exact* terms as to *enable* any person skilled in the art...to make and use” it. In addition to enabling others to “practice” the invention, the written description requirement also assures that the claims are sufficiently “definite” to fairly apprise the world of the boundaries of the intellectual property [79].

The “definiteness” and especially the “enablement” aspects of the “written description” requirement, if literally enforced, would impose a nearly insurmountable obstacle to biological inventors because of the inherent difficulty of verbally describing a new plant variety and how to make it. The PTO and the courts, however, have construed the Patent Act to require only a reasonable description of the new plant variety and to allow an alternative procedure by which applicants may deposit the biological material necessary to make the invention in a public repository [80].

B. The Nature and Scope of Rights Conferred By a Patent

A patent gives its owner a “right to exclude” others from the intellectual property staked out by the claims [81]. Specifically, a patent entitles its owner to sue in federal court [82] to stop anyone who, without permission, “make[s], use[s], or sell[s]” a thing or process that “infringes” the patent monopoly or be compensated monetarily [83].

A thing or process can infringe a patent in two ways: either “literally” or under the “doctrine of equivalents.” Literal infringement means that a thing or process is more or less perfectly described by the words of the patent claims, properly interpreted [84]. The doctrine of equivalents, by contrast, is rooted in the ancient legal principle, traceable through the civil law tradition back to Aristotle, that a thing may be within the ambit of a legal text if it is

within its spirit, even if it is not within its letter (and vice versa). The test for infringement by equivalence is whether the accused thing or process performs substantially the same function in substantially the same way to achieve substantially the same result as the thing or process described by the claims [85]. Pointing to an insignificant difference between the claimed invention and the accused thing or process will not do. To avoid infringement, then, one must steer clear of not only the literal terms of the claims, but also the fuzzy zone of equivalents that lies at the periphery of the claims.

There is one final thing worth noting about the nature and scope of patent protection. Some patents are “invalid” (meaning the applicant did not in fact meet all the statutory requirements), and even valid patents may be “unenforceable” in court. The validity of a patent under the statutory criteria is subject to challenge either by asking the PTO to reconsider its decision to issue the patent [86] or as a defense in the context of an infringement suit [87]. In other words, an accused infringer can escape liability either by denying infringement [88], or by establishing, say, that the claimed invention lacks novelty or that the written description is inadequate [89]. And even if a patent is valid, an accused infringer still may be able to escape liability by offering evidence that some “inequitable conduct” has tainted the patent, as when the applicant withheld important prior art from the PTO and thus cannot “in equity” enforce his “legal” rights under the patent [90].

C. The PVPA Compared and Contrasted with the PPPA and the Patent Act

The nature and scope of protection available under the three statutes and the requirements for securing such protection are similar, but significant differences exist on both scores.

1. Nature and Scope of Protection

The PVPA contains four provisions that exempt activities generally considered infringing under the patent statutes. First, in the *breeders’ exemption*, the PVPA permits unlicensed use and reproduction of protected varieties “for plant breeding or other bona fide research,” including the development of other new varieties [91]. Second, the PVPA exempts any otherwise infringing activity from its prohibition as long as it is “done privately and for noncommercial purposes” [92]. Neither the Patent Act nor the PPA, by contrast, contain any express, general exemption for research or private use. And although the courts have recognized an implied exception for such uses, it is relatively narrow, allowing at most a *purely “experimental”* or *de minimis* use of a patented invention. Research conducted with a view toward eventual commercial development is not allowed [93]. The remaining exemptions in the PVPA—the *farmers’ exemption* [94] and the *crop* exemption [95]—are a bit more complicated.

The farmers’ exemption allows farmers (if they are not in the seed business themselves), to replant their fields with seed produced by plants grown in earlier years from protected seed [96]. It even used to allow such farmers to sell this “saved seed” to other farmers in lieu of replanting their own fields (as long as the other farmers were not in the seed business either).

In a 1995 decision, *Asgrow Seed Co. v. Winterboer* [97], the Supreme Court resolved an ambiguity concerning what limit, if any, the PVPA placed on the quantity of saved seed one farmer might sell to another under the farmers’ exemption. The court ruled that the farmers’ exemption impliedly limits the quantity a farmer may sell to no more than what he could have planted on his own land. This closed what otherwise could have become a rather large loophole—particularly for plants, such as the soybeans involved in *Asgrow Seed*, that produce vast quantities of seed. Shortly before the court rendered its

decision in *Asgrow Seed*, Congress amended the PVPA to bring it into line with the revised, 1991 UPOV Convention. These amendments narrowed the farmers' exemption even further, requiring farmers to secure the permission of the certificate holder before making any "brown bag" sales [98].

Under the crop exemption, a farmer may sell saved seed to anyone, in any quantity, as long as it is used for "nonreproductive purposes" such as food, animal feed, or producing industrial products, such as oil or ethanol [99].

The second major difference in the protective scope of the three statutes lies in the definition of infringement. Although the two patent statutes both define infringement in general terms as the "mak[ing], us[ing], or sell[ing]" of an infringing article [100], the PVPA is much more specific.

Relative to making, the PVPA's prohibition is limited to reproduction by seed or tuber [101]. On the other hand, it is deemed an infringement to commit any of the other acts listed—such as selling—even if the plants involved were "made" by asexual reproduction [102]. For using, the PVPA narrowed the definition to include only the use of a protected variety "in producing (as distinguished from developing) a hybrid or different variety therefrom" [103]. The PVPA also expressly expanded the concept of "sell[ing]" to include any sort of transaction—i.e., "market[ing]," "buy[ing]," and "exchang[ing]" [104]. The PVPA also added "import[ation]" and "exportation" [105], as well as the "condition[ing]" and "stock[ing]" of protected varieties, to the trilogy of infringing acts [106].

As for the temporal scope of the monopoly, the term of both patents and plant variety certificates is the same 20 years), except that trees and vines receive an extra 5 years worth of protection under the latter [107]. However, the clock starts running on a patent the day the application is filed, whereas the term of a plant variety certificate is still measured from the date of issue. Because that could be some time after filing, the effective term of a given plant variety certificate may be more than 20 (or 25) years.

2. Requirements for Securing Protection

The plant-specific statutes relax the basic requirements for securing protection under the Patent Act in four principal ways. For "newness," as indicated in the foregoing, Congress eliminated the nonobviousness requirement. Congress also eliminated the utility requirement in the plantspecific statutes.

Under the PPA, an applicant need demonstrate that its asexually reproduced variety is "new and distinct" [108]. The PVPA further requires, for the sexually reproducible varieties that are the subject of its protection, that applicants to show that the claimed variety is "stable" and "uniform" [109]. The PVPA also defines these terms with great precision [110].

For "novelty" or "anticipation," inventors under both patent statutes are held to the same standard [111]. The problem of self-anticipation by the inventor has less of a bite, however, when applied to plant patents. This is because only *enabling* disclosures count for such purposes, and it is often quite difficult, if not impossible, to recreate a plant invention without seeds or cuttings, no matter how detailed the written or pictographic description of the plant and breeding procedure might be [112].

The PVPA is both specific and more relaxed than the PPA. It expressly provides that the presence of a variety in a printed publication makes it an anticipating "public variety" [113]. And in the 1994 amendments, Congress eliminated the concept of self-anticipation by deleting the second part of the "public variety" bar which, similar to the second part of the analogous provision of the patent law [114], had required applicants who publicly disclosed a new variety in a printed publication to file for protection within a year or be barred [115].

For “written description,” the PPA requires only that “the description [be] as complete as is reasonably possible” [116]. The Court of Customs and Patent Appeals [117] interpreted this to mean “that there is no requirement for a how-to-make [i.e., enabling] disclosure in a plant patent application” [118]. At the same time, the court underscored that the applicant must make a reasonable effort to describe the new variety, affirming the PTO’s rejection of an application claiming a new variety of Bermuda Grass where “the characteristics chosen to define the new plant [were] meaningless unless compared with predecessor plant varieties” [119]. In such cases, “it is incumbent upon the applicant to provide information of such a character that a meaningful comparison can be made” [120].

Similar to the PPA, the PVPA provides only that an application must contain a “description of the variety setting forth its distinctness, uniformity, and stability and a description of the genealogy and breeding procedure, when known” [121]. It then expressly recognizes what was insinuated into the patent law by judicial decision—that “photographs or drawings or plant specimens” may be used to address any definiteness concerns. Indeed, unlike the PPA, but like the Patent Act, the PVPA requires an enabling disclosure. The applicant must deposit “a viable sample of basic seed, including any propagating material, necessary for propagation...in a public repository” [122].

3. Claiming Agricultural Biotechnology Under the Patent Act

To show how the rules discussed in the foregoing are applied in particular cases, the balance of this chapter surveys the few recent published decisions involving efforts to patent agricultural biotechnology under the most challenging and protective of the three statutes—the Patent Act.

The first is a 1992 decision involving an application claiming “a method of combatting plant insect pests” by “applying to the plant environment or plant seed plant-colonizing bacteria” containing DNA that encodes for a protein toxic to insects, but harmless to humans [123]. This invention like it promise to reduce reliance on chemical insecticides and the attendant risks to health and the environment.

The patent examiner rejected the application on the ground that it was obvious in view of the prior art. The Board of Patent Appeals and Interferences (which reviews initial decisions made by examiners) disagreed. After “carefully review[ing] all the references cited by the examiner in their entirety,” the Board was “unable to find a suggestion therein to do what [the applicants had] done, namely incorporate the gene into the chromosome of bacteria capable of proliferating in the plant environment and applying that bacteria to the environment or seed of the plant” [124].

A year later, in 1993, the board had occasion to address an application claiming a “recombinant DNA molecule” with a “DNA sequence encoding a polypeptide displaying the biological activity of swine growth hormone” and a method for producing this polypeptide [125]. Identifying the gene for swine growth hormone allowed the manufacture of a synthetic hormone that could be used to make larger pigs—a use that had been “limited since extracting swine growth hormone from pituitary glands of swine [had not been] adequate to provide the needed commercial quantities.”

This time the board agreed with the examiner that in view of what was known about human, bovine, and rat growth hormones—including the high degree of similarity between them—it would have been obvious to a person of ordinary skill in the art to identify the swine growth hormone gene. Although the application was thus rejected, a recent court decision in a similar case disagreed that the state of the biotechnological art generally was advanced enough at that time to permit a person of ordinary skill to find a gene if given a partial or even an entire DNA sequence [126].

Despite the availability of specialized protection under the PPA and the PVPA, the list of agriculture-related inventions patented under the Patent Act includes plants too, for as the board has noted, some plant inventors view the various exemptions in the PVPA, as well as the PPA's limitation to asexual reproduction, as "loopholes" [127]. However, although the utility requirement is easily satisfied, and even definiteness and enablement problems may be avoided with little difficulty, the nonobviousness requirement remains a formidable obstacle to obtaining a patent on plants developed through conventional breeding.

This can be seen in another recent case involving an application claiming a new variety of soybean with greater yield and resistance to root rot than existing varieties. The patent examiner rejected the application on enablement grounds because its description of how to make the invention—by crossing two varieties—omitted "significant information about the breeding process, the selection pressures for disease resistance," and so forth. The "language used... was so indefinite that one skilled in the art [would be] unable to identify that plant variety and distinguish it from other varieties."

On appeal, the board disagreed on both points. First, the board noted that the applicant had offered to make the seeds of his new variety publicly available by placing them in the depository of the American Type Culture Collection. This cured what otherwise might have been a failure to satisfy the enablement requirement. Second, the board seemed to be relaxing the written description requirement along the lines of the PPA and the PVPA. It was enough that an application "sets forth a reasonable description of the characteristics of the seed and plant including, flower color, plant type, maturity group, bacterial resistance, nematode resistance," and the like.

The board concluded from evidence put forward by the applicant that such a description "is accepted by the art as descriptive of the characteristics of a soybean variety." Nevertheless, the board ultimately upheld the examiner's rejection on the ground that it would have been obvious in view of the prior art to achieve rot resistance by crossing a certain prior art plant with rot-resistant varieties and the degree of resistance that the applicant had achieved was not so unexpected that there was no motivation or suggestion to combine. In another case decided about the same time, an applicant unsuccessfully sought protection for a cotton cultivar the applicant claimed "possesse[d] the okra leaf character in combination with a high yield, high ginning out turn, good quality fiber and with resistance to 19 common races of the wide spread disease, bacterial blight" [128]. Once again, the nonobviousness requirement proved to be the rub. New plant varieties developed through genetic engineering, rather than conventional breeding, however, would seem to be less somewhat likely to run afoul the nonobviousness requirement because a true inventive step may well be involved in the engineering.

III. CONCLUSION

The differences between the three modes of protection outlined in this brief survey are significant and must be carefully considered by plant variety developers. For although one may protect a new variety under either the Patent Act or one of the plant-specific statutes (depending on whether the variety is sexually reproducible or not), that is not to say that one may protect the *same* variety under both a utility patent and a plant-specific title of protection. There is a judge-made rule against "double patenting"—that is, obtaining two patents on the same invention or an additional patent on an obvious variation first (unless the additional patent is limited to the term of the first patent) [129]. Under this rule, one cannot obtain both a utility patent and a plant patent on the same variety. The courts have yet to decide whether this rule applies equally to plant variety certificates. It is also not entirely clear how they

would, because the difference in the scope of protection patents and variety certificates is sufficiently different to be considered as distinguishing from double-patenting [130].

NOTES

1. See Note, Elizabeth Joy Hecht, *Beyond Animal Legal Defense Fund v. Quigg: The Controversy Over Transgenic Animal Patents Continues*, 41 Am. U.L. Rev. 1023, 1026–27 (1992); Reid G. Adler, *Controlling the Applications of Biotechnology: A Critical Analysis of the Proposed Moratorium on Animal Patenting*, 1 Harv. J.L. & Tech. 1, 2, and n. 3, 20 and nn.122–25 (1988).
2. Biotechnology has its origins in the mid-19 century, when Charles Darwin identified natural selection as the evolutionary mechanism in his seminal work *On the Origin of Species* (1850). Gregor Mendel independently discovered the role of genetics in such selection with his pea experiments in 1865 (although that knowledge did not come to light until 1900). Adler, *supra*, at 22 and n.142. In 1869, Miescher discovered deoxyribonucleic acid (DNA) which, in 1944, was identified as the medium of genetic communication. The stage was finally set for the biotechnological revolution when, in 1953, Crick and Watson discovered the structure of DNA. *Id.* at 22 & n.146. The secrets of the genetic code were thus unlocked.
2. Reid G. Adler, *Can Patents Coexist with Breeders' Rights? Developments in U.S. and International Biotechnology Law*, 1986 Int. Rev. Ind. Propr. Copyright L. 195, 207 and n.80 (reprinted with permission from the Max-Planck Inst., Munich, Germany). The terms *biotechnology*, *genetic engineering*, and *recombinant DNA technology* denote the process of identifying the gene sequence thought to cause the expression of a particular trait in an organism, splicing together that sequence (perhaps using material from other species), and microinjecting or otherwise introducing the composite gene into an embryonic organism then develops the trait as it matures and contributes the gene in the creation of offspring. See Adler, *supra* note 1, at 2 n.3, 5 n.19; see also Hecht, *supra* note 1, at 1024–28 and nn.4, 13–17. This technique was first practiced successfully in microorganisms in 1974, and in plants and animals in 1982. Adler, *supra* note 1, at 3–4.
3. See Adler, *supra* note 1, at 19–20 and nn.113–18; see also U.S. House Report No. 699, 103d Cong., 2d Sess. 9 (1994) (accompanying the 1994 amendments to the Plant Variety Protection Act (PVPA)), reprinted in 1994 U.S. Code, Cong. Admin. News 2423 [hereinafter "House Report"] (emphasizing the genetic security and environmental benefits of stimulating plant variety development through intellectual property protection).
4. "Industrial property" in European parlance.
5. For a more detailed statement of the rationale behind intellectual property protection, see, e.g., *In re LeGrice*, 301 F. 2d 929, 934–35 (C. C. P. A. 1962); Adler, *supra* note 1, at 220–22.
6. Steven Holtzman, CEO of Embryogen Corporation, quoted in Gladwell, *Mouse Patent May Bolster Research Efforts*, The Washington Post, Apr. 13, 1988, at F1.
7. See, e.g., Thomas Zeleny, *Property Rights in Living Things: Difficulties with Reproduction and Infringement*, 2 San Diego Justice J. 209 (1994); Margaret J. Lane, *Patenting Life: Responses of Patent Offices in the U.S. and Abroad*, 32 Jurimetrics J. 89 (1991) (discussing the inherent difficulties of meeting traditional patent criteria with claims to organisms); Marsha L. Montgomery, Note, *Building a Better Mouse—And Patenting It: Altering the Patent Law to Accommodate Multicellular Organisms*, 41 Case W. Res. L. Rev. 231 (1990); JoAnne E. Seibold, *Can Chakrabarty Survive the Harvard Mouse?*, 2 U. Fla. J.L. Public Policy 81, 90–93 (1989) (same).
8. See *Animal Legal Defense Fund v. Quigg*, 932 F.2d 920 (Fed. Cir. 1991) (dismissing suit by animal rights activists challenging policy allowing animal patenting invalid on procedural grounds). See generally Hecht, *supra* note 1; Adler, *supra* note 1; *id.* at 5–8 (summarizing the arguments of the animal rights movement relative to animal patenting).
9. *Gottschalk v. Benson*, 409 U.S. 63, 67 (1972) (citing *Funk Bros. Seed Co. v. Kalo Innoculant Co.*, 333 U.S. 127 (1948)) (discussed *infra* note 24).
10. See generally Adler, *supra* note 2; Thomas E. Jurgensen, *Of Plants, Patents, and Breeder's Rights: Some Proposals for International Unification of Proprietary Protection of Plant Biotechnology*, 12 J. Agric. Tax. L. 291 (1991).
11. Done Apr. 15, 1994, ch. 5 (patents), reprinted in 1 Law & Practice of the World Trade Organization annex 1C (Joseph F. Dennin ed., 1995).

12. This one remaining prohibition is technically based on the Thirteenth Amendment to the U.S. Constitution, which outlawed slavery in the wake of the Civil War.
13. TRAIP Agreement, *supra* note 11, art. 27(3)(b).
14. *New York Trust Co. v. Eisner*, 256 U.S. 345, 349 (1921).
15. 35 U.S.C. §§ 101 *et seq.* (1994).
16. U.S. Const. art. I, § 8, cl. 8.
17. 35 U.S.C. § 101 (emphasis added); *accord* TRIPS Agreement, *supra* note 11, art. 27 and n.5 (“patents shall be available for any inventions, whether products or processes, in all fields of technology, provided they are new, involve an inventive step and are capable of industrial application”; “inventive step” and “capable of industrial application” are synonymous with the terms “nonobvious” and “useful”).
18. The PTO is the agency Congress established to administer the patent law, subject to judicial review. Congress created a specialized court, the Court of Customs and Patent Appeals (CCPA), in 1929 to conduct review in these technically complex cases. *See generally* The Honorable Giles S. Rich, A Brief History of the Court of Customs and Patent Appeals (1980).
In 1982, Congress merged the CCPA and another specialized tribunal, the Court of Claims, into a single court—the U.S. Court of Appeals for the Federal Circuit. Congress also expanded the patent jurisdiction of the new court to include appeals from all U.S. District Courts in patent infringement suits. That jurisdiction had been committed to the various regional courts of appeals. It was hoped that creating a single court to hear all patent-based cases would cure the serious conflict of law that had developed between the regional circuits. *See* Federal Courts Improvement Act of 1982, Pub. L. No. 97-164, § 402, 96 Stat. 37; S. Rep. No. 275, 97th Cong., 2d Sess. 3–6, *reprinted in* 1982 U.S. Code, Cong. & Admin. News 11, 13–16, Titanium Metals Corp. v. Banner, 778 F.2d 775, 779 (Fed. Cir. 1985).
19. 1889 Dec. Comm'r Pat. 123, *quoted in* *Diamond v. Chakrabarty*, 447 U.S. 303, 311 (1980).
20. *See id.* at 125.
21. *Id.*
22. 447 U.S. 303 (1980), *aff'g* 596 F. 2d 952 (C. C. P. A. 1979).
23. *See* 596 F. 2d at 968–69.
24. 447 U.S. at 311. An earlier Supreme Court itself had contributed to this belief in a case involving agricultural biotechnology. The applicant in *Funk Bros. Seed Co. v. Kalo Inoculant Co.*, 333 U.S. 127 (1948), had discovered a way of making an inoculant of nitrogen-fixing bacteria from the genus *Rhizobium* that was effective on a multiplicity of different plant types. This had not been possible before, because the presence of different species of the bacteria—each of which was effective on only one type of plant—generally inhibited nitrogen-fixing activity. The applicant overcame this obstacle by identifying certain strains of each species that could be successfully mixed together.
The Court concluded that the PTO had properly rejected the application after observing that the inhibitory qualities of the bacteria “are the work of nature,” and “the discovery of [such] phenomena” is not patentable. *Id.* at 130. The Court’s conclusion seems to be a *Latimer*-esque non sequitur, however, because the applicant had claimed an artificial agglomeration of natural phenomena that clearly could be considered, as a matter of ordinary English, a biological “composition of matter.”
25. 447 U.S. at 313.
26. 227 USPQ (BNA) 443 (Bd. Pat. App. & Interf. 1985).
27. *Ex Parte Allen*, 2 USPQ2d (BNA) 1425 (Bd. Pat. App. & Interf. 1987) (allowing claim to a genetically-engineered polyploid oyster), *aff'd without published op.*, 846 F.2d 77 (Fed. Cir. 1988). The first patent actually granted on a multicellular animal was the famed “Harvard Mouse” Patent, U.S. Patent No. 4,736,866 (1988), which claimed any nonhuman mammal, such as a mouse, genetically engineered in the prescribed manner to increase its susceptibility to cancer).
28. *See supra* note 1.
29. *See Adler, supra* note 2, at 197; *see also* *In re LeGrice*, 301 F. 2d 929, 937–38 (C. C. P. A. 1962) (describing the use of this technique in breeding roses).

30. See *Chakrabarty*, 447 U.S. at 311 & n.8.
31. See *id.* at 312.
32. See *Chakrabarty*, 596 F.2d at 982–83 & n.18.
33. 35 U.S.C. §§ 161–164 (1994).
34. See *In re LeGrice*, 301 F.2d 929, 932–33 (C.C.P.A. 1962) (citing the legislative history of the PPA in a case involving a rose plant patent).
35. *Id.* at 944.
36. 35 U.S.C. § 161.
37. *Id.* § 162.
38. *Id.* § 163.
39. See *Chakrabarty*, 447 U.S. at 312; see also *LeGrice*, 301 F.2d at 937–38 (“because of the infinite number of possible combinations between genes and chromosomes,” “the actual creation of a new plant...is not subject to controlled reproduction”); 35 U.S.C. § 161 (defining “new variety of plant” as a legion of clones—“whoever invents or discovers *and asexually reproduces*” a “distinct and new” individual gets “variety” protection).
40. See Adler, *supra* note 2, at 198.
41. See *id.* at 198, 210.
42. See generally *id.* at 211–12 (explaining the difference between reciprocal rights and “national” treatment); 7 U.S.C. § 2403 (1994) (“nationals of a foreign state in which they are domiciled shall be entitled to so much of the protection here afforded as is afforded by said foreign state to nationals of the United States for the same genus and species. House Report, *supra* note 3, at 9.
43. See *id.* at 198 & nn. 22–23.
44. See *id.* The treaty entered into force in 1968, and its original signatories were France, The Federal Republic of Germany, Belgium, and Holland. It has been amended three times—in 1972, 1978, and 1991, and currently has 25 members. Thirteen of them (including the United States) have signed the revised 1991 version.
45. See generally *id.* at 212.
46. See *id.* at 210 (Italy and Sweden under general patent statutes; the United States and South Africa under plant patent statutes).
47. See *id.* (Czechoslovakia, Austria, and Holland).
48. *Id.* (France and the Federal Republic).
49. See *id.* at 211; see also *id.* at 213 & n.106 (quoting UPOV Article 5[3] [breeders “shall” be allowed to use protected material in developing other varieties and marketing them, but cannot repeatedly use protected material to produce a new variety commercially]).
50. See *id.* at 208 n. 83 and 210–12 (quoting UPOV Article 2).
51. “The necessary changes having been made.”
52. See Adler, *supra* note 2, at 209 & n.90. In 1994, Congress amended the PVPA “to make such Act consistent” with the 1991 revisions to the UPOV Convention. The PVPA Amendments Act of 1994, Pub. L. No. 103–349, 108 Stat. 3136; see House Report, *supra* note 3, at 7–8.
53. See House Report, *supra* note 3, at 9.
54. See *id.*
55. See Adler, *supra* note 2, at 198–99.
56. 7 U.S.C. §§ 2401–2582 (1994).
57. For a more expansive analysis of this tension, see Adler, *supra* note 2, at 196, 201–15.
58. See *id.* at 207.
59. 35 U.S.C. §§ 101.
60. *Id.*
61. *Id.* §§ 102–103.
62. *Supra* note 11, art. 27 (quoted above in the text accompanying note 13).
63. See *id.* § 102(a) (patent not available if “the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent”) (emphasis added); see also *id.* § 102 (b) (patent not available if “the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than 1 year prior to the date of the application for patent”) (emphasis added).

- Note that § 102(a) deals with anticipation by others, whereas § 102(b) deals with self-anticipation and is essentially a promptness requirement, allowing an inventor only a year after placing the invention in the public domain to file for a patent. See generally 1 Chisum, *supra* note 27, ch. 3.
64. A patent has two basic parts, the “claims” and the “specification.” See 35 U.S.C. § 112 paras. 1–2 (technically, the claims are part of the specification, but they appear in a separate section at the end and are usually referred to as if they were separate). The claims alone define the “metes and bounds” of the patented invention and thus the scope of the monopoly. See *Aro Mfg. Co. v. Convertible Top Replacement Co.*, 365 U.S. 336 (1961). The rest of the specification by contrast, is discursive and contains all of the information that the inventor is required to disclose as well as any other information he thinks might be worth including, such as background information describing the need for the invention, the state of the prior art, and so forth.
65. See 1 Chisum, *supra* note 27, § 3.02[1], at 3–12 (collecting cases); Donald S. Chisum, *Prior Invention and Patentability*, 63 J. Pat. and Trademark Off. Soc. J. 397 (1981); Donald S. Chisum, *Anticipation, Enablement and Obviousness: An Eternal Golden Braid*, 15 Am. Int. Prop. L. Assoc. O.J. 57, 58 (1987).
66. See 1 Chisum, *supra* note 27, § 3.02[1], at 3–6 (collecting cases).
67. See 35 U.S.C. § 103; *Graham v. John Deere Co.*, 383 U.S. 1 (1966). See generally 1 Chisum, *supra* note 27, ch. 5.
68. See e.g., *In re Oetiker*, 977 F.2d 1443, 1447 (Fed. Cir. 1992) (note, however, that relative to obviousness, “the reference must either be in the field of the applicant’s endeavor or, if not, then be reasonably pertinent to the particular problem with which the inventor was concerned”) (citations omitted). See generally 2 Chisum, *supra* note 27, § 5.03[1]–[3].
69. Oetiker, 977 F.2d at 1447. See generally 2 Chisum, *supra* note 27, § 5.04[1]; Kenneth R. Adamo, *The Power of Suggestion: Teaching, Reason or Motivation and Combined-Reference Obviousness*, 76 J. Patent Trademark Off. Soc. 177 (1994); James W. Badie, “Motivation” or “Obvious to Try”: *Is There a Difference?*, 75 J. Patent Trademark Off. Soc. 54 (1993) (pointing out differences in the “motivation,” “obvious to try,” and “suggestion” tests and arguing that the PTO should return to the suggestion test because the motivation test is unclear); Robert W. Harris, *Critique of the Federal Circuit’s Suggestion Test for Obviousness*, 72 J. Patent Trademark Off. Soc. 990 (1990).
70. See generally Nonobviousness: The Ultimate Condition of Patentability (J. Witherspoon ed., 1980).
71. See generally Jeremy Cubert, *U.S. Patent Policy and Biotechnology: Growing Pains on the Cutting Edge*, 77 J. Pat. & Trademark Office Soc’y 151 (1995); Karl Bozicevic, *Patenting DNA—Obviousness Rejections*, 74 J. Patent Trademark Office Soc. 750 (1992); Brian C. Cannon, *Toward A Clear Standard of Obviousness for Biotechnology Patents*, 79 Cornell L. Rev. 735 (1994); see also *infra* text accompanying notes 126–27.
72. 35 U.S.C. § 101. See generally 1 Chisum, *supra* note 27, ch. 4.
73. U.S. Const. art. I, § 8, cl. 8.
74. See *Bedford v. Hunt*, 3 Fed. Cas. 37 (No. 1217) (C.C.D.Mass. 1817) (Story, J.) (“It is sufficient, that it has no obnoxious or mischievous tendency[] [and] that it may be applied to practical uses.... If its practical utility be very limited, it will follow, that it will be of little or no profit to the inventor.... The law, however, does not look to the degree of utility....”); see also *Ex Parte Murphy*, 200 USPQ (BNA) 801 (Bd. Pat. App. and Interf. 1977) (reversing examiner’s rejection of claim to new slot machine on the ground that such machines were legal in some places and morality, as such, is not the proper concern of the patent law). Note that the argument the examiner made in *Murphy* resembles the moral argument that the animal rights movement has leveled against animal patenting. See generally 1 Chisum, *supra* note 27, § 4.03.
75. See 1 Chisum, *supra* note 27, § 4.02[2] [c] (discussing *Brenner v. Manson*, 383 U.S. 519 (1966), which suggested this possibility, but did not have to reach the question because the applicant had not expressly asserted the utility of his claimed mouse-tumor-inhibiting compound in cancer research). See generally 1 Chisum, *supra* note 27, § 4.02[2] [g] (the utility requirement in the biotechnology field); Christopher A. Michaels, *Biotechnology and the Requirement of Utility in Patent Law*, J. Patent Trademark Off. Soc. 247 (1994); Maebius, *Novel DNA Sequences and the Utility Requirement: The Human Genome Initiative*, 74 J. Patent Trademark Off. Soc. 651 (1992).
76. See *In re Brana*, 1995 U.S. App. LEXIS 6362, at *21 (Fed. Cir. 1995) (“Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation

- of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans.”).
77. See generally 2 Chisum, *supra* note 27, ch. 7.
78. See TRIPS Agreement, *supra* note 11, art. 33. Previously, the term of U.S. patents—unlike that of most European patents—had been 17 years from the date of *issue*. This was considered less desirable than the European practice, because it allowed an applicant who could drag out the application process through dilatory procedural tactics to emerge from it many years later, after all sorts of infringing technology had grown up within the scope of the claims, like a hostile submarine in the middle of a slow-moving convoy (hence, the name “submarine patents”). And while most patent applications are prosecuted within 3 years, so that the effective, term remains at least 17 years, pioneering technologies—of which DNA-related inventions are a prime example—may take longer. Proponents of a date-of-issue system point out that these are precisely the inventions that ought not to be discouraged by the effective truncation of the patent term.
79. 35 U.S.C. § 112 para. 1 (emphasis added). The application also must “set forth the *best mode*” of the invention (e.g., the best materials to use) “*contemplated* by the inventor.” *Id.*
80. See *Ex Parte* ***, 27 USPQ2d (BNA) 1492 (Bd. Pat. App. & Interf. 1992) (discussed *infra*); see also *In re Argoudelis*, 434 F. 2d 1390 (C.C.P.A. 1970) (claim to antibiotic compound derived by process employing live microorganism enabled by deposit of microorganism in public repository). The rule of *Argoudelis* has been codified in regulations the PTO has promulgated to govern the examination of patent applications. See Manual of Patent Examination Procedure § 608.01 (p). This subject is discussed in Adler, *supra* note 2, at 215–19; Adler, *supra* note 1, at 13 & nn. 70–74; see also Lance Leonard Barry, *A Picture is Worth A Thousand Words: Vas-Cath, Inc. v. Mahurkar*, 76 J. Patent, Trademark Off. Soc. 5 (1994); William E. Player, *Does Ex Parte Humphreys Suggest A Double Standard?*, 75 J. Patent Trademark Off. Soc. 853 (1993) (noting that commercial availability is considered enough for chemical compounds, but not for genetic probes); Richard Warburg, *Enablement of Biotechnological Inventions: The Deposit Requirement*, 24 Suff. U.L. Rev. 951 (1990); Hampar, *Patenting of Recombinant DNA Technology: The Deposit Requirement*, 67 J. Patent Trademark Off. Soc. 569 (1985).
81. See Adler, *supra* note 1, at 11–12. See generally 4 Chisum, *supra* note 27, § 16.02.
82. See 28 U.S.C. § 1338 (1994). The loser has a right to appeal to the U.S. Court of Appeals for the Federal Circuit, *see id.* § 1295(a)(1) (1994); *supra* note 18.
83. See 35 U.S.C. § 281 (1994). See generally 5 Chisum, *supra* note 27, ch. 20. This does not mean, however, that a patent owner necessarily has an absolute right to make, use, or sell the claimed invention, since the patent holder is still subject to the rest of the law. Thus, a patent holder claiming a drug cannot market the drug without first gaining approval of the Food and Drug Administration (FDA). Nor can a patent holder practice an invention that entails the release genetically engineered pesticidal or herbicidal microorganism into the environment without the approval of the Environmental Protection Agency (EPA) or the Department of Agriculture (USDA), or that would involve transgenic experimentation on nonfarm animals without the approval of peer reviewers mandated by federal guidelines. See Adler, *supra* note 1, at 11–12 and nn.59–63.
84. See, e.g., *Builders Concrete, Inc. v. Bremerton Concrete Prods.*, 757 F.2d 255, 257 (Fed. Cir. 1985). See generally 4 Chisum, *supra* note 27, § 16.01.
85. See, e.g., *Graver Tank & Mfg. Co. v. Linde Air Prods. Co.*, 339 U.S. 605 (1950). See generally 4 Chisum, *supra* note 27, § 18.04.
86. This is called a “reexamination” proceeding. See 35 U.S.C. §§ 301–307 (1994) (any person may bring prior art to the attention of the PTO and request that they reconsider their decision to issue a patent in view of that art); *see also Patlex Corp. v. Mossinghoff*, 758 F.2d 594 (Fed. Cir. 1985) (holding that this administrative procedure does violate the right of trial by jury in civil cases guaranteed by the Seventh Amendment, even where an accused infringer seeks reexamination in the midst of a lawsuit).
87. See 35 U.S.C. § 282 (1994). See generally 5 Chisum, *supra* note 27, § 19.02.
88. *Id.* § 282(1).
89. *Id.* § 282(2)–(3).
90. See 5 Chisum, *supra* note 27, § 19.03–04.
91. See 7 U.S.C. § 2544 (1994); *see also id.* § 2541(3) distinguishing between “use” of the new variety in “producing” a new variety and “developing” a new variety).

92. *Id.* § 2541(e).
93. See 4 Chisum, *supra* note 27, § 16.03[1].
94. See 7 U.S.C. § 2543.
95. *See id.*
96. *Id.*
97. 115 S. Ct. 788.
98. See Pub. L. No. 103–349, § 10, 108 Stat. 3136, (deleting exemption); House Report, *supra* note 11, at 14–15.
99. *See id.*
100. See 35 U.S.C. §§ 161, 271 (1994).
101. See 7 U.S.C. § 2541(a)(3).
102. *See id.*
103. *Id.* § 2541(a)(4).
104. *See* § 2541(a)(l).
105. *Id.* § 2541(a)(2).
106. Note also that under the Patent Act, there is no limitation on the number of distinct inventions that an applicant may claim in one patent. *See* 35 U.S.C. § 112 (employing the plural word “claims”). Under the PPA, by contrast, an applicant may claim only one new plant. *See id.* § 162 para. 2 (1994).
107. *See* House Report, *supra* note 3, at 8; 7 U.S.C. § 2483(b).
108. 35 U.S.C. § 161.
109. 7 U.S.C. §§ 2402(a)(3)–(4) (1994).
110. *See id.*; *see also id.* § 2401(b)(5) (further defining “distinctness”); *id.* § (a)(1)–(a) (defining other terms, such as “variety,” “breeder,” and “sexually reproduced”).
111. See 35 U.S.C. § 102(b) (set out *supra* note 61); *id.* § 161 para. 2 (“The provisions of this title relating to patents for inventions shall apply to patents for plants, except as otherwise provided.”); *In re LeGrice*, 301 F.2d 929, 933, 944 (C.C.P.A. 1962) (Section 102 applies to plant patents as well as utility patents).
112. *See LeGrice*, 301 F.2d at 937–38, 944–45.
113. See 7 U.S.C. § 2401(b)(6).
114. 35 U.S.C. § 102 (b).
115. *See* House Report, *supra* note 3, at 11 (discussing changes to Section 42 (a) (2) of the PVPA, 7 U.S.C. § 2402 (a)(2)).
116. 35 U.S.C. § 162. Also unlike the Patent Act, the PPA allows only one claim per patent. Since each independent claim is a free-standing monopoly, many related inventions may be claimed in a single utility patent application.
117. *See supra* note 18.
118. *In re Greer*, 484 F.2d 488, 490–91 (C.C.P.A. 1973) (citing *In re LeGrice*, 301 F.2d 929, 944 (C.C.P.A. 1962)).
119. *Id.* at 491.
120. *Id.*
121. 7 U.S.C. § 2422 (2).
122. *Id.* § 2422 (3). For further discussion of the differences between the patent statutes and the PVPA, see Adler, *supra* note 2, at 199, 205–07.
123. *Ex Parte Obukowicz*, 27 USPQ2d (BNA) 1063 (Bd. Pat. App and Interf. 1992).
124. *Ex Parte Movva*, 31 USPQ2d (BNA) 1027 (Bd. Pat. App. and Interf. 1993).
125. *In re Deuel*, 34 USPQ2d (BNA) 1210 (Fed. Cir. 1995).
126. *In re Deuel*, 34 USPQ2d (BNA) 1210 (Fed. Cir. 1995).
127. *Ex Parte ****, 27 USPQ2d (BNA) 1492 (Bd. Pat. App. & Interf. 1992).
128. *Ex Parte Thomson*, 24 USPQ2d (BNA) 1618 (Bd. Pat. App. & Interf. 1992).
129. *See generally* 3 Chisum, *supra* note 27, ch. 9.
130. For a discussion of whether double protection should be allowed, see Straus, *Patent Protection for New Varieties of Plants Produced By Genetic Engineering—Should “Double Protection” Be Prohibited?*, 15 Int. Rev. Ind. Prop. Copyright L. 426 (1984).

Biotechnology in Agriculture: Ethical Aspects and Public Acceptance

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I. BIOETHICS AND BIOTECHNOLOGY

A. Bioethics

Bioethics considers the ethical issues raised in biology and medicine, and especially those raised by human activity in society and the environment using biotechnology [1,2]. The word *biotechnology* simply means using living organisms, or parts of them, to provide goods or services. The word can apply to agriculture in the past thousands of years, but it is often used to apply to new techniques. We should not forget that all civilizations were formed needing food, clothes, and medicines, and in that sense biotechnology is not new. What is new is that we can now make new varieties much more quickly, and with greater variation—and some foodstuffs made from plants bred using genetic engineering are already being sold in parts of the world.

Bioethics considers issues affecting all living organisms and the environment, from individual creature to the level of the biosphere in complexity. All living organisms are biological beings, and share a common and intertwined biological heritage. The term bioethics reminds us of the combination of biology and ethics, topics that are intertwined.

Bioethics especially includes medical and environmental ethics. The word was applied mainly for issues of medical ethics in the 1970s and 1980s, but the 1960s and 1990s saw much more attention on environmental ethics. We must include both, medical ethics includes any factor affecting health, and ecological and environmental ethics must include human interactions, as these interactions are one of the dominant ecological interactions in the world. Agricultural systems include economic, environmental, and human interactions. To resolve the issues, and develop ideals or principles to help us do so, we must involve anthropology, sociology, biology, religion, psychology, philosophy, and economics; we must combine the scientific rigor of biological data, with the values of religion and philosophy to develop a world view. Bioethics is, therefore, challenged to be a multisided and thoughtful approach to decision making so that it may be relevant to all aspects of human life.

Some academics have tried to define more precisely what bioethics is, and basic principles. There are two basic approaches, one being descriptive and the other being prescriptive. One describes how people makes decisions, and the other suggests the process that can be used to make decisions. However, bioethics is not to prescribe the correct “answer,” only the process that is used to decide. To make good choices, and choices that we can live with, improving our life and society, is certainly a good thing. However, what is good for one person may not be good for the broader society, and the global nature of agricultural economics and environmental impact, makes us think of the global arena. The choices that need to be made in the modern biotechnological and genetic age extend from before conception to after death—all of life. Later I will discuss some bioethical principles for agricultural biotechnology and bioethics, but first we need to consider the cultural background and public acceptance.

B. Historical and Cultural Background

Bioethics is both a word and a concept. The word comes to us only from 1970, yet the concept comes from human heritage thousands of years old. It is the concept of love, balancing benefits and risks of choices and decisions. This heritage can be seen in all cultures, religions, and in ancient writings from around the world [1]. The relations between human beings within their society, within the biological community, and with nature and God, are seen in prehistory; therefore, we cannot precisely define the origins of bioethics. Human civilization has been tied to agriculture for many millennia, and the concept of bioethics first emerged in the relationships that people had with nature, a nature that could be cultivated to provide for human needs.

In the conclusion of an earlier book, *Shaping Genes* [2], I said that we have much to learn from the issues raised by genetic technology, not just the nature of our genes, but the nature of our thinking about what is important in life. New technology can be a catalyst for our thinking about these issues, and we can think of the examples such as genetic engineering, which have been stimuli for research into bioethics in the last few decades. However, the issues raised are not fundamentally different from those of the past [3], and I would reject the use of the word “genethics” which has been the title of at least two recent books [e.g., 4].

Agriculture has long been connected to economics, and until the industrial age most economies depended basically on agriculture. Economic factors are an inseparable part of society, and trading between adjacent regions has been a major source of cultural mixing, today as in past centuries. The world has become smaller with modern trade and communications, and this is certainly one factor in the growing trend for internationalism. This is epitomized in the General Agreements on Tariffs and Trade (GATT), signed in 1993. Therefore, we need international approaches that will survive in the global market competition, without exploiting particular groups of people.

There are a variety of different ethical traditions, and these are also part of our social heritage, although most have a more spiritual base. Any theory of bioethics that will be applied to peoples of the world must also be acceptable to the common trends of major religious thought. The spiritual divisions of humanity are less mixed than the social ones, and these have been used as transnational boundaries in the past, and also today. The Islamic countries, Catholic countries, and loosely called Christian countries, are major regions of the world. Asia has more diversity of religion; for example, Buddhism in Sri Lanka is different from that in Japan. Within Asia there are also many Christians and Muslims, and most of the world’s religions. For those who want to read more on the approaches of different cultures to agricultural biotechnology issues, I refer to other works on the Christian [5,6], Islamic [7], Jewish [8], and Buddhist [9] approaches.

These different traditions should be respected to make this universal bioethics also cross-cultural ethics; respected to the extent that they do not conflict with fundamental human rights, which should be protected, and recognized. One of the assumptions of modern bioethics is that all human beings have equal rights, as defined in the Covenants on Human Rights [10]. We can argue for the foundation of human rights from secular philosophy or religion. Different societies have different goals, as do different persons. This diversity is to be valued; we should never expect all persons to balance the same values in the same way all the time.

The limits to tolerance are already broadly outlined in international covenants, such as the Declaration of Human Rights, and international treaties on environmental protection, which include limits on the permitted damage to the common environment, such as the convention on ozone-damaging chemicals, and on deep-sea dumping. Agriculture is dependent on water and environment, which are sometimes shared resources between different countries. Most maritime nations have declared 200-mile limits within which they claim prior rights to exploit marine resources, and the many examples of overfished species shows the need for international fishing strategies. We also have economic treaties, such as GATT, defining the limits of unfair trade. However, as will be discussed later, economic priorities conflict with environmental protection, and we need better resolution of this conflict in practical bioethics.

Issues such as justice and stewardship of nature have been debated for millennia. Differences in approaches are clear from early historical discussions of these issues; for example, there have always been people supporting and opposing euthanasia or animal use. These differences and similarities are seen within any group of persons within every society. Basically data from opinion surveys and observation suggest that the diversity of thinking within any one group is much greater than that between any two groups; therefore, we can attempt to look at basic universal principles that can be used in deciding these issues [1]. The social environment in which persons grow up, and the education strategies, are becoming more similar with time, suggesting that a universal approach is even more possible now than it was a century ago. In agriculture, several of the major divisions are overuse of animals, and the exclusion of certain animals by religious dietary laws tend to follow cultural boundaries more than medical issues, such as euthanasia, which are divided in all cultures.

The Judeo-Christian-Islamic view of the relation of humans and nature is that they are both continually dependent on God. People have been told to subdue, cultivate, and take care of the earth, to multiply and to have dominion over the created order (Genesis 1:28, 2:15). Biotechnologists could consider they are to continue the “good” work of creativity [2,6]. However, we find interpretations of these scriptures differ within followers of each religion, and rather than stressing one particular view the bioethical tradition is that of tolerance for the views of others. Some persons interpret biotechnology as playing God and others as serving God, so it is difficult to draw religious boundaries.

C. Public Opinion and Ethics

Bioethics is not just an academic endeavor or an applied part of philosophical ethics, it is rooted in the daily life and attitudes of all people; hence, the title of my recent book, *Bioethics for the People by the People* [1]. One way to examine the reasoning persons have is to ask them in surveys of opinion. Scholars may go through literature and historical studies, but often these studies are selected by their choices, which may not represent the public. We need to look at more than history, and more than policies that governments have developed; we need to reach into the hearts of persons. In 1993 an International Bioethics Survey was performed across ten countries of the world [1]. Some of the relevant results are described in Section II, with comparisons with surveys in North America and Europe.

Worldwide there have been quite a number of surveys focusing on biotechnology [11]. Surveys, including the International Bioethics Survey will come under criticism for attempting to look at bioethical decision making and reasoning using opinion surveys [1]. Opinion surveys look at opinions, and not the persons as a whole. However, the actions of individual persons and also society, can be predicted by surveys—with a real margin of error that can be determined only after surveys are conducted. The written survey permits more thinking on issues than an interview does. Also multiple-choice answers can be leading; hence, the use of many open choices in these surveys. Open responses, free questions, were used, but with a finite set of categories and an “other” category for the unusual ideas. We must be careful not to overgeneralize, it is important to look at all the data, and test the data from surveys with the data we obtain from literature, customs, and observation.

Surveys may be misused, but they could be used to form policy that respects the persons and personal choices that are expressed, because some countries in the world do not allow this expression of informed choice. Surveys are just a beginning and from addressing some of the issues exposed, it is hoped that further research in bioethics based on the perspective of “from the people for the people” may be implemented worldwide. We also need to ask why persons choose the answers that they do [1,12].

II. PUBLIC PERCEPTION OF BENEFITS AND RISKS OF BIOTECHNOLOGY

A. International Comparison of Public Acceptance

A fundamental question of bioethics is do people in different countries share the same thinking and reasoning? The basic method of comparison is to estimate the diversity of views within each community and to compare these differences with other societies [1]. If they make decisions in the same way, then we could call this universalism, and it also raises the possibility of further universalization of ethics. People in the world are increasingly being given the same media coverage of technology, and education also has many similarities—as even this book shows. Persons may reach different conclusions, but if the process of thinking is similar, this is still some type of universal approach. If persons do not have the same way of deciding, then what we must aim for is cross-cultural understanding, perhaps with some degree of universalism.

The use of surveys is only one part of the overall approach we can use to look at cultures; however, the data from surveys must be explained by any description of the real world. Another part of the data is the use of the products, and we can see current practices in agricultural biotechnology by the preferences of farmers, consumers, and what sort of products companies produce. Analysis of all these factors is important and, for the consumption of products of new biotechnology, we must see the results of the sales of these products in supermarkets, and their acceptance by the farmers who first use them.

There have been many surveys conducted on acceptance of biotechnology, including agricultural issues [11]. However, there have been few in developing countries [1]. There are various strategies being used to study public opinion. The first type is the use of fixed response questions, to chose from set answers, and this has been done in the United States. The major study in 1986 was the Office of Technology Assessment study [13]. In 1992 there was a study by Hoban and Kendall [14], looking at agricultural issues. There has also been comparative studies of scientists in the United States and in Europe, looking at their perceptions of the public image of genetic engineering [15,16]. In 1993 there was a survey conducted by the Canadian Institute of Biotechnology in Canada [17]. The Eurobarometer is a regular public

survey in Europe, including different questions each time, and is conducted in all 12 countries of the European Community. In 1991 Eurobarometer 35.1 looked at biotechnology and genetic engineering, and in 1993 Eurobarometer 39.1 repeated the same questions [18]. The Eurobarometer poll is limited because of the relatively small number of questions, and also the set format of the questions, but it is the most comprehensive in terms of sample response, randomness, size, and number of countries. There is some diversity within Europe, in biotechnology policy, public acceptance, and regulations.

Although these surveys provide some assessment of public acceptance, they generally use simple, set questions. Recent survey strategies in Europe look at reasoning more than just statistics [19,20], which may shed more light on the factors that will affect policy development. Martin and Tait [20], conducted surveys of selected groups of the United Kingdom public. They conclude that groups with an interest in biotechnology have probably already formed attitudes to it, which are unlikely to significantly change. They looked at industry and environmental groups, and at local communities, which are major players in the development of policy at both national and local levels. They also suggest that persons with the least polarized attitudes are most open to multiple information sources. Consumer research in The Netherlands conducted by SWOKA—an Institute for Consumer Research—has involved two major studies of what persons in The Netherlands think about eating foods made through biotechnology [19,21].

In New Zealand there was a study using both set and open questions in 1990 [22]. In Japan there have been several studies, the most comprehensive of these being a study that I did in 1991, among public, academics, scientists, and high school teachers, in which I also reviewed all the previous studies in Japan [23]. In these surveys I used open questions, and found that some arguments that are often used in biotechnology debates, such as eugenic fears or environmental risk, are not the major concerns voiced by persons in open questions. The more common concerns are interference with nature or general fear of a less concrete nature. Also the survey found that many persons perceive both benefit and risk simultaneously, and they are attempting to balance these; also educated persons show as much concern, in fact, biology teachers considered there was more risk from genetic engineering than did the ordinary public.

B. International Bioethics Survey

The survey was performed in 1993 in ten countries of the world, in English in Australia (A), Hong Kong (HK), India (IN), Israel (IS), New Zealand (NZ), the Philippines (P) and Singapore (S); in Japanese in Japan (J); in Russian in Russia (R); and in Thai in Thailand (T). Translations were checked by professional translators and questions were pretested [1]. The countries chosen in the International Bioethics Survey were chosen for two reasons: one being as representatives of the world, and the other in terms of convenience of access. Unfortunately, there is no African, South American, nor Islamic country among the countries chosen. The countries chosen include India, a country of mixed religion and the major so-called developing country, although it has an agricultural and social history much longer than most countries. Russia represents the former communist world, another possible dominant force in shaping opinion. The Philippines is a Catholic country. Thailand is a Buddhist country and represents South East Asia. New Zealand and Australia, with some comparisons to North America, and to past European surveys, represent Christian and Western countries. Hong Kong and Singapore represent the Chinese influence, and some comparison to mainland Chinese attitudes was also made. A small sample from Israel was also included, as one Middle Eastern country.

Three population samples were chosen for these International Bioethics Surveys, public, university students, and high school teachers. The questionnaires consisted of six A4-sized pages, with a one-page introductory letter including a form for the public and teachers to

request a summary of the survey results. The public and student questionnaires were identical. The teacher's survey included some of the same questions, but half of the questions were about teaching and curriculum in bioethics and genetics [1]. The surveys to public and teachers were sent with stamped return envelopes, and persons were asked to respond within each country.

Questionnaires were distributed randomly nationwide by hand to the public in Australia and New Zealand (with the assistance of Nobuko Macer, Eubios Ethics Institute), India (by Jayapaul Azariah and Hilda Azariah, University of Madras), Japan (with the assistance of Yuko Kato and Nobuko Macer, University of Tsukuba) and Thailand (by Peerasak Srivines and Prasert Chatwachirawong, Kasetsart University). The samples were chosen by a clustered random-sampling method, involving selection of a representative cross section of the community on maps, then inside those areas every second house on both sides of every second street had a questionnaire delivered into the letterbox. In India and Thailand some members of universities and institutes were also asked to deliver questionnaires, and this explains the higher representation of more educated persons in the samples than the general population ([Table 1](#)).

The surveys in Israel (with assistance of Frank Leavitt, Ben Gurion University) and Russia (with the assistance of Vijay Kaushik, Russian Academy of Sciences) included half public and half academics (including philosophers, molecular biologists, general humanities and science, and medical graduates), and included random delivery to institute members and to household, in the manner described in the foregoing. The Russian sample included 56% from Moscow, 22% from Ufa, and 22% from Peterzavodsk and, therefore, does not represent the rural section of the Russian population. The sample size from Israel is small and this sample is included as only a preliminary observation.

The International Bioethics Survey focused on agricultural biotechnology, and medical genetics, with some other questions looking at environmental attitudes and attitudes to disease. The questionnaires included about 150 questions in total, with 35 open-ended questions. The open questions were designed not to be leading, to look at how persons make decisions—and the ideas in each comment were assigned to different categories depending on the question, and these categories were compared among all the samples. In total nearly 6000 questionnaires were returned from ten countries during 1993 [1].

Student samples were obtained with the kind assistance of the persons indicated. The surveys were distributed by staff of the universities, and returned to a following class or department mail box. The samples include: medical schools in Australia (Peter Singer, Monash University), Japan (Michio Hirayama and Norio Fujiki, Fukui Medical School, N=127; Hideo Hayashi, University of Tsukuba, N=308), New Zealand (D. Gareth Jones, Otago University), the Philippines (Angeles T. Alora, University of San Tomas); a medical laboratory course in Hong Kong (Maureen Boost, Hong Kong Polytechnic); and biology students in India (Jayapaul Azariah and Hilda Azariah, University of Madras), Singapore (Lim Tit Meng, University of Singapore, N=23; Ong Chin Choon, Singapore Polytech, N=227) and Thailand (Peerasak Srivines and Prasert Chatwachirawong, Kasetsart University).

The high school teacher surveys were national, using randomly selected schools from published school lists (with the assistance of Shiro Akiyama, Yukiko Asada, Nobuko Macer, and Miho Tsuzuki, University of Tsukuba). All 438 high schools in New Zealand were surveyed. Two copies of the questionnaire, with two stamped return envelopes, were sent along with covering letter to the principal requesting that they randomly give one each to a biology and a social studies teacher. The samples of respondent included more biology teachers than social studies teachers.

General information gathered in the surveys included sex, age, marital status, children, education, religion, importance of religion, race, income, and rural or urban locality, and some

Table 1 Sample Characteristics and Awareness of Biotechnology in the International Bioethics Survey

% of N Characteristics	Public								Medical/biology students								High school teachers						
	NZ	A	J	J91	India	Thai	R	Israel	NZ	A	J	India	Thai	P	S	HK	NZb	NZs	Ab	As	Jb	Js	
Number	329	201	352	551	568	689	446	50	96	110	435	325	232	164	250	104	206	96	251	114	560	383	
Response rate (%)	22	13	23	26	57	36	43	<20	60	70	66	65	50	70	80	52	61	28	47	21	37	26	
Female	59	55	48	47	39	52	64	62	59	50	33	47	58	54	77	55	36	38	52	37	12	8	
Urban	77	71	73	-	78	46	90+	80	85	89	49	85	58	87	96	88	31	73	75	79	63	66	
Mean age (years)	47.4	45.2	41.7	39.8	30.6	37.2	36.3	33.4	20.8	18.1	21.1	21.8	21.3	21.1	19.3	21.0	40.8	42.5	41.8	42.0	40.7	40.0	
Married	59	62	66	66	45	59	54	62	3	0	1	2	0.4	1	0.4	0	83	86	79	70	77	75	
No child	33	39	40	35	55	22	41	48	97	100	100	98	96	100	99	100	22	15	24	24	30	28	
Education																							
High school	43	36	37	37	4	2	13	16	29	94	54	7	4	0	23	71	1	0	1	1	0	0	
2-year college	18	15	19	22	6	3	18	20	48	4	6	13	18	0	77	3	1	2	0.4	1	0.2	1	
Graduate	25	28	31	31	31	35	37	39	20	2	38	27	60	50	0	6	64	58	59	57	78	82	
Postgraduate	9	16	10	7	52	59	28	25	3	0	0	51	13	47	0	8	30	37	39	41	21	17	
Other	5	5	3	3	7	1	4	0	0	0	2	2	5	3	0	4	4	3	0.4	0	0.8	0.3	
Awareness of pesticides																							
Not heard of it	2	5	3	4	5	0	2	4	2	5	5	6	0	1	7	13	0	0	0	0	0.4	0.3	
Heard of it	48	47	61	58	44	34	54	60	60	56	73	41	59	76	67	78	5	6	5	10	24	40	
Could explain	50	48	36	38	51	66	44	36	38	39	22	53	41	23	26	9	95	94	95	90	76	60	
Awareness of biotechnology																							
Not heard of it	23	19	6	3	10	2	8	18	13	25	5	7	6	13	0.4	8	0	6	0	8	1	1	
Heard of it	62	56	65	65	53	57	62	62	54	54	69	53	71	68	45	74	12	51	11	38	11	50	
Could explain	15	25	29	32	37	41	30	20	33	21	26	40	23	19	55	18	88	43	89	54	88	49	
Awareness of genetic engineering																							
Not heard of it	9	9	9	6	17	13	14	8	0	3	8	10	17	4	1	7	0	4	0	1	1	15	
Heard of it	62	49	74	68	46	58	60	82	26	43	67	40	63	60	51	79	7	41	9	43	25	67	
Could explain	29	42	17	26	37	29	26	10	74	54	25	50	20	36	48	14	93	55	91	56	74	18	

Abbreviations used in all tables: NZ, New Zealand; A, Australia; J, Japan; J91, Japan 91 survey (Macer, 1992); Thai, Thailand; R, Russia; P, Philippines; S, Singapore; HK, Hong Kong; b, biology teachers; s, social studies teachers.

Source: Ref. 1.

data are in [Table 1](#). Results of the other questions, further background, and more examples of open comments have been published [1]. In this paper the word *significant* implies a statistical significance of $P < 0.05$. The funding for these surveys came principally from the Eubios Ethics Institute, with some assistance from the ELSI (Ethical, Legal, and Social Impact issues) group of the Japanese Ministry of Education, Science and Culture Human Genome Project, and The University of Tsukuba. The high school samples in Japan are supported by the Ministry of Education, and are part of a longer-term project to develop high school materials to teach about bioethical issues in the biology and social studies classes.

C. Knowledge and Awareness of Biotechnology

The level of interest science and technology was measured using a 5-point scale, "extremely interested, very interested, interested, not interested very much, not at all interested." In general most respondent answered that they had some interest, with few saying they were not. Another measure may be the response rate, which was generally between 20–30%. However, other data suggest knowledge of science is not so closely correlated with response rate [1,23].

Although the principal purpose of the survey was to investigate opinions about genetic engineering, in Q5 some other controversial and noncontroversial developments were listed and the results for each can be compared. Persons were first asked about their awareness of the techniques with the following question:

Q5. Can you tell me how much you heard or read about of these subjects?

1. Not heard of 2. Heard of 3. Could explain it to a friend

Agriculture pesticides

In vitro fertilization

Computers

Biotechnology

Nuclear power

AIDS

Human gene therapy

Genetic engineering

The results of the awareness question for pesticides, biotechnology, and genetic engineering are shown in [Table 1](#). It is interesting that biotechnology was generally one of the most unfamiliar terms, next to gene therapy. The awareness of biotechnology in Japan, however, was one of the most familiar, consistent with other surveys, [23]. In a 1988 survey of 2000 persons in the United Kingdom, only 38% of respondents had heard of biotechnology [24], considerably fewer than in the countries in this survey. In the United States in 1992, 25% said that they had heard "nothing" about biotechnology, 38% said "a little," 30% "some," and 8% "a lot" [14]. This suggests that the awareness of biotechnology in the United States is at least no higher than in these Asian countries, and could be lower.

The awareness of gene therapy was the lowest among the eight developments. Genetic engineering was generally the least familiar among the other areas, with pesticides, in vitro fertilization, computers, and nuclear power being most familiar. We can compare this result with a question in the United States in 1986 [13] in which 32% said they did not know the meaning of genetic engineering, whereas 66% said that they thought they knew the meaning of the word. In the International Bioethics Survey, the term genetic engineering, was significantly more familiar in all samples. The awareness was significantly related to educational attainment in most samples. There was no question requiring respondents to explain their understanding of technologies, as was used in some surveys [13,18]. An indirect

measure of the depth of knowledge was the comments that were given in response to open questions.

The samples with the greatest awareness were generally biology teachers, next were medical students (New Zealand, Japan, Australia, and Philippines), followed by the other groups, social studies teachers, biology students, and the public. For all developments and in all samples, there was a positive correlation between awareness and the expressed level of interest in science from the results of the earlier question. Following questions, discussed in the next section, asked them whether they thought each development would have a benefit or not, and their perceptions about the risks of technology by asking them how worried they were about each development. We can expect the awareness to grow with the increased use of biotechnology products, and we actually find awareness that genetically modified organisms are being used to produce foods and medicines is already quite high (see [Table 4](#)).

D. Benefits and Risks of Biotechnology

In all countries of the International Bioethics Survey there was a positive view of science and technology; it was perceived as increasing the quality of life by most persons in all countries. Less than 10% in all countries saw it as doing more harm than good [1]. Persons were asked about the benefits and risks of specific developments of technology, and both benefits and risks were cited by many respondents. The areas of science and technology that were chosen for this survey included several controversial subjects, and this question (Q6, Q7) was modified from one used in Couchman and Fink-Jensen [22] and Macer [23], with the request for a reason. The topic of computers (a neutral control subject) and nuclear power were added to the four biological areas, as follows:

Q6. Do you personally believe each of these scientific discoveries and developments is a worthwhile area for scientific research? Why?

1. Yes 2. No 3. Don't know

Q7. Do you have any worries about the impact of research or its applications of these scientific discoveries and developments? How much? Why?

1. No 2. A few 3. Some 4. A lot

In vitro fertilization

Computers

Biotechnology

Nuclear power

Agricultural pesticides

Genetic engineering

The results for biotechnology are shown in [Table 2](#), to illustrate the analysis. For this question, the comments were assigned into one category, and the results are shown. People do show the ability to balance benefits and risks of science and technology, consistent with earlier surveys [23,25]. People do not have a simplistic view of science and technology, and can often perceive both benefits and risks. This balancing of good and harm is necessary for bioethics, and I have called this one indicator of the bioethical maturity of a society.

When specific details of an application are given there is generally greater acceptance, suggesting persons have some discretion [1,23]. It also suggests that if details are given, the public will show greater acceptance of an application, especially for human gene therapy [1,26]. This is particularly seen in questions looking at environmental release of genetically modified organisms (Q31) which were taken from the OTA survey [13], with comparisons with a question of Hoban and Kendall [14]:

Table 2 Perceptions of Benefit and Risk and Open Comments About Biotechnology

Reasons	Public						Medical/biology students							High school teachers							
	NZ	A	J	India	Thai	R	Israel	NZ	A	J	India	Thai	P	S	HK	NZb	NZs	Ab	As	Jb	Js
Q6. Do you personally believe biotechnology is a worthwhile area for scientific research? Why?																					
Yes	46	59	74	75	90	73	72	56	47	78	77	85	71	90	65	94	62	93	73	95	87
No	5	3	7	5	1	4	10	4	0	3	6	2	4	3	6	0	3	0.4	0	2	5
Don't know	47	38	19	20	9	22	18	40	53	19	17	13	25	7	29	6	35	7	27	3	8
N	315	196	332	526	680	456	50	94	105	423	309	232	157	218	105	203	94	249	110	556	381
Not stated	52.7	49.5	53.0	46.8	37.4	74.5	80	60.6	58.1	51.8	41.1	31.0	50.3	63.9	66.7	27.3	58.5	32.1	45.5	46.6	57.7
Economy	0.6	0	0.3	1.9	3.5	3.9	0	0	0	1.2	2.6	2.2	1.3	2.8	1.9	4.9	0	2.0	0.9	0	0
Science	6.0	10.2	7.8	9.1	14.7	5.6	4	6.4	2.9	7.6	9.1	22.4	5.1	3.2	1.9	10.7	10.6	14.5	8.2	15.5	9.5
Cure disease	1.6	6.1	1.2	2.7	0.7	0	0	3.2	5.7	1.9	4.0	0.4	2.6	3.6	1.0	3.4	2.1	7.2	2.7	1.8	1.3
Humanity	10.2	9.5	14.8	19.0	11.6	6.9	6	2.1	7.6	15.8	19.7	9.5	17.8	13.3	12.4	26.3	12.8	16.5	12.7	4.3	0.5
Inc. efficiency	1.3	0.5	0	3.0	3.4	0.2	0	0	1.0	0	7.1	2.6	3.2	1.6	0	1.5	0	1.6	0.9	1.1	0
Good for environ.	2.2	0.5	1.2	3.2	12.2	1.7	0	1.1	5.7	2.4	1.0	15.5	3.2	3.2	2.9	5.4	0	1.6	0.9	2.2	1.1
Agriculture	4.4	4.1	9.6	7.8	7.7	0.8	0	4.3	3.8	9.2	11.7	8.1	2.6	4.8	6.7	11.2	4.3	8.0	6.4	11.0	4.7
Help if careful	4.4	5.6	2.7	2.3	5.0	0.2	8	2.1	1.9	1.9	1.9	2.6	3.8	2.0	0	9.3	5.3	14.1	14.6	13.9	16.0
Dangerous	0.9	1.5	3.9	0.4	0.4	2.3	0	0	0	3.1	0.3	0.9	0.6	0.8	1.0	0	0	0	0.9	0.5	0.3
Playing God	2.2	1.5	3.0	0.4	0.3	0.7	0	0	1.0	0.5	0.3	0.4	2.6	0.4	0	0	2.1	0	0.9	0.4	1.3
Don't need	1.6	0.5	0.6	0	0.3	0.2	0	3.2	0	1.0	0	0	0	0	0.4	1.0	0	0	0	0.4	0.3
Unknown	11.8	9.7	1.8	2.7	1.9	3.2	2	7.5	12.4	3.8	1.6	3.0	5.8	0	3.8	0	3.2	0.4	3.6	0.4	2.1
Q7. Do you have any worries about the impact of research or applications of biotechnology ? How much? Why?																					
No worries	33	33	37	59	61	39	35	36	54	31	56	52	35	44	32	42	31	23	29	22	22
A few	32	25	44	19	30	25	30	34	26	51	22	37	27	29	40	37	38	37	25	49	46
Some	25	25	14	15	8	26	26	25	13	15	15	8	30	18	19	17	27	31	28	21	22
A lot	10	17	5	7	1	10	9	5	7	3	7	3	8	9	9	4	4	9	18	8	10
N	280	187	381	500	674	456	48	91	95	420	307	230	154	243	104	204	93	236	102	554	383
Not Stated	41.4	41.2	53.4	58.6	49.2	81.4	77.1	51.7	57.9	62.9	60.6	36.5	64.9	71.2	74.1	50.0	63.4	41.1	6.9	58.1	63.2
Don't know	14.3	15.5	2.2	5.0	3.6	2.2	4.2	12.1	11.6	3.1	2.9	5.7	2.6	2.5	1.9	0	3.2	1.3	0	0.2	1.3
Interfere nature	5.0	2.7	2.8	4.3	0.9	1.1	2.1	2.2	4.2	4.3	2.0	0	0.7	0.8	0.9	1.0	5.4	1.7	6.9	1.8	4.4
Fear/feeling	8.6	4.8	6.9	4.3	4.2	5.0	0	3.3	4.2	7.1	6.2	3.0	5.8	2.9	2.9	6.4	5.4	4.7	7.8	2.5	1.6
Ethical	1.1	3.2	1.3	0.8	0.3	0.7	0	2.2	3.2	0.5	0	0	1.3	0.8	1.0	2.5	3.2	4.2	0	4.3	3.4
Social effect bad	1.1	2.7	0.3	1.0	0.7	0.7	0	0	1.1	1.0	1.0	0.9	1.3	1.2	0	0	0	0.9	4.9	1.6	1.8
No control/waste	1.8	3.7	1.7	0.3	2.7	1.9	0	2.2	1.1	2.1	1.0	4.3	2.6	0.4	1.9	4.4	0	7.2	1.0	9.0	8.6
Bad health	1.1	0	0.9	1.2	1.0	0.4	0	1.1	1.1	1.7	0.7	0.9	1.3	1.2	3.9	0.5	0	0.9	1.0	0.7	1.0
Dangerous	1.8	2.7	1.9	0.6	1.6	0.9	0	2.2	3.2	0.7	2.0	1.3	1.3	0.8	0	2.5	0	0.4	2.0	1.4	0.8
Ecology	2.1	0.5	2.2	3.0	2.0	1.5	0	3.3	3.2	5.0	3.3	5.2	0	2.9	1.9	3.4	1.1	5.9	2.0	13.7	8.4
Human misuse	13.2	14.4	8.7	5.8	5.8	2.6	14.6	13.2	2.1	9.1	5.9	3.0	3.9	4.5	1.9	17.7	10.8	22.9	18.6	4.0	3.1
Eugenics	0.4	1.1	0.6	0	0.5	0.2	2.1	0	0	0	0.3	0.4	0.7	0.4	0	0.5	1.0	1.3	1.0	0.8	0
Can control	8.2	7.5	6.5	16.8	27.5	1.5	0	6.6	7.4	2.6	14.3	38.7	13.6	10.3	9.6	11.3	6.5	7.6	4.9	2.4	2.4

Source: Ref. 1.

Q31. If there was no direct risk to humans and only very remote risks to the environment, would you approve or disapprove of the environmental use of genetically engineered organisms designed to produce...?

1. Approve 2. Disapprove 3. Don't know

Tomatoes with better taste

Healthier meat (e.g., less fat)

Larger sport fish

Bacteria to clean up oil spills

Disease-resistant crops

Cows which produce more milk

The results are in [Table 3](#). The approval of the Calgene FlavrSavr modified tomato which has delayed ripening for general cultivation in the United States was given by the U.S. Department of Agriculture (USDA) in 1993, and it was approved for general commercial food consumption by the Food and Drug Administration (FDA) in 1994, and sold in the summer 1994 in some parts of the United States. The results show that it would be generally supported around the world.

The healthier meat question is relevant to efforts to make less fatty meat, both by hormones in pigs, and other animals. In the United States in 1992, 45% said "acceptable," 32% "unacceptable," and 23% "don't know" to a similar question [14]. In a related question on cows with increased milk, and in the United States in 1992, 36% said "acceptable," 41% "unacceptable," and 23% "don't know" to a similar question [14]. This has become reality in 1994 with the general use of bovine growth hormone (BST; bovine somatotropin) in the United States dairy industry, a hormone made by genetic engineering that can increase milk yield by 10–20%. It also received less support in the International Bioethics Survey than the goal of less fatty meat, which is consistent with the existing milk surplus in some countries. In a recent telephone survey in the United States conducted by Hoban [27], consumers gained confidence about consuming milk produced from cows treated with BST after receiving scientific facts attributed to respected agencies (e.g., AMA, FDA, NIH).

The most support is seen for disease-resistant crops, and bacteria to clean oil spills. These are two uses of genetic engineering that most agree with ([Table 3](#)). The sports fish is an example of genetic engineering for fun—and it is reassuring that many persons reject such genetic engineering. The highest degree of support for the sports fish is in the United States where 53% approved in a 1986 survey, while 73% approved of bacteria to clean oil spills or disease-resistant crops [31]. The general support for products of genetic engineering seems to be high, especially if they are claimed to be more healthy. In the Canadian study, comparisons between chemicals and genetically engineered organisms usually found less support for chemical methods [17]. All these examples of genetic engineering are already made.

In the 1991 survey in Japan an open question looking at awareness, benefits, and risks of genetic manipulation of microbes, plants, animals, and humans, was asked [23,26]. The responses made by the public, teachers, and scientists were compared with results for New Zealand [22], and few differences were observed. As in the United States, human genetic manipulation is associated with the most risks, and plant genetic manipulation with the least, but unfortunately they did not compare open comments [13].

E. Food Concerns and Human Health

A question that was repeated from the 1991 Japanese and 1990 New Zealand surveys in the International Bioethics Survey was:

Table 3 Approval of Environmental Release of Genetically Modified Organisms

Q31. If there was no direct risk to humans and only very remote risks to the environment, would you approve or disapprove of the environmental use of genetically engineered organisms designed to produce...? Yes- Approve No- Disapprove DK Don't know

% NZ	Public						Medical or biology students						High school teachers											
	A	J	J91	India	Thai	R	Isrl.	USee	NZ	A	J	India	Thai	P	S	HK	NZb	NZs	Ab	As	Jb	Js		
Tomatoes with better taste																								
Yes	49	54	69	-	73	83	35	40	-	54	53	71	77	88	68	74	58	67	51	60	47	67	55	
No	35	35	20	-	20	10	45	44	-	21	36	15	17	5	27	17	32	22	35	25	40	21	29	
DK	16	11	11	-	7	7	20	16	-	15	11	14	6	7	5	9	10	11	14	15	13	12	16	
Healthier meat (e.g., less fat)																								
Yes	54	60	57	-	66	84	35	44	-	74	71	65	68	88	75	72	62	72	63	71	57	60	47	
No	30	31	26	-	22	9	43	42	-	20	23	18	18	4	21	17	27	18	31	18	32	24	33	
DK	16	9	17	-	12	7	21	14	-	6	6	17	14	8	4	11	11	10	6	11	11	16	20	
Larger sport fish																								
Yes	22	19	22	19	48	58	13	20	53	28	23	24	50	64	54	44	42	26	16	22	22	19	16	
No	61	65	54	50	27	25	61	58	43	63	65	52	31	20	40	39	37	59	68	64	67	64	67	
DK	17	16	24	31	25	17	26	22	4	9	12	24	19	16	6	17	21	15	16	14	11	17	17	
Bacteria to clean up oil spills																								
Yes	75	82	71	75	74	87	63	70	73	92	89	76	74	85	78	86	70	85	82	91	84	77	63	
No	11	11	13	7	14	5	20	12	23	1	4	10	13	6	19	6	23	7	11	3	8	12	15	
DK	14	8	16	18	12	8	17	18	4	7	7	14	13	9	3	8	7	8	7	6	8	11	22	
Disease-resistant crops																								
Yes	70	78	66	75	78	91	54	50	73	81	81	67	81	91	82	83	72	85	70	83	70	71	55	
No	16	13	17	6	13	4	25	28	23	7	13	13	11	5	15	8	14	10	20	7	18	15	22	
DK	14	9	17	19	9	5	21	22	4	12	6	20	8	4	3	9	14	5	10	10	12	14	23	
Cows that produce more milk																								
Yes	36	39	44	-	75	84	23	38	-	55	44	49	72	86	70	57	54	59	49	57	43	56	38	
No	45	42	32	-	19	7	38	40	-	31	35	29	19	5	26	25	34	28	37	25	39	26	35	
DK	19	19	24	-	6	9	39	20	-	14	21	22	9	9	4	18	12	13	14	18	18	18	27	
Q1d. Genetically modified plants and animals will help agriculture become less dependent on chemical pesticides.																								
++ Agree + Agree strongly = Neither - Disagree - Disagree strongly																								
++	16	18	11	9	19	18	10	12	-	12	11	7	16	13	21	17	11	25	7	23	14	8	3	
+	48	47	31	39	37	51	34	35	-	52	61	42	34	52	49	55	50	59	46	55	46	37	23	
=	22	25	45	45	5	15	37	33	-	26	19	43	11	17	20	23	32	10	35	16	26	44	58	
-	12	9	10	6	12	13	14	16	-	8	9	7	27	15	9	5	7	6	11	6	11	9	12	
--	2	1	3	1	8	3	5	4	-	2	0	1	12	3	1	0.4	0	0	1	0.4	3	2	4	

Source: Ref. 1.

Q14. If any of the following were to be produced from genetically modified organisms, would you have any concerns about using them? How much? Why?

1. No 2. A few 3. Some 4. A lot

Dairy products

Vegetables

Meat

Medicines

Less concern was shown about medicines, that about vegetables and dairy products and most concern about meat ([Table 4](#)). A variety of reasons were cited ([Table 5](#)). The results of that question found concerns similar to the surveys in New Zealand [22] and Japan [23]. One of the main concerns was that the products would be unnatural, but there were also a variety of other comments as shown in the table. Comparison with the surveys in The Netherlands [19,21] allows development of methods to explore persons' concerns. In the 1992 U.S. study, price was also a critical factor, rather than whether the product was better quality [14]. However, there is a significant fraction of the food market in most countries from the products that claim to be of higher quality, and the best test of consumer preference is from sales statistics, for example from the sales of the "tasty" tomato released in the United States in 1994.

In the International Bioethics Survey, four specific questions used in the U.S. survey of Hoban and Kendall [14] were used to explore the acceptance of food products made from cross-species gene transfer. In all the countries in this survey ([Table 6](#)), plant-plant gene transfers (Q9) were most acceptable, with animal-animal (Q11) next, and animal-plant (Q10) or human-animal gene transfers (Q12) were least acceptable (p. 212–213). A variety of reasons were cited, which are reproduced elsewhere [1]. Some people made comments that suggested they were looking at secondary aspects, such as whether they liked potato or chicken. In the United States, the proportion accepting these were 66 (Q9), 39 (Q11), 25 (Q10), and 10% (Q12) [14], and the trend was also seen in Canada [17]. The generally higher fears about animal genetic engineering, and meat, is also seen in Europe [18,19]. In another question, over half the respondents in all samples in the International Bioethics Survey said that they had stopped eating a food because of concerns over its safety [1].

Many genetic diseases may be able to be treated by correcting the defective genes, which is called gene therapy. *Gene therapy* is a therapeutic technique in which a functioning gene is inserted into the cells of a patient to correct an inborn genetic error or to provide a new function to the cell. Over 70 human gene therapy trials have been approved in numerous countries. The responses to the questions about gene therapy show people do have significant discretion over therapeutic and cosmetic applications of gene therapy [1]. This is encouraging for ethicists, and is similar to the discretion shown in the question about use of genetic engineering to make a sports fish, compared to a disease-resistant crop. There was lower support for enhancement uses (improving physique, intelligence, making more ethical) than for treating disease, suggesting some discretion. There was extremely high support for use of gene therapy to cure disease, both as somatic cell (fatal or late onset) or inheritable (fatal or nonfatal); and high support as an acquired immunodeficiency syndrome (AIDS) vaccine.

There was least discretion against enhancement uses in Thailand and India, which may suggest that the economic strife and infectious disease makes people more pragmatic about the use of any therapy to treat disease. However, in other countries also, the success of cosmetic surgery suggests that once it is possible, the 20–30% who accept genetic engineering to improve intelligence, may do so in practice. Whether this is acceptable is a much bigger question and may require stricter control than today's cosmetics as heritable changes affect future generations.

Table 4 Concerns About Consuming Foods Made from Genetically Modified Organisms

	Public							Medical or biology students							High school teachers							
	NZ	A	J	J91	India	Thai	R	Israel	NZ	A	J	India	Thai	P	S	HK	NZb	NZs	Ab	As	Jb	Js
Q13. Before today, were you aware that genetically modified organisms, such as bacteria, plants, and animals, are being used to produce food and medicines? 1 Yes; 2 No																						
Yes	80	78	61	75	82	56	77	65	97	84	77	88	89	88	93	79	-	-	-	-	-	
Q14. If any of the following were to be produced from genetically modified organisms, would you have any concerns about using them? How much? Why? 1 No; 2 A few; 3 Some; 4 A lot																						
Dairy products																						
No	27	31	16	48	48	48	37	41	44	48	32	53	48	28	34	21	57	24	49	26	36	17
Few	22	21	40	52	26	28	63	22	36	26	46	33	35	24	23	43	22	24	21	30	38	36
Some	20	23	26	-	14	18	-	25	13	20	15	7	11	25	31	22	13	32	16	21	16	28
Lot	31	25	18	-	12	6	-	12	7	6	7	7	6	23	12	14	8	20	14	23	10	19
Vegetables																						
No	33	34	24	59	47	54	45	47	60	47	37	55	51	30	36	31	60	25	51	29	39	21
Few	19	22	42	41	27	28	55	20	23	29	42	29	29	21	26	37	25	22	22	27	38	40
Some	19	19	19	-	14	13	-	23	12	18	13	8	15	22	28	21	7	33	15	22	14	24
Lot	29	25	15	-	12	5	-	10	5	6	8	8	5	27	11	11	8	20	12	22	9	15
Meat																						
No	25	29	14	45	47	43	31	37	38	41	28	48	35	26	26	20	53	22	46	25	33	15
Few	18	18	39	55	25	29	69	18	30	22	43	32	35	20	21	36	22	22	23	24	37	34
Some	22	23	28	-	13	19	-	18	19	25	19	12	22	25	30	27	14	34	16	23	19	31
Lot	35	30	19	-	15	9	-	27	13	12	10	8	8	29	23	17	11	22	15	28	11	20
Medicines																						
No	38	46	22	50	47	52	54	45	73	59	44	57	40	36	40	26	61	26	57	33	32	16
Few	23	21	39	51	28	25	46	10	18	19	36	29	28	15	20	28	23	30	22	35	39	31
Some	18	20	20	-	13	15	-	20	5	17	15	7	16	21	25	24	10	31	13	21	17	29
Lot	21	13	19	-	12	8	-	25	4	5	5	7	16	28	15	22	6	13	8	11	12	24

Source: Ref.

Table 5 Reasons Given for Feelings About Consuming Foodstuffs Made from Genetically Modified Organisms (Q14)

Q14 reasons		Public							Medical or biology students							High school teachers						
%	Q?	NZ	A	J	India	Thai	R	Isr	NZ	A	J	India	Thai	P	S	HK	Nzb	Nzs	Ab	As	Jb	Js
N		306	195	329	514	681	440	50	94	108	426	296	231	160	246	103	196	96	250	106	531	354
Not stated	Dairy	36	36	48	53	43	68	74	38	39	53	59	26	63	58	59	41	29	37	38	48	49
	Vege	38	37	49	53	44	78	74	41	42	54	57	28	61	60	57	44	30	36	38	50	50
	Meat	35	34	48	53	45	76	74	37	39	53	63	27	63	57	57	39	29	35	36	49	48
	Meds	37	37	47	54	43	79	76	39	40	52	61	28	61	59	52	42	31	35	40	47	48
Unknown health effect	Dairy	6	7.7	10	6.0	5.4	7.8	0	3.2	5.6	12.2	3.4	5.2	8.5	3.7	7.7	3.6	15.6	12.3	4.4	8.9	11.9
	Vege	5	6.7	8	5.8	5.0	6.8	0	2.1	6.5	11.3	2.4	4.3	9.2	5.7	9.7	3.6	14.6	13.2	4.8	9.2	11.9
	Meat	6	7.7	10	5.9	5.5	6.8	0	4.3	6.5	12.0	2.0	6.1	7.2	4.9	7.8	4.1	14.6	14.2	4.8	9.0	12.4
	Meds	7	6.7	9	6.0	5.5	6.3	0	1.1	6.5	7.3	2.0	5.2	6.5	3.7	9.7	4.6	13.5	10.4	2.8	9.4	11.9
Long-term risk	Dairy	3	1.5	5	0.8	1.8	2.7	0	3.2	0.9	0.9	0.7	1.7	1.3	2.0	0	5.1	11.5	13.2	6.8	4.0	3.7
	Vege	3	1.5	5	0.6	1.8	2.0	0	2.1	0.9	0.9	0.3	1.3	1.3	1.6	1.0	5.1	11.5	13.2	6.4	4.0	3.4
	Meat	3	1.5	5	0.8	1.9	2.2	0	3.2	0	0.9	0.3	2.2	1.3	1.6	1.9	5.1	9.4	13.2	6.8	4.0	3.4
	Meds	3	1.5	4	0.6	1.9	2.0	0	1.1	1.9	0.9	0.3	3.1	1.3	0.8	0	4.1	6.3	11.3	5.2	3.8	2.5
Quality	Dairy	6	2.1	9	4.1	5.7	4.2	0	4.3	2.8	2.8	3.8	7.8	4.6	3.3	1.9	7.7	2.1	4.7	6.8	7.3	2.0
	Vege	6	3.1	6	3.3	4.1	1.7	0	2.1	2.8	2.6	5.8	7.4	3.9	2.4	1.9	6.6	2.1	3.8	6.8	6.4	2.0
	Meat	7	3.1	8	3.9	5.2	2.2	0	4.3	2.8	3.8	4.1	9.7	5.2	5.3	6.8	7.1	2.1	2.8	7.6	7.0	2.0
	Meds	7	1.5	5	2.7	3.2	1.2	0	1.1	1.9	3.1	2.0	6.1	2.6	2.4	1.9	6.1	3.1	1.9	5.6	7.3	1.7
Safety	Dairy	11	11	11	3.5	20.1	2.2	6	12.8	11.1	9.9	2.1	21.7	3.3	10.2	4.8	11.7	12.5	9.4	14.0	12.8	10.0
	Vege	11	11	11	3.7	19.7	2.0	6	13.8	11.1	10.6	2.0	21.2	3.9	10.1	3.9	11.7	12.5	9.4	13.6	12.8	9.6
	Meat	11	11	11	3.7	17.1	2.0	6	12.8	11.1	9.9	2.4	16.2	3.3	10.9	2.9	11.2	11.5	9.4	14.0	13.0	9.6
	Meds	12	10	10	4.7	19.5	1.7	6	13.8	11.1	9.4	3.0	20.1	5.9	10.2	3.9	12.2	10.4	11.3	16.8	13.4	10.5
Side effects	Dairy	1.3	2.1	1.2	1.0	4.4	2.0	2	4.3	7.4	1.2	1.7	9.1	5.2	6.5	4.8	4.4	6.3	3.8	1.6	0.4	0.6
	Vege	1.3	3.1	1.2	1.2	4.1	1.0	2	5.3	7.4	1.2	2.0	9.1	5.2	5.7	3.9	3.1	6.3	3.8	1.2	0.6	0.9
	Meat	1.3	3.1	1.2	1.0	4.6	1.2	2	5.3	7.4	1.4	2	11.4	5.2	6.1	3.9	3.6	6.3	3.8	1.2	0.6	0.6
	Meds	1.6	5.6	5	2.9	5.5	1.5	4	3.2	10.2	2.6	0	13.5	6.5	6.1	3.9	4.6	8.3	5.7	1.6	3.4	3.4
Unknown research	Dairy	1.6	0	2.1	0	2.4	2.2	2	0	0	1.2	4.4	0.4	0.7	0.4	0	4.6	5.2	1.9	1.6	4.0	2.3
	Vege	1.3	0	2.1	0	2.2	1.5	2	0	0	1.2	0	1.3	1.3	0.4	0	2.0	5.2	1.9	1.6	4.1	2.3
	Meat	1.3	0	2.1	0	2.2	1.5	2	0	0	1.4	0	0.9	1.3	0.4	0	2.0	5.2	1.9	1.6	4.3	2.6
	Meds	1.6	0	1.8	0	1.9	1.7	2	0	0	0.9	0	0.9	2.0	0.8	1.0	1.5	5.2	1.9	1.6	4.3	2.5
Unnatural	Dairy	18	16	6	7.8	1.9	2.9	6	7.4	8.3	5.6	5.1	2.2	2.0	2.9	6.7	5.6	7.3	7.5	4.0	4.7	7.6
	Vege	19	15	7	8.3	1.9	1.2	6	6.4	9.3	4.9	5.4	0.9	2.6	3.2	3.9	5.1	7.3	7.5	4.0	4.3	8.2
	Meat	20	14	7	7.5	2.5	2.2	6	7.4	10.2	5.4	5.1	0.9	3.9	5.3	4.9	5.1	9.4	6.6	4.4	4.7	7.9
	Meds	13	9	5	4.5	0.9	1.7	4	3.2	0.9	3.3	3.7	0.9	1.3	2.0	1.9	5.1	7.3	2.8	3.2	3.4	6.8

Source: Ref. 1.

Table Continues

Table 5 (Continued)

Unethical	Dairy	5.8	4.6	1.5	0.6	1.2	1.3	6	8.5	12.0	0.2	1.0	1.3	0	0.8	1.0	4.1	1.0	2.8	0.9	1.1	2.0
	Vege	3.6	3.6	1.2	0.6	1.5	0.7	6	3.2	9.3	0.2	0.7	0.9	0	0.4	1.0	2.0	1.0	0.8	0.9	0.6	1.7
	Meat	7.5	8.7	1.8	4.9	2.2	1.5	6	10.6	14.8	0.5	5.4	1.8	0	2.0	1.0	6.1	2.1	3.6	3.8	0.9	2.3
	Meds	4.2	2.6	1.2	0.6	1.2	0.7	0	6.4	9.3	0.2	0.3	0.9	0	0.4	0	2.0	1.0	0.8	0.9	0.6	1.4
Environment	Dairy	1.3	0.5	0.6	1.8	0.3	0	0	2.1	5.6	2.1	0.3	1.3	0.7	2.4	0	6.6	5.2	9.6	6.6	4.0	3.1
	Vege	1	1	1.5	1.7	1.0	0	0	2.1	5.6	2.3	0.3	1.7	0.7	2.0	0	7.7	5.2	11.2	7.5	4.5	3.1
	Meat	1	1	1.5	1.6	0.7	0	0	2.1	5.6	2.1	0.3	2.2	0.7	2.0	0	6.6	6.3	10.0	6.6	4.3	3.4
	Meds	1	0	0.6	1.4	0.4	0	0	1.1	2.8	1.9	0.3	1.8	0.7	2.4	0	4.1	3.1	7.6	5.7	4.0	2.8
Economic abuse	Dairy	1.3	3.1	0.3	0	0.3	0.2	2	3.2	0	0	0	0.9	0	0	0	4.1	2.1	2.4	3.8	1.5	2.8
	Vege	1.3	3.1	0.3	0	0.3	0	2	3.2	0	0	0	0.9	0	0	0	3.6	2.1	2.4	3.8	1.7	2.6
	Meat	1.3	3.6	0.3	0	0.3	0	2	3.2	0	0	0	0.9	0	0	0	3.6	2.1	2.4	3.8	1.7	3.1
	Meds	1.3	2.6	0.6	0	0.4	0	0	3.2	0	0	0.3	0	0	0	0	2.6	3.1	12.6	3.8	1.5	2.8
Other	Dairy	3	3	7	3.9	2.8	0.5	2	2.1	1.9	6.6	3.1	3.9	2.6	2.4	5.8	6.6	3.1	5.2	2.8	6.0	4.0
	Vege	3	3	6	5.2	3.4	0	2	4.3	1.9	5.9	3.4	5.2	2.6	3.3	5.8	5.6	3.1	4.8	2.8	4.9	2.8
	Meat	3	3	5	3.9	2.5	0.4	2	2.1	1.9	5.6	3.7	2.2	3.3	2.0	3.9	6.1	3.1	5.2	2.8	4.9	3.1
	Meds	3	3	5	3.7	2.1	0.2	2	4.3	1.9	5.6	2.7	2.2	1.3	2.0	3.9	7.1	6.3	6.0	5.6	4.1	3.7
Not needed	Dairy	1.3	3.6	0.3	2.6	1.0	2.0	2	7.4	3.7	0	1.0	2.2	2.0	1.6	1.9	1.0	4.2	1.2	2.8	0.9	1.4
	Vege	1.3	3.6	0	1.9	1.3	1.7	2	6.4	3.7	0	0.7	2.2	2.0	1.2	1.0	1.0	4.2	1.2	2.8	0.8	1.4
	Meat	1.3	5.1	0	2.5	1.5	1.7	2	7.4	3.7	0	1.7	2.6	1.3	1.6	1.9	2.6	5.2	1.2	2.8	0.8	1.7
	Meds	1.3	2.1	0	1.6	0.6	1.2	0	2.1	0.9	0.2	0.7	2.6	0.7	1.6	0	0	2.1	0.8	1.9	0	0.6
Lack Information	Dairy	7	5.1	8	1.6	2.4	1.2	2	3.2	1.9	4.2	1.4	3.5	1.3	0.8	3.9	3.1	6.3	5.6	9.4	4.1	8.8
	Vege	6	5.1	8	1.4	2.2	0.5	2	3.2	1.9	4.2	1.0	3.9	0.7	0.8	2.9	3.1	6.3	5.6	9.4	4.1	8.2
	Meat	7	5.1	8	1.4	3.0	0.5	2	3.2	1.9	4.5	1.4	5.3	0.7	0.8	2.9	3.1	6.3	5.6	9.4	4.3	8.2
	Meds	7	5.1	7	1.4	1.9	0.5	2	3.2	1.9	4.5	1.0	4.4	0.7	0.8	1.9	3.1	6.3	5.2	9.4	4.5	8.5
Needed	Dairy	2.9	6.2	2.4	11.5	4.1	1.5	4	8.5	5.6	2.8	13.9	11.3	4.6	2.9	2.9	2.6	1.1	2.4	0.9	2.3	0.6
	Vege	4.2	8.2	2.7	11.8	4.6	1.2	4	9.6	5.6	3.1	16.3	10.0	7.1	3.6	5.8	2.6	1.1	3.2	0.9	2.3	0.9
	Meat	2.3	6.7	2.4	10.0	3.6	1.0	4	7.4	5.6	2.8	9.2	10.1	4.6	2.8	3.9	2.6	1.1	2.8	0.9	2.1	0.9
	Meds	9.5	17.0	5.0	15.0	7.2	1.7	10	21.3	16.7	5.6	18.2	8.7	11.7	9.8	15.5	7.1	2.1	4.8	3.8	3.2	2.0
Increase food	Dairy	0.7	0	0	4.1	0.7	0.2	0	0	0	0	6.8	0	2.6	2.4	0	2.0	0	2.4	0	0	0
	Vege	0.7	0.3	0	3.9	0.6	0.5	0	0	0	0	5.4	1.3	1.4	2.8	1.0	2.0	0	2.0	0	0	0
	Meat	0	0	0.3	2.7	0.7	0.5	0	0	0	0	1.0	0	2.6	1.6	0	2.0	0	2.4	0	0	0
	Meds	1.8	0	0	1.9	0.6	0.2	0	0	0	0	1.4	0	1.3	0.8	0	3.1	0	2.4	0	0	0.3
Better	Dairy	3	4.1	1.5	2.7	2.5	0.7	0	0	3.7	1.4	0.3	2.2	2.0	4.5	0	5.1	3.1	6.4	2.8	1.5	1.1
	Vege	4	2.1	2.1	2.1	2.2	0.7	0	4.3	1.9	1.6	1.0	0.4	1.3	2.0	1.0	4.6	3.1	6.8	2.8	1.3	1.4
	Meat	1.3	2.1	1.5	1.6	1.8	0.4	0	0	1.9	1.2	0.7	0.4	1.3	1.2	1.0	4.6	2.1	6.4	2.8	1.3	2.0
	Meds	3	4.1	5	1.8	4.0	1.0	0	7.4	1.9	5.2	1.4	2.2	2.0	2.8	1.0	4.6	5.2	6.8	5.7	2.1	1.1
Already done	Dairy	3	4.1	1.5	2.7	2.5	0.7	0	0	3.7	1.4	0.3	2.2	2.0	4.5	0	5.1	3.1	6.4	2.8	1.5	1.1
	Vege	4	2.1	2.1	2.1	2.2	0.7	0	4.3	1.9	1.6	1.0	0.4	1.3	2.0	1.0	4.6	3.1	6.8	2.8	1.3	1.4
	Meat	1.3	2.1	1.5	1.6	1.8	0.4	0	0	1.9	1.2	0.7	0.4	1.3	1.2	1.0	4.6	2.1	6.4	2.8	1.3	2.0
	Meds	3	4.1	5	1.8	4.0	1.0	0	7.4	1.9	5.2	1.4	2.2	2.0	2.8	1.0	4.6	5.2	6.8	5.7	2.1	1.1

Source: Ref. 1.

Table 6 Genetic Engineering and Cross-Species Gene Transfer

	Public							Students							
	NZ	A	J	India	Thai	R	Israel	NZ	A	J	India	Thai	P	S	HK
Q9. Genes from most types of organisms are interchangeable. Would potatoes made more nutritious through biotechnology be acceptable or unacceptable to you if genes were added from another type of plant, such as corn? Why?															
Acceptable	56	56	39	56	82	45	50	86	75	51	58	78	65	79	76
Unacceptable	27	23	25	21	4	24	24	9	9	18	18	7	17	8	11
Don't know	17	21	36	23	14	31	26	5	16	31	24	15	18	13	13
Q10. Would such potatoes be acceptable or unacceptable to you if the new genes came from an animal? Why?															
Acceptable	19	23	11	29	48	16	22	49	42	16	27	48	17	25	25
Unacceptable	60	54	40	42	19	42	52	32	24	37	39	19	58	48	48
Don't know	21	23	49	29	33	42	26	19	34	47	33	33	25	27	27
Q11. Would chicken made less fatty through biotechnology be acceptable or unacceptable if genes were added to the chicken from another type of animal? Why?															
Acceptable	29	40	20	40	68	32	26	50	42	30	42	68	42	41	42
Unacceptable	46	40	41	27	10	35	46	25	27	35	24	13	36	27	31
Don't know	25	20	39	33	22	33	28	25	31	35	34	19	22	32	27
Q12. Would such chicken be acceptable or unacceptable if the genes came from a human? Why?															
Acceptable	10	16	6	16	29	10	14	20	20	11	18	30	7	14	19
Unacceptable	78	66	53	52	44	66	64	65	53	52	41	44	81	65	70
Don't know	12	18	41	32	27	24	22	15	27	37	41	26	12	21	11

Source: Ref. 1.

There is clear support for attempting to do good, and no apparent public rejection of therapy targeted on genes. About three-quarters of all samples supported personal use of gene therapy, with slightly higher support for use of gene therapy in children [1]. The major reasons were to save life and increase the quality of life. Few persons gave a reason such as "improving genes". About 5–7% rejected gene therapy considering it to be playing God, or unnatural. There was very little concern about eugenics (0.5–2%), confirming the results of a different open question in 1991 [23,26].

F. Environmental Concerns

Some environmental concerns were seen in the responses to the general questions on genetic engineering and biotechnology (see Tables 2 and 5). A specific question was also given before these questions:

Q1. To what extent do you agree or disagree with the following statements?

1. Agree
2. Agree strongly
3. Neither
4. Disagree
5. Disagree strongly

"d. Genetically modified plants and animals will help agriculture become less dependent on chemical pesticides."

The results are in Table 3. In the 1991 survey in Japan [23] 49% of the public agreed that genetically modified plants and animals would help Japanese agriculture become less dependent upon pesticides, whereas 49% of teachers and 56% of scientists agreed; 71% of the company scientists agreed with this statement. Only 7% of scientists and the public disagreed with this, and 13% of teachers disagreed. This question statement is a major argument of those calling for the development of genetic engineering in agriculture, and the result suggests that it is supported by a majority of persons, although still many are not sure about how they feel (see Table 3).

The way that genetic engineering is being used to introduce pest resistance to plants is to

transfer genes to selectively kill insect pests in plants. The research has been conducted on many species of plants, with success, especially transferring the gene encoding the insecticidal protein of the bacteria *Bacillus thuriengensis* [2]. This protein selectively kills certain insects, whereas other insects are not killed. When chemical insecticides are used, all insects may die, so this method is an advantage. The protein is not toxic to other animals, so it is also much safer than chemicals. Other examples of environmental benefits are known, and it is likely that this will gain public support of biotechnology.

In 1990 European public opinion poll conducted in the United Kingdom, France, Italy, and Germany, by Gallup for Eli Lily [28], the respondents were asked to choose the largest benefit that they saw coming from biotechnology, from between one of four possible benefits. Over half rated cures for serious diseases as the most important benefit. Another option was reducing our dependence on pesticides and chemical fertilizers, which 26% of Italians, 24% of French, 22% of British, and 16% of Germans, chose as the largest benefit. The respondents were asked a similar question about their largest concern: 40% of French, 35% of Germans, and 25% of British and Italian respondents chose eugenics, and slightly lower proportions overall chose environmental harm, 34% in Britain, 33% in France, 22% in Italy and 21% in Germany. Potential health hazards from laboratory genetic research were named by 29% in Italy, 17% in France, 11% in Britain, and 10% in Germany. Overall one-third of respondents feel that biotechnology is ethical and one-third feel that it is unethical, and one-third think it is in between, "neither."

Therefore, it appears that in all countries medical advances, and the ability to cure genetic disease are the major benefits people see from genetic engineering and biotechnology. Environmental concerns are a close second concern, and this is consistent with the International Bioethics Survey when we consider concrete concerns. However, from the results of the open question, we also see numerous concerns about what is natural, or ethical (see [Tables 2](#) and [5](#)). The benefits are divided, depending on the organisms that are considered. Microorganisms are seen for both medical use and general use to produce useful substances through fermentation. Plants and animals are seen for their obvious agricultural importance, and genetic manipulation is perceived for its ability to aid the breeding of new varieties and to increase production of food [23].

There were also two open question asking what images persons had of life and nature. The diversity of comments was similar in all countries, with similar proportions expressing different ideas—which is very interesting for looking at what we think of nature or life. The question on nature followed several question on genetic engineering, so it is not surprising that many (about one-quarter) included a comment that nature is something that should not be touched by human beings, and about one-tenth mentioned ecological problems [1]. The ethical limits of genetic engineering may in the end be decided by aesthetic perceptions of "nature," rather than environmental risk itself. This is very difficult to define, and this survey is an attempt to begin a search among ordinary people around the world on what these limits might be. We all have some limit, whether it be blue roses or chicken with four legs—and we also realize these limits change through time. A simple definition of bioethics, as I said earlier could be love of life. The images of life, therefore, are most illuminating in the pursuit of bioethics.

G. Source of Information and Trust in Authorities

Another issue of ethics is who should make decisions, and who do persons trust. A question on the level of trust that persons had in authorities for information on the safety of biotechnology products was asked, as shown in [Table 7](#). There was most trust in the government in Hong Kong and Singapore, and least in Australasia, Japan, Russia, United States and

Table 7 Trust in Authorities

(Q29) Suppose that a number of groups made public statements about the benefits and risks of biotechnology products. Would you have a lot of trust, some trust, or no trust in statements made by...?

	Public							Students							
	Trust	NZ	A	J	India	Thai	R	Israel	NZ	A	J	India	Thai	P	S
Government agencies															
A lot	5	8	8	25	33	5	24	7	7	4	25	28	20	34	37
Some	52	61	48	47	63	39	38	65	68	37	49	66	62	58	55
No	43	31	44	28	4	56	38	28	25	59	26	6	18	8	8
Consumer agencies															
A lot	24	13	12	23	43	33	28	28	8	8	23	41	17	6	25
Some	58	61	65	57	54	44	42	58	54	60	51	55	68	63	58
No	18	26	23	20	3	23	30	14	38	32	26	4	15	31	17
Companies making biotechnology products															
A lot	5	4	6	21	8	6	20	3	4	5	25	13	15	7	8
Some	44	52	43	47	70	31	28	49	53	38	54	75	57	66	57
No	51	44	51	32	22	63	52	48	43	57	21	12	28	27	35
Environmental groups															
A lot	21	20	15	47	-	53	54	18	14	7	52	-	57	35	45
Some	68	64	60	44	-	37	36	73	73	52	37	-	42	60	50
No	11	16	25	9	-	10	10	9	13	41	11	-	1	5	5
University professors															
A lot	25	30	12	38	42	35	42	50	54	10	47	29	46	30	47
Some	65	60	61	53	57	50	48	48	43	62	39	69	52	65	47
No	10	10	27	9	1	15	10	2	3	28	14	2	2	5	6
Medical doctors															
A lot	33	30	12	48	60	55	46	55	58	10	55	55	68	42	48
Some	60	64	58	43	38	35	50	44	40	64	37	44	29	54	49
No	7	6	30	9	2	10	4	1	2	26	8	1	3	4	3
Farmers or farm groups															
A lot	6	9	6	-	7	-	28	6	6	7	72	7	18	6	6
Some	69	69	50	-	67	-	50	70	70	50	15	76	71	54	43
No	25	22	44	-	26	-	22	24	24	43	13	17	11	40	51
Dietitians or nutritionists															
A lot	24	21	6	-	25	-	40	28	21	5	68	25	42	20	20
Some	66	69	54	-	67	-	50	65	69	56	18	65	53	66	71
No	10	10	40	-	8	-	10	7	10	39	14	10	5	14	9

Source: Ref. 1.

Europe. Despite the lower trust shown in the government in Russia, they had a level of trust in medical doctors. The result is most striking when we compare it with Japan, in which doctors were not trusted. In fact, it appears Japanese do not trust anyone very much, but the biggest difference with the other countries was that doctors and university professors were mistrusted, especially so by medical students. Whereas Russians show great trust in doctors and environmental groups, and a high level of trust in professors. Companies were least trusted everywhere. Farmers were also not trusted (unlike the United States where in 1992, 26% had a lot of trust, 68% had some trust, and 6% had no trust in farmers) [14].

The lack of trust in companies or governmental regulators is also seen in European [18] and North American surveys [13,14,17,29]. This lack of trust is a concern. The most trusted

source of information are environmental groups [20,29]. The main source of information is the media in all countries [1], but people are becoming more selective in what they believe.

H. Economic Concerns and Patenting Life

The question of patenting live organisms and genetic material is a contentious issue in many countries. In the United States and many other countries, normal criteria for accepting patents apply to any subject matter: that is, the invention requires the attributes of novelty, nonobviousness, and utility, and the invention should be deposited in a recognized depository. In 1985 the US Patent Office awarded a patent for a maize variety, in 1987 they ruled that polyploid oysters were patentable subject matter, and in 1988 they awarded a patent for a mouse. The mouse contains an activated oncogene and has been called "Oncomouse." It is very sensitive to carcinogens, and is being used in testing the safety of substances. The patent extends to all transgenic animals containing an activated oncogene. This patent decision triggered much debate about the ethical issues over patenting [30].

Although accepting the same patentability criteria, some countries have specifically excluded certain types of invention, for example the European Patent Convention excludes the patenting of varieties of plants or animals. An EC directive supporting the principle of patents for genetically engineered animals is still being debated, and Denmark excludes animal patents in law.

A patented product that reaches the commercial market gives the inventor some compensation for the time he or she spent in research for the development. The system is self-sustaining, if patents are awarded, companies will invest time into research, but if not, there is less incentive for companies to conduct research and less total research is performed. There may be a greater amount of total knowledge because there is more total research performed. However, property rights are not absolutely protected in any society because of the principle of justice, for the sake of "public interest," "social need," and "public utility," societies can confiscate property. There are also exemptions to patent law if the object is "offensive to (general) public morality," which could prevent the patenting of some animals for ethical reasons.

There may be better alternatives to patenting plants and animals. In 1961 the Convention on the International Union for the Protection of New Varieties of Plants (UPOV Convention) established international "plant variety rights," and by 1989 there were 19 member countries, which include more than 70% of the world seed market of all countries with a market economy [31]. The requirements include stability, homogeneity, novelty, and distinctiveness. The varieties must be generally distributed and researchers have exemptions, as do farmers from the payment of royalties on seed that they save from their harvest.

However, there is still no reward given to the farmers who for millennia have established crop varieties, which plant breeders use as starting materials. In 1983, at a UN Food and Agriculture Organization (FAD) conference, representatives from 156 countries recognized that "plant resources were part of the common heritage of mankind and should be respected without any restriction." Since then, an international network of gene banks has begun to be established that will provide genetic material worldwide. These also preserve genetic material from species that are becoming extinct because of environmental destruction. In 1994 the Biological Diversity Convention was ratified, and it specifically protects the intellectual property rights of indigenous biological organisms.

In 1991, a controversy arose when a single patent application for 337 human genes was made in by the US National Institutes of Health (NIH) in the United States. In February 1992, they made another application for patents on 2375 genes. The U.S. applications were

later rejected for technical reasons, rather than ethical ones, and the decision was not appealed. The French government, and Japanese genome researchers announced that they would not apply for similar patents because of ethical reasons. England's Medical Research Council (MRC) applied for a similar patent on more than 1000 genes, but England joined France in calling for an international agreement to waive any of these patents if they should be granted, and withdrew their application when the NIH did. The human genome is common property of all human beings, and no one should be able to patent it en masse, if at all [32]. The policy issue is still being debated [33].

The scientist Craig Venter left the NIH to start the company Human Genome Sciences, which includes The Institute for Genomic Research (TIGR), Gaithersburg, which continued the work of the NIH to create a database of cDNA sequences from as many human genes as possible. In late 1994, they released terms of the way researchers can access the 150,000 cDNA sequences they compiled into a database, which include 35,000 of unknown function [34]. Users must sign an option agreement, and companies will have 6 months to try to make joint shares in commercial developments. The release of the cDNA sequences from TIGR to the general community is under the condition that they can take first look for 30

Table 8 Support for Patenting

Q30. People who create something original can obtain financial reward for their efforts through patents and copyright. In your opinion, for which of the following should people be able to obtain patents and copyright? 1 Approve 2 Disapprove 3 Don't know

	Public										Students									
	NZ	NZ90	A	J	J91	India	Thai	R	Israel	NZ	A	J	India	Thai	P	S	HK			
New Inventions, such as consumer products																				
Yes	90	93	93	85	91	79	78	52	76	93	90	92	72	75	89	76	65			
No	4	7	2	6	3	12	9	15	10	2	3	2	15	14	6	9	15			
DK	6	-	5	9	6	9	13	33	14	5	7	6	13	11	5	15	20			
Books and other information																				
Yes	82	85	80	68	73	74	69	77	76	88	88	82	68	65	88	64	62			
No	8	15	8	14	8	16	17	6	14	6	5	7	18	16	8	17	14			
DK	10	-	12	18	29	10	14	17	10	6	7	11	14	19	4	19	24			
New plant varieties																				
Yes	63	71	55	49	60	60	85	59	64	56	49	52	61	83	65	66	47			
No	22	29	24	19	16	21	8	12	22	26	33	19	23	10	26	17	31			
DK	15	-	21	32	24	19	7	29	14	18	18	29	16	7	9	17	22			
New animal breeds																				
Yes	48	59	45	41	49	54	82	54	58	50	39	45	59	78	61	56	42			
No	33	41	29	26	22	25	10	16	24	32	37	24	28	12	29	26	28			
DK	19	-	26	33	29	21	8	30	18	18	24	31	13	10	10	18	30			
Genetic material extracted from plants and animals																				
Yes	31	51	38	35	37	50	73	52	56	33	32	30	51	78	52	55	40			
No	41	49	34	30	27	25	12	13	28	41	39	39	27	11	36	21	29			
DK	28	-	28	35	36	25	15	35	16	26	29	31	22	11	12	24	31			
Genetic material extracted from humans																				
Yes	23	-	31	32	28	42	60	45	46	22	28	25	46	70	44	47	37			
No	50	-	43	36	33	34	18	17	36	57	46	48	31	17	44	26	30			
DK	27	-	26	32	39	24	22	38	18	21	26	27	23	13	12	27	33			
A medical treatment or drug to cure AIDS																				
Yes	59	-	60	60	-	66	81	94	62	43	54	56	71	83	74	73	60			
No	28	-	26	21	-	22	13	3	28	40	34	26	18	13	19	20	22			
DK	13	-	14	19	-	12	6	3	10	17	12	18	11	4	7	7	18			

Source: Ref. 1.

days before publication at papers, with an option for a 30-day extra delay. SmithKline Beecham (of the UK) has control of Human Genome Sciences, linking pharmaceuticals with the human genome. In a countermove, another giant company, Merck, has made arrangements with the University of Washington to develop an open cDNA database, available for all researchers without charge. Merck is building on the chance to make a good image with the scientists, but TIGR argues that they want some reimbursement for the 200 million dollar investment in obtaining the sequences and making the database available. However, it is likely that they already have selected some genes for commercial development, and the profits from several hormones could compensate for all the work. The relevance for agriculture is that now such genome companies are applying the techniques to agricultural organisms, and have the resources to sequence all the novel cDNA from a single organism within a year. We should not forget the parallel with farmer's ownership of plants and animals, in which "life" is owned, but the originators of patents did not envisage the ownership options on such substantial portions of a genome of one organism.

The policy should be made considering all the economic, environmental, ethical, and social implications, and it should be internationally consistent. Public opinion could force a policy change in the patenting of genetic material, even if it is judged to be legally valid. Public opinion was examined in the International Bioethics Survey in a question shown in [Table 8](#). The question revealed negative attitudes to patents on life, especially of human genes, consistent with the 1990 results in New Zealand [22] and 1991 results in Japan [23].

III. BIOETHICAL PRINCIPLES FOR AGRICULTURAL BIOTECHNOLOGY

A. Autonomy of Choice Versus Justice

Respect for the autonomy of individuals is a fundamental principle of ethics, and it is found in early times in those religions that recognized freedom of belief. If we respect autonomy of human beings, we should respect their right to have at least some property, or territory, and control over their own body. In agriculture this means respect for freedom of growing what crops a farmer chooses, and eating what food we like, within social constraints (e.g., human flesh is a general taboo in most cultures).

People's well-being should be promoted, and their values and choice respected, but equally, which places limits on the pursuit of individual autonomy. We should give every member in society equal and fair opportunities, this is justice. Utilitarianism (the greatest good for the greatest number) is useful for general proportions, but it is very difficult to assign values to different persons' interests and preferences. Society should also include the future of society; future generations are also an essential part of society. Therefore, we should protect the environment for the future generations [2].

B. Balancing Benefits and Risks: Love

One of the underlying philosophical ideas of society is to pursue progress. The most cited justification for this is the pursuit of improved medicines or increased stable food supply, which is doing good. A failure to attempt to do good, is a form of doing harm, the sin of omission. This is the principle of beneficence. This is a powerful impetus for further research into ways of improving health and agriculture, and living standards.

Biotechnology is challenging because, similar to most technology, both benefits and risks will always be associated. A fundamental way of reasoning that persons have is to balance doing good against doing harm. We could group these ideals under the idea of love. We need to share benefits of new technology and risks of developing new technology to all people.

People in developing countries should not be the recipients of risks passed onto them by industrialized countries, despite the economic pressure to allow this. We can think of the dumping of hazardous wastes in developing countries in return for financial reward, but the environmental and human health consequences of dumping toxic waste cannot be measured. Industrialized societies have developed safeguards to protect citizens, and some of these involve considerable economic cost. Although it may not be possible for developing countries' governments to impose the same requirements, they should not attempt lower standards—rather they should use data obtained in countries with strict and sufficient safeguards of health, with the aid of intergovernmental agencies. Any basic human right should be the same in all countries, and this is one of the roles of the United Nations. Ethically this would support the implementation of minimum international standards for regulation of biotechnology [35].

The precise outcome of interventions in nature or medicine is not always certain. It has taken major ecological disasters to convince people in industry or agriculture of the risks. Introducing new organisms to the environment is also associated with risk. If we introduce very different gene combinations into the environment they could have major consequences, which may be irreversible [2]. The new genes may enter other organisms, or the new organisms themselves may replace existing organisms in the ecosystem. The ecological system is very complex, minor alterations in one organism can sometimes have effects throughout an ecosystem. Field trials and experimentation are an ethical prerequisite before full-scale use of new organisms, as is the scheme used by the USDA in the United States, and quarantine regulations used throughout much of the world.

C. There Is Ethical Value in Life

Many want to protect nature, not because of its value or property, but simply because it is there. The concepts and images that the words "life" and "nature" imply are similar in different countries [1]. Many people value nature, and this has suggested some to suggest that it may even be part of our sociobiological traits, a "biophilia" [36]. The scientific understanding of biological knowledge allows us to understand the reason a flower blooms, but we may still value its beauty. This aesthetic value is also a part of bioethics, yet is difficult to quantify.

Related to this is the concept of biodiversity, which is now legally recognized as a "good" in the Biological Diversity Convention, primarily because of economic potential, but it is also protected because of aesthetic value. *Biodiversity* is a word used to picture the great diversity of living organisms on the planet. Just as the individual processes of life are dynamic, so is the composite of the lifeforms. The idea of dynamism also implies a balance. The dynamic nature is implied in both science—the second law of thermodynamics—and religion, in the Biblical doctrine of creation and preservation; and Asian religions with "harmony" [1,2]. There are various religious stories to support preservation of biological diversity, the most famous of which is the story of Noah, which is shared by the Judeo-Christian—Islamic traditions. Noah preserved all the domestic and wild animals from environmental catastrophe, a catastrophe that it says was caused by the actions of humans.

We need to ask whether there is ethical value in having different species? A related idea is that of "species integrity" (i.e., species should not be mixed), which was examined by several

questions in the International Bioethics Survey (see [Table 6](#)). Modern biologists generally think of species as reproductive communities or populations. There is no universal or absolute rule that all species are discretely bounded in any generally consistent manner. One species may exchange little or no genetic material with related or adjacent species, whereas others may do so all the time. Species exist in nature as reproductive communities, not as separate creatures.

The cross between a horse and a donkey, the mule, is certainly accepted in many cultures. The greatest public concern is over the mixing of human and animal genes. Some people object to the insertion of human growth hormone or hemoglobin genes in pigs. These animals may be used to make medically useful proteins, and could be considered just an extension of the modern dairy industry that tries to increase milk production in cows. To challenge the integrity of a species requires more than a single-gene change. There is also research to produce transgenic animals that can be organ donors for humans. This is technically difficult, but perhaps possible—but at first, people may consider this concept “playing God.” However, eating animals, or having inbred dog varieties is considered acceptable, which suggests that it will be within the bounds of common morality to use animals for organ donors. One could argue that medical need is a greater reason than the desire to eat meat, so that this will be accepted, and there is more ethical justification for this type of “bioreactor” agriculture than for meat production.

However, genes may be altered if it presents a better alternative to the other options available for providing food for other members of the human race, we must consider alternatives. There is no inherent “sanctity of the genes,” however, we may value to maintenance of existing species and natural nature beyond our agricultural use, which is consistent with worldwide public opinion [1].

D. Footholds on Slippery Slopes

In most interventions in life, there are slippery slopes. The idea is that because we perform some action, we will perform another. Controls that were adequate for initial exploration may fail under increased pressure. Although we may not do any direct harm with the application in question, it could result in progressive lowering of standards toward the ill-defined line beyond which it would be doing harm. The inability to draw a line is no measure of the nonimportance of an issue; rather, some of the biggest fundamental questions in bioethics and life are of this nature.

Some persons, from all countries, say that some developments of science and technology, such as genetic engineering, are interfering with nature because “nature knows best.” However, we have some good reasons to interfere with parts of nature; for example, we try to cure many diseases that afflict humans or other living organisms and we must eat. A negative science fiction image has been easily promoted and is appealing to the human imagination. The fascination with creating “new forms of life” is coupled with a fear of how far it might be taken. There are many movies that play on scary themes, from *Frankenstein* to the 1993 blockbuster movie *Jurassic Park* brought genetic engineering into the imagination of many. These are very powerful in shaping public acceptance and perceptions.

E. Animal Regulations

Animals have long been used for agriculture, and are likely to continue to be used. Genetic engineering and biotechnology continue the trend to look for increased efficiency in terms of the products. They will be further used in the production of useful substances, such as proteins

for medical use. Some religious rules also consider the appropriateness of different uses of animals [2].

The moral status of animals, and decisions about whether it is ethical for humans to use them, depend on several key attributes; the ability to think, the ability to be aware of family members, the ability to feel pain (at different levels), and the state of being alive. Causing pain is considered bad, and it is the major guiding principle for animal treatment [1,37]. If we do use animals we should avoid pain.

If we believe that we evolved from animals, we should think that some of the attributes that we believe humans have, which confer moral value on humans, may also be present in some animals [38]. Although we cannot draw black and white lines, we could say that because some primates or whales and dolphins appear to possess brain features, family behavior, and grief over the loss of family members similar to those of humans, they possess higher moral status than animals that do not exhibit these. Therefore, if we can achieve the same end by using animals that are more “primitive” than these, such as other mammals, or animals more primitive than mammals, then we should use the animals, at the lowest evolutionary level suitable for such experiment, or for food production (which is by far the greatest use of animals), or use plants or cells.

Animals are part of the biological community in which we live, and we have to consider the ethical implications of whether they possess autonomy. If we consider biological relations it is natural to ask the question whether animals share behavior similar to that of humans. When we look at animals, we see that some animals exhibit nonselfish behavior, called altruism. Some even give when there is no hope to receive any genetic benefit, helping unrelated individuals. Therefore, we must ask the question: Is altruism the basis for love, and does it make an ethical difference [1]? In practice, we need to build a society that minimizes the departure from the ideal of doing no harm, and respects the choices that people make.

People will continue to eat animals, and practical ethics must improve the ethical treatment for all animals. One area of concern is whether animals should be in a field or in a caged box, or factory farm. There have been several countries that have banned the use of battery-caged hens. It has been illegal to use battery cages in Switzerland since 1992. The possible boredom of animals on factory farms [39] may be another ethical argument against their use. People need to decide how much more they are prepared to pay for better treatment of animals, such as the costs of eliminating battery farming, or the costs in not using new animal treatments that produce cheaper milk or meat, such as bovine growth hormone. The consequences of these decisions on the difference communities involved in agriculture also needs to be considered, along with a variety of external factors.

F. Sustainability and Balancing Ideals

Rather than calling these factors principles, we could call them ideals [1]. These ideals all need to be balanced, and the balance varies more within any culture than between any two. Surveys combined with observations of policy and behavior in different countries allow us to look at how these principles and ideals are balanced. An examination of history also shows how the balancing act has varied in different times and places. In another paper I have discussed what is ethical biotechnology [40].

In the midst of growing awareness of environmental change and damage we should be aware of the need for sustainable living. Sustainable living involves not just efficient agriculture, but also minimizing our energy use and pollution. It involves changing public policy and the very way people think. In the long-term, the most important approach is a lasting change of

present human attitudes to those that are compatible with sustainable life. We need lifestyle change most, but biotechnology may aid the process of sustainability. Part of this change may be changing preferences for agricultural products.

We must ensure that efficient and sustainable agriculture is encouraged, but recognize that it is only part of a broader solution. *Sustainable agriculture* could be defined as the appropriate use of crop and livestock systems and agricultural inputs supporting those activities that maintain economic and social viability, while preserving the high productivity and quality of the land. We need to improve agricultural efficiency to succeed; however, current research interests in biotechnology are not necessarily the best way to provide sustainable agriculture. Large corporations are developing new techniques that may require constant application. For example, biological weed control is more cost-effective and has a higher-success rate than that achieved in searching for useful agrochemicals, yet development is limited because it may not make commercial profits.

Some of the criticism is against technology in general, and needs balanced consideration. For example, there are valid criticisms about the development of herbicide-tolerant plants, that biological control is better, but they do have immediate environmental advantages in some cases. For example, maize growers make four to six herbicide applications a season, but if the crop was tolerant to a broad-spectrum post-emergence herbicide only one application would be needed. Reducing herbicide use and switching to biodegradable products is consistent with sustainable agriculture and is an important practical step in that direction, as long as commercial interests do not prevent the eventual widespread use of the ideal, biological control [2].

IV. THE FEATURE OF BIOETHICAL BIOTECHNOLOGY

A. Scientific Responsibility

The ethical role of scientists is defined by several levels of moral community: the scientific community itself, the local community, the national society, and the global society. Scientists are involved in various different relations, but first they are participants in society, having the same responsibilities as any citizen. Scientists are also part of a profession. If the scientific profession or community does not censor themselves others will do so.

A basic human responsibility is honesty, which unfortunately is broken by some. The United States established a special Office of Research Integrity to investigate cases of alleged scientific misconduct. They define that “research misconduct is plagiarism; fabrication or deliberate falsification of data, research procedures, or data analysis; or other deliberate misrepresentation in proposing, conducting, reporting, or reviewing research” [1], Just less than half the cases brought to the office have resulted in guilty charges being upheld against scientists.

There are social responsibilities in the choice and use of research. Some basic areas of responsibility include those related to the acquisition of scientific knowledge, awareness of the potential for misapplication of their findings, to make society a partner in the management of knowledge, to bear in mind the interests of present and future generations, and the need to actively value human beings and other life.

Other persons are involved in the application of science in the world. Companies have been responsible for about 80% of the releases of GMOs in the world [35]. As commercial seeds and animals are passed on to farmers, the farmers will assume increasing responsibility for sensible farming practice, which is usually also in their long-term interests (e.g., monitoring of pest resistance to insecticidal proteins).

B. Teaching the Ethical, Environmental, and Social Issues Science Raises

Unless the broader dimensions of applied science are taught, society will be unable to make balanced decisions about the use of technology. In all countries in the International Bioethics Survey, there is strong support for teaching students about the ethical and social issues associated with science and technology [1], and such issues are already introduced into the curriculum to varying degrees in Australia, New Zealand, and Japan, as measured in the International Bioethics Education Survey. The general attitudes toward the teaching of bioethics were extremely positive. It is interesting that more biology teachers thought bioethics should be taught in biology classes, whereas social teachers thought they should teach it.

There is more inclusion in Australia and New Zealand teachers than in Japan. There should be research into how these issues are being best taught, the most suitable issues, the suitable classes, and the most effective delivery. They are relevant to both science and social studies classes. The next stage in the education project is the development of materials to aid the teaching of these issues, and the responses obtained were used to make such materials. Teachers are testing some materials and are developing them for use at appropriate times in existing courses.

Public education is a special responsibility of scientists, who have the best knowledge of the technology, even if they may not know so much of the effect. Recently, surveys of scientists in the United States [15] and Europe [16], engaged in recombinant DNA research, found that more saw public attention on genetic-engineering research as beneficial or neutral than harmful to their research. The view in America was more positive than in Europe. Further public education programs to stress the benefits of biotechnology have also been called for by others in North America and Europe [14]. Their goal is to reduce what is seen as a high level of concern about the technology, which seems unobtainable given the views of educated groups that have been surveyed. Persons who have high familiarity with such techniques, such as scientists and high school biology teachers, are also concerned about such technology [23], and the emotions concerning acceptance of technology are varied and complex [1,23]. Rather than attempting to dismiss feelings of concern, society should value and debate these concerns to improve the bioethical maturity of society.

In fact, in the International Bioethics Survey the most positive samples toward enhancement genetic engineering were from Thailand and India, which as a country in general, may not be as highly educated as Japan or Australasia. Even if those samples did include more graduates than the other countries, the trend is also seen in an educated sample from a medical school in China [41] that used questions used in Japan in 1991 [23]. The results of another survey in China on science and technology in 1989 also found extremely positive attitudes toward technology, with only 2% saying that science and technology did more harm than good, with 82% saying more good and 12% saying the same [42]. In the International Bioethics Survey, Thailand was most similar to China, with 3% saying more harm, 54% more good, and 42% the same [1]. The effect of education on attitudes to biotechnology needs to be examined, by comparisons with other influences on opinion, such as experience of pollution, food supply, medical benefit, and so on.

C. Safety and Risk Are Bioethical Concerns

In some discussions of the effect of biotechnology safety and risk are considered separately from bioethical concerns. However, as shown in the foregoing, the origin of concern about safety and effect is the ethical principle of do no harm. People of various cultures, ages,

educational training, occupation, and outlook on life perceive both benefits and risks from developments of science and technology [1,23]. Technology that touches life is perceived to be just as worthwhile as technology that does not directly affect living organisms, but people may perceive more risks from technology that directly affects living organisms than from those physical science developments that do not. This is true of countries with a long history of technological use, such as Europe; a dependency on agriculture, such as New Zealand or Australia; an industrial economy, such as Japan; a mixed economy as the United States; or a developing economy, such as China, India, or Thailand.

D. Conclusions

People do show the ability to balance benefits and risks of science and technology. People in the countries surveyed do not have a simplistic view of science and technology, and can often perceive both benefits and risks. These data also generally find that most of the total diversity in all samples is in any one country or group. In every society, there are persons who want to use new genetic techniques and those who do not. The issue goes deeper than religion or culture, and suggests that these issues will always be divided. Individuals in different countries share attitudes similar to these questions, but still the social systems in Asia and Oceania are different. Despite the similarity in the views of individuals, the social system in Japan and some other countries is constructed differently, and may not represent the views of the public [43].

The results are also being used in an attempt to develop a method for assessing the general “bioethical maturity” of different societies, which includes the ability to balance benefit and risk; and discretion between enhancement and therapy; and the balance between autonomy and freedom on restriction [1]. The social consequences of biotechnology depend on the society that we make—but they are international. We can conclude that bioethical concerns are already one factor affecting the implementation of biotechnology in agriculture, because they are part of the “equation” that people use when deciding whether to accept the products. It is in the interests of the whole of society that persons make good decisions considering the total current and future effect of their choices, and scientists have a role in education to enable this.

NOTE

Most of the publications by Macer referred to in this paper are available on the world wide web <<http://www.biol.tsukuba.ac.jp/~macer/index.html>>.

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Biotechnology and Food Production: Relevance to Developing Countries

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I. AGRICULTURAL AND FOOD PRODUCTION IN DEVELOPING COUNTRIES: AN OVERVIEW

A. Asia

By the year 2000, the per capita land availability of India's projected 1 billion inhabitants will have shrunk to 0.14 hectares (ha). For food grains alone, needs are estimated at 240 million tonnes for the year 2000.

With the exception of Brunei and Singapore, agriculture plays a prominent role throughout Southeast Asia. In Indonesia, it involved more than 60% of the population, and contributed 24% to the gross domestic product (GDP) and more than 60% to the value of nonoil exports by the early 1990s. Paddy production accounted for more than 40% of agricultural output, land use, and employment in 1990, with production increasing from 12 million tonnes in 1969 to 29 million tonnes in 1989, self-sufficiency in rice production having been reached in 1985 [Dart et al., 1991]. If between 1981 and 1991, 33 million were added to the Indonesian population, rice production doubled to 44 million tonnes [Margolin, 1993a].

In Malaysia, in 1990, agriculture and forestry represented 18.7% of the GDP; by the year 2000, they will not represent more than 13.4%, compared with 37% for industry [Pomonti, 1991a]. Malaysia is also a leading exporter of cocoa.

By the early 1990s, in the Philippines, the poorest among the ASEAN (Association of Southeast Asian Nations) member states, coconut and sugarcane accounted for about 30% of the cultivated acreage and for about 50% of agricultural exports, on which about 15 million small holders depended [Pistorius and Smits, 1990].

In Thailand, agriculture still employed more than half of the active population and provides 32% of exports [Margolin, 1993b]. In 1990–1991, the production of major agricultural commodities was 18.4 million tonnes of rice, 25 million tonnes of cassava, 5 million tonnes of sugar, and 3.7 million tonnes of maize. Worldwide, Thailand has been the leading rice

exporter (49% of cultivated land) and the foremost exporter and second most important producer of cassava after Brazil [Bhumiratana, 1990].

In Vietnam, agriculture represented half of the GDP, one-third of exports, and occupied 75% of the active population in the early 1990s [Margolin, 1993b]. About 10.4 million farming households were restricted to only 0.1 ha of arable land per person. Sixty percent of land was privately owned in the South, whereas in the North, 95% of farms have been turned into collective farms [Pomonti, 1991b]. A prominent player on the international rice market since 1989, Vietnam exported 1.8–2 million tonnes of rice in 1993 [Pomonti, 1993].

Southeast Asia also supplies the near totality of natural rubber, palm oil, copra, pepper, and a large proportion of cocoa, sugar, cassava, and coffee [Margolin, 1993b].

Agricultural productivity varies from one country to another: Cambodia has not yet recovered to 1960s levels, whereas in Laos, Myanmar, and the Philippines, per capita agricultural production has receded by at least 10% in a decade; in Vietnam, rice production doubled between 1990 and 1992, after it became the world's third-largest rice exporter in 1989. Malaysia imported 30% of its rice needs, the government supporting rice cultivation exclusively in the few irrigated areas; its powerful plantation sector (5 million ha, compared with 650,000 ha of paddy fields), as well as its industrial development, has enabled Malaysia to reduce its food bill from 10 to 5% of imports. Thailand has almost doubled its agricultural area in 30 years, but yields are still low: 2.1 tonnes of rice per hectare, compared with 3.1 tonnes per hectare in Vietnam, 4.4 tonnes per hectare in Indonesia, and 5.7 tonnes per hectare in China. On the island of Java or in Vietnam, there is a lack of cultivable land, whereas Malaysia is experiencing a lack of manpower or an increase in its cost [Margolin, 1993b].

In addition to necessary diversification or reconversion, an increase in efficiency and productivity (e.g., through lower production costs and improved quality), with the help of biotechnologies, could be an appropriate answer to the difficulties agriculture is facing in Southeast Asia.

B. Latin America

Although agrarian reforms has eliminated the unequal dependence status and the contributions in kind made by farmers to their landlords, application of these reforms has been uneven. In Central America, land redistribution is still largely incomplete. In Brazil, only one-tenth of the program has been achieved and small holdings represent only 9% of total agricultural acreage. In Colombia, farms of more than 200 ha represent 40% of total cultivated land, compared with 46% before redistribution [Herzlich, 1992]. In Ecuador, land concentration has decreased significantly, although large farms exist in the Amazonian part of the country, over 4 million ha, in particular for the cultivation of oil palm and soybeans; agriculture represents 15% of the GDP and 40% of the total population live in rural areas [Niedergang, 1992].

To meet the needs of growing populations, many governments have blocked agricultural prices and have maintained export taxes which, paradoxically, has encouraged high-profit crops and livestock husbandry. As a result, landless farmers and rural dwellers have migrated to the slums of large cities [Herzlich, 1992]. Furthermore, instead of transforming all agriculture, successive governments have focused on a few crops and livestock husbandry geared toward exports. This was the case in Mexico, which nevertheless acknowledged exceptional agricultural growth in the 1970s [Baudin, 1992].

In Chile, in 1978, 60% of latifundia expropriated previously were more or less reconstituted, but they represented a mere 40% of cultivated lands. The nonrestituted land was bought by the wealthiest farmers, as well as by corporations, which introduced an intensive exploitation model, based on modern techniques and cheap labor. At the end of

the 1980s, the latifundium had completely lost its dominant position, whereas modern farms, called "Californian," often of a medium-size, were thriving and using intensive-cropping systems. Chilean agriculture had become the driving force behind national economic growth [Baudin, 1992].

In Brazil, during the 1980s, the country boasted a cultivated acreage of about 40 million ha; it produced 70 million tonnes of grains and more than 5 million tonnes of meat. Development of a capital and technology-intensive agriculture was not confined to the south and southeast; it had displaced traditional crops, such as coffee, soybeans, and wheat, to the cerrados, thus incorporating central Brazil into the new agricultural frontier. The cerrados region covers 180 million ha (21% of national territory), of which 50 million ha are potentially suitable for crop production.

Livestock husbandry had moved toward intensive management practices, in particular for swine and poultry. Once an importer of chicken meat, Brazil became the world's second largest exporter in the mid-1980s. The extension of citrus orchards has made Brazil the leading world exporter of concentrated orange juice, with an annual turnover of 1.5 billion dollars. Furthermore, Brazil has become the world's second-largest producer of soybeans and the leading exporter of soybean oil and meal.

Thus, in about 30 years, Brazil has built up its agroindustrial complex and agribusiness, which corresponds to more than one-third of GDP, to 40% of total employment and 60% of the trade balance. However, grain needs are estimated to be about 115 million tonnes in the year 2000. Consequently, in its proposal for a Programme for Agroindustrial Competitiveness (PCA), the Brazilian Government has stressed that the Brazilian Agricultural Research Enterprise (EMBRAPA) will emphasize the scientific and technological capability in such areas as biotechnologies and genetic engineering, informatics applied to agriculture and livestock husbandry, agroecology, and genetic resources.

C. Arab States

Near Eastern countries have become large importers of foodstuffs owing to their high population growth rate, the stagnation of agricultural production, and the decline in food output. The value of agricultural imports for the Near Eastern countries reached 17 billion dollars in 1990, whereas the agricultural exports amounted to about 2.5 billion dollars, with a resulting deficit of 14.5 billion dollars [Salamé, 1993].

Land reclamation has been instrumental in the development of nontraditional agriculture in Egypt. Over the 1980–1990 decade, some 26,400 ha of desert land were reclaimed, and in 1992, reclamation was adding 17,600 ha of cultivable land annually [Mackie, 1992].

The 1977–1987 decade witnessed a major agricultural expansion in Saudi Arabia. In 1992, Saudi Arabia was entirely self-sufficient relative to bakery products and was the sixth world wheat exporter: 2 million tonnes out of the 4 million tonnes produced. A major problem was that of water resources.

Agricultural land covers about 7.5 million ha in Algeria, 8.8 million in Morocco and 4.8 million in Tunisia [Daaloul et al., 1990]. In Morocco, more than 57% of the total population lives in rural areas; 40% of the total active population belong to the agricultural sector and, in rural areas, 68% of the population derive their income from agriculture.

Agricultural production needs to be supplemented by imports of cereals, sugar, vegetable oils, milk, and meat, although Morocco had achieved self-sufficiency to a large extent (10 million tonnes of cereals in 1994, a record after 2 years of drought and mediocre harvests; e.g., 3 million tonnes in 1993).

Several programs concerning crop breeding, agricultural techniques, and plant protection have led to important results; however, there has been a need for further research to enhance productivity and to meet national needs.

D. Sub-Saharan Africa

The United Nations Economic Commission for Africa has been prompted to state that the 1980s was a lost decade for the development of sub-Saharan Africa because production of agricultural foodstuffs per capita dropped by 20% down to 200 kg/person in 1990; a cash crops' recession (except tea) and marked decrease in export earnings; subsidized food in the cities, but decreasing remuneration of farmers; failure of many agricultural development projects; and ineffectiveness of agricultural-assistance projects. The 1990s could be another lost decade for the development of Africa, judging from the pessimism of forecasts for agricultural and food production, industrialization, and participation in international trade.

If food security were to be ensured everywhere, cereal needs were estimated at 410 million tonnes of maize, or its equivalent, for the year 2020, whereas production would reach only 165 million tonnes; the resulting deficit of 245 million tonnes would have to be met by imports [Fottorino, 1992].

Among the major causes of this situation was the lack of sufficient attention given to the farmers' situation; they had to feed cities in exchange for low prices for their commodities, while political leaders focused on costly industrial projects; in the absence of incentives and adequate agricultural policies, compounded by the lack of appropriate techniques and inputs, the African farming communities tended to revert to self-subsistence agriculture [Fottorino, 1992]. Sub-Saharan Africa also remained, to a large extent, on the outer rim of the "green revolution" spreading throughout Asia and benefiting from hundreds of years of knowledge of irrigation techniques and the presence of major rivers. The green revolution played a role only in Kenya and Zimbabwe, where it contributed to extending and improving maize crops [Gherardi, 1993].

However, in 1990, agriculture still represented 32% of the GDP in sub-Saharan Africa [Chalmin, 1992].

II. AGRICULTURAL BIOTECHNOLOGIES: ACHIEVEMENTS, COOPERATION, AND PROSPECTS

A. Asia

1. China

Another culture was being practiced in about 1000 institutions throughout China. A high number of microspore-derived plant species were obtained by Chinese scientists, a world record. These species included wheat (*Triticum aestivum*), maize, hot pepper (*Capsicum annuum*), and sugar-beet (*Beta vulgaris*). The acreage of haploid paddy rice and wheat was extended to millions of hectares, with outstanding economic profit [Zhaoxiang and Yongchun, 1990]. The use of embryo rescue technique and in vitro culture of a hybrid between *Triticum aestivum* and *Agropyron elongatum* led to a new wheat variety "Xiaoyan no. 6," which, in 1990, was grown on 38 million ha, with a yield of 16 million tonnes.

It became possible to propagate more than 100 crop species, by in vitro tissue culture. In vitro micropropagation is also used for the clonal multiplication of banana: in the Guangdong province, 3–4 million banana plantlets are produced annually by tissue culture, and 1 million plantlets have been exported. Meristem culture is also being carried out to produce virus-free

seedlings of potato, strawberry, garlic, and asparagus (*Asparagus cochinchinensis*). Seedlings of virus-free potato have been commercialized and cultivated on about 300,000 ha (i.e., 10% of the total acreage) in the early 1990s. A propagation and extension system for virus-free potato production has been established in several provinces, and yields have been increased by up to 100 or 200% [Guang-Nan Wu, in a paper submitted to the workshop on "Assessment of biotechnology for food production in Vietnam," Hanoi, 9–12 December 1991].

In the Guangxi province, the sugarcane cultivar "Gueitang 11," with a high content of sucrose, has been mass-propagated and grown on more than 30,000 ha in 4 years, instead of the 10 years generally needed when conventional propagation is used.

At the Shanghai Institute of Biochemistry, plant genetic engineering uses the introduction of alien DNA by the pollen tube after pollination, with a view to developing resistance to diseases in rice, wheat, soybeans, and cotton. This simple technique of genetic transformation is an indication of the unsophisticated approach chosen in some Chinese laboratories to obtain good results in crop breeding. At the National Laboratory of Protein Engineering and Plant Genetic Engineering of the University of Beijing (Beida), research on transgenic tomato and rice are being carried out with a view to transferring genes coding for resistance to fungal blast and rice rust, respectively [Borry, 1992].

At the Laboratory of Plant Cell Engineering of the Beijing Academy of Agricultural Sciences, research projects are being carried out on

Anther culture aimed at selecting new winter wheat and rice varieties

Culture of protoplasts and cell fusion (a winter wheat variety has been regenerated from protoplasts)

Somaclonal variation and embryo cultures in maize and rice

Interspecific crosses to breed disease resistance in winter wheat

Chinese strains of *Bacillus thuringiensis* and *Bacillus sphaericus* were applied successfully to vegetables, maize, rice fields, and pine trees, covering over 18 million ha. For more detailed information, see Sasson [1993].

The need to increase China's present grain yields can be met by using both conventional breeding and plant biotechnologies. The rice genome project, supported by the National Commission of Sciences and Technology, is an important, long-term research project, with major potential applications. The Rockefeller Foundation also supports about 15 laboratories for rice biotechnology research.

2. India

It is anticipated that over the 1993–2000 period, about 8 billion rupees would be invested in health care, 2 billion rupees in agriculture, and some 4 billion rupees in other areas, mainly in most conventional biotechnology-derived products, investments in recombinant products being rather small [Ghosh, 1993].

It is anticipated that the consumption of hybrid seeds would rise from 90,000 tonnes in 1992 to 115,000 tonnes in 1995, and about 165,000 tonnes by 2000. Increasing quantities would be produced locally, to exploit the potential of hybrid vigor in paddy, wheat, rapeseed, and mustard [Ghosh, 1993].

Up to 1993, 22 approvals had been granted giving a capacity of 110 million tissue culture-derived plantlets per year for the whole country [Ghosh, 1993].

During 1988–1989, the Department of Biotechnology (DBT) of the Indian Ministry of Science and Technology launched the Oil Palm Demonstration Projects, with a view to reducing edible oil imports, which accounted for 104 million dollars in 1990–1991, and providing a

regular source of income to the farmer, as this crop species is perennial. Many state governments, private entrepreneurs, and financial institutions have drawn up ambitious expansion programs for oil palm in Indian [Kumar et al., 1993].

A program for tissue culture of coconut was launched in 1986–1987. Large-scale plantations using in vitro plants are anticipated after evaluating field performance. In 1995–2008, massive-scale production units will be set up to supply high numbers of plantlets of high-yielding coconut varieties to replace traditional low-yielding ones [Kumar et al., 1993].

In 1984–1985, the A.V. Thomas group (AVT) initiated commercial production of cardamom through tissue culture. On average, tissue culture-derived plantlets show an increase of 63% in yield, the mean estimated yield being 360 kg/ha. AVT has also applied tissue culture to tea, for it owns 2000 ha of tea plantations; the company has been successful in increasing tea yield to 2520 kg/ha, compared with the average South Indian tea yield of 2,060 kg/ha [Kumar et al., 1993].

Unicorn Biotek is a recent example of a tissue culture-based company moving modern biotechnologies from laboratory to marketplace. In addition to a project on the large-scale production of virus-free banana plantlets, supported by the Department of Biotechnology, Unicorn Biotek, produced and exported 120,000 *Spatiphyllum* (a foliage ornamental) and 2000 rose plantlets over 12 months at competitive prices in the world market. In addition to selling to clients in Belgium, The Netherlands, Denmark, and the United States, the company is also present on the local market: farmers in Andhra Pradesh, Karnataka, and Maharashtra bought 20,000 disease-free strawberry plantlets from Unicorn Biotek; they can buy higher numbers of high-yielding and disease-free plantlets from the company, which has improved productivity and lowered the price of tissue culture-derived plantlets. Although Unicorn Biotek is a good illustration of efforts by India's more than 40 tissue-culture companies (in 1993) to bring commercially viable products to the market place, it faces competition from major Southeast Asian companies, and also from other Indian companies [Knudsen, 1993].

Together with the Biotech Consortium of India, Ltd., the Department of Biotechnology (DBT) has assisted in setting up two pilot plants for the mass production of biocontrol agents. The DBT plans to promote small production units in villages by providing training and credits to entrepreneurs. The major focus is to control one of the world's most damaging pests, the cotton bollworm, recorded in 96 crop species and 61 weeds and wild species in India [Kumar, 1992].

Sandoz India, Ltd. produced a *Bacillus thuringiensis* Berliner-based biopesticide in September 1992. Similarly, Hindustan Lever, Ltd., the Indian subsidiary of Unilever NV, produced a *Bacillus thuringiensis israelensis*-based biopesticide using molasses as a culture medium. It has been successfully tested against insects attacking cabbage, pigeonpea, maize, safflower, and cotton. The insecticide is also active against black flies and mosquitoes and therefore, can be used in malaria vector control.

The biopesticides extracted from neem (*Azadirachta indica*) of the Meliaceae family, are attracting increasing attention from scientists and companies throughout the world. Neem extracts reportedly control more than 200 species of insects, mites, and nematodes, and major pests such as locusts, rice, and maize borers, pulse beetles, and rice weevils [Khanna, 1992].

In 1993, about 1000 tonnes of *Rhizobium* inoculants were produced, but it was forecast that, by the year 2000, the consumption of *Rhizobium* fertilizers may rise to 8,000–10,000 tonnes/yr, covering 50–60% of the 30 million ha of land used for leguminous crops. For the blue-green algae (BGA), used for rice cultivation, more than 400 production ponds have been set up for growing BGA for field trials and nearly 1100 field demonstrations have been organized in different parts of India to popularize the use of BGA fertilizers. The latter increase rice yield by 7–9%, enabling a reduction in chemical fertilizer use of up to 30%.

Large-scale use of BGA, which is considerably cheaper than chemical fertilizers, could have a substantial influence, because about 40 million ha of land have been devoted to paddy cultivation [Ghosh, 1993].

Promising results have been obtained in conventional research and development for increased production of sugarcane, wheat, and rice, but more attention has to be directed to oil seeds, pulses, and coarse grain cereals. Despite shortcomings in packaging, storage, and transportation of plantlets, India has the potential of becoming a major international trading partner of horticultural and floricultural products. For more detailed information, see Sasson [1993,1994].

3. Indonesia

The Agency for Agricultural Research and Development—Badan Litbangtan (AARD)—supports the main program on agricultural biotechnologies in Indonesia, and has set up a National Center for Agricultural Biotechnology at the Bogor Central Research Institute for Food Crops (BORIF), nominated as a national center of excellence in biotechnologies by the Ministry for Research and Technology.

Initial research emphasis at the National Center is placed on seed technology, and, as part of the Rockefeller Foundation's International Rice Biotechnology Program, cooperation has been established with the International Rice Research Institute (IRRI, Los Baños, Philippines), concerning the regeneration of plants from calli of javanica rice. This regeneration is a prerequisite for any selection based on somaclonal variation, protoplast fusion, or plant transformation [Dart et al., 1991].

The Australian Center for International Agricultural Research (ACIAR) scheme for collaboration between laboratories in Australia and Indonesia on projects of mutual benefit became highly productive in terms of both scientific achievements and technology transfer. An ACIAR project involving the New South Wales Department of Agriculture concerns the selection of *Rhizobium* strains for soybean inoculation, the legume species that is grown after rice and in acid soils in newly developed fields in transmigration areas. Another project sponsored by the ACIAR concerns the improved diagnosis and control of groundnut stripe virus. This project aims to protect groundnut from the virus by transforming the legume species with the viral coat protein gene using microprojectiles, followed by the regeneration of transformed groundnut calli [Dart et al., 1991].

The Dutch Government supports the setting up of the Lembang Horticulture Research Institute (LEHRI). Biotechnologies are being used at the LEHRI for the major commercial crop species, garlic, potato, and asparagus; the research agenda for garlic and shallot (*Allium* spp.) includes virus elimination, diagnostic kits for virus identification in plant tissue; cabbage and tomato are also being studied (virus-free seeds, multiplication of elite clones, somaclonal variation). A new research institute in Solok is expected to focus on citrus, papaya, mango, and banana (i.e., on the production of disease-free planting material, varietal improvement, postharvest activities and marketing) [Dart et al., 1991]. For more detailed information, see Sasson [1993].

The first oil palm clones derived from somatic embryogenesis were outplanted in the field in 1987. About 1 million plants were sold in 1993. Although clonal plants are sold at five times the price of seedlings, there is a large market in Indonesia trying to catch up with Malaysia in overall palm oil production [Dart et al., 1991].

Some 3 million ha of coconut are under production, with generally low yields. Hybrids are being produced and their cloning by tissue culture has been hindered by difficulties in regenerating plantlets from the cultures. There are commercial incentives to develop clones of

the Kopyor mutant coconut tree which produces a nut with special flesh, used in ice cream, confectionery products and preserves, and is worth 10–15 times more than the ordinary nut [Dart et al., 1991]. Two coconut projects in the Lampung province of southern Sumatra involve researchers from the French International Cooperation Centre for Agricultural Research for Development-CIRAD's Department of Perennial Crops: one government project for small plantations funded by the World Bank and one private project run with the help of the Multi Agro Corporation on coconut and cocoa.

A facility at the Faculty of Agriculture of the Gadjah Mada University in Yogyakarta produces *Rhizobium* inoculants (with the trade name Legin) for government-sponsored crop improvement projects. The production capacity is approximately 20 tonnes/yr, an amount sufficient to inoculate about 111,000 ha of soybeans. The full capacity of the facility is probably five times this production. Rhizogin is the trade name of another *Rhizobium* inoculant prepared and marketed by the private company, Rhizogin-Indonesia, the production capacity of which is sufficient to inoculate approximately 440,000 ha/yr with the recommended dose of 180 kg inoculant per 65 kg of soybean seeds. It is supplying about 70% of the inoculum needed for soybean cultivation in the country. With additional labor and investment in bioreactor capacity, the factory could double or treble its output and unit costs could be reduced markedly [Saono, 1991].

Integrated pest management and biological control of plant pests are likely to have a high return on investment. Between 1986 and 1989, the government phased out pesticide subsidies and banned 57 broad-spectrum formulations of insecticides. A national integrated pest management program was set up, coordinated by the National Development Planning Board; as a result, the spraying frequency per field dropped from 2.2 to 0.8 times per season, resulting in a 50% decline in insecticide consumption, while rice production increased by 12%. The Lembang Horticulture Research Institute (LEHRI) and the Indonesian Government's extension service teamed up with the Swiss chemical company Ciba-Geigy AG in a pilot project on reducing pesticide consumption in cabbage cultivation; farmers have found that three to six applications of pesticide are adequate, without any loss of yield, compared with the 15 sprays normally applied. The project also relies on the use of natural predators: for cabbage, a parasitoid wasp *Diadegma eucerophaga* was chosen, which lays eggs in the diamond backmoth larvae, preventing them from reaching maturity. Encouraged by the promising results, Ciba-Geigy AG has extended the program to potatoes, then to onion and tomato cultivation [Kumar, 1992]. The Dutch Government is supporting an extension program of integrated pest management to complement that of the FAO on rice.

Indonesia gives a high priority to the development of biotechnologies, with direct support from the Ministry of State for Research and Technology and the Departments of Agriculture and Education. Plant tissue culture and micropropagation techniques are well established in several laboratories, and large-scale commercial production of planting material has been achieved for oil palm [Research Institute for Oil Palm at Marihat]. Similar developments for other plantation crop species are likely in the mid-1990s. The use of the same technique to eradicate viruses is also likely to support commercial production of planting material of potato and several horticultural crop species, and selected strains of mycorrhizae are expected to become available for commercial inoculant production [Dart et al., 1991].

4. Malaysia

One of the major areas of research and development is the clonal propagation of high-yielding, oil palm varieties, carried out by the Palm Oil Research Institute of Malaysia (PORIM) and the Federal Land Development Authority. The market is sizeable, as more than 1.6 million ha

of plantations existed in the early 1990s. The problem of abnormal flowering has been solved and cloned superior oil palms has been marketed since 1992. All Malaysian laboratories working in this area, including the PORIM, are producing such clones. They were also increasingly involved in the mass propagation of other crop species, with a view to diversifying production and meeting local needs.

The Malaysian Agricultural Research and Development Institute (MARDI) have established a Biotechnology Center devoted to the in vitro culture of the following crop species: coconut (cloning of selected plants, in vitro germination of embryos, callogenesis, and somatic embryogenesis); cocoa (organ culture); papaya (cloning of selected varieties); anther culture of rice hybrids and somaclonal variation of rice; pepper (production of pathogen-free plants to control *Phytophthora* disease); strawberry (meristem culture); and pineapple (culture of axillary buds to propagate the crosses of Sarawak and Singapore Spanish varieties, suitable for the canning industry) [Bordier, 1990]. A cocoa clone derived from in vitro micropropagation was released at the MARDI Cocoa Research Station near Teluk Intan, Perak [Zakri, 1991]. For more detailed information see Sasson [1993].

The Southeast Asian lowland swamps are home to the sago palm (*Metroxylon sagu*). The three leading world producers—Malaysia, Indonesia, and Papua New Guinea—are interested in cooperation aimed at developing sago plantations and using sago starch extensively. Japan is also supporting this cooperation through the creation of a Sago Research Fund.

In the early 1990s, Sarawak had a total area of 19,720 ha of sago palm, including both semiwild and cultivated stands. Sago flour production increased by about 60% between 1984 and 1990 to 84,991 tonnes. Since 1984, there has been an upturn in the export of sago starch: from 3,406 to 27,502 tonnes, earning the State of Sarawak 11.4 million dollars in revenue [Zulpilip et al., 1991].

A potential area for sago plantation exists in Malaysia, which has 2.4 million ha of peat land, of which 1.66 million ha are in Sarawak. Because of its natural adaptation to peat soils of low nutritional value and high acidity, sago seems to be the only crop species that could grow on these soils without reclamation. Furthermore, sago being little prone to natural disasters, such as drought, pest and disease infestations, and flooding, is considered by farmers a minimal-risk crop species. A langorous cycle makes its cultivation ideally suited to a part-time activity, leaving the farmer time between planting and harvesting to seek other income-earning opportunities [Zulpilip et al., 1991].

With the increased demand from industry for its products and a 9- to 15-year growth cycle, quality varieties or clones of sago palm will need to be planted on a large scale. Although sago palms can be propagated from suckers, the number is limited; therefore, clonal propagation through in vitro techniques is the most suitable means both for producing the vast amount of planting material required for extensive plantations and for improving the quality and vigor of palms [Alang et al., 1993].

Since 1983, research has been conducted at the Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Pertanian Malaysia, Serdang, Selangor, toward clonal propagation of sago palm. Several hundred clonal plantlets were produced over a period of 18–24 months from culture initiation; the protocol for inducing embryogenesis from explants and developing embryoids into plantlets was being improved [Alang et al., 1993].

To sum up, with more than 10 multimillion dollar modern sago factories in the early 1990s, a 200-ha Sago Research Station, and 16,000 ha at the planning stage, Sarawak became the world leader in sago development, research, and the exploitation of the palm. There is a need for market promotion, a standardized grading system, and quality regulation of sago flour, because the prospects for Sarawak's sago industry in the international market depends

not only on price competition, but also on consistent quality and a reliable supply of sago flour [Zulpilip et al., 1991].

5. Philippines

The most active tissue culture work in the Philippines is being carried out at the Institute of Plant Breeding in micropropagation, germplasm conservation, disease elimination, and crop improvement. The laboratory works on vegetables (tomato and white potato), fruit species (rambuttan, pineapple, papaya, citrus, banana, and durian), plantation crops (sugarcane, rattan, bamboo, ramie), cereals (corn, wheat), legumes, and ornamentals (*Anthurium*, orchids, *Mussaenda*). The tissue culture program of the Institute of Plant Breeding was expanded in 1989–1990 under a new Cellular and Molecular Plant Biology Programme [Zamora and Barba, 1990].

Yams are micropropagated by culturing shoot tips and nodal segments. Micropropagated bananas (cultivars Lakatan, Saba, and Bungulan) are evaluated for plant and yield traits, and are similar to sucker-derived bananas. However, growth is faster among micropropagated bananas than sucker-derived bananas. Flowering also occurs earlier among micropropagated plants [Zamora et al., 1989]. Elimination of mosaic and banana bunchy top viruses by isolation of meristems from heat-pretreated plants and subsequent in vitro culture became possible [Zamora and Ramos, 1989].

From the shoot tip-grafting technique developed by Spanish researchers [Navarro et al., 1975], the meristem-budding technique has been adapted to suit locally available citrus and to produce plants free of tristeza and leaf-mottling diseases [Zamora et al., 1988]. The technique has been extended to the Bureau of Plant Industry for the production of disease-free citrus plants.

At the National Institute of Biotechnology and Applied Microbiology, research activities include producing monoclonal antibodies to detect citrus tristeza virus, banana mosaic virus, banana bract mosaic virus, banana bunchy top virus, and papaya ringspot virus [Zamora and Barba, 1990].

There are several research and commercial tissue culture laboratories in the Philippines. These research laboratories have developed tissue culture techniques for disease elimination and micropropagation in ornamentals and in food crop species, ranging from vegetables to plantation crops. Most commercial laboratories are carrying out orchid tissue culture, very few of them have gone into plantation and food crop species. The disparity in cost of micropropagated plants versus conventional propagules and the resources available to small farmers limit the use of micropropagated plants [Zamora and Barba, 1990]. For more detailed information, see Sasson [1993].

Scientists at the International Rice Research Institute (IRRI) estimated that the tiny nitrogen-fixing fern *Azolla* was used on less than 2% of the world's total rice cultivating area of 150 million ha, whereas its beneficial effect has been demonstrated. *Azolla* can double its biomass every 2–5 days and supply nitrogen to the paddy field. IRRI experiments showed that it could reduce the total weed mass by 72%, thereby diminishing the need for applying expensive herbicides. *Azolla* contains between 22 and 37% protein and can be consumed as animal feed, but also directly by humans, as in omelettes and burgers [Komen, 1990].

6. Thailand

Thailand has led the world in ornamental flowers, such as orchids, and in many processed fruits and vegetables, such as canned pineapple, fruit juice, and concentrates, and canned baby corn. Plant tissue culture was introduced into the Thai orchid industry in 1964, and the

continuity of research and development has kept Thai orchids popular worldwide. Thailand also continues to be the world leading exporter of rice and cassava. It is also very strong in frozen products, particularly those arising from aquaculture (shrimps and prawns), but also new vegetable varieties such as green soybean and okra.

Current research initiatives under the leadership of the National Center for Genetic Engineering and Biotechnology are aimed to foster the development of biotechnologies and accelerate the transfer of these technologies from local as well as overseas sources. Recent achievements included:

1. The setting up of companies to produce biofertilizers from algae and *Rhizobium*
2. The establishment of a new firm for the production of phycocyanin and shrimp larvae feed from *Spirulina*
3. The commercial production of virus-free potato tubers for the supply of fast-food chains and snack producers who were spreading over Asian countries
4. The development of a heat-tolerant shiitake mushroom, now produced by small farmers in the north and northeast of Thailand

The National Corn and Sorghum Research Program was organized in 1966 as a cooperative effort involving Kasetsart University, the Ministry of Agriculture and Cooperatives, and the Rockefeller Foundation. In 1983, the International Center for the Improvement of Maize and Wheat replaced the Rockefeller Foundation in this venture. This program provided Thai farmers with improved maize varieties and crop-culture practices, resulting in a 100-fold increase in maize production within two decades. In addition to conventional plant breeding, Thai researchers, in particular at Kasetsart University, have applied biotechnologies to the improvement of crop species.

This is particularly true in horticulture: tissue culture, used for genetic preservation of outstanding cultivars, rapid propagation, and mutation breeding, has been applied to several fruit crops.

The Department of Agriculture in the Ministry of Agriculture and Cooperatives, together with staff from several departments at Kasetsart University, have contributed to the development and extension of straw mushroom (*Volvariella volvacea*) production technologies throughout Thailand. According to the survey conducted by the Department of Agricultural Extension from 1965 to 1968, the ten largest spawn makers in Bangkok altogether produced an average of 832,250 cans of straw mushroom spawn per year. Straw mushroom cultivation has become an important biotechnology-based industry and a supplementary source of income for many farmers. Shiitake mushrooms (*Lentinus edodes*) also represent a major food item, but they are mostly sold in a dried form. The development of systems for growth in simple indoor facilities can create a worldwide market for fresh mushrooms. For more detailed information, see Sasson [1993].

The King of Thailand, Bhumibhol Adulyadej (Rama IX), has been a promoter of rural development in his country and over 1000 so-called Royal Projects testify to his prominent role in this respect. Some of these contained a biotechnological component. The King's goal was to curtail poppy-growing and bring legitimate livelihood to Thailand's hilly tribes through crop substitution. These crop species would lure farmers away from opium production and also arrest the destruction of forests and watersheds.

Public and private agencies assisted in research and development as well as in the transfer of crop production and processing technologies. Contributions of the National Science and Technology Development Agency included variety selection, massive propagation, and variety improvement of cut flowers by tissue culture, development of virus-free potato tubers and

strawberry runners, heat-tolerant shiitake mushrooms, and high-efficiency wastewater treatment for the food factories.

Necessary infrastructure was not omitted (e.g., village roads, electricity grid, and small irrigation devices and systems). Foreign assistance came from the United Nations agencies, New Zealand, United States, and Taiwan. The project buys the farmers' produce, then grades, packages, and markets it. It has turned a profit for the villagers, also by processing their jams and wines, frozen strawberries, canned vegetables, dried fruits, and flowers for export. Poppy cultivation has declined by 85% as the farmers became vegetable, fruit, coffee, and flower growers.

The Thai Department of Agriculture, in Bangkok, produces legume inoculants distributed by the Thai Department of Agriculture Extension and dealers in the private sector. There had been a considerable growth in the use of these inoculants: from 3.36 tonnes in 1977 to about 200 tonnes in 1992.

7. Vietnam

Rice breeding deserves the highest priority. Anther culture and the derived haploid lines have been used in Vietnam since 1980 in the institutes of the Ministry of Agriculture and Food Industries. Cooperation with China, where millions of hectares have been planted with hybrid rice cultivars derived from haploid breeding, could help Vietnam catch up.

Potato has become a major food crop in Vietnam since the 1970s, after the new, higher-yielding rice varieties of the green revolution had enabled the Vietnamese farmer to grow a winter crop between two rice crops. A rapid propagation procedure was developed through the collaborative efforts between scientists of the National Council for Scientific Research (NCSR) Institute of Biotechnology in Ho Chi Minh City and farmers in the Dalat region. The so-called flash out system (FOS) combines *in vitro* techniques (the starting point was potato plantlets of a selected variety grown on an artificial medium cut into microcuttings that give rise to plantlets) and conventional vegetative propagation (apical and axillary bud cuttings that give rise to new plantlets). The subsequent large-scale commercial production of potato planting material could progressively replace the conventional tuber planting and save thousands of tonnes of "seed" tubers, which could be consumed instead of being stored. Furthermore, in 2 years, at Dalat, potato yield doubled from 9 to 18 tonnes/ha, owing to the quality of the planting material, which means that imports of European potato tubers can be avoided. Many farming families are involved in this profitable agribusiness, while contributing to the rapid dissemination of the most appropriate potato varieties. One farming family of four can produce and sell 500,000–1 million potato plantlets per year [Nguyen Van Uyen, 1991, personal communication]. The objective is to raise annual potato production from 500,000 tonnes in 1991 to 1 million tonnes for both domestic consumption and export [Commandeur and Pistorius, 1992]. The flash out system could be extended to other tuber or root crop species, such as cassava, sweet potato, and vegetables.

A cooperative research program on banana was designed by Vietnamese and French partners. The objective of this 5-year (1992–1996) research program is to provide Vietnamese producers with healthy clonal planting material, belonging to higher-yielding and more resistant banana cultivars, at a reasonable cost.

A private joint venture to set up a plant tissue culture laboratory and greenhouses for plant propagation at Thu Duc, near Ho Chi Minh City, has been established between a Taiwanese company, Pan Viet Co., and the NCSR Institute of Biotechnology. Since beginning operations in May 1991, the laboratory has produced 20,000 banana plantlets per day. The medium-term goal is to plant 20,000 ha on land leased by the company to the Government of Vietnam,

to export between 800,000 and 1 million tonnes of bananas annually. Estimated at about 60 million dollars, the joint venture will lead to exports worth about 200 million dollars/yr. This kind of successful development could be extended throughout the Mekong River Delta, where there is a great potential for large-scale banana plantations for export, as well as for other crop species.

Sugarcane was the first monocotyledonous crop species to be multiplied through the flash out system. In this case, the tillers are cut into three to five portions that are used (instead of apical buds in the case of potato) to propagate the plant. The technique is used to accelerate the release of new varieties in about 2 years. For more detailed information, see Sasson [1993].

At the Institute of Agricultural Sciences, Microbiology Section, Hanoi, Nguyen Kim Vu and his co-workers selected efficient and effective strains of *Rhizobium* for soybean and groundnut inoculation. Field experiments carried out for several years in various regions of Vietnam have shown that inoculation with these strains could increase seed yields from 40 to 290 kg/ha. Since 1988, the same group has been testing a preparation made of *Azospirillum* (called azogin) in eight northern provinces of Vietnam and has demonstrated that it could replace one-third of the inorganic nitrogen fertilizer applied. At the Institute of Biology of the National Center for Scientific Research, Hanoi, blue-green algae has been under study since the early 1980s. Rice plants inoculated with *Anabaena sphaerica* and *Nostoc muscorum* result in a yield increase of 44 and 29%, respectively, compared with plants that received no blue-green algal fertilizer [Commandeur and Pistorius, 1992],

Six different institutions are working on the inoculation of cereal crop species (rice and maize), to stimulate the growth of the plants through enrichment of their rhizosphere with useful bacteria.

8. Asia Network for Small-Scale Agricultural Biotechnology (Appropriate Technology International)

Appropriate Technology International (ATI) is a private, non-profit-making development assistance organization, headquartered in Washington DC, with projects in Asia, Africa, Latin America, and the Caribbean. The ATI, in cooperation with the NifTAL Project (Nitrogen Fixation by Tropical Agricultural Legumes, College of Tropical Agriculture, University of Hawaii), SATE (Small Enterprise Development and Appropriate Technology Europe), and the Department of Biology and Society, Free University, Amsterdam, has developed a biotechnology network as part of a program referred to as the Lab to Land Program. The network focuses on small-scale agricultural biotechnologies, according resource-poor farmer the double role of beneficiary and participant.

The Asia Network for Small-Scale Agricultural Biotechnology (ANSAB), with its headquarters in Kathmandu, Nepal, is the nucleus of the Lab to Land Program, initially linking Bangladesh, China, India, Indonesia, Nepal, the Philippines, Sri Lanka, Thailand, and Vietnam. It was established during the first ANSAB workshop (30 March–1 April 1992), held in Kathmandu and attended by 70 participants from the nine Asian countries.

The ANSAB has a multifaceted membership, including farmers, nongovernmental organizations, researchers and biotechnologists from both the public and private sectors, private sector businesses, financial institutions, and policy makers. The unifying factor of this diverse membership is the concern for resource-poor farmers.

The ANSAB workshop identified the following mature agricultural biotechnologies in the nine participating countries ([Table 1](#)).

The biotechnologies initially selected for the program were in various stages of commercialization in different countries. Barriers to commercialization efforts often existed, such as a lack of research

Table 1 Biotechnologically Mature Agricultural Countries as Identified by ANSAB

Country	Mature agricultural	Biotechnologies	Priority-wise
Bangladesh	Biofertilizer	Mushroom cultivation	Bamboo propagation
China	Mushroom cultivation	Biofertilizer	Plant tissue culture
India	Biofertilizer	Mushroom cultivation	Seedling raising (trees and vegetables)
Indonesia	Biofertilizer (blue-green algae)	Biofertilizers (<i>Rhizobium</i> and mycorrhizae)	Biopesticide
Nepal	Plant tissue culture	Mushroom cultivation	Biofertilizer (<i>Rhizobium</i>)
Philippines	Biofertilizer	Biopesticide (<i>Trichoderma</i>)	Biofertilizers (<i>Rhizobium, Azospirillum, mycorrhizae</i>)
Sri Lanka	Biofertilizer (soybean <i>Rhizobium</i>)	Biofertilizer (<i>Azolla</i>)	Plant tissue culture (coconut, fruit trees)
Thailand	Plant tissue culture (asparagus and strawberry)	Plant tissue culture (for reforestation)	Biofertilizers (<i>Rhizobium, blue-green algae, mycorrhizae</i>)
Vietnam	Biofertilizer	Biopesticide	Plant tissue culture

and development support or agricultural extension, poor technology-transfer mechanisms, lack of entrepreneurial and technical skills, lack of venture capital, high product cost or poor product quality, and subsidies for alternative products (such as chemical fertilizer). By providing a regional context for considering these issues, it was expected that the sharing of experience, technology implementation, and regional transfer would be significantly improved.

Vietnam has been a pioneer in developing low-cost, simple methods for the rapid micropropagation of disease-free potato varieties (see Sec. II.A.7). The Vietnamese example illustrates the practicality of small-scale operations in tissue culture with direct benefit for resource-poor farmers. It also points to the need for close cooperation between research centers, farmers, and sources of capital.

Soils from South and Southeast Asia that frequently tested low for *Rhizobium* and legume seed would benefit from inoculation (although phosphate fertilizer application might also be needed, and low soil pH had to be corrected, for good response). Legume yield increases of 10–90% following inoculation were reported in South Asia. In the Philippines, inoculation trials gave an average yield increase of 124% for soybeans, 29% for mungbeans, and 37% for groundnuts; in spite of this potential, use of inoculant is very low.

The ATI is seeking to develop the commercial small-scale and decentralized production of *Rhizobium* inoculants to increase soybean yields. Launched in 1987, the project was designed to benefit about 10,000 small holders in the northern provinces of Thailand, which produce about 70% of the total soybean harvest. The expected benefits were an increase in income of 17–42%, because of higher yields, together with a reduction in the use of chemical nitrogen fertilizers; a decrease in soybean and nitrogen fertilizer imports; and improved soil fertility through the nitrogen compounds remaining in the soil after harvest [Pistorius and Smits, 1990]. In collaboration with the Thailand Institute for Scientific and Technological Research and the Thailand Rural Reconstruction Movement, the ATI sponsored trials to demonstrate the use and commercial viability of small-scale, decentralized production of a new blue-green alga inoculant. Yield increases in initial field trials during 1988–1989 for both off-season and in-season rice farming ranged from 16 to 39% [ANSAB Newslett., 1992–1993].

The Bangladesh company, Biolink, in collaboration with the Department of Soil Science of the Bangladesh Agricultural University, has been producing *Rhizobium* inoculants since 1991. The company produces about 7 tonnes of different kinds of inoculants annually, the annual capacity being 10 tonnes. Agricultural extension programs might further enlarge the market [Haque, 1993].

In Nepal, ANSAB assisted with field trials of the high-yielding, late blight-resistant potato variety MS 42.3. This variety was received in 1986 from the International Potato Center, Lima, Peru, in the form of a test-tube plantlet. MS 42.3 yields an average 32.4 tonnes/ha (i.e., a 22% higher yield over the average yield of Kufri Jyoti and Cardinal, the two most popular commercial improved varieties). Furthermore, MS 42.3 being resistant to late blight disease, does not require spraying, which has cost Nepalese farmers 90–105 dollars/ha [ANSAB Newslett. 1992–1993].

These trials represent a further step toward improved potato “seed” production and supply in Nepal, where potatoes—the fourth staple crop species after rice, maize, and wheat—are cultivated on an estimated 86,000 ha. However, the average yield of 8.5 tonnes/ha is one of the lowest in the world. The multiplication of the MS 42.3 variety, called sand rooting, bypasses rooting of microshoots in sterile media; instead, microshoots are directly rooted in clean but nonsterile sand, with more than a 90% survival rate. Minituber production from sand-rooted potato plantlets results in significantly lower costs: cultivation costs for minituber production average 20 dollars/ha, compared with normal potato tuber production (1.5 tonnes) cost of 96 dollars/ha. Storage costs of minitubers are also much lower [ANSAB Newslett. 1992–1993].

In June 1992, the ATI financed a study of the mushroom market in Nepal, Bangladesh, and India by S.T. Chang, professor in the Department of Biology at the Chinese University of Hong Kong and president of the International Mushroom Society for the Tropics. Nepal has developed a fairly good technology for the production of white button mushrooms (*Agaricus bisporus*) and oyster or abalone mushrooms (*Pleurotus* spp.). However, the current yield of 200 kg/tonne of substrate for *Agaricus* and 400 kg/tonne for *Pleurotus* can be raised to 250 and 500 kg/tonne, respectively, through the improvement of sterility and cropping techniques. The spawn production system, primarily a governmental activity currently, is expected to be developed privately. The present domestic market in Nepal for button mushrooms is estimated at 260–500 tonnes/yr, and growth potential is good owing to increasing tourism. The estimated production of fresh button mushrooms in 1991 was 80–100 tonnes, so that Nepal imports the balance as canned mushrooms from both China and India. The low cost of skilled labor, the favorable climate, the abundance of substrate, and the presence of direct air service to potential importing countries provides a good basis for a mushroom export industry in Nepal [ANSAB Newslett. 1992–1993].

In Bangladesh, the Department of Agriculture Extension has developed cultivation methods for oyster mushrooms, straw mushrooms (*Volvariella volvacea*), and *Auricularia* sp., as well as the procedures for spawn production of these species on a commercial scale. A 1991 economic study of straw mushroom production concluded that rural landless women were encouraged and attracted by mushroom cultivation as a part-time work activity. A domestic market has been developing and one company has planned to set up a modern facility for export of button mushrooms [ANSAB Newslett. 1992–1993].

In 1991, the total production of all types of mushrooms in India approached 7000 tonnes. Button mushrooms accounted for 80–85% of total production, followed by oyster mushrooms (9%) and straw mushrooms. Production was also moving to the plains owing to the availability of cheap substrate materials and ready access to markets. Average yields ranged from 60 to 80 kg/tonne of substrate in 6–8 weeks cropping periods. In modern medium-to-large cultivation

operations using environmentally controlled conditions, the average yield is 150–160 kg/tonne of compost [ANSAB *Newslett.* 1992–1993].

B. Latin America and the Caribbean

Almost 73% of total biotechnology research has been directed toward in vitro tissue culture, micropropagation, and clonal multiplication of crop species. Projects using genetic engineering account for about 9% of the research endeavors [Bijman et al., 1990]. By 1990, 40% of research and development projects concerned root and tuber crop species, in particular, potato, cassava, and sweet potato projects being carried out in international agricultural research centers, such as the International Potato Center (CIP, Lima, Peru), the International Center of Tropical Agriculture (CIAT, Cali, Colombia), and the Tropical Agricultural Research and Training Center (CATIE, Turrialba, Costa Rica), which play a leading role in the dissemination, development, and transfer of these biotechnologies.

Research on plantation crop species is of lesser importance and largely concerns coffee. The use of tissue culture for micropropagation of banana and plantain is a routine in most countries of the region. Research and development activities concerning ornamentals and fruit species have shown a fast growth rate [Bijman et al., 1990; Buttel, 1986; Sorj et al., 1989].

Genome mapping; gene identification, isolation, and transfer; anther culture, and haploid breeding are less frequent and often at an experimental stage. About 15% of laboratories are classified as highly efficient institutions, developing and applying advanced biotechnologies in priority areas for the genetic improvement of crop species. Conversely, 25% of them lack the funds and trained personnel to carry out frontline research.

1. Mexico

The Research Center for Genetic Engineering and Biotechnology of the National Autonomous University of Mexico was created in April 1982 at Cuernavaca, State of Morelos; by 1986, research activities were in full swing. Basic research covered the following areas:

1. Molecular biology of nucleic acids
2. Biochemistry of proteins and peptides
3. Genetic improvement of microbial strains of basic and industrial interest
4. Fermentation, scaling-up, and bioengineering of processes (pilot-plant studies)
5. Enzyme engineering

In plant biotechnologies, the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN) at Irapuato, has been the only center working on plant genetic engineering and molecular genetics of biological nitrogen fixation. In addition to work on chili pepper, beans, and other food crop species, a major research project on the transformation and regeneration of amaranth is being carried out. In addition to their role in improving the nutritional quality of amaranth, the genes coding for proteins of amaranth can be transferred into other grain crop species [Komen, 1992].

At the Fruticulture Center, Postgraduate College, Chapingo, temperate, subtropical, and tropical fruit crop species are being micropropagated, with a view to producing plantlets on a large scale for plantations. In addition, micropropagation is used for the multiplication of rootstocks that are tolerant to adverse soil conditions (drought, salinity) for apple, or for pathogen-free plant production (blueberry, raspberry, pineapple, banana, citrus, and grapes). Peach plants with high tolerance to cold and unseeded grape plants have been obtained from immature embryos. A procedure was developed to rescue hybrid embryos of *Carica* spp. tolerant to papaya ring spot virus and plantlets have been obtained.

In Mexico, about 2.6 million people depend on coffee cultivation and production. Coffee rust affects 90% of coffee plantations. The Mexican Government has taken measures to facilitate the distribution of disease-free *in vitro* micropropagated plantlets to the needy planters to protect labor in this sector and, at the same time, maintain export earnings.

There are several modern businesses with a total annual production of more than 1.2 million micropropagated plants, and a few of these are planning to direct their activities toward foreign markets. For more detailed information, see Sasson [1993].

2. Costa Rica

In Costa Rica, the world's second-largest banana producer after Ecuador and leading supplier to the European Union, banana has become the top export item. Costa Rica undertook a vast program aimed at increasing the banana area from 32,000 to 45,000 ha, or even more, in 1995, so that annual production should reach 1.8 million instead of 1.5 million tonnes. In its laboratories located at Guapiles, amidst the plantations, the national banana corporation, Corbana, a semipublic company, is carrying out research on organic fertilizers and the large-scale production of nematode-free plantlets. This biotechnological approach has eliminated the need for nematocide use in the soil of Corbana's plantations since 1987–1988. The combination of a fallow period and use of nematode-free plantlets could lead to the eradication of the soil-living and root-invading worms. The control of the fungal disease, black cercosporiosis, is a high research priority and cooperation has been initiated with the French International Co-operation Center for Agricultural Research for Development-CIRAD [Bertrand de la Grange, 1992].

The Research Center for Cellular and Molecular Biology of the University of Costa Rica, in San José, has broad experience in molecular biology and diagnosis of plant viruses. Research also focuses on virus resistance in beans, the expression of viral proteins in bacteria, and molecular biology of *Rhizobium*. The Tropical Agricultural Research and Training Center (CATIE), Turrialba, is considered the most important center in agricultural higher education in Central America and the Caribbean. The Tropical Crops Improvement Programme concentrated on coffee, cocoa, and Musaceae (plantain, sweet and cooking bananas). Research is also carried out on cassava, sweet potato, cocoyam, taro, and other crop species of local importance.

The American Cocoa Research Institute (ACRI), the research arm of the Chocolate Manufacturers' Association, provides financial support to biotechnological research in countries of Central and South America, particularly at CATIE: yield enhancement, plant resistance and integrated pest control, and cocoa processing. Work to develop nucleic acid probes for fungal diseases of cocoa is being carried out by Penn State University scientists, in collaboration with researchers at CATIE, under the Cocoa Molecular Biology Program, set up by the ACRI [Brenner, 1992].

3. Colombia

Research on plant tissue culture forms part of the Agro-Food Plan of the Universidad Nacional, carried out by the Faculties of Sciences and Agronomy. In addition to micropropagation and somatic embryogenesis of banana and plantain varieties, in particular of those varieties resistant to black cercosporiosis, radioactive methods are used to induce mutants. Vegetative propagation was the only means of multiplying the babaco (*Carica pentagona*, Caricaceae), a palatable papaya hybrid. Micropropagation is being studied as the successor to conventional, fastidious means of propagation. The Biotechnology Institute is leading research in somatic embryogenesis of cocoa and plantain [Luz Marina Dueñas, 1990].

At the Colombian Institute for Agricultural Research, three projects are progressing aimed to incorporate biotechnologies into conventional agricultural research programs:

micropropagation and cloning of fruit species (e.g., citrus, mango, maracuja-passion fruit tree, guava, papaya, and pineapple); improvement of food and plantation species (potato, rice, banana, and cocoa); and molecular diagnosis of plant pathogens (in potato, garlic, sweet potato, citrus, guava, and papaya).

In 1932, the National Federation of Coffee-Growers of Columbia was set up. The Federation supported the National Coffee Research Center (CENICAFE), which developed a rustresistant coffee variety after 20 years of investigation. Promising results were also obtained in somatic embryogenesis, regeneration of plantlets, culture of protoplasts, and biochemical studies on cell cultures. Efforts are being made to achieve the genetic transformation of coffee, with a view to incorporating the gene(s) for *Bacillus thuringiensis* toxin(s) to make coffee plants pest-resistant. Somatic hybridization is also meant to overcome the difficulties concerning conventional hybridization between wild and cultivated coffee species, owing to the incompatibility between diploid and tetraploid species.

At the Faculty of Agriculture of Caldas University, Manizales, a program has developed on the propagation and breeding of Musaceae. An interinstitutional plantain research group was created following a meeting at this Faculty on 18 June 1993. The French International Cooperation Center for Agricultural Research for Development-CIRAD's Department of Fruit and Horticultural Crops was requested to provide technical support and to seek funding sources [CIRAD News, October 1993, 6]. For more detailed information, see Sasson (1993).

4. Brazil

By the late 1980s, the National Center for Genetic Resources and Biotechnology (CENARGEN) research teams were able to master the techniques for gene isolation, cloning, transfer, and expression in bacteria and plant cells. An agreement was concluded with the American biotechnology corporation Agracetus to transfer and express the gene for methionine (from the Brazil nut, *Bertholletia excelsa*) in common beans (*Phaseolus vulgaris*). CENARGEN has strengthened its capability in plant tissue culture and regeneration. The center also focused its activities on the biological control of pests by baculoviruses and *Bacillus sphaericus*, and such fungi as *Metarhizium*, *Beauveria*, and *Nomuraea*.

In addition, in plant tissue culture, the EMBRAPA's National Research Center for Temperate Climate Fruit Species and National Research Center for Vegetables has obtained significant results with the production of virus-free plantlets of strawberry, potato, and other species. The National Research Center for Wheat has obtained double-haploid lines of wheat derived from anther cultures and has tested the productivity of these lines in different regions. Resistance to disease has also been transferred to wheat from wild relatives. At the support unit for the National Research Programme on Soil Biology, highly efficient *Rhizobium* strains have been isolated for the inoculation of common beans and soybeans; equally important are the research activities of this unit on atmospheric nitrogen fixation by various strains of *Azospirillum* and *Acetobacter* in the rhizosphere of grasses.

Brazil also has established a strong cocoa research program that, until 1981, was funded by a levy of 10% on exports of cocoa beans and products. This country has the largest national cocoa germplasm collection in the world and plant collection is under way.

The CIRAD collaborated with the EMBRAPA on the production of high-quality oil palm seeds at the Rio Uruba oil palm research station. Launched in 1992 with a special grant from the French Ministry of Foreign Affairs, this activity aimed to produce between 500,000 and 1 million seeds in 1993. This project would augment oil palm cultivation not only in Brazil, which imports 100,000 tonnes of palm oil annually (1992), but also in neighboring countries [CIRAD News, Oct. 6 (1993)]. In addition, the CIRAD has achieved a first by creating

tropical hybrid rice, in cooperation with a private company in Brazil and French Guyana. This work is based on the utilization of cytoplasmic male sterility in indica rice. At the end of 1992, 200 new hybrid lines were available for evaluation in 1993 [CIRAD, 1993].

The main public research and training institutions involved in biotechnologies are concentrated in Brazil's central-southern region (i.e., in the states of São Paulo and Rio de Janeiro). Others are found in the states of Minas Gerais, Paraná, and Rio Grande do Sul. In the state of São Paulo, there is strong emphasis on plant biotechnologies in relation to agricultural development and technologies concerning biomass transformation (production of bioenergy and recycling of sugar industry wastes and by-products). In the same state are the most advanced institutions in molecular biology, such as the Institute of Biomedical Sciences of the University of São Paulo and the Center for Molecular Biology and Genetic Engineering of the University of Campinas (UNICAMP).

The maize project involves the Institute of Biomedical Sciences, the University of Campinas Center for Molecular Biology and Genetic Engineering (Laboratory of Plant Molecular Biology), and the seed corporation Agroceres S.A. The joint project associating the CIRAD's Department of Annual Crops and Rhône-Poulenc Agrochimie in the production of maize hybrids in Brazil began to bear fruit in 1993 [CIRAD News 5:6 (July 1993)].

At the College of Agriculture Luiz de Queiroz, the University of São Paulo's faculty of agriculture, the O.J. Crocomo group first started using plant tissue culture in June 1971, and was one of the first teams to introduce these techniques in Brazil. In September 1988, the creation of the Center for Agricultural Biotechnology (CEBTEC) confirmed the quality of the group's research and development work:

1. Propagation of virus-free strawberry plants derived from meristem cultures of selected varieties, for commercialization by the private company Citoplant; papaya propagation; embryo culture for the propagation of the native palm species *Acrocomia aculeata* (macauba) from Minas Gerais, the oil of which could be used to produce soap, cosmetics, and fuel.
2. Shoot-tip micrografting of citrus species, with a view to producing super seedlings free of diseases and pathogens; this project, initiated in 1990, was funded by the company Citrovita, part of the industrial conglomerate Votorantim.
3. Interspecific hybridization in bean, to develop hybrids resistant to the common bean mosaic virus and tolerant of water stress. The project, funded by the European Union, was carried out in collaboration with the Faculty of Agronomy of Gembloux, Belgium.

The Center for Molecular Biology and Genetic Engineering of the UNICAMP, inaugurated in 1990, comprises four laboratories dedicated to plant molecular biology, microbiology, virology, and medical genetics. The Plant Molecular Biology Laboratory is working on the seed storage proteins of maize, sorghum, and an Asian grass, *Coix lacryma-jobi*; the genes coding for these proteins have been cloned to develop transfer vectors and to achieve their expression in seeds.

At the Virology Laboratory, in collaboration with a small Brazilian company specializing in pharmaceuticals and antiviral substances, with a pilot plant in Campinas, a mutant strain of baculovirus was developed for the biological control of the soybean caterpillar (*Anticarsia gemmatalis*) and the sugarcane borer (*Diatraea saccharalis*, Lepidopteran). Commercial production of this strain in live insects has been achieved, as well as its purification and formulation as an aqueous solution sprayed from airplanes or tractors in sugarcane and soybean fields. Production of a polyvalent virus, active against two or three insect pests (e.g., Lepidopteran defoliators), is expected. Collaboration exists between the Virology Laboratory

and similar research units in France (National Institute for Agricultural Research Plant Protection and Pest Control Units), United States (University of Florida, Gainesville), and Canada (York University, Toronto).

At the Federal University of Rio de Janeiro, the following work has been carried out at the Laboratory of Plant Physiology of the Institute of Biophysics:

1. Somatic embryogenesis of *Arracacia xanthorrhiza*, a native plant of Peru, the rhizome or root of which is used by Nestlé-Brazil in baby foods because it is rich in starch, carotene, and vitamin C; cell suspensions were grown to obtain large quantities of embryos which could be coated for broader and easier use by farmers.
2. Meristem cultures of sweet potato cultivars to eliminate viruses and pathogens, followed by field trials around Rio de Janeiro to check the agronomic traits of the clones; yields were raised from 9 to 19 tonnes/ha; this simple procedure of "cleansing" the cultivars is, therefore, considered very efficient.

At the Institute of Biology, the Laboratory of Plant Genetic Engineering (Department of Genetics) initiated plant tissue culture in 1983 and plant genetic engineering in 1987. Genetic transformation is carried out on economically important species, such as rice, groundnut, potato, tomato, egg plant, and grapevine. Attempts have been made to introduce the genes coding for herbicide tolerance, insect resistance, or increase in seed nutritional value.

By the mid-1980s, it was realized that the Brazilian industry for the production of legume inoculants (soybeans) lacked an appropriate technology ensuring maximum efficiency of the inoculant used. The total production of packets (200 g) for 1990 was estimated at about 17 million (i.e., some 3500 tonnes) produced by seven inoculant manufacturers; 95% of these packets were inoculants for soybeans. In June 1990, the Brazilian Network of Laboratories for *Rhizobium* Strains Recommendation (certification) was established. It involved 11 research institutions and the seven private inoculant manufacturers. The objective was to improve the mediocre quality of the inoculants produced in Brazil, caused by companies giving priority to the low price of the inoculant at the expense of quality.

Sementes Agroceres, S.A., a large national private agrofood corporation, dedicating 4% of its turnover to research and development, had diversified its activities in vegetable seeds in 1968. It focused on the production of potato "seed" through in vitro tissue culture, and collaborated with Biótica S.A., an Argentinian private company headquartered in Buenos Aires, part of the Polichaco Group. The project was initiated in 1987. Sementes Agroceres S.A. used one of its subsidiaries to develop the basic production of potato seed. In Argentina, the field multiplication of potato seed sent by Agroceres was done by Biótica in collaboration with the National Institute for Agricultural Research. As a result of the project in 1991, the first 35,000 cases of certified potato seed were distributed on the Brazilian market by Agroceres. Production tests carried out in Paraná confirmed the higher yields from the biotechnology-derived potato seed: 38 tonnes/ha, compared with a national average of 13 tonnes/ha. Efforts toward increasing productivity and reducing production costs for field multiplication by Biótica led to promising results: a cost reduction from 90,000 dollars/ha in 1988 to 10,000 dollars/ha in 1992, the medium-term objective being 6000 dollars/ha [Castelhano Bruno and Silva Waack, 1992].

5. Argentina

The priority areas of the National Biotechnology Program were biochemical engineering; nitrogen fixation; plant tissue culture and plant molecular biology; production of vaccines; and production of diagnostic reagents.

In addition to research carried out at the University of Buenos Aires, the National University of La Plata, the National University of Rosario, and the National University of Cuyo are involved in biotechnology research on potatoes and other tuber or root crop species. At the University of La Plata, work focuses on potato, rice, and soybeans: gene mapping, selection of cultivars resistant to diseases, and environmental stresses [Bijman et al., 1990].

Tecnoplant S.A., a leading in vitro micropropagation private company in Argentina and Latin America, has been working since 1985 on the selection and massive propagation of vegetable and fruit species. The company sells banana, kiwi, apple, cherry, pear, vine, strawberry, raspberry, and blackcurrant plantlets in pots, ready for planting in the field, or already with roots for immediate use by the customer. It also "cleans" varieties delivered by customers, carrying out under contract the propagation of Colombian and Costa Rican banana varieties, asparagus, strawberry, and other berries. At the request of large-scale producers and planters, the company has developed new lines for massive propagation.

Tecnoplant S.A. also provides advisory services for micropropagation, multiplication, management, harvest, and postharvest technologies; marketing; and such. In 1992, Tecnoplant S. A. became the Division of Plant Biotechnology of Bio Sidus S.A., Argentina's second largest pharmaceutical company and leading national laboratory (e.g., producing and marketing human recombinant alfa-2, interferon, and human recombinant erythropoietin).

6. Chile

Most plant biotechnology research has concentrated on cash crop species: citrus, jojoba, wine and table grapes, kiwi fruit (University of Chile); kiwi (Compañía Chile de Tabaco); sweet cucumber (*Solanum muricatum*), citrus, carnation (Universidad Católica de Valparaíso); blackberry, papaya, *Eucalyptus*, ferns, and other ornamentals (German Gallardo Comp.); bulbs and blackberry (Tecviv Ltd); garlic and potatoes (Universidad Austral); papaya, potatoes (Universidad Pontificia de Chile); potatoes (University of Talca); blackberry, asparagus, and various fruits (University of Concepción) [Bijman et al., 1990].

At the Universidad Pontificia de Chile, the research program includes improvement and conservation of virus-free potato germplasm, mass production of healthy elite individuals, large-scale propagation of varieties resistant to stress and pathogens, and vegetative and sexual multiplication of little-known fruit species and Andean endangered species. Joint programs with other universities on genetic engineering are being developed [Bijman et al., 1990]. For more detailed information, see Sasson [1993].

7. The Caribbean

According to Ali (1992), with the exception of Cuba, the Caribbean is, in terms of production, still overwhelmingly in the first phase of biotechnologies in the following areas: plant selection and improvement, baking, baker's yeast manufacture, brewing, alcohol production, mushroom cultivation, methane generation, and anaerobic and aerobic waste treatment.

At the Scientific Research Council, Kingston, Jamaica, the use of leafy cuttings, miniset technology, and tissue culture proved to be superior to yam propagation methods. Yam was threatened by a disease discovered in the 1960s known as internal brown spot. Research on controlling the disease commenced at the University of the West Indies and was subsequently pursued at the Caribbean Agricultural Research and Development Institute (CARDI). During the 1980s, CARDI established a plant tissue culture laboratory in Barbados, with a view to developing a virus-tested yam multiplication project. In the same laboratory, more than 32 varieties of cassava, imported between 1984 and 1988 from the International Center of Tropical Agriculture, are conserved. After the cassava bacterial blight disease reached Barbados in

1987, breeding work was extended to the improvement of disease resistance in this crop species [Pistorius et al., 1990].

In Trinidad and Tobago, at the University of the West Indies, St Augustine, biotechnological research is being carried out on sugarcane to improve yield and resistance to disease and pests, with a view to counteracting a plunge in international sugar prices through lower production costs and increasing productivity. Food crops such as plantain are also the subject of priority research, with emphasis on plant tissue culture, development of bioinsecticides and mycorrhization [Pistorius et al., 1990].

In Cuba, at the National Center for Scientific Research, haploid sugarcane lines have been generated, with some giving higher yields than their parent lines under natural conditions; efforts have also been made to isolate cell lines resistant to salt, aluminium toxicity, and microbial toxins. Microbial biotechnologies include the large-scale cultivation of microorganisms for the decomposition of lignocellulosic biomass and the production of high-value compounds, such as amino acids, citric acid, and enzymes. Furthermore, yeast grown on molasses, used as fodder for livestock, has been partially substituted for imported soybeans [Pistorius et al., 1990].

Transgenic potato plants resistant to the most current viruses were obtained at the Center for Genetic Engineering and Biotechnology, and field trials were carried out in 1992. The Cuban scientists were of the opinion that genetic engineering was the fastest approach to "cleansing" and improving potato planting material, together with the development of effective diagnostic kits for the identification of the various viruses. The increasing production of virus-free potato planting material has enabled Cuba to save half of the foreign currency spent on importing virus-free potato tubers (seed). In addition to potato plantlets produced in vitro, potato microtubers are also produced in vitro and can be used as planting material.

At the Biotechnology Institute Las Vilas, Santa Clara, clonal multiplication of banana following in vitro micropropagation from axillary buds has produced about 5 million plantlets annually, with less than 1% genetic variation. This was performed in unsophisticated facilities at 25°–30°C; the yield from tissue culture-derived plantlets was 30% higher than that from banana trees propagated by conventional vegetative means. A Porto Rican sugarcane variety grown for 40 years in Cuba has also been massively propagated.

8. Cassava Biotechnology Network

Many constraints concerning cassava breeding and improvement, which remained difficult to resolve through conventional research, are now amenable to biotechnologies within an international collaborative program in the form of a network involving both developing and developed country organizations.

In 1994, cassava (*Manihot esculenta*) was the primary carbohydrate food for more than 500 million persons throughout the world. In 1990, an estimated 150 million tonnes were produced, 22% of this in Latin America. The starchy roots of cassava produce more calories per unit of land than any other crop except sugarcane. Cassava leaves provide vitamins and proteins when eaten as a vegetable, a common practice in sub-Saharan Africa.

Tolerant of drought and low soil fertility, cassava is grown primarily in marginal agroclimatic zones and by small farmers. Cassava can be processed into different forms for a wide range of uses, much of this processing being done locally, providing jobs and income in rural areas. Cassava production, processing, and marketing are often women's activities.

Of the 98 species of *Manihot* designated in the most recent monograph of the genus, 17 are native of North America. The main center of species diversity is in Brazil, with a secondary center located in Mexico [Bertram and Schaal, 1993].

In September 1988, the Cassava Biotechnology Network (CBN) was formed. The CBN was supported by the Special Programme Biotechnology and Development Cooperation, Directorate General for International Cooperation, Netherlands. Coordination was provided by the International Center of Tropical Agriculture (CIAT, Cali, Colombia) and, as a voluntary service to the research community, by members of the CBN Steering Committee.

In 1994, the cassava biotechnology research projects under way were as follows:

1. Numerous projects on tissue culture and micropropagation in Barbados, Cameroon, Cuba, China, Indonesia, Nigeria, Panama, Peru, Samoa, Venezuela, Zaire, and others; CIAT and IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria)
2. Nine projects on cassava regeneration in China, France, Netherlands, United Kingdom, United States, Zimbabwe; CIAT and IITA
3. Seven projects on transformation of cassava in Brazil, Canada, United Kingdom, and United States; CIAT and IITA
4. Six projects on molecular mapping, markers and fingerprinting in France, United Kingdom, and United States; CIAT and IITA
5. Three on virus resistance in The Netherlands, United States, and Zimbabwe
6. Seven on cyanogenesis in Denmark, The Netherlands, Thailand, and United States; CIAT and IITA
7. Two on photosynthesis in Australia and the United States
8. Two on cryopreservation in France; CIAT
9. Numerous projects on cassava processing in Argentina, Brazil, Colombia, Congo, France, Ghana, India, Nigeria, South Africa, Tanzania, United Kingdom; CIAT

The cassava genetic-mapping project, or Manihot Genome Project, funded by the Rockefeller Foundation, has been carried out by Angel, Tohme, and Bonierbale at the CIAT. The project is expected to contribute to construction of a saturated genetic linkage map of cassava useful in various genetic studies and cultivar improvement.

A 1991 meeting, supported by the Rockefeller Foundation and organized by the FAO, explored the possibility of applying advanced genetic techniques to improve the storage life of cassava. The project envisaged called for three 5-year phases. Phase 2 would culminate in region-specific field tests of transgenic cultivars; and phase 3 in the diffusion of transformed, locally preferred cultivars with longer storage life in the target environments [*CBN Newslett.* 1(1):15(1993)].

The route of regeneration of cassava plantlets from tissue culture used by almost all groups was somatic embryogenesis from cassava leaf lobes. The International Laboratory for Tropical Agriculture (ILTAB)/Scripps group achieved a breakthrough in improving the efficiency of somatic embryogenesis and plant recovery in cassava (Mathews et al., 1993).

In 1994, several groups were involved in cassava transformation in Canada, United States, The Netherlands, United Kingdom, Colombia, and Brazil. Cassava calli were easily transformed using *Agrobacterium*; somatic embryos were transformed by *Agrobacterium* and by particle gun, and chimeric embryos expressing the *gus* gene were produced, as well as chimeric plantlets [Fauquet, 1993].

C. Arab States

1. Egypt

Research in plant biotechnologies is being carried out at the Cell Research Section of the Agricultural Research Center, Giza, Cairo. In 1990, the Ministry of Agriculture and Land

Reclamation decided to create a National Agricultural Genetic Engineering Laboratory (NAGEL), comprising the following major units: nitrogen fixation, germplasm development and storage, molecular biology and cytogenetics, fermentation and food processing, and a computer center. The NAGEL's main research objective is to transfer agronomically desirable traits, such as tolerance to salt and drought, and pest resistance between species or genera, using embryo rescue and tissue culture techniques. Callus initiation and plant regeneration are being studied in wheat, barley, maize, sorghum, rice, alfalfa, and broad bean (*Vicia faba*). Current research also includes protoplast fusion for interspecific or intergeneric crosses that are impossible to achieve through conventional breeding methods.

At the NAGEL, Eweida et al. [1992] were able, for the first time, to regenerate three commercial potato cultivars (*Solanum tuberosum*) from leaf and tuber disks, with a chimeric gene encoding the coat protein of potato X virus. The Egyptian researchers then carried out the trials on the transgenic potato plants to test their resistance to the X virus when challenged with it [in *Proceedings of the 1992 Miami Bio/Technology Winter Symposium, Advances in Gene Technology: Feeding the World in the 21st Century*, vol. 1, Miami, 20–24 January 1992, p. 58],

A 4-year date palm production improvement program was initiated in 1988. It included the selection of 1000 pollinators in Aswan, New Valley, and Assiut sites, the selective pollination of female trees, and the continuous selection of natural offshoots that could be used in tissue culture experiments. Imported tissue culture-derived date palm plantlets were also tested in greenhouses and nurseries on several sites. The planting of tissue culture-derived date palm saplings was carried out in 1991 and 1992.

Interest in the application of rhizobial inoculants in Egypt had arisen in the 1930s, and the first inoculum production was achieved in 1939 in the Ministry of Agriculture, and the National Research Center had been involved in biological nitrogen fixation research and development since the early 1960s.

The Faba Bean Nile Valley Project in Sudan and Egypt, conducted in association with the International Center for Agricultural Research in Dry Areas and funded by the International Fund for Agricultural Development, contributed to the understanding of the rhizobia needed for efficient crop production. Exchange of strains and evaluation of cultures and rhizobium inoculants of faba bean were achieved.

At Ain-Shams University Faculty of Agriculture, a Microbial Resources Center (MIRCEN) was created in 1978 as part of the world MIRCEN network set up by UNESCO, the United Nations Environment Programme and the International Cell Research Organization. The Soil Biotechnology Unit, Department of Microbiology, Faculty of Agriculture, Cairo University, Giza, Cairo, was established in late 1990 to carry out research on nitrogen fixation and biofertility of desert soils. Priority was given to investigations on "integrated nitrogen fixation by symbiotic and associative systems," such as intercropping of grain-legume species (inoculated with the specific rhizobia) and grasses (inoculated with associative nitrogen fixers), and mixed canopy of fodder species, including nitrogen-fixing trees (*Leucaena* and *Sesbania*) and grasses (rye grass, sorghum, barley, and Sudan grass). The unit maintains a collection of rhizospheric microorganisms and plant growth-promoting rhizobacteria, as well as of *Frankia* strains for the inoculation of *Casuarina* spp.

A research project on nitrogen fixation in agricultural and natural ecosystems was carried out for 7 years, in collaboration with the Department of Agricultural Microbiology, Institute of Atomic Science in Agriculture, Wageningen, The Netherlands. Another research project, part of the Egyptian Major Cereals Improvement Project, was carried out in collaboration with the Agricultural Research Center, Giza, Cairo, and New Mexico University, on nitrogen fixation in cereals and soybeans as affected by nonsymbiotic and symbiotic microbial strains and mycorrhizal inoculants. Inoculation of wheat and maize seeds with efficient strains of

Azotobacter and *Azospirillum*, respectively, in the presence of organic amendment (2.0 tonnes/feddan; 1 feddan=0.44 ha), could save half the normal field amount of nitrogen fertilizer, while at the same time obtaining higher yields of both cereals; inoculation of soybean varieties (Clark and Caland) with a mixture of *Bradyrhizobium japonicum* strains, in the presence of organic amendment (2.0 tonnes/feddan), 15 kg nitrogen units/feddan, and rock phosphate (33.0 kg/feddan), resulted in high grain yields [N.Magdoub, 1990, personal communication].

Large-scale production of vesicular-arbuscular mycorrhizae (VAM) spores was carried out within the framework of a collaborative project between the Unit of Biofertilizers, the Agricultural Research Center, and Dundee University, United Kingdom. VAM inoculation has been effective in reducing the preemergence damping off and severity of root-rot disease in soybeans. The most recent project in biofertilization used *Crotalaria* sp. and *Sesbania rostrata* as green manures [Y.Z.Ishac, 1990, personal communication].

2. Saudi Arabia

The Date Palm Research Center is considered one of the leading research centers in date palm micropropagation in the Arab States. In vitro-derived date palms have reached the production stage, confirming that the in vitro plantlets are genetically similar to the mother variety [Abu El-Nil, 1986a,b; Abu El-Nil et al., 1986; Al-Ghamdi, 1987]. Other tissue culture laboratories for date palm propagation were set up at King Saud University in Riyadh and at the Ministry of Agriculture and Water.

The results obtained in Saudi Arabia, as well as in Morocco, Algeria, and Tunisia, have shown that in vitro micropropagation can be very successful. However, future research is needed to develop date palm clones resistant to the bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis* (*Fusarium* wilt). In the date-producing Arab States, the application of tissue culture techniques to improve date palm varieties, coupled with processing, packaging, and marketing efforts, will lead to major changes in the export capacity of high-quality dates. For more detailed informations, see Sasson [1993].

3. Morocco

In 1981, there was only one tissue culture laboratory; a decade later it coexisted with ten research laboratories and five commercial laboratories; cooperation has been established between the public and private sectors. Of the three Maghreb countries, plant biotechnology transfer to the commercial sector is the most advanced in Morocco.

Research and development groups working on the following crop species have been identified: cereals, citrus, date palm, banana, strawberry, potato, and the argan tree. Close collaboration has been established between the different services of the Ministry of Agriculture involved in tissue culture techniques and also with the growers' associations. Such a close relation is paying off; an increasing number of farmers are requesting more tissue culture-derived plants of potato, citrus, strawberry, banana, and date palm [M.Aaouine, 1990, personal communication].

Under the National Institute for Agricultural Research (INRA), Morroco's main agricultural research institution, an important research program was carried out on the date palm, at the Institute's Regional Center in Marrakech. This program was initiated to control the bayoud disease, caused by *F. oxysporum* f.sp. *albedinis*; since its appearance at the end of the 19th century, the disease has wiped out two-thirds of Morocco's palm groves. Disease control is of vital importance, because the date palm provides a living for millions of people and is an effective means against desertification.

Date palm clones resistant to, or tolerant of, the pathogenic fungus were isolated by the

INRA scientists as part of a wide-ranging breeding program. Some of these clones were producing good quality dates. An in vitro micropropagation project was designed with a view to producing healthy plantlets on a large scale through meristem and axillary bud cultures; somatic embryogenesis was also obtained. The micropropagation techniques have been transferred to the industrial production units of a private company, also responsible for distributing the plantlets to farmers.

A 4-year collaborative project has been carried out by the three Maghreb countries, with the assistance of the European Economic Community and a French laboratory, with a view to enhancing the in vitro propagation of date palm and developing clones resistant to the bayoud disease. A collaborative project on date palm, also aimed at controlling bayoud disease, was implemented by the Agronomic and Veterinary Institute Hassan II (Rabat) tissue culture laboratory, in Agadir and Purdue University, West Lafayette, Indiana.

Virus-free citrus plantlets were commercially produced, using shoot-tip grafting in vitro. Shoot multiplication and rooting of new rootstocks was obtained, with a view to producing virus-free trees on selected resistant rootstocks with improved yield and a higher quality of fruit. This was achieved by the Société de Développement Agricole, the objective of which was to produce about 200,000 virus-free scions annually to meet national needs for grafting and replanting.

Virus-free potato tubers were produced using multiplication of nodal cuttings and microtuber production, with a view to establishing a national program for certified tubers, eliminating tuber imports, and improving yield and quality of local potato varieties. This was achieved by the partly state-owned Société de Gestion des Terres Agricoles. In 1990, according to the FAO's estimates, Morocco exported 59,000 tonnes of potatoes.

Virus-free strawberries are being produced on a large scale, with a view to ending imports, producing new cultivars rapidly, and improving yield and fruit quality. Similarly, commercial propagation of pineapple using tissue culture techniques has been achieved and aimed to meet local demand for planting material. Virus-free plants have been obtained for sweet potato and grapes, whereas commercial propagation has been achieved for banana, olive, pistachio, prune, jojoba, and verbena. Some relatively salt-tolerant banana cultivars have been propagated through tissue culture, and drought-tolerant tomato cultivars have been regenerated in vitro. The private corporation Domaines Royaux de Meknès own an industrial tissue culture unit, which markets more than 50,000 plantlets of date palm, 250,000 plantlets of banana, and 400,000 plantlets of verbena annually [M.Aaouine, 1990, personal communication].

A project using biotechnologies to improve cereal and food legume varieties is under way under the aegis of the National Institute for Agricultural Research, in cooperation with the International Center for Agricultural Research in the Dry Areas. The project, carried out in Settat, near Casablanca, aims to contribute to a wider program concerning genetic improvement of wheat (soft and durum), barley, triticale, lentils, chickpeas, and field beans. Androgenesis and plant regeneration from callus has been achieved with cereals (wheat and barley), with a view to selecting stress-tolerant genotypes and improving the yield and nutritional value of cultivars.

The argan tree (*Argania spinosa*) is an endemic wild tree growing in southwestern Morocco over an area of about 828,000 ha in semiarid and arid zones. Because it is well adapted to very dry and low-fertility soils in regions where the annual rainfall is less than 200 mm, this tree plays a prominent role not only in the overall economy of the region (Souss), but also in the protection of the environment. The tree belongs to the Sapotaceae family, the representatives of which are found mainly in the tropics, some of them having an important economic or industrial role (e.g., the sheabutter tree or karite, *Butyrospermum parkii*, and

the gutta percha tree). The argan tree is the farthest northern representative of this family [Nouaim et al., 1991].

The seed is used to produce an edible oil, but argan oil production represents only 1.6% of total annual edible oil consumption in Morocco. Oil extraction is rather rudimentary and not complete: about 100 kg of fruits and 8–10 h of work are needed to produce 1–1.2 L of oil. Its low concentration in linoleic acid and high content of linolenic acid, confer a high nutritive value on argan oil, considered as hypocholesterolemic. The properties of argan oil for skin protection against ageing led Galenic Laboratories of manufacture a series of products under the label "Argane," and the company Colgate Palmolive produced a soap containing 1% of argan oil called "Antinea." These high-added-value products could foster interest for the argan oil and argan tree [Nouaim et al., 1991].

The argan tree is above all considered a fruit tree and secondarily a fodder and a forest tree. Well-maintained plots, even in arid zones, could produce up to 800 kg of fruits per hectare (i.e., at least 250 kg of dry pulp, 200 kg of broken shells, 20–25 kg of oil cake, and more than 8 L of edible oil, which is sold at a high price) [Nouaim et al., 1991].

The acreage of the argan forests has been decreasing; population and livestock growth, the spreading of intensive crops, as well as the infringement of regulations in protected areas, are the main causes of deforestation. It has been estimated that at the current deforestation rate, the argan tree will almost entirely disappear in the year 2051 [Nouaim et al., 1991].

Such gloomy prospects led the Moroccan Forestry Service to undertake reforestation programs as well as projects to control sand formation. In the early 1990s, about 3000 ha were reforested and some 4700 ha of sand dunes were fixed. The main regeneration process of the argan tree is by offshoots; the second regeneration process is by seeds. At the French National Institute for Agricultural Research (INRA), in Dijon, an in vitro predevelopment unit has been working on in vitro culture of the argan tree since 1989. French scientists noticed that the plantlets obtained from in vitro cultures are true to the mother plant and that, in particular, the characteristics of the taproot are preserved [Nouaim et al., 1991]. At the French INRA and the Faculty of Sciences of Agadir, Morocco, scientists observed that the argan tree roots contain endomycorrhizae, which play an important role in the transfer of water and phosphorus, both of which are limiting factors in the arid soils underlying the argan forests; furthermore, as the roots of the tree have no absorbing hairs, the fungal mycelium can replace them and even explore a large volume of soil. Both the taproot and lateral roots contain endomycorrhizae. It was thus suggested to inoculate seedlings and tissue culture-derived plantlets with endomycorrhizae such as *Glomus mosseae* or *Gigaspora margarita*, so that the young saplings could benefit from the physiological advantages offered by these symbiotic fungi [Nouaim et al., 1991].

To sum up, the value and importance of plant biotechnologies have been recognized in Morocco; growers are increasingly requesting pathogen-free plantlets of banana, date palm, strawberry, and potato. Furthermore, owing to Morocco's lower labor costs and the absence of taxes on farmers' income, foreign corporations are investing in this area. In the coming years, plant biotechnologies will be applied to a wider range of crop and forestry species, and private sector involvement will increase.

At the Faculty of Sciences, Mohammed V University, Rabat, research has been carried out since 1980 on nitrogen fixation by grain legumes. Research in the early 1990s concentrated on the effect of several limiting factors on nitrogen fixation: soil acidity, water stress, and iron deficiency. Furthermore, the research program has been extended to subterranean clover and to actinorhizian trees (*Casuarina* sp.). The main objective of this research work is both to help raise grain legume yields, while decreasing inputs of nitrogen fertilizer, and to contribute to reforestation schemes with nitrogen-fixing tree species.

At the Agronomic and Veterinary Institute Hassan II, the research program of the Soil Microbiology Laboratory includes the following themes:

1. Ecology of *Rhizobium* spp. in different Moroccan soils
2. Characterization of *Rhizobium* spp. natural populations
3. Competition and survival of *Rhizobium* strains introduced into Moroccan soils
4. Determination of inoculation need for the main grain and fodder legumes cultivated in Morocco, and establishment of a map showing the regions where legume inoculation is or is not needed
5. Salt tolerance of the *alfalfa-Rhizobium* symbiosis in the Tafilalet oases
6. Introduction, in collaboration with the Forestry Department, of new nitrogen-fixing tree legumes, such as *Leucaena leucocephala*

Extension and development activities have resulted in on-farm demonstration inoculation trials on soybeans, chickpea, and berseem (*Trifolium alexandrinum*) in various regions of Morocco; training field technicians in legume inoculation; and organizing extension days with farmers to familiarize them with legume inoculation techniques.

4. Tunisia

The creation in 1983 of the National Center for Biotechnology, in Sfax, was the result of a national plan for biotechnology research, oriented mainly toward agriculture, the agrofood industry, and health. A national commission for biotechnology coordinates the research carried out at the Sfax center, the Pasteur Institute (for health aspects), the Faculty of Sciences, the National Institute for Scientific and Technological Research, the National Institute for Agricultural Research of Tunisia (INRAT), and the National Institute for Agriculture of Tunisia (INAT) [Daaloul et al., 1990].

At the INRAT, in vitro shoot-tip micrografting is being used in citrus to produce virus-free plants; meristem and bud cultures have been applied to peach, almond, pistachio, and jojoba; and a laboratory has been created for date palm in vitro micropagation and multiplication, with FAO assistance.

At the INAT, in vitro tissue culture has been applied to potato, artichoke, strawberry, and ornamentals; all in vitro culture stages have been mastered, including the rooting of plantlets; collaboration has been undertaken with an agricultural development institution to scale up the process to the commercial stage. Crop breeding, especially toward stress- and disease-resistance in barley and wheat, also rely on plant biotechnologies [Daaloul et al., 1990].

Biological nitrogen fixation research on grain legume species was initiated in 1980 at the Laboratory of Agronomy of the INAT, on native rhizobia that induce broad bean nodulation. Later on, similar work was carried out on pea, chickpea, groundnut, and common bean. Inoculation trials showed positive effects on broad bean, field bean, pea, and chickpea. These effects might be due to both the *Rhizobium* and the phosphate added to the peat carrying the inoculum.

Introduction of alfalfa, which could contribute to the intensification of cereal cultivation over 300,000 ha of fallow fields, was carried out in cooperation with Australia. Such an innovation seemed to be successful in zones receiving more than 150 mm of annual rainfall, but sufficient seed supply was needed. Preliminary inoculation trials, carried out with the assistance of the International Center for Agricultural Research in Dry Areas and the International Development Research Center (Ottawa), showed a positive effect of the inoculum on alfalfa and on *Medicago hispida*.

Acacia cyanophylla has been used in Tunisia since the 1930s by foresters, because it was a good pioneer plant species which, after 12–18 years, could be replaced on the rehabilitated

soils by higher-value species or citrus. It was also used as a fodder tree on very large areas: about 47% of fodder units produced annually in this country originated from *A. cyanophylla*. Nitrogen fixation research on *Acacia cyanophylla* was initiated in 1983 and a culture collection of native *Rhizobium* isolates was constituted. The majority of these strains belong to the *Bradyrhizobium* genus. The overall purpose of the research work is to isolate and multiply highly efficient *Rhizobium* strains, as well as endomycorrhizae that could improve *A. cyanophylla* phosphorus nutrition and to clone the most effective plants using tissue culture. The most efficient symbiotic associations thus identified could be used, first in field trials, then in plantations.

D. Sub-Saharan Africa

In 1994, in vitro micropropagation was being conducted for the following cash crop or plantation species: oil palm (Ivory Coast, Nigeria); date palm (Algeria, Egypt, Morocco, Tunisia); cocoa (Nigeria); coffee (Kenya, Zimbabwe); tea, camphor tree, and pyrethrum (Kenya); sugarcane (Kenya, Zimbabwe); olive tree (Egypt, Morocco); strawberries (Egypt, Morocco, Tunisia); pineapple (Zimbabwe); tobacco (Zimbabwe), forest tree species (Egypt, Kenya, Senegal, Tunisia); and ornamentals (Kenya, Mauritius).

For food crop species, the following are being micropropagated: banana (Burundi, Cameroon, Egypt, Gabon, Ghana, Morocco, Nigeria, Senegal, Tunisia); plantain (Cameroon, Gabon, Ghana, Nigeria); tuber plants (Algeria, Burundi, Egypt, Kenya, Mauritius, Morocco, Tunisia, Zimbabwe); cassava (Burundi, Cameroon, Congo, Gabon, Nigeria, Zimbabwe); taro (Cameroon, Ivory Coast, Gabon, Ghana); yams (Cameroon, Ivory Coast, Gabon, Ghana, Nigeria); sweet potato (Burundi, Kenya, Zimbabwe); rice (Senegal); soybeans, maize, and groundnut (Zimbabwe).

Many African staple crops (e.g., cassava, sweet potato, yam, and plantain) are vegetatively propagated and so far have received little research funding (orphan crops). Meristem culture, shoot-tip and node-cutting cultures are routinely used to eliminate viruses from improved cassava clones and to micropropagate them. The virus-free improved clones have been distributed by the International Institute of Tropical Agriculture (IITA) to national agricultural research institutions for evaluation and testing. The in vitro reduced growth storage method was applied to conserving clonal germplasm: a total of more than 2000 accessions are being maintained at the IITA.

Embryo culture techniques for the germination of mature and immature cassava embryos are being developed. In IITA scientist has reported that production of plantlets from callus produced from cassava leaf has become possible using several cassava varieties resistant to the Africa cassava mosaic virus.

1. Senegal

On 20 March 1992, the Research Unit on In vitro Culture (URCI, Unité de Recherche en Culture In Vitro) was inaugurated in Dakar, as a joint laboratory between the Senegalese Institute for Agricultural Research (ISRA) and the French Scientific Research Institute for Development in Cooperation (ORSTOM). The unit, located at the ORSTOM/ISRA Center in Dakar, aimed to make available a biotechnological tool for improving forest species through in vitro tissue culture. Set up with funding from the French Ministry for Cooperation, the laboratory's first objective was to create a seed pool of selected clones for producing improved seeds for reforestation. In a second stage, the laboratory will apply biotechnologies to improving other plant species (e.g., horticultural and large-scale cultivation crop species). It is expected

to play a regional role through welcoming foreign researchers seeking to become better acquainted with plant tissue and cell culture.

2. Ivory Coast

The Ministry of Scientific Research has taken steps to facilitate the integration of plant biotechnologies into the national agricultural research program. In 1991, a committee consisting of scientists and representatives of the main agricultural companies was set up to investigate a plan for plant biotechnology development in the Ivory Coast [Mulongoy et al., 1993].

Plant biotechnology research at the National University in Abidjan includes micropropagation of virus-free yam and plantain. Other research interests include yam improvement through the production of haploids, invertase activity in relation to yam quality during storage, and isoenzyme characterization of yam and eggplant to document the diversity of these genera [Mulongoy et al., 1993]. A project of close collaboration between the Research Institute of Plant Biology of the University of Montreal, and the National University, Abidjan, with funding from the International Development Research Center (Ottawa), aims to transfer plant tissue culture techniques for in vitro micropropagation and clonal multiplication of yams. The research project has led to the production of yam microtubers in vitro; it has been followed by laboratory and field work in the Ivory Coast, oriented toward substituting the traditional vegetative propagation methods with a technique more suited to the large-scale distribution of superior yam clones.

The first laboratory for oil palm in vitro culture was set up in the early 1980s at La Mé by the French Institute of Research on Oil and Oilseeds, now part of the CIRAD's Department of Perennial Crops. Ivory Coast was the second-largest producer of palm oil in Africa and, therefore, was interested in improving multiplication techniques for oil palm. In the 1970s, 50,000 plantlets of oil palm, resulting from 1000 crosses were produced. Plantlets of elite varieties were distributed to farmers and to Palmindustrie, a governmental company. At La Mé, the oil palm germplasm was stored in vitro at low temperature or in liquid nitrogen [Mulongoy et al., 1993].

In 1993, palm oil represented more than 20% of the world production of vegetable oil and ranked second behind soybean oil. A larger share of world production depends on increasing productivity through the utilization of higher-yielding oil palm varieties or clones tolerant to the principal diseases. In West Africa, yield was no higher than 20–23 tonnes of fresh fruit bunches per hectare, even when there was a sufficient water supply, because solar radiation was insufficient in the face of the important cloud blanket. The same oil palm varieties yielded 30 tonnes/ha or even more in Southeast Asia, where solar radiation was higher than 30% (CIRAD, 1993).

In the Ivory Coast, a diagnosis of the status of the 130,000 ha of oil palm groves has been carried out by the CIRAD at the request of Palmindustrie. The study shows that industrial exploitations replanted with plant material bred over the 1970s and 1980s yielded about 25% more than the first generation at the same age. It has been demonstrated that the best clones of oil palm (derived from tissue culture) yield 25–30% more than the average hybrid progeny from which they originated. More than 2500 ha have been planted worldwide with oil palms derived from somatic embryos. Some flowering abnormalities are explained by the variations in the amounts of endogenous plant hormones. The identification of molecular markers would allow the early elimination of calli likely to give rise to abnormal clones. On the other hand, reducing the production cost of plantlets is considered a priority objective: research is being carried out on developing embryogenic suspensions in a liquid culture medium;

a process of this kind has the advantage of offering substantial savings in labor and laboratory space (CIRAD, 1993).

As West Africa is the natural center of origin of the oil palm, there is a large genetic collection of both wild and highly developed breeding lines, as well as a long tradition of oil palm research. Much of the data required for selection of parents for production of progenies suitable for cloning are already in the archives. The African countries are in a strong position to introduce new genetic variation, including disease resistance, and to compete with Southeast Asian material derived from the relatively narrow base of the Deli Duras. On the other hand, there is a need to acquire the DNA technology for clonal monitoring and for oil palm genetics to aid the breeding program [Jones, 1992].

In the breeding of high-yielding cocoa varieties, hybrids of selected clones have been identified in the Ivory Coast by the CIRAD in collaboration with IDEFOR (Forestry Institute); capable of producing up to 3 tonnes of marketable cocoa per hectare, they are widely used. Productivity improvement is also sought through the creation of varieties resistant to diseases (*Phytophthora* spp.) and pests. The genomic sequence of the swollen shoot virus has been identified in collaboration with the French National Institute for Agricultural Research, paving the way to new diagnostic methods [CIRAD, 1993].

Research work is being conducted at CIRAD's center in Montpellier on the genetic diversity of cocoa, using DNA markers in about 300 clones. Restriction fragment length polymorphism (RFLP) and random-amplified polymorphic DNA (RAPD) markers are used to establish a map of the cocoa genome. In the medium term, genes for resistance to diseases and for organoleptic qualities of cocoa are expected to be identified. In collaboration with Francereco (Nestlé group), the conditions for obtaining somatic embryos from petals have been determined in nine genotypes. In 1992–1993, plants derived from somatic embryos were being grown in pots in greenhouses [CIRAD, 1993]. For more detailed information, see Sasson [1993].

3. Nigeria

Of the 24 government research institutes in 1994, about 10 were involved in some kind of biotechnology research, largely related to agriculture and food processing. The National Center for Genetic Resources and Biotechnology, created in 1986 on the Moor Plantation, Ibadan, is coordinating research activities carried out in 9 satellite laboratories.

Crop species studied extensively (including at the International Institute of Tropical Agriculture, IITA, Ibadan) are cassava, yam, groundnut, cowpea, sorghum, maize, rice, cocoa, and rubber. Since 1977, the IITA has been working on hybrid maize and, by 1983, Nigerian research institutes had followed suit, resulting in on-farm testing in 1984. That same year, the first private company, Agricultural Seeds Co., became involved in producing hybrid maize seed. Furthermore, Pioneer Hi-Bred Seed Nigeria initiated production of hybrid maize in 1990 and, in 1992, bought up the Agricultural Seeds Co. In early 1992, hybrid maize covered an estimated 100,000 ha of the country's 2 million ha planted with maize [in the *IITA Annual Report, 1991*].

For about four decades, Nigerian researchers had been evaluating rice germplasm in investigations aimed at developing resistance to blast disease and brown leaf spot disease, both prevalent in Nigeria. New, resistant varieties were developed and distributed to farmers in the early 1990s, both in Nigeria and across sub-Saharan Africa. The development of high-salinity tolerant rice might change some 2 million ha of Nigerian coastal marshlands into paddy fields [Ogunseitan, 1991].

With use of combinations of tissue culture and other techniques, the research goal was to create as large a collection of soybeans as possible that were sufficiently promiscuous

(nodulating freely with natural *Rhizobium* present in Nigerian soils), photoperiod-insensitive, nonshattering, higher-yielding, with good seed longevity [Ogunseitan, 1991],

Nigeria is the leading yam producer in sub-Saharan Africa. In the face of spiraling population growth, intensive cropping systems are needed. It would be necessary to lower production costs devoted essentially to planting material, billowing, weed control, and stick setting (especially in forest areas), not to mention those linked to harvesting tasks, to ensure the competitiveness of yam versus other crop species. Research work should also play a vital role in selecting plants with a different stem morphology, tuber traits, growth periods, and multiplication rates. Postharvesting losses are heavy. Their mitigation could significantly reduce the need to extend the cultivated acreage and the resulting drop in soil fertility. At the Laboratory of Plant Tissue Culture and Biotechnology, Department of Botany, University of Nigeria, Nsukka, Enugu State, plantlets were regenerated from explants of a few edible yam species derived from stem nodes (with or without leaf), internodes, and meristems. Furthermore, methods for the culture of zygotic embryos have been devised to recuperate embryos from plants with a high abortion rate.

Cocoa is still the leading agricultural export of Nigeria, the sixth-largest cocoa producer. The Cocoa Research Institute of Nigeria (CRIN) was set up in 1953 on the Moor Plantation, Ibadan. Although cocoa was introduced into Nigeria about a century ago, active research began only about 30 years ago. With the advent of biotechnologies, in vitro propagation and germplasm conservation benefited from their application. During the third Nigerian breeding program, somatic embryos were obtained by Esan in 1975 from cultured cotyledon and hypocotyl tissues of very young cocoa embryos. Attention focused mainly on micropropagation and in vitro germplasm conservation of cocoa at the CRIN. For more detailed information, see Sasson [1993].

4. Cameroon

At the Jay P. Johnson Biotechnology Laboratory of the Institute for Agricultural Research, Buea, Cameroon, culture media have been developed for the regeneration of yam plantlets (*Dioscorea rotundata*) from stem nodes, as well as for the induction of multiple shoots and rooting of microcuttings derived from these shoots. For macabo (*Xanthosoma sagittifolium*), a method for inducing secondary shoots on the initial bud has been devised, followed by the rooting of the resulting shoots and production of plantlets.

The project on the genetic improvement of plantains and other bananas aims to improve banana production and to increase exports. In 1993, production increased from 30,000 to 100,000 tonnes and laboratories were built for phytopathology and plantlet production at an affordable price for planters. The project's board of directors brought together representatives of the Institute for Agricultural Research and the CIRAD's Department of Fruit and Horticultural crops (CIRAD-FLHOR). Funding amounted to about 2.7 billion CFA over 5 years.

The Regional Center for Banana and Plantain, an original Euro-African regional cooperation structure, was set up in 1989 on the joint initiative of the Government of Cameroon and the CIRAD. It was then extended to include the Central African Republic, Congo, and the Institute for Agricultural and Livestock Research, jointly run by Burundi, Rwanda, and Zaire. On 7 July 1993, the directorate of the CRBP was inaugurated in Njombe, Cameroon [CIRAD News, October 1993, 6]. For more detailed information, see Sasson [1993].

5. Kenya

The Kenya Agricultural Research Institute (KARI) was set up in 1902 and since the early 1950s, 15 stations for agricultural research and extension have been established

throughout the country under the aegis of the government. Early research work was initiated on the breeding and selection of cereal species, particularly wheat and maize, followed by work on coffee and tea. Most of the applications of plant biotechnologies are found in the research carried out on these crop species.

Most research on potatoes is being carried out at KARI's National Plant Quarantine Station, Muguga, which screens and multiplies the material supplied by the International Potato Center. Further large-scale propagation through tissue culture is undertaken by the KARI's National Potato Research Station located in Tigoni. New varieties are disseminated by the Agricultural Research Corporation. It is estimated by the International Labor Organization that the adoption of plant biotechnologies for the propagation and cultivation of potatoes might increase labor productivity by 24%, while doubling the contribution of potato production to national income [Olelombo, 1991; Pistorius, 1991].

At the Crop Department of the University of Nairobi, research is being carried out on meristem culture and somatic embryogenesis of several crop species, with a view to breeding pest-resistant plants. Viruses have been identified in the same department by the enzyme-linked immunosorbent assay (ELISA) technique, increasingly used in research stations and in quarantine and germplasm conservation units. The International Potato Center has introduced the technique into the region to detect potato viruses. In addition to the work carried out on citrus and strawberries, research has also been under way on cassava, sugarcane, and coffee. Maize tissue culture is also performed at Kenyatta University, near Nairobi [Clark and Juma, 1991].

Coffee is the greatest export-earning commodity in Kenya, ahead of tourism and tea. Coffee breeding in Kenya began in 1971, stimulated by losses resulting from the coffee rust and coffee berry disease. At the Ruinu Coffee Research Foundation, a small team of researchers developed tissue culture methods for micropropagation purposes. Somatic embryogenesis has been obtained on secondary calli derived from leaf explants. Regeneration of coffee plantlets from tissue cultures is still difficult to achieve on a large scale and with a high success rate [Olelombo, 1991; Pistorius, 1991]. In 1986, the Coffee Research Institute released a new coffee variety, Ruinu-11, with a short stature, derived from Catimor. The current breeding program is directed toward resistance. There is also interest in developing RFLP and isoenzyme markers to assist coffee breeding. New coffee-breeding programs are being conducted on two segregated populations: one from a cross between Kenyan Catimor and Colombian Catimor, and another resulting from a cross of robusta tetraploids with arabica.

Tea cloning also plays an important role in the cultivation of this extending cash crop: in 1991, about 10% of the large-scale tea plantations were planted with clonal material obtained through tissue culture. Small farms, representing 67% of total acreage, are all planted with clonal material [Olelombo, 1991; Pistorius, 1991]. For more detailed information, see Sasson [1993].

6. Zambia

Zambia's use of legume inoculants has been increasing rapidly and the demand has challenged the facilities' ability to keep pace. Established in 1984, the Mt. Makulu Research Station Inoculum Production Facility has grown rapidly and promoted the application of biofertilizers over inorganic nitrogen use. An estimated 8000 ha of soybeans were inoculated during the first year, followed by annual increases of up to 40,000 ha of soybeans being inoculated with the appropriate *Bradyrhizobium* strains. The average production increase in legume inoculants on a peat-base carrier for several species has been 10–15% since 1987. A total of 75,000

packets, each 250 g (18.75 tonnes), were supplied during the 1991–1992 production season, of which 95% were for soybeans.

7. Zimbabwe

In Zimbabwe, over the 1980–1990 period, agriculture had contributed an average 15% to the gross domestic product, and 55% of the active population are working in agriculture, which represented 42% of foreign currency earnings [Fritscher, 1992].

Zimbabwe, by far the most important forestry and agricultural producer in the Southern African Development Coordination Conference (SADCC) region, applies a hydroponic method of raising seedlings of forest species on a large scale. The so-called speedling technology consists of germinating and raising seedlings in polystyrene cells, under tightly controlled regimens of temperature, irrigation, and fertilization. After having been developed in the United States, speedling nurseries first appeared in South Africa during the 1970s, when several commercial vegetable producers adopted and invested in this technology. By the 1980s, very large nurseries were operating, servicing not only the agricultural and horticultural sectors, but also the South African forestry industry, the largest in Africa [Tiffin and Osotimehin, 1992].

Although the speedling technology was not developed in Zimbabwe, its success was based on local experimentation and know-how, local materials, and entrepreneurial self-reliance. In 1989, Delta Corporation approached Brookfield Seedlings which, along with Tabex (a major Zimbabwean tobacco and horticultural conglomerate) and other business investors, took over its tissue culture laboratory and established it on-site at Brookfield. In 1991, the laboratory started to function commercially and aimed to develop disease-free cuttings of coffee, strawberries, bananas, citrus, sweet potato, and other economically important crop species. The laboratory also led to a new venture in the production of mushroom spores for domestic consumption, instead of importing them from Europe [Tiffin and Osotimehin, 1992]. For more detailed information, see Sasson [1993].

8. Republic of South Africa

In late 1992, the Foundation for Research Development (FRD)—a government-sponsored body responsible for developing South Africa's human resources in science, engineering, and technology—compiled information on current biotechnological activities in the country, carried out by academic and research institutions, as well as industrial companies. A survey was conducted in which 50 companies involved in biotechnologies were asked to state their top priority for development:

1. Agricultural and plant biotechnologies (20%)
2. Production of food and protein substitutes (18%)
3. Medical biotechnologies and diagnostics (12%)
4. Pharmaceutical and therapeutic substances (8%)
5. Veterinary and animal biotechnologies (8%)
6. Environmental biotechnologies (8%)
7. Molecular biology (4%) [FRD, 1992].

Research groups in universities and “technikons” work mainly in the new biotechnologies, the majority being interested in medical biotechnologies (49%) and plant biotechnologies (45%) [FRD, 1992]. There seems to be general recognition that agricultural biotechnologies should have high priority to improve food production and that certain areas of health and the environment should also be considered priority areas.

Under the Agricultural Research Council, the Vegetable and Ornamental Plant Institute (VOPI), Pretoria, has maintained over 1000 plant types of over 80 species with acknowledged economic and horticultural value. In addition to being a reliable source of disease-free potatoes, sweet potatoes, garlic, and flower bulb species, the VOPI has a semicommercial tissue culture facility.

The Agricultural Research Council has cooperation links with the International Potato Center (CIP, Lima, Peru), the African Potato Association in Tunisia, and the Gene Bank in Lusaka, Zambia. It has been involved in research activities in plant breeding (Kenya) and sweet potato (Malawi).

At the Stellenbosch Institute for Fruit Technology, the Division of Plant Biotechnology and Pathology produced the first genetically engineered crop species, the strawberry cultivar Seleka, with herbicide resistance. Research has concentrated essentially on improving resistance to bacterial and fungal diseases, leaf roll virus, plum poxvirus, ripening inhibitors, cultivar typing, plant transformation, and plant tissue culture. Enhanced resistance has been incorporated into plants against bacterial canker, fire blight, and spots of stone fruits caused by *Pseudomonas* sp. and *Xanthomonas campestris var. pruni*; apple blisters caused by *Pseudomonas syringae*; and blossom blasts of Forella pears. Diagnostic kits were developed to detect bacterial and viral diseases in passion fruits. To these achievements could be added the regeneration of apricot, apple, and pear plants. Research is also being conducted on the interaction of apple and plum plantlets and vesicular-arbuscular mycorrhizae.

Transgenic Royal and Gala apples, virus-resistant apricots, herbicide-tolerant strawberry cultivars (Seleka, Trobella, and Tioga) have been developed since this line of research was initiated in 1988. Cooperation exists with the Bayer Corporation, and the South African Committee for Genetic Experimentation is overseeing the release of genetically engineered plants. The Institute provides on-farm services and advice.

The South African Sugar Association Experiment Station (SASEX), which has links with 35 countries (including Malawi, Swaziland, and Zimbabwe), has developed new sugarcane varieties, using both conventional breeding and biotechnologies. Current research focuses on resistance to the sugarcane stem borer, *Eldana saccharina*. The SASEX's excellent tissue culture facilities lend themselves to the training courses it runs for both local scientists and those from neighboring countries. In addition, SASEX has hosted regional courses for farmers and specialists from Fiji, Sri Lanka, Thailand, Brazil, and other countries, as well as for farmers from South Africa, Malawi, and Swaziland.

At the University of Cape Town, life science and biotechnological research is well established and recognized worldwide. The following projects are being carried out:

1. Development of isotope tools for assessing drought tolerance in cotton- and tobacco-breeding programs
2. Gene targeting within the Poaceae family to improve resistance in cereal crop species to drought, disease, and herbicides
3. Genetic engineering to control the maize streak virus
4. Development of a biopesticide against the sugarcane stem borer, *Eldana saccharina*
5. Production of flavors and fragrances from indigenous plant species

9. Mauritius

At the University of Mauritius several grain legume species have been selected for pulse production locally to cut down on imports: *Vigna mungo*, *Phaseolus vulgaris*, *Lablab purpureus*, *Glycine max*, *Lens culinaris*, and *Cicer arietinum*. Research work is being carried

out on the selection of effective and persistent strains of *Rhizobium* for these legume species to be grown under various soil conditions and fertilizer [Manrakhan, 1990].

After screening selected species of economic importance to Mauritius, their suitability for successfully cloning through tissue culture has been studied. In 1985, a Food and Agricultural Research Council (FARC) was set up with a view to linking research with commercial exploitation. It set up a cell and tissue culture laboratory, initially for *Anthurium* and horticultural crops in which Mauritius has earned a solid export reputation, and for several vegetables and other ornamentals, mainly for export purposes [Manrakhan, 1990].

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Biotechnology and the Environment*

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I. INTRODUCTION

Environmental biotechnology is the use of living organisms or their products to understand, monitor, and manage the environment. In one sense, this emerging field of biotechnology is simply an extension of natural processes, having been practiced by humans for centuries. Native Americans used many signs of nature to understand and monitor their environment, and they used fish and other organic nutrients to amend soil for improved agriculture. Long before scientists could manipulate *nif* DNA, agronomists understood the benefits of crop rotation and planted legumes to rejuvenate fields. Modern wastewater treatment is nothing more than manipulation of natural biosorption and biodegradative mechanisms, compressing waste stabilization in both time and space, and the ancient art of composting has been extended to the degradation of explosives. Many such examples exist, and they all serve to illustrate the point that living organisms or their products have had, and will continue to have, profound effect on the environment. Indeed, were it not for microbial life forms, Earth would be a very different and hostile planet today, completely lacking in oxygen and supporting an atmosphere of toxic gases devoid of an ozone layer.

Microbiologists and, more recently, ecologists and environmental scientists have long speculated about the possibility of exploiting life processes for the purposes of understanding, monitoring, and managing the environment. The scientific age brought new insight to the complex interactions between life forms and their environment, and many living monitors, usually called indicators (or bioindicators), were developed to serve as sentinels of environmental change or

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deterioration. Management of the environment, including the achievement of goals for sustainable development [1,2], has been more problematic, but will become possible through the judicious application of many technologies, including biotechnology. With the advent of molecular biology and its commercial spin off, biotechnology, there now exists the potential to more effectively understand, monitor, and even control or manage the environment. Examples of environmental biotechnology applications already enjoying some degree of success include the following:

1. Isolation, amplification, and visualization of genes from environmental samples, for the *detection* of specific microorganisms or metabolic processes or for *monitoring* environmental processes;
2. *Bioremediation*: the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous wastes;
3. *Bioprevention or bioprocessing* of industrial waste streams to remove toxic chemicals or other hazardous wastes, or to convert wastes into useful materials such as commodity chemicals and alternative fuels
4. *Biorestitution* of altered habitats, including creation of wetlands, woodlands, and disease-resistant plants and animals

These and other emerging biotechnology research and development ideas hold great promise for application to environmental problems and to opportunities for sustainable development. Although many ideas will no doubt quickly transition to valuable commercial products or processes, others will boarder on the realm of science fiction. In either event, new ideas must be scientifically investigated for feasibility, and such basic research support will come largely from federal government agencies.

II. INVESTMENTS AND MARKET TRENDS

In 1994, it was estimated that total biotechnology sales in the United States were 7.7 billion dollars, a 10% increase over the previous year [3]. By 1995, biotechnology revenues in the United States had reached 12.7 billion dollars, an impressive 65% increase over 1994 [4]. The vast majority of products sold were, and continue to be, pharmaceuticals. Total annual biotechnology sales in the United States are expected to reach 50 billion dollars in the next decade [5], and the industry is now responsible for approximately 100,000 high-skill jobs generated by 1300 U.S. biotechnology firms [3]. Clearly, biotechnology has captured a significant portion of the U.S. marketplace, and all projections point to a sustained honeymoon for this "high-tech" industry.

Market projections for the environmental component of biotechnology are less definitive. A current estimate for the overall environmental market worldwide is 300 billion dollars annually [2], although this estimate may be too low, because the *Environmental Business Journal* recently placed the U.S. share alone of this emerging industry to be 170–200 billion dollars in 1994 [6]. In 1994, the Organization for Economic Cooperation and Development (OECD) estimated that the world market potential for the biotechnology component of environmental technologies will reach 75 billion dollars by the year 2000 [7]. Whatever the environmental biotechnology market becomes over the next decade, the global community fully comprehends its potential, as evidenced by discussions and agreements at the United Nations Conference on Environment and Development in Rio de Janeiro in 1992 [8] and by recent international investment trends.

In fiscal year (FY) 1995, Japan, through its Ministry of Agriculture, Forestry and Fisheries and its Environment Agency, invested 200 million dollars in biotechnology research and

infrastructure [9] related to aquaculture, agriculture, alternative energy sources, and bioprevention technologies. The Science and Technology Agency of Japan also set aside 4.9 million dollars in FY 1995 to support young researchers (under 35 years of age) at RIKEN and the Research Development Corporation of Japan (JRDC) [9]. These and other biotechnology investments in Japan are in keeping with Japan's desire to increase its international technological competitiveness, including its recent decision to enter the bioremediation marketplace.

Europe is also hoping to capture a major part of the global environmental biotechnology market. In 1995, Glass et al. [10] placed overall environmental spending in Europe (solid and hazardous waste management, site remediation, air and water pollution control, and various other consulting, engineering, and analytical services) at 84–94 billion dollars. In general, bioremediation and other environmental biotechnologies accounted for only a small percentage of this market. The major exception to this generalization is The Netherlands. In 1995, the Dutch government estimated that the biotechnological component of their 5.4 billion dollar environmental market was 1.4 billion dollars [11]. Germany has a major interest in environmental biotechnology, focused on the cleanup market and driven by increasing government regulation. The German market for bioremediation is presently estimated to be 100 million dollars [10], and the largest markets are contaminated soil and groundwater, followed by offgas treatment and specialized wastewater treatment.

In 1995, the U.S. National Science and Technology Council (NSTC) released two reports that focused on environmental technologies that will create new jobs while improving and sustaining the environment in the United States [2,5]. Bioremediation alone has been estimated by the National Research Council to become a major environmental biotechnology industry with annual U.S. sales exceeding 500 million dollars by the year 2000 [12]. Unfortunately, federal environmental biotechnology investments in the United States have not been commensurate with potential payoffs and with these reports. In FY 1994, the last year for which detailed biotechnology budget numbers are available [13], the United States invested 90 million dollars in environmental biotechnology research and development. This amount represented 2% of the total FY 1994 4.3 billion dollar federal investment in biotechnology research and infrastructure [13].

Continued investment in biotechnology research and development, as it pertains to environmental problems and opportunities, is critical to all nations. Such investments will contribute to environmental cleanup and restoration and to the goals for a sustainable future for the United States [1] and for its many international partners in the global marketplace.

The purpose of this chapter is to (1) highlight specific examples of successful environmental biotechnology applications, and (2) identify research and development trends in environmental biotechnology. In almost every instance, these applications and trends will benefit agriculture, by providing improved ways to understand, monitor, and manage the environment. The environmental technologies categorized in *Technology for a Sustainable Future* [1] were avoidance, monitoring, and assessment; control; remediation; and restoration technologies. These four categories are examples of either monitoring or management strategies, and it is implicit that environmental understanding is required for each technology to be successful. Biotechnology can and will be used to accomplish all of these environmental technologies, and it will also help provide a better understanding of the environment.

III. CORPORATE SUCCESS STORIES

A recent report in *The Scientist* [14] reiterated the plight of small biotechnology companies, which include most of the environmental biotechnology firms. It is becoming increasingly difficult for companies to secure financing, even in the midst of an overall growth climate for

the industry, both in the United States and globally [15]. Most small companies are in the development and demonstration stage, lacking a validated product to sell. Consequently, investors are shying away from these small firms, forcing buyouts by larger, more stable companies, or mergers between two or more small start-up companies.

Even so, there are many examples of successful commercial applications of biotechnology being used to investigate and solve environmental problems. Some are at the pilot plant stage, and promise to move soon into full production or application. Others are fully operational, and are competing well with traditional technologies. Most of these involve bioremediation, especially bioprevention and biofiltration, although *in situ* bioremediation of waste sites is beginning to gain a place in the portfolio of accepted remediation technologies. The following examples represent a sampling of "corporate success stories."

Phytotech, Monmouth Junction, New Jersey, is the first biotechnology company in the world that is devoted to the development and use of plants to accomplish remediation of contaminated sites, a process that has been designated phytoremediation. Toxic metal pollution of soils and sediments is a major environmental problem, and one that is tractable to cleanup by plants [16]. Phytotech recently conducted field trials in New Jersey and in the Mariupol and Chernobyl regions of the Ukraine, using a metal-accumulating cultivar of the mustard plant *Brassica juncea* [16]. Good accumulation of Pb, Cr, Cd, and Ni from soil was demonstrated, and *B. juncea* also removed ⁹⁰Sr from soil in Chernobyl. Accumulation of ⁹⁰Sr by *B. juncea* was three times greater than accumulation by *Zea mays*, and the final concentration of ⁹⁰Sr in *B. juncea* shoots was 12 times greater than that in Chernobyl soil. Phytotech is presently investigating several variables that could affect accumulation of metals and radionuclides, including pH, chelating agents, soil microorganisms, dissolution of soil oxides, and addition of ions, such as phosphate and calcium. Plant extraction (phytoextraction) may never completely remove metals and radionuclides from soil, but this approach appears to be one of the more promising technologies presently available. It is economical, it will probably leave topsoil in a usable condition for the future, it reduces the amount of contaminant volume to be landfilled or incinerated, and it covers the soil surface during the cleanup process. Disadvantages include the slow nature of the process and the availability of metal-contaminated plants to herbivores—both insects and vertebrates.

Energy BioSystems, The Woodlands, Texas, is presently evaluating a 5-barrel/day, continuous-operation pilot plant designed to remove organically bound sulfur from diesel fuel [17; R.E.Levy, Energy BioSystems, personal communication]. This pilot plant will be used to validate the process and to scale up to a commercial level of 10,000 barrels/day or more. It is estimated that biocatalytic desulfurization will cost 50% less than a traditional hydrodesulfurization unit to install and 10–15% less to operate. The rate of sulfur removal by this *Rhodococcus-based* bioprocess is presently 45%, and a goal of the pilot plant is to move that rate to near extinction. Clearly, this environmental biotechnology application is well poised to have a profound effect on sustainable development and on reducing sulfur emissions to the atmosphere.

Paques BV, Balks, The Netherlands, is a world leader in the development of closed, compact reactors for both aerobic and anaerobic treatment of industrial and domestic wastewaters. Recently, Paques developed a process for the biological removal of zinc and sulfate from the wastewater stream of Budelco, a major Dutch zinc smelter. The process is based on sulfate-reducing bacteria that are present in a submerged, fixed-film reactor. As of this writing, the water treatment plant has been operational for over 3 years. It has a capacity of 300 m³/h and removes metals with an efficiency of 99.7% and reduces sulfate below the required 200 mg/L limit. An additional benefit from this biotreatment process is that Budelco has been able to produce commercially useful zinc and sulfuric acid from the residual process slurry.

British Nuclear Fuels, Preston, United Kingdom, is in the demonstration and scale-up stage for many environmental biotechnologies relating to microorganism-metal interactions, removal of radionuclides from the environment, and cleanup of nonradioactive heavy-metal pollution. One of their most innovative technologies involves the use of naturally occurring microorganisms to decontaminate concrete and steel surfaces contaminated with actinides or fission products. Contaminated concrete structures are being placed in systems that will promote a biogenic sulfuric acid attack similar to that which occurs in concrete sewer pipes. Formation of a sulfur-oxidizing biofilm (primarily thiobacilli) is encouraged on contaminated concrete surfaces, and the biofilm traps biogenic sulfuric acid between the biofilm and the concrete. Initial studies have shown that up to 10 mm of surface can be removed from concrete samples in less than 6 weeks. Surfaces treated in this manner can then be more readily removed, treated, and disposed of by convention techniques. This decontamination biotechnology is now being demonstrated as an integrated process in both the United Kingdom and the United States by a Cooperative Research and Development Agreement (CRADA) effort between British Nuclear Fuels and the Department of Energy's Idaho National Engineering Laboratory [J.Benson and H.Eccles, BNFL, personal communication].

IV. RESEARCH AND DEVELOPMENT TRENDS

A. Extremozymes

Enzymes that catalyze chemical reactions in the presence of reaction conditions that would denature or otherwise deactivate most biomolecules have been termed *extremozymes* [18,19]. These remarkable enzymes have been isolated from bacteria and archaea that live in extreme environments such as hypersaline waters, deep sea thermal vents, geothermal marine sediments, antarctic seawater, sewage sludge digesters, acid mine drainage, hot springs, and toxic waste sites. The properties of extremozymes, depending on the enzyme, allow them to function at pH extremes, temperature extremes, high salt concentrations, high pressure, and high organic solvent concentrations. Clearly, there is a place for extremozymes in industry, but there is also a place in the many facets of environmental biotechnology. Extremozymes can be used to process toxic waste streams and to catalyze reactions in waste tanks that constitute extreme habitats. Collaborations between microbiologists, enzymologists, and chemical engineers will most certainly lead to novel applications of extremozymes, immobilized or otherwise poised for catalysis. Pure cultures and consortia of extremophiles (or normal microbes that have been genetically engineered to produce extremozymes) will also be useful in processing waste mixtures that contain high salt, high organic solvent, and high metal concentrations, and extremes in pH, pressure, or temperature. In 1996, a new company, Recombinant BioCatalysis, Inc., was formed with the express purpose of cloning and expressing extremozyme genes—including genes isolated from microorganisms that have not yet been cultured—in common mesophilic heterotrophs. This revolutionary start-up company will clone genes from a variety of extremophiles, including hyperthermophiles and psychrophiles, for commercial applications [20].

B. Bioremediation

The use of living organisms to reduce or eliminate hazards resulting from accumulations of toxic chemicals or other hazardous wastes in the environment has been defined as *bioremediation* [5]. Bioremediation has great appeal for many reasons. It is environmentally benign, in the sense that it involves natural biotransformation processes, does not require destructive manipulation of the waste site (e.g., excavation or incineration), and does not usually produce toxic end products. Bioremediation is also more cost-effective than most

traditional approaches; start-up costs are usually high, but operational costs are typically very low, resulting in overall lower costs [7,21,22]. Unfortunately, the reliability and predictability of bioremediation has not been validated [23]. There are many basic science and engineering roadblocks that must be overcome before bioremediation becomes both predictable and reliable.

Little information is available on gene stability, expression, and exchange in natural environments; most genetic research has been conducted in the laboratory under ideal conditions. Likewise, physiological research has been neglected, and more information is needed on enzyme induction and repression, metabolic pathways, cometabolism, and other factors involved with metabolism of toxic chemicals in natural environments. Especially critical is understanding how organisms function when presented with mixed wastes that often include chlorinated solvents, polycyclic aromatic hydrocarbons, heavy metals, radionuclides, chelating agents, and fuel hydrocarbons. These materials solubilize membranes, uncouple reactions, alter enzyme active sites, and in general, wreak havoc on biological processes.

Another area for attention is community ecology; very little is known about how microbial communities respond to wastes *in situ*. Metabolically competent bacteria may not respond to toxic wastes for a multitude of reasons. Such bacteria could be weathered onto substrate, dormant, or both; they could be capable of degrading one substrate, but remain inactive because of inhibition by other wastes. Presumably, in some situations metabolically competent bacteria are totally absent. All of this involves better understanding of ecology (e.g., species interactions, bioavailability, attachment, and taxis) and geology (e.g., macro- to microheterogeneity, transport, and weathering), not to mention better methods for on-line, real-time process monitoring and verification. Additional information in all of these areas would allow engineers to optimize biological and geochemical processes and get them to the marketplace where they could be used to clean up toxic waste sites, eliminate toxic chemicals from waste streams, and even contribute to new waste-free production methods for industry.

C. Bioprevention

Bioprevention is the use of biological processes to remove toxic chemicals or other hazardous materials from industrial waste streams, or to convert wastes into useful materials, such as commodity chemicals and alternative fuels. Many of the processes being studied and optimized for bioremediation applications can also be applied to bioprevention, and these include organic chemical degradation reactions, and heavy-metal and radionuclide transformations (oxidation-reduction reactions). Other bioprevention processes have been termed “green technologies” because they provide for new environmentally friendly approaches to industrial products and processes.

There now exist several biotechnology alternatives to production processes that in the past involved toxic or otherwise environmentally harmful chemicals. For example, paper manufacturers are now using xylanase enzymes to bleach pulp and, thereby, reduce the amount of chemical bleaching. Mannanases produced by the filamentous fungus *Trichoderma reesei* are also being used to facilitate pulp bleaching (i.e., to reduce the amount of chlorine needed to achieve brightness) [24]. The textile industry has also employed carbohydrases to process fabrics; for example, the application of cellulases to denim to achieve the effects of stone washing, surface polishing, and softening [24]. Biological detergents now represent the largest industrial application of enzymes, and considerable research is now underway to improve and extend the application and activity of these “green” biochemicals [24].

D. Bioenergy

The search for sources of energy, the recovery of energy, and the delivery and use of energy, all have had, and will continue to have, profound influence on the environment. Strip mining for coal created acid mine pollution of streams, manufactured gas plants left behind a legacy of polycyclic aromatic hydrocarbon (PAH)-contaminated soils and aquifers, the need to convey electricity brought about the development of polychlorinated biphenyl (PCB)-containing transformers, hydroelectric power plants changed the course of streams and forever altered fisheries, numerous supertankers have spilled their contents onto shorelines with catastrophic results, burning fossil fuels increased atmospheric CO₂ and contributed to global climate change, use of nuclear energy has created intractable waste problems, and the list goes on. Although none of these threats can be eliminated in the near future, current research in the area of bioenergy holds great promise for providing cleaner and sustainable sources of energy that will reduce or eliminate many of these liabilities.

Biotechnology may someday reduce CO₂ levels in the atmosphere. There are presently two approaches to biological scrubbing of CO₂ from fossil fuel emissions and from the atmosphere itself. First, photosynthetic microorganisms, primarily microalgae, are being investigated for their ability to convert CO₂ to carbohydrates, ranging from simple sugars to complex polysaccharides. Several candidate organisms have been studied, some of which may produce commercially useful polysaccharides [25]. The Japanese have isolated several marine microalgae that fix CO₂ with high efficiency, and they have coupled pilot algal culture systems with CO₂-emitting stacks to reduce emissions [26]. One problem with this approach is that the biomass produced, including the biopolymers, such as sugars and polysaccharides, is readily biodegradable and, therefore, could soon be converted back to CO₂ and H₂O. A solution to this problem is to dry the biomass and use it as a fuel source in power plant boilers, and this too is being investigated by Japanese scientists [26]. The second approach to scrubbing is to develop systems that will biologically remove CO₂ from the carbon cycle. One such approach promotes the growth of marine coccolithophorids at the expense of concrete and atmospheric CO₂ [26]. Coccolithophorids combine bicarbonate ions (from artificial weathering of waste concrete) with CO₂ to produce the calcium carbonate coccoliths associated with these marine algae. Ultimately, coccoliths settle, become part of the ocean floor sediment, and, therefore, serve as a long-term sink for inorganic carbon.

Biohydrogen is the term being used to describe photosynthetic production of hydrogen gas. When H₂ is combusted for energy, H₂O is the only product. Clearly, this alternative energy source would reduce the CO₂ emissions associated with fossil fuel combustion and could represent a sustainable source of clean energy for the future. Biohydrogen involves visible light energy as the driver of reduction processes that are catalyzed by either hydrogenase or nitrogenase to produce H₂. The system receiving the most attention is the ability of heterocystous cyanobacteria to split water into H₂ and O₂ [27,28], although a mutant strain of the green alga *Chlamydomonas reinhardtii* is now being investigated by scientists at the Oak Ridge National Laboratory [29]. Much work needs to be done before production efficiencies can be maximized and cost effectiveness realized. Even so, there is great interest in biohydrogen as a sustainable alternative source of energy.

Biodiesel is another biological approach to alternative energy, and there are presently two ways to produce “bio-based” diesel fuel. First, vegetable oils and waste oils from restaurants and other food processors are now being used as sources for diesel fuel. In 1993, this type of biodiesel was being used extensively in Europe, with a production capacity of 32 million gal/yr (30). Soybean-derived diesel is presently undergoing commercial trials in the United States. Early indications are that particulate emissions are reduced with biodiesel, and there is no

organosulfur component in biodiesel. However, the low cost of petroleum-derived fuels in the United States will not soon create a market for biodiesel. The second method of production involves culturing marine microalgae to obtain lipids that can be converted to biodiesel, and the National Renewable Energy Laboratory (NREL) has conducted extensive research in this area. For example NREL investigators have recently cloned a gene (acetyl-CoA carboxylase) from the marine diatom *Cyclotella cryptica* that they believe is involved with the rate of fatty acid biosynthesis [30]. It is hoped that this and related genetic-engineering approaches can be used to enhance the ability of microalgae to produce lipids for biodiesel.

E. Biosensors

The use of biomolecules and biological processes to activate a molecular transducer that, in turn, will signal the occurrence of an event or the presence of a material has evolved into a sophisticated industry. Biosensors were first used in clinical applications, and included fluorescent antibodies (immunoglobulins conjugated to fluorochromes) and enzyme-based immunoassays (immunoglobulins bound to enzymes that catalyze substrate to produce a colored product) to detect pathogenic microorganisms and microbial toxins. Today, many routine tests in the clinical laboratory are based on biosensors.

Biosensor technology has expanded to environmental interrogation, and now includes many different approaches, ranging from antibody-based detection of proteins, to gene probes for specific microorganisms, to the use of acetylcholinesterase for pesticide detection. A new generation of biosensors is based on a technology called *reporter genes*. Reporter genes have largely resulted from recombinant DNA technology, and involve the insertion of a gene or genes into the DNA of an organism that one wishes to monitor or track in the environment. The first and most successful of the reporter genes was *lacZ*, a gene in the lactose (*lac*) operon that codes for the production of β -galactosidase. When *lacZ* is expressed, it hydrolyzes disaccharide-like compounds to produce highly visible colored products. Other reporter genes include the ice nucleation gene (*inaZ*), the β -glucuronidase (*gusA*) gene, and the *xy/E* gene from the TOL plasmid [7,31].

A novel reporter that is currently enjoying increased popularity is the luciferase or bioluminescence system of marine *Vibrio* species. When *lux* genes are inserted into an operon of interest, downstream from the promoter of the operon, the recombinant organism emits visible light when the operon is induced. A project at the University of Tennessee's Center for Environmental Biotechnology and the Oak Ridge National Laboratory's Center for Biotechnology is focusing on a *lux* gene construct (*Pseudomonas fluorescens* HK44) that naturally degrades the PAH naphthalene. In the presence of naphthalene, HK44 emits a visible light that can be quantitatively measured with fiber-optic photometry, and the bioluminescence is directly proportional to both the biomass of HK44 and substrate (i.e., naphthalene) concentration. A mathematical model (second-order rate equation) has been developed that accurately describes the activity of HK44, and model predictions have been within 3% of actual degradation rates measured for a wide range of substrate/biomass ratios and operating conditions. This bioluminescence reporter technology is being field tested in large lysimeters (contained 8-ft-diameter \times 10-ft-deep [2.4-m \times 3-m] stainless steel cylinders developed by the Department of Energy for studying radioisotope leaching in landfills) at the Oak Ridge National Laboratory Y-12 Facility. Both free HK44 cells and cells contained in biosensor probes [32] will be evaluated. The lysimeters will provide a scale that is sufficient for efficacy, cost, and risk analysis of this biotechnological approach to toxic waste remediation. If successful, bioluminescence reporter technology will be a valuable tool for on-line, real-time bioremediation process monitoring and validation. It may also prove useful as a surrogate for

bioavailability testing, because the substrate must move into the bacterial cell and induce an enzyme system for bioluminescence to occur.

F. Biodiversity

Recent evidence suggests that there are countless “yet-to-be-cultured” eubacteria and archaea present in the biosphere, awaiting developments in microbial culture technology so that they can be isolated and described. Bull et al. [33] estimated the total number of bacterial species to be 40,000. In a recent paper on microbial diversity, Tiedje [34] pointed out that different lines of evidence suggest that between 300,000 and 1-million species of bacteria inhabit the Earth, yet *Berkeley's Manual of Systematic Bacteriology* (four volumes containing 2784 pages) lists only 3100. Extrapolation of this hypothesis suggests that *Berkeley's Manual*, at least in contemporary format, could ultimately reach 400–1300 volumes in length! Soil alone may contain 10,000 species per gram, based on the heterogeneity of DNA extracted from soil samples [34]. The oceans of the world contain countless picoplankton that are yet be cultured. For example, the cold water archaea, first detected in seawater samples from depths ranging from 100 to 500 m [35,36], are now believed to constitute a substantial percentage of the total bacterial biomass present in oceans throughout the world [37]. These archaea have not been isolated in pure culture, grown, and phenotypically described; they have been detected only by means of polymerase chain reaction (PCR) amplification, characterization, and classification of their ribosomal RNA. Woese recently noted that approximately 300 archaeal species have been described in the literature, and believes that this number will balloon (C.R.Woese, personal communication). Boone has stated that methanogens alone have accounted for five to ten new species per year for the last 10 years (D.R.Boone, personal communication). It is doubtful that all of these yet-to-be cultured archaea and eubacteria are dormant in their respective habitats; some must be active and involved in biogeochemical cycling in unknown ways. This hypothesis is supported by extensive circumstantial evidence; for example, data provided by vitalstaining techniques such as the AODC, direct viable counting [38], and INT staining [39]. Given their presumed relative abundance, and community structure shifts in response to environmental disturbance [40], yet-to-be-cultured archaea and eubacteria no doubt transform vast quantities of geochemicals in the biosphere. Clearly, the diversity of microbiota as it becomes known will provide the basis for many new environmental biotechnologies, including biotechnologies for understanding, monitoring, and management.

V. BIOTECHNOLOGY FOR SUSTAINABLE DEVELOPMENT

It has been the intent of this chapter to articulate the place of biotechnology in the bridge to a sustainable future. Environmental technologies have been identified as the building blocks for that bridge [1,2], and they are key to the achievement of long-term environmental, energy, and economic goals, not only for the United States, but for all nations of the world. *Sustainable development* has been defined as “development that meets the needs of the present without compromising the ability of future generations to meet their own needs” [1]. Without doubt, biotechnology is well poised to play a significant, if not the lead, role in that development.

I was in my final year of graduate school, when the first Earth Day was celebrated on April 22, 1970. The tools of molecular biology were beginning to come on line, but modern biotechnology had not yet been born. There was growing concern over environmental problems, and both policy makers and industry were making inroads toward solving past problems and anticipating future technology’s effects [2]. Most attention, however, was focused on “end-of-pipe” equipment for controlling discharges to the atmosphere and waterways; little or no attention was being given to biological treatment and prevention, and it was the

prevailing attitude in 1970 that new biological processes being studied in the laboratory (e.g., pesticide and PCB degradation) could not be successfully introduced to established ecosystems (e.g., farm fields and river bottoms). The ensuing 25 years have witnessed profound changes in this philosophy, and biotechnology has come of age.

On Earth Day 1995, the place of biotechnology in developed nations was well established. For the first time, a biotechnology company had been awarded the Presidential National Medal of Technology, the highest honor given in the United States for achievement in science and technology. Amgen had received the award in December 1994, for bringing two widely successful medicines (epoetin alfa [EOGEN] and filgrastim [NUPOGEN]) to market. Clearly, biotechnology had become a major driver in the pharmaceutical industry and on Wall Street. As outlined in this review, biotechnology is also rapidly becoming a major driver in the environmental marketplace—nationally and globally. Only a sampling of the emerging environmental biotechnologies have been discussed in this chapter, but they illustrate a trend. The future of environmental understanding, monitoring, and management lies in biotechnology, and through aggressive application and attention environmental biotechnology will help build the bridge to a sustainable future for all humankind.

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Prospects and Limitations of Agricultural Biotechnologies: An Update

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I. INTRODUCTORY REMARKS: BY JEFF SCHELL

It is now generally accepted that the practice of plant breeding will move forward as progress is made in knowledge and technology.

Breeders in the future will not only be forced to hone their traditional skills but will also have to integrate them with knowledge and experience to coincide with more recent advancements and technologies derived from cellular and molecular concepts and approaches. For worldwide crops such as potato, rice, maize, and in the future wheat, as well as for high value vegetable crops, the future of breeders depends upon the use of a combination of breeding practices (traditional breeding, genetic engineering and tissue culture). In addition to an increasing reliance on breeding, agriculture will also depend on biocontrol to complement and to make possible the use of chemicals that are compatible with intelligent management of the natural resources essential for a sustainable yet highly productive agriculture.

The various chapters in this book document these points very convincingly and relatively exhaustively. Thus most aspects of agriculturally relevant biotechnology are covered. Plant tissue culture techniques not only provide essential ways for the clonal propagation of many agriculturally important crops (e.g., woody plants, ornamentals and vegetables) but they are also the basis for the production of transgenic plants and are at the forefront of recent studies in plant physiology, developmental biology and biochemistry. Spectacular progress in our knowledge of plant sciences, including those relevant to agriculture and breeding, can be

expected from the integration of more traditional sciences with molecular and cellular concepts and techniques. A prime example is seen in the impact molecular genome analysis is already having on traditional breeding (via so called map-based breeding) but also on research and molecular breeding (e.g., via map based gene-cloning).

This rosy description of the future of genetic engineering is rightly tempered in this book by a realistic assessment of the many problems that still need to be faced before what is predicted can be accomplished.

It was therefore wise to ask scientists well known for their "no-nonsense" approach such as Ingo Potrykus and his colleagues to write about genetic engineering of crop plants.

To quote from their introduction: "it has been possible to develop the state-of-the-art of gene transfer to plants to a level at which many of the major crop plants are accessible to the technique and for which we have good reason to believe that there is no basic biological principle that will prevent gene transfer to a specific crop plant." Yet these authors clearly identify the considerable research and development that is still needed before one can, in many but not all instances, consider large scale applications.

A recurrent point made and well documented in various chapters of this book is that genetic engineering—because it is not limited by the natural barriers that ensure the maintenance of species and prevent transfer of genes between unrelated organisms—is an extremely powerful way to increase the genetic variability available to breeders.

The present rise in interest to acquire new knowledge via fundamental research can best be illustrated by reminding ourselves of recent progress in our understanding of the molecular physiology of development, of the mechanism of action of old and newly discovered phytohormones, and of growth signals used by microorganisms that are pathogens or symbionts of plants (such as for instance lipo-chitooligosaccharides). We can expect to see more chapters on these topics in future books on biotechnology.

Biotechnology can contribute to making traditional agriculture more productive while at the same time more sustainable (see different chapters dealing with plant protection against biotic and abiotic stresses). It can also help achieve the goals of "non-food" and/or cash-crop agriculture as has been described in the various chapters dealing with crop improvement and metabolite production.

Hopefully sufficient attention will be paid to regional crops of importance by local populations in developing countries.

Finally topics such as "Biofertilization" and "Bioremediation" are convincingly dealt with. Topics such as "Biotechnology of Farm Animals" and "Marine Biotechnology" as well as the unavoidable but all important "Legal and Public Aspects" round out the all inclusive title of *Agricultural Biotechnologies*.

Some topics have already come to blossom and illustrate well the potential of present day plant sciences. There is the breakthrough in the cloning and characterization of resistance genes along with significantly improved understanding of the nature of avirulence genes and elicitors. Many hypothetical models predict that resistance genes should be involved in the signal transduction pathways linking pathogen derived signals with the regulated expression of defense genes and of programmed cell death (hypersensitivity reaction). The first available data on resistance genes show that this concept may well be correct in its very general sense, even if all resistance genes do not code for elicitor receptors.

We may safely predict that in the near future we will know more details of the molecular mechanisms underlying plant-pathogen resistance mechanisms. Several elicitor receptors will soon be isolated and characterized along with genes involved in localized pathogen-induced programmed cell death. Plant breeders will make use of isolated resistance genes to produce crops with improved resistance to a variety of pathogens and pests.

The content of this book fully justifies the conclusion that Biotechnologies are bound to play an ever growing role in agriculture not only in the long-term future, but also presently, and in the near future.

Fortunately the progress in applied plant biotechnology corresponds with and is in fact stimulating fundamental scientific progress. This is important because the real driving force behind this science is the success of applied plant biotechnology such as: 1) plant protection based on the expression of several different introduced genes coding for different principles acting on the same targets and thereby providing a possible solution to the problem of the emergence of resistant pathogens and pests, 2) new quality traits as well as future crops producing tailor-made non-food products (lipids, carbohydrates, and biodegradable thermoplastics). Only if plant biotechnology becomes a commercial as well as an environmental success will sufficient support be available for this fascinating research.

II. PLANTS AND AGRICULTURE: BY INDRA K.VASIL

Human population has grown steadily since the advent of agriculture, nearly 12,000 years ago, which ensured a continuing and reliable supply of food. The most rapid growth occurred during the past two centuries during which time agriculture became highly industrialized, public health improved, and there were increases in incomes and international trade in food grains. Despite the age-old human concerns about balancing population and food supplies and many population control measures the world's population is projected to continue to grow into the early decades of the 21st century, stabilizing at 9–11 billion sometime during 2030–2050. Much of this growth will occur in the already overpopulated, underdeveloped, and poorer regions of Africa, Asia, and Latin America, which will be home to nearly 90% of the human population.

Food shortages were common during the early and middle years of the 20th century. The introduction of Green Revolution varieties of wheat, rice, and maize during the mid-1960s reversed this trend and helped avoid major food shortages by keeping increases in food productivity slightly ahead of population growth. During this period, both China and India, the two most populous nations with chronic food shortages, became net food exporters. Realistically, however, such increases in food productivity can not be sustained indefinitely. It is not surprising, therefore, that increases in food productivity have begun to decline during the past few years. Furthermore, improved economic conditions in China and India have created greater demand for better and varied food products, particularly poultry and meat, which require greater supplies of feed grains. As a result, both China and India have now become net importers of food. World food reserves have declined from a high of 77 days to less than 50 days.

With the current trends of population growth and agricultural production, the demand for food in the most populous parts of the World will double by the year 2025, and nearly triple by 2050. Increases in food productivity of this magnitude can not be brought about in such a short period of time by conventional breeding, especially when some of our most important crops are approaching the physiological limits of productivity, nor by increasing the amount of arable land, which accounts for 97% of all food production in the world. Arable land, which is finite and comprises about 3% of the earth's surface, is deteriorating and decreasing as a result of soil erosion, salinization, over cultivation, and acidification. These factors, combined with expected increases in population, will actually decrease the global per capita arable land from the current 0.28 hectare to 0.17 hectare by the year 2025. In addition, fresh water supplies, essential for modern high-input irrigation agriculture, are becoming limited

by increased human and agricultural use, and polluted by agricultural run-off and widespread use of agrochemicals.

It is feared that the resulting food shortages in the overpopulated parts of the world during the 21st century may lead to widespread social, economic, and political unrest, making food security the single-most serious threat to international peace and security. At the same time, it is well known that countries with efficient agricultural systems generally have high living standards, strong economies, lower rates of population growth, and democratic forms of government. Increasing food productivity in a sustainable manner will, therefore, not only provide adequate nutrition to the expanding humanity, but will reduce population growth, protect the environment, promote economic development, and ensure social and political stability.

The challenge for the agricultural sector during the next few decades is therefore clear; double food production by 2025, and triple it by 2050, on less per capita land, with less water, under increasingly challenging environmental conditions. The situation is further complicated by the fact that in spite of the heavy use of agrochemicals, modern agriculture still loses nearly 42% of crop productivity to competition with weeds and to pests and pathogens, and an additional 10–30% to post-harvest losses due to a variety of factors, especially in the developing countries where storage conditions are poor.

During the 20th century traditional plant breeding has brought about enormous increases in crop productivity. However, plant improvement by hybridization is slow, and is restricted to a very small gene pool owing to natural barriers to crossability. Beginning in the early 1980s, advances in plant cell culture and genetic transformation have overcome these barriers by making it possible to transfer defined genes into all major food crops, including cereals, legumes, cassava, potato, and many vegetables and fruits. The entire global gene pool—whether it be plant, animal, bacterial, or viral—is available for utilization. The first genes that have been integrated into crop species provide resistance to non-selective and environment friendly herbicides, and many pests and pathogens. Increasingly large acreages of transgenic maize, soybean, potato, tomato, and cotton are being commercially grown for human use and consumption. Carefully planned introduction of such crops on a worldwide scale would greatly help in reducing or even eliminating the enormous crop losses attributed to weeds, pests, and pathogens. The use of such crops will also have a beneficial effect on the environment by significantly reducing the use of agrochemicals. Other genes for improving crop productivity, and manipulating starch/protein/oil quality and quantity, resistance to environmental stresses such as temperature and drought, are also being isolated and studied. In the foreseeable future, these will be used to produce second generation transgenic crops.

It is clear that during the next few decades a wide variety of transgenic crops will become integrated into agricultural systems in the industrialized countries. Introduction of such crops into the developing countries, which need them most, will be slow and largely through the efforts of multinational biotechnology companies, because most of the developing countries currently lack the scientific and industrial infrastructure to develop and introduce these technologies into their agriculture. In the long term however, much of the increase in food production to meet the dual challenge of population growth and food demand must occur in the developing countries. This will impact greatly on their overall economic development, which will help control the relentless increases in population. It is critical, therefore, that scientific and technical manpower and infrastructure be created in developing countries to take advantage of the remarkable advances in agricultural biotechnology. Assistance should be provided to the developing countries by international organizations such as the United Nations Educational, Scientific and Cultural Organization (UNESCO), the Food and Agriculture Organization of the United Nations (FAO), and the World Bank through its many

international agricultural research centers, like those in Mexico and the Philippines, where Green Revolution originated. International agriculture in the latter half of the 20th century was dominated by the Green Revolution. Considering the power and potential of agricultural biotechnology, it is likely that the Gene Revolution will dominate the agriculture of the 21st century. Like the Green Revolution, it may help accelerate the rate of food production, save lives from hunger, create livelihoods in rural households, save large tracts of land that would otherwise be needed for food production, reduce birth rates, ensure low food prices, stimulate broad-based economic growth, expand world trade, and help create a sustainable agricultural system for future generations. Finally, it should be understood that although plant breeding has long been, and will continue in the future, to be indispensable for plant improvement, it must now be complemented and supplemented by molecular breeding and genetic transformation, in order to establish a sustainable agricultural system for the 21st century.

III. ANIMAL BIOTECHNOLOGY: BY NEAL L.FIRST

Animal Biotechnology has developed rapidly from the early 1980s when the first transgenic mice and first in vitro produced bovine embryos occurred. Today animal breeding companies are using marker assisted selection to provide earlier and improved selection of breeding animals. Gene mapping efforts are annually identifying a large number of candidate genes for testing of usefulness in marker assisted selection.

The propagation of genetically valuable or transgenic animals is enhanced by in vitro production of embryos. There are already more than a dozen commercial companies selling or producing for customers in vitro produced embryos. Cloning of embryos of cattle and sheep has been shown to be possible, but low efficiencies have thus far prevented commercial use. Animal production and genetic selection systems can be made more efficient by sexing the sperm used for in vitro production of embryos. The flow sorting of sperm cells to separate X from Y bearing cells has been successful in most species tested and in cattle and swine has resulted in offspring of the desired sex. While effective with in vitro embryo production, the sexed sperm are of less than normal viability and sperm numbers and not yet useful in artificial insemination or the freezing of sperm.

The artificial insemination and embryo transfer industries are today heavily dependent on use of cryopreserved sperm and embryos. However the cryopreservation of oocytes and the combination of some biotechnologies such as embryo biopsy and sexing with cryopreservation are still in the research stage. Transgenic cattle, sheep, swine, and poultry have been produced primarily by microinjections of DNA into pronuclei of eggs or by use of viral vectors. The intended use has been to change animal growth, disease resistance, wool production, or to produce new products in milk. The efficiencies thus far have been much lower than in mice. Therefore there has been little commercial use of transgenic technology except for the involvement of several companies in production of pharmaceutical products in milk.

The use of cultured embryonic stem cells and nuclear transfer to make offspring has resulted in calves and lambs. This technology offers promise of making large numbers of nearly identical offspring or more efficient gene transfer or selection. However thus far the technology is not sufficiently efficient for commercial use. Growth hormones and growth promoting factors are being developed for improved efficiency of animal growth. Growth hormones are commercially in use at present for promotion of milk production in cattle.

The anatomy and physiology of birds and the late stage of egg laying prevents the introduction of DNA into pronuclei as in mammals. Thus other methods such as oocyte microinjection of DNA, viral introduction of DNA and introduction of DNA into primordial germ cells are being used in avian gene transfer research. Oocyte microinjection, blastoderm transfection

and primordial germ cell manipulation have all been successful in producing transgenic birds. The production of transgenic birds appears possible but is not yet in commercial use.

Patented applications of recombinant DNA biotechnology to animal agriculture became apparent in the early to mid 1980s with development of recombinant vaccines effective against calf scours and against rabies. Today numerous vaccines in use are produced by recombinant technology. Biotechnology has also revolutionized diagnostic techniques by introducing higher sensitivity and specificity. Monoclonal antibodies, DNA probes, Southern and Western blotting, DNA and RNA amplification through PCR, constitute an integral part of the diagnostic arsenal at the disposal of the advanced laboratories. Based on these techniques rapid animal-side field test used by the clinicians furnish results in real time, permitting immediate decisions as to the proper treatment, vaccination, or stamping-out policy. High technology based diagnosis is expected to become a routine in every diagnostic laboratory forming a sound basis for control and treatment of animal diseases.

In the short time of approximately fifteen years, animal biotechnology has moved from an unknown and unstudied science to a strong discipline of science and extensive commercial application. The science and its commercial use are rapidly progressing. The ultimate benefactor will be humans through improved food supply, nutrition and health care products available to humans around the world.

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Printed in the United States of America

ISBN: 0-8247-9439-7

MARCEL DEKKER, INC.

NEW YORK • BASEL • HONG KONG

ISBN 0-8247-9439-7

