

BIOTECHNOLOGY  
INTELLIGENCE  
UNIT

PLANT BIOTECHNOLOGY AND  
PLANT GENETIC RESOURCES  
FOR SUSTAINABILITY  
AND PRODUCTIVITY

Kazuo N. Watanabe  
Eija Pehu



Academic Press

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INTELLIGENCE  
UNIT**

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PLANT GENETIC RESOURCES  
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AND PRODUCTIVITY**

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# **BIOTECHNOLOGY INTELLIGENCE UNIT**

## **PLANT BIOTECHNOLOGY AND PLANT GENETIC RESOURCES FOR SUSTAINABILITY AND PRODUCTIVITY**

**R.G. LANDES COMPANY**  
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## FOREWORD I

Global concerns about food, environment and health have been growing continuously since World War II. Global peace must be attained by providing security of resources and metaphysical happiness to all people living on this planet beyond governmental politics, nationalities, ethnic conflicts and social matters. As technologies develop, many conventional problems of food, environment and health have been alleviated. However, the continuous increase in the global population has been providing a screaming alert on global hunger into the middle of the next century. It is true that industrialization has contributed greatly to improving the standard of living, but it is also obvious that environmental issues have emerged from industrialization. Global agricultural production has increased four times in this century. This increase in production capacity has been achieved through the development of various technologies and by intensive applications of large quantities of agro-chemicals such as fertilizers, insecticides, pesticides, herbicides, etc. In other words, high input agriculture has resulted in high output of useful products and problematic residues. Agriculture with such a high investment of resources has resulted in environmental destruction, health concerns and a prominent increase in the difference between rich and poor. Present technologies and strategies which employ them are not sufficient to overcome irreversible environmental destruction. Economic and political goodwill efforts of governmental decision makers may partially solve the problem. On the other hand, implementing various strategies employing new technologies with available resources is the key factor to meeting global needs, as well as furthering the development of super-technologies and the exploitation of resources.

Plant sciences are major subjects in food, environment and associated health issues; plant biotechnology and plant genetic resources have been utilized in innovative ways to alleviate various pitfalls. Research and development in plant sciences has advanced dramatically in the past three decades. Development of particular products has also progressed by application of plant tissue and cell culture techniques, such as micropropagation. Moreover, specific technologies and products have alleviated production constraints in agriculture by such means as biochemical and molecular diagnostics, plant hormones and biological pesticides, as well as improvement in conventional knowledge and techniques in agriculture. Exploitation of plant genetic resources provided the success of the Green Revolution in the 1960-70s. Further findings on various important traits and useful biochemical substances from plant genetic resources are expected to improve conventional crop breeding and non-conventional uses for crop breeding and pharmaceutical and food industries.

Development of genetically modified organisms by cutting and pasting genes from unconventional sources will provide vast potential for dramatically improving food production and solving environmental and health issues. However, an enormous amount of plant genetic resources still remains which is unprotected and unexplored. Investigations of genetic resources will lead to their protection and the implementation of environmentally friendly approaches in implementing R&D.

Thus, strategic discussion of the uses of plant biotechnology and plant genetic resources is important and shall provide a unique case for solving various components of global problems. This book is edited by two scientists who are very active in plant biotechnology and plant genetic resources, Dr. Kazuo Watanabe, Kinki University, Japan and Cornell University, USA and Dr. Eija Pehu, University of Helsinki, Finland. Both have made outstanding contributions to plant germplasm utilization and plant biotechnology transfer for international communities. The contributors of this book are from academic institutions, international organizations, government agencies and private biotechnology industries all over the world. This book shall be regarded as one of the best information sources on the present status of plant biotechnology and plant genetic resources. This book is unique in providing philosophy and ideas in employing plant biotechnology and available genetic resources rather than describing the technology and botany of the genetic resources, and shall be kept as a text for instructing beginners and for sharing ideas with researchers and policy makers. I trust that this book will contribute to the development of ideas on balancing sustainability and productivity in agriculture and the environment for the 21st century.

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## FOREWORD II

Global society is faced with three major challenges: hunger; environmental degradation; and population growth. Part of the solution must be found in the technologies that utilize our genetic resources to increase productivity in a manner that is suitable. "Plant Biotechnology and Plant Genetic Resources for Sustainability and Productivity" represents a major accomplishment in moving us toward that objective.

This volume is the product of a partnership among scientists throughout the world. Nations' economies are now linked to the point where we now must think in terms of one global system that includes raw materials (primarily food), value-added and processed products materials, knowledge and the capacity to access and influence the global food system. Entering a global economy also has increased our awareness of a global ecology and the interdependence of all life on Earth.

Successful solutions to complex problems require powerful collaborations of world-class scientists in a broad range of disciplines. This book brings together molecular geneticists, plant breeders, entomologists, economists, production specialists and others to treat issues related to plant biotechnology and plant genetic resources. It provides an overview of agricultural productivity, the environment and human health. It also addresses major contemporary concerns about plant genetic resources and plant biotechnologies, and reviews the present status of the utilization and/or potential utilization of plant genetic resources and plant biotechnology in representative plant species. It is a valuable resource for scientists and policy makers as the world faces unprecedented challenges in the sustainability and productivity of the global food and fiber system.

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## PREFACE

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This book is organized as a follow-up of the work by Altman and Watanabe (1995) which was published by RG Landes as the first book in its Biotechnology Intelligence Unit series. The aim of this book is to promote discussion on the strategies for the best uses of modern biotechnology and precious plant genetic resources to alleviate components associated with global constraints in hunger, environment and health. This book consists of three major sections: 1) Overview on the potential of plant genetic resources and plant biotechnology for agriculture, environment and human health; 2) major contemporary concerns on plant genetic resources and plant biotechnology; and 3) present status on availability, utilization and/or potential of plant genetic resources and plant biotechnology in representative plant species. Contributions are made from public and private research and development organizations, policy makers, technology recipients and scientists involved with plant genetic resources. Global aspects are considered and contributions are made from North and South America, Africa, Europe and the Pacific Rim. Foreword sections are written by two distinguished senior professors, Dr. A. Komamine, President of the Japanese Society of Botanical Research and Dr. W.R. Coffman, Associate Dean of the College of Agriculture and Life Science, Cornell University, Ithaca, NY, USA.

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**Dr. Eija Pehu** is a professor in agronomy and Head of the Department of Plant Production, University of Helsinki. She earned her Ph.D. in Horticulture from Virginia Polytechnic Institute and State University and M.Sc. degree from the Department of Crop Production, University of Helsinki. Dr. Pehu has maintained two interests throughout her career, one in applied plant biotechnology and crop production, and the other in international development. She has published extensively in cellular biology and genetic engineering of crops, and also in tropical agriculture and international development. Her major interests in development are the socio-economic impact of technology transfer, participatory approaches and gender dimension in development. Dr. Pehu was honored by a Distinguished Woman Leader Award from Virginia Polytechnic Institute and State University for her outstanding achievements in intellectual leadership and mentoring of young professionals.

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## CHAPTER 1

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# PLANT BIOTECHNOLOGY AND PLANT GENETIC RESOURCES: A GLOBAL PERSPECTIVE

K. N. Watanabe and K. V. Raman

The use of plant biotechnology to enhance crop productivity and sustainability is a high priority worldwide. We discuss the importance of applying this technology along with the use of plant genetic resources to provide a long-term solution for enhancing crop productivity and to promote agricultural and environmental sustainability. Other associated technologies are also discussed. The present book draws on the work of several researchers from around the world to summarize state-of-the-art research in these two areas. This chapter highlights the overall content of the book by summarizing the diverse views presented by the contributing authors and identifying some of the major global issues affecting food production.

### ALLEVIATING GLOBAL POPULATION INCREASE AND HUNGER

#### POPULATION INCREASE AND UNDERNOURISHMENT

Population increase and undernourishment continue to be of major global concern. The global human population is expected to reach 10 billion in the year 2040 with a 90% population increase in developing countries.<sup>1-2</sup> Many countries currently do not have an adequate supply of food for their population, leading to a lack of economic policy, an imbalance of resources allocation and further shortage due to continuing population increase.<sup>2</sup>

Currently, statistics reveal that the global population increase is attributed to a constant increase in the developing world, especially in Asian countries such as China and India.<sup>1</sup> With such increases in population, the production and supply of food must be balanced with the need to avoid hunger. Unfortunately, all current data in food production and land use indicate that production, supply and demand do not meet. Thus, there is a food shortage in many places of the world.<sup>3-4</sup>

Population increases are usually due to: 1) economic dependency; 2) landlessness and maldistribution of land; 3) capital-intensive instead of labor-intensive industrialization; and 4) export-oriented growth. Besides the population increase, national policies on agricultural growth and economic development alter the supply and demand of available foods within many areas of the developing countries,<sup>5</sup> and this could be considered one of the major factors causing hunger. Population growth could

be lowered by income increases or income redistribution, which might result in more economic security.<sup>6,9</sup> However, this cannot be achieved on a global basis as the factors involved are diverse and complex. Science and technology would alleviate the situation but would not be a full solution for stopping the population increase. Consistent efforts in policy-making on economics and birth control, overall development of country/region and education associated with birth control and health might also help to slow global population growth.

The imbalance between high birth and high death rates, which once essentially complemented each other and kept population growth at bay, appears now to be the main cause of population growth in many developing countries.<sup>6</sup> Improvement of living conditions, health care, education and social factors involving the role of females in the family<sup>9</sup> will contribute to population control.

A paradox of hunger exists in many developing countries. While food production is rather self-sustaining for the population, the food is often used as an export commodity to gain valuable foreign currencies.<sup>7,10-12</sup> Furthermore, food can be used as a weapon in strategic food policy in a major food-producing and exporting country against competitive/hostile food-dependent and importing nation(s). International agricultural assistance to food-demanding and developing countries often is driven by the specific interests of the industrialized nation(s). Even humanitarian aid against hunger may be influenced by such strategic decisions.<sup>9</sup> The policies and priorities made by multilateral international organizations also affect the amount and quality of aid provided to particular regions. A fair balance, without influencing the specific agenda(s) of donors and their contributions, must be the key in providing truly equitable assistance to the countries that need urgent support.<sup>13</sup>

Agricultural market economics also influence food availability by the amount and types of crops available to the poor. For example, the United States of America (U.S.A.) is one of the major food exporting countries. Fluctuations in the availability and price of a particular U.S.A. commodity, such as grains, alter the international market. Thus, increased prices could cause more hunger because it becomes impossible for poor people to afford the food since the market price is inflated. This economic problem can be exacerbated by an imbalance in the unilateral supply of certain foods to rich, food-importing countries such as Japan.<sup>14</sup> This further increases the price and decreases the availability of commodities in certain poor regions in the world.<sup>15</sup>

In accounting for the above biased policy and economic aspects, food could be available in these countries, but the people there are so poor that they cannot afford enough food and they are exposed to undernourishment. Roughly 20% of the total world population is facing such a dilemma. For example, about one third of the population in Africa (170 million) and 20% of the population (530 million) in the Far East, including China, face hunger.<sup>16</sup> In several African countries, the per capita food production has been decreasing over three decades.<sup>17</sup> This clearly implies an increase in the number of undernourished in the total population of that region.

Malnourishment continues to be a major problem, especially for infants in many developing countries.<sup>1</sup> This is mainly attributed to poverty. Technology could alleviate the components of these fatal problems. For example, vitamin A deficiency could be greatly reduced by developing cultivars such as sweet potatoes with high vitamin A content. Currently, this crop is a major staple in East and West Africa and in many Asian countries. Similarly, genetic engineering could be applied to developing crops that are high in essential amino acids, such as methionine in legumes and lysine in grains. Such crops are advantageous to infants in poor families, who depend on a limited variety of food materials. Using genetic engineering, there is also a potential for the reduction of toxic substances such as cyanides in cassava, glucosinulates in rapeseeds and glycoalkaloids in potatoes. Such crops would help avoid food-poisoning and provide more diverse uses.

## SUSTAINABILITY ISSUES

Since civilization and agriculture have existed on this planet, the rapid increase in human population has been associated with an improved ability to produce foods. The food production capacity has risen in two major ways: 1) an increase of land use and 2) an increase of the total amount of food produced per season on the presently cultivated land.<sup>6</sup>

The World Bank<sup>1</sup> estimated in 1995 that there are 1.5 billion ha of cultivated land. In 1990, farmland per person in developed countries was estimated at 1.5 ha. In contrast, in the developing countries that have 80% of the total world population, the arable land is 0.7 ha per capita.<sup>18</sup> The differences between developed and developing countries have grown over the last three decades.<sup>17</sup>

For example, China, with nearly one quarter of the world population (1.2 billion), has to sustain itself with only 10% of the total land used (150

million ha) for agriculture in the world.<sup>19</sup> In contrast, Japan, a major food importer, has 0.36 ha per capita, which is nearly three times more arable land per capita than that in China. Furthermore, this country has the purchasing power to acquire foreign food products. In contrast, China has weak purchasing power.<sup>20</sup> While these kind of statistics vary depending on the sources, even taking 50% of the reports into account shows that there is a need for more arable land per capita in China in order to increase food production.

Additional arable land in the world could be increased by 25% above the present level of 1.5 billion ha, which then would total 2 billion ha within one hundred years.<sup>3-4</sup> However, the population increase will be 100% in a quarter century or so, thus the farmland per capita will continue to decrease rapidly. This implies that land productivity must be enhanced to secure food globally. Also, improving the use of presently available land should be encouraged due to concerns about the environmental effects of exploiting new lands, which obviously destroys or at least changes natural resources. A paradox of needs in development and environmental protection is a major dilemma for all nations and communities.

The global environmental change involving weather and climate also affects food production. Factors such as global warming, desertification, natural disasters and human-caused incidences such as slash-and-burn methods of farming can seriously affect food production. The greenhouse effect may cause a rise in global temperature, leading to an increase in carbon dioxide and, in turn, to an increase in the growth of many plant species; but, this could also drastically reduce both the quantity, or biomass, and quality of substances produced from these plants.<sup>21</sup> Overall, these environmental changes could also alter food production and these ongoing environmental changes may be irreversible.

Increasing productivity in a sustainable manner is a real challenge. The increase of the food supply must be achieved by raising the performance of land area. Mechanization, use of inorganic and industrial fertilizers, pesticides, introduction of new crops and improved varieties are some major technologies that enhance productivity. On the other hand, there are many factors that could reduce the productivity of land. A few major ones are: 1) land degradation attributed to erosion and agro-chemical use; 2) energy costs; 3) genetic erosion and uniformity of crops; and 4) international and national policies.<sup>3-4</sup>

Providing that the fertility of the land and the availability of technology to augment the produc-

tion were the same as the world average, many countries such as China would still need to have more arable land or increase productivity per land area to be self-sufficient food producers. Besides this rough estimate, various other factors influence the productivity of the land. These factors can be minimized by providing cash for purchasing foreign food products. On the other hand, the capital available for importation is very limited to cover the gap between supply and demand within a country.

### PLANT BIOTECHNOLOGY ASSISTS SUSTAINABILITY AND ENVIRONMENTAL PROTECTION

In addition to food production, environmental and sustainability issues are also major global concerns. The use of agro-chemicals and fossil-energy-dependent production systems does not promote sustainable cropping systems. Sustainability can be achieved by the application of new knowledge, resources and technologies, such as the use of plant biotechnology. However, economic policy and social aspects greatly influence the movement toward such synchronization of productivity and sustainability. It now appears that the contribution of new science and technology plays an important role in this area. According to Caldwell:<sup>22</sup>

Science now has a significant role in shaping perspective and priorities on life and biosphere. The influence of science is often indirect and its movement throughout the various sectors of society and throughout the world has historically been slow and uneven. There are indications, however, that response to scientific findings has been accelerating.

Plant biotechnology could provide tools which meet with research and development (R&D) in the major components of sustainable agriculture and natural resource management. These tools include: water (and watershed) management; soil quality and erosion (nutrient, salt, drought, residue fallow, crop, and erosion); agro-ecosystem; integrated nutrient management for crop protection; and integrated pest management (IPM).<sup>23</sup> Especially in the IPM area, bio-pesticides would further facilitate sustainability and environmental protection. These bio-chemicals include mycoinsecticides, symbiotic nematode-bacteria complexes against insects, a toxic protein to insects by *Bacillus thuringiensis* bacteria, myco-herbicides, insect viruses (=baculoviruses), and symbiotic rhizobacteria.<sup>23-24</sup> These bio-control agents are specific to the pest and have no side effect on the

environment or other organisms, including humans. Furthermore, these agents do not leave residues in the environment or on agricultural food products, and they are regarded as environmentally friendly and safe.

Thus, plant biotechnology is helpful for maintaining sustainability and protecting the environment. This is mainly attributed to two areas: 1) reduction of agro-chemicals through the use of proper management strategies in agriculture, together with improved cultivars which are generated via biotechnology tools; and 2) monitoring the environment by different diagnostic tools to assist in the reduction of pollution and associated environmental changes. Productivity can be promoted and, simultaneously, sustainability can be enhanced using plant biotechnology.

It must be admitted that the overall enhancement made by various technologies facilitated the world increase in grain production.<sup>25</sup> This lowered commodity prices due to saturation of the market. However, this does not reflect a meeting of real needs, but a meeting of the demands of capital-rich nations. A global imbalance of demand and supply in the food markets may be favored by ample supplies. On the other hand, avoiding worldwide hunger would involve different issues. Political and economic aspects at the global and regional levels are important in determining who will have the food.<sup>7</sup> However, emerging constraints on natural resources and environmental issues in conjunction with increasing world population, especially in developing countries, continue to be major concerns. Even when these issues are favorable, the population structure in many societies changes toward a greater proportion of seniors than juveniles, and this reduces the availability of labor.<sup>26</sup> Such a change also hampers the production system, with a decrease in the proportion of the younger generation that engages in food production.

## **TECHNOLOGY AND ITS ROLE IN INCREASING FOOD PRODUCTIVITY**

Progress in conventional technology is considered crucial for increasing food productivity and availability. However, advances in conventional technology will not be adequate to meet the demands placed on agriculture. A reorientation is needed in order to realize the opportunities for technical change opening up through advances in microbiology, biochemistry and biotechnology.<sup>27</sup> Hereafter, we focus on topics associated with crops, plant biotechnology and genetic resources, which are the major subjects of this book. We will not discuss in depth animal husbandry or fishery, which are also important food sources.

Increased productivity or yield per area can be achieved at a certain level by presently available technologies and strategies such as crop management, chemicals and breeding.<sup>28</sup>

Crop management can be easily enhanced in communities with a modest educational level and on a large scale with commercial growers. However, its success depends on: 1) the availability of resources including infrastructure such as machines; 2) the number and level of extension specialists; and 3) long-term support of policy makers and the public sector. This approach requires specific knowledge and extension programs which address marginal self-sustaining farmers, and would not apply immediately to poor people.

When considering concerns of the effects on the environment and health, together with increasing regulations on agro-chemical use, the use of agricultural chemicals is not expected to be an emerging solution. In addition, hunger is associated with poverty, and thus the majority of self-sustaining farmers who need to boost their food production would not be able to afford such agro-chemicals. Thus, this only applies to resource-rich areas/communities, and would not help the poor regions which actually need more food.<sup>29</sup>

Present science, art and technology in breeding could meet the requirements of needed agricultural food production and safeguard the environment.<sup>29-30</sup> Plant breeding helps alternative agriculture and environmental quality by reducing the use of agro-chemicals that influence water and soil and leave residues in crops and, consequently, humans.<sup>31</sup> Furthermore, plant breeding will reduce the use of fossil petroleum products in present agricultural systems by employing available energy-efficient varieties. It can also provide energy such as biomass for biogas, bio-alcohol and oil from seeds.<sup>32</sup> Thus, plant breeding could provide more farm profitability and yield stability, improved food quality and safety, as well as better environmental quality and safety and erosion control.<sup>28</sup> Newly bred cultivars meet the changing requirements of the agriculture system and various consumers. Consequently, the development of new cultivars assists both food productivity and environmental sustainability.

With the appropriate combination of other conventional technologies and approaches, breeding would strongly impact food production. Indeed, the Green Revolution of the 1960-70s was mainly due to the creation of high-yield cultivars, an appropriate crop management system and agro-chemicals.<sup>33</sup>

It should be noted that the use of plant genetic resources has contributed greatly to breeding and developing new cultivars.<sup>34-35</sup> The best cultivar examples are those used in developing countries during the Green Revolution and including the semi-dwarfing genes, *Rht1* and *Rht2*, from Japanese landrace wheat, which helped increase production in many developing countries, and *sd-1* from a Taiwanese rice variety, which contributed to a significant increase in rice yield in many Asian countries. The details of such contributions and future prospects of plant genetic resources are presented in chapter 4 by Rao and Iwanaga. In relation to another major topic of this book, plant biotechnology, it should be noted that plant genetic resources and their management are now greatly associated with the application of plant biotechnology. This is addressed in a latter part of this book as well by Rao and Iwanaga.

Breeding or cultivar development can be further boosted by the appropriate use of genetic resources and biotechnological tools. These tools are reviewed by Altman and Watanabe,<sup>36</sup> Gatehouse et al,<sup>37</sup> Kung and Wu,<sup>38-39</sup> Skerritt and Appels,<sup>40</sup> and Stacey and Keen.<sup>41</sup> Specific examples on commodity plant species are given in this book. They include: rice, chapter 7; grain legumes, chapter 8; maize, chapter 9; potato, chapter 10; and Andean crops, chapter 11. Some examples are also given in locally or internationally important plant species in industry including: tobacco, chapter 12; flowers, chapter 13; date palm, chapter 14; oil seed rape and vegetable *Brassica*, chapter 15; medicinal plants, chapter 16; and trees, chapter 17.

Biotechnology offers various ways of altering basic mechanisms of organisms and it provides the means of developing crops for specific environments. This is a major departure from traditional agriculture where the environment is tailored, as far as possible, to suit the crop.<sup>42</sup> Biotechnology, especially its major component, genetic engineering, could result in an improvement of crops via: 1) quality control; 2) enhancement of nutritional availability; 3) pest and disease resistance; 4) herbicide resistance; 5) increased productivity; and 6) tolerance to environmental stresses such as drought.

As an extreme example, sucrose phosphate synthase (SPS) gene isolated from corn<sup>43</sup> could boost the yield 30-fold in potatoes by inserting the gene via genetic engineering.<sup>44</sup>

Plant biotechnology also contributes to pre-harvest and post-harvest technology, including storage, processing and transportation systems. Losses of food, such as perishable horticultural products lost during and after the harvest due to

improper management and their short lifetime, can be avoided with the use of plant biotechnology. In addition, biotechnological approaches such as genetically manipulating the ripening/decay of fruits and vegetables could significantly increase the portion of edible harvest and aid storage of such perishable materials under harsh, resource-poor conditions. Now, many genes which contribute to delayed ripening technologies using anti-sense technology are available in plant biotechnology, and products such as the Flavr Savr<sup>TM</sup> tomato have been commercialized by Calgene in the U.S.A.<sup>45</sup>

## PLANT BIOTECHNOLOGY: AN AID FOR INCREASING FOOD PRODUCTIVITY AND ENHANCING ENVIRONMENTAL PROTECTION

Plant biotechnology has been one of the most important topics with respect to food security, environmental issues and health concerns. With the overall development of biotechnology and its application to agriculture, present plant biotechnology could alleviate various global problems in sustainability, environmental health and productivity in agriculture. The Green Revolution in the 21st century has been predicted due to advances in agro-biotechnology.<sup>36,46</sup> The specific role of the technologies in augmenting food production is presented in chapter 3 by Persley. Once these technologies are shared among various communities and nations, the impact could be far greater than that seen during the Green Revolution of the 1960s.<sup>36,46-48</sup>

## GLOBAL EXPENDITURES ON BIOTECHNOLOGY

The resources invested in 1995 in the exploitation and development of biotechnology could be estimated at US \$40 billion worldwide.<sup>49</sup> The investment in biotechnology in Japan was estimated at US \$8 billion in 1995; this is about 20% of the total expenditures in the world. North American investment for 1995 was estimated at US \$20 billion, which is 50% of the global expenditures in biotechnology. In Europe, 30% of global expenditures, equivalent to US \$12 billion, were invested. The private sector has been investing more than half of the total and has been making significant progress toward commercialization of biotechnological products for the global market.

While an enormous amount of funds has been invested in plant biotechnology, the developmental outcomes such as products/uses, are limited due to issues of policy making, regulatory issues, safety concerns, patenting and bioethical aspects. These aspects are discussed by Persley in chapter 3.

### THE STATUS OF PLANT BIOTECHNOLOGY IN U.S.A.: A FEW EXAMPLES

The commercialization of genetically engineered organisms (GMOs) is prominent in the U.S.A., and many agricultural products derived from genetic engineering are now marketed widely there. Some examples are: 1) the Flavr Savr™ Tomato made by modifying the gene expression for ripening; 2) herbicide resistant soybeans made by inserting the resistance gene; 3) canola oil made by genetically modifying biochemical pathways to decrease saturated fatty acid contents; 4) "self-vaccinated" vegetables for virus resistance; and 5) potatoes, corn and cotton with insect resistance derived from bacterial genes. Several other crops are now in the final stages of approval.<sup>24</sup>

### STATUS OF PLANT BIOTECHNOLOGY IN JAPAN

Japanese authorities are now considering importing genetically engineered plants and products for commercial markets in Japan. The products from the genetically modified soybean with built-in herbicide resistance developed in the U.S.A., have been approved for importation. Thus, safety issues and bioethics associated with GMOs are not only concerns in the U.S.A. but also in Japan. Japanese regulatory agencies have their own policy for work in this area. The Japanese public has a modest exposure to the matter via various media. The Japanese public and governmental regulatory agencies have their own view on agro-biotechnology and most often the public in general is unaware of the risks and benefits of this technology. In the U.S.A., the media and other public awareness programs have greatly facilitated the public release of products. In Japan, public awareness and further understanding of the necessity of GMOs need to be enhanced by exchanging experiences involving different authorities, scientists, industry and consumers from other countries.

### FACILITATING AND PROMOTING REGULATORY ISSUES AND USE OF PLANT BIOTECHNOLOGY IN DEVELOPING COUNTRIES

Plant biotechnology has been used for research purposes in modestly developed countries neighboring Western Europe, North America (U.S.A. and Canada) and Japan. Often countries such as Mexico, Philippines, Thailand, Malaysia etc. have been exporting agricultural products to biotechnologically developed countries. The products made by biotechnology in these modestly developed countries could be of major concern to the public in the technologically advanced nations as well as to the domestic consumers in the developing countries. A few developing countries have estab-

lished their own biosafety regulations regarding the environmental release of GMOs and food safety. The Convention on Biological Diversity and several other international agencies are attempting to promote agreement on biosafety guidelines at an international level. An international enactment of regulatory policies could, therefore, take place soon. However, there is a need to share knowledge about the risks associated with the use of this technology, and open discussions on safety need to be encouraged. Experience and alternative views could be provided to interested developing countries concerned with understanding this technology from Europe, North America, and Japan through various interactions.<sup>24,36</sup>

### ADVANCES IN PLANT BIOTECHNOLOGY

The classical use of biotechnology has been the day-to-day use of fermentation technology using microorganisms. This type of biotechnology was been discovered in and has been utilized since prehistoric times. A common definition of biotechnology could be adapted from the following statement by the Crop Science Society of America: "Development of products by a biological process requiring engineering technologies, such as fermentation or controlled environments, or utilizing current technologies (such as recombinant DNA techniques) for the modification and improvement of biological systems."

Plant biotechnology can be divided into four groups: 1) Cell, tissue and organ culture; 2) diagnostic tools; 3) molecular markers; and 4) genetic engineering.<sup>36</sup> In addition, biological agents such as biopesticides could be included as supplementary components with plant biotechnology. The present status of R & D differs among these four groups. However, there is has been a gradual shift toward finding applications in development. A particularly good example is found in the area of vegetative propagules of commercially used plant species: micropropagation by clean in vitro facilities has boosted pathologically healthy, uniform and high-quality seedling production in many vegetatively propagated plant species such as potato, asparagus, trees, etc. Together with diagnostic tools such as virus indexing by ELISA (enzyme-linked immunosorbent assay) or NASH (nucleic acid spot hybridization) kits,<sup>40</sup> the commercial production of many vegetatively propagated species is becoming a reality, at least in the developed world, and now modestly developed countries are also adapting such technologies for their industrial development.<sup>36</sup>

Diagnostic tools such as NASH and ELISA help monitor pest and disease infestations in crops and improve genetic resources with samples from seed

programs, production fields, storage facilities, markets, etc. These could provide parameters for the precise uses of pesticides or alternative approaches for pest control. Furthermore, detection kits for agro-chemical residues and for naturally occurring toxins which exist in soil, water, field and crops are available in the form of polyclonal and/or monoclonal-antibody based kits.<sup>40,50-52</sup> The mechanism of these kits is simple and does not require expensive equipment such as high performance liquid chromatography (HPLC), and thus applications could reach small subsistence farmers. These kits give appropriate uses of chemicals and thereby enhance environmental health. The food safety of agricultural products is also guarded by such detection kits. Natural and other toxins are identified and this facilitates the commercialization of healthy toxin-free food.

Molecular markers based on nucleic acids are now essential tools in plant breeding. These markers assist in the introgression and selection of genetic traits, the management of plant genetic resources and in measuring biodiversity.<sup>53</sup> With the applications of molecular markers, plant breeding gets revived for the 21st century. This will greatly facilitate crop genetic improvement processes and reduce associated costs.<sup>54</sup>

Molecular markers based on polymerase chain reaction (PCR) provide further acceleration in breeding and improve genetic resources management in efficiency due to their simple application and cost effectiveness.<sup>55-56</sup> Safer use of molecular marker technology was facilitated by non-radioactive probing and blotting of such markers.<sup>57</sup> These techniques now make the adoption of technology much easier to various programs including those in the developing world, as well as those in safety-conscious communities in developed countries. PCR-generated markers enhance genetic resource management very rapidly by allowing a more efficient collection of genetic information essential for crop improvement.<sup>53</sup>

Genetic engineering is still at an experimental stage compared with the three above areas, and requires that several major issues be resolved before a large scale application of product use can occur.<sup>39</sup> Current work could provide new ways of using artificial genes to alleviate various problems in crop production and contribute indirectly to solving environmental problems associated with modern agriculture (e.g. reducing agro-chemicals by generating pest-resistant cultivars).<sup>38-39,58</sup> However, the release of such products has been slow and only recently have a few products been successfully commercialized.

Plants can also be used as sources for production of useful chemicals such as edible vaccines against human diseases; for example, potatoes and bananas can produce vaccines by the insertion of artificial genes.<sup>59-60</sup> Thus, genetic engineering could contribute directly to improving human health, especially in poor regions which stagnate due to various political, social and economic factors.<sup>61</sup>

#### FROM A LABORATORY TECHNOLOGY TO A USEFUL AND PUBLICLY ACCEPTED TECHNOLOGY

The utilization of genetically modified organisms (GMOs) produced from genetic engineering is still in an initial phase in plant biotechnology. However, the innovation of incorporating non-host genes from totally different organisms should not be underestimated and should be considered as an alternative option for genetic improvement besides the use of conventional genetic resources from related species.

Rapid development of this technology has also led to further advances in many areas such as transforming particular plant species, which was impossible until only recently.<sup>62</sup> This basic research area is now becoming industrialized, at least in the U.S.A.<sup>63</sup> While conventional plant breeding significantly improves productivity, further improvement in plants is essential to boost not only productivity but their balance in continuity for the utilization of varieties. In addition to technical issues, there are several major political and social aspects that must be addressed here for plant biotechnology to be useful and accessible to the public.

#### Regulations/Safety issues

The commercialization of genetically engineered organisms (GMOs) is prominent in the U.S.A., and a few agricultural products derived from GMOs are now marketed widely there.<sup>24</sup> A more gradual approach is taken by the Europeans, and an even slower and cautious approach by the Japanese. Many developing countries are now developing their own regulatory policies or are being persuaded to accept these products due to necessity in importing the goods.

In the U.S.A., several agencies have been involved in getting approvals on safety issues concerning agricultural products developed by recombinant DNA methods, including public hearings to acquire an understanding of general public concerns. Details on some of these regulatory aspects are referred to in Altman and Watanabe,<sup>36</sup> Krattiger and Rosemarin<sup>64</sup> and Tzotzos.<sup>65</sup>

## **Intellectual Property Rights (IPR)**

One of the pitfalls in the utilization of this high technology and its transfer is attributed to issues of intellectual property rights, particularly on patents of proprietary technologies claimed by the private sector. This issue is a common concern for many parties, including international aid organizations.<sup>66,67</sup> Lesser discusses this in detail in chapter 5. Several cases involving IPR on biotechnology have been presented and the scientific community has expressed concerns on R&D associated with this technology.<sup>68</sup> Resolving IPR issues is now a major priority and several developing countries are emphasizing training on patents, royalty, negotiation and franchising.

## **Social Concerns**

Risk perception and ethical issues associated with exploitation and use of biotechnology are discussed by Macer in chapter 6. These aspects are important for the delivery and use of biotechnology in order to face and alleviate the present productivity and sustainability problem.<sup>69</sup> The public sector has been surveyed about their concerns about biotechnology, and overall opinion polls have shown that this sector has not yet accepted this technology.<sup>70</sup> The private sector also independently provides open information and discussion in various media; for example, Ciba-Geigy, recently debated issues and ethics present in genetic engineering.<sup>71</sup>

In addition to governmental and commercial efforts in safety issues, the National Agriculture Biotechnology Council (NABC), an independent non-profit organization hosted at Cornell University, NY, U.S.A., has periodically provided opportunities for public discussion.<sup>72-73</sup> NABC consists of universities, concerned scientists, growers, industry, NGOs and consumers. NABC forums on public concerns have focused on agricultural biotechnology related to biosafety and food safety issues on GMOs. Furthermore, bioethics associated with science and development are also a main subject of the council.

Additionally, an international consortium approach modeled after NABC has been formed.<sup>72-73</sup> The principal objectives are as follows: 1) to provide an open forum for persons with different interests and concerns so that they may come together to speak, to listen, to learn and to participate in meaningful dialogue and evaluation of the potential impacts of agriculture biotechnology; 2) to define issues and public policy options related to biotechnology in food, agricultural and environmental areas; 3) to promote increased understanding of the scientific, economic, legislative and

social issues associated with agricultural biotechnology by compiling and disseminating information to the interested public; 4) to facilitate active communication among researchers, administrators, policy makers, practitioners and other concerned people to ensure that all viewpoints contribute to the safe development of biotechnology for the benefit of society; and 5) to sponsor meetings and workshops and to publish and distribute reports that provide a foundation for addressing issues.

Giving plant biotechnology and its products to any community presents a complex challenge. An integral part of this challenge are biosafety and food safety issues. Biosafety, food safety and the regulation of biotechnological activities have been at the forefront of the biotechnology debate for almost a decade now. Discussions on biosafety issues have been going on in many arenas at the same time. This has led more than once to the development of quite divergent opinions, depending on which professional body is most prominent in a particular group of experts. The result is confusion in the mind of policy makers, technology developers and end-users, who are confronted with contradictory data, advice and feelings. The solution to this state of affairs can only come through more interaction between members of different professional bodies and non-professional end-users from the general public involved in the debate, and by improving the information given to policy makers on all aspects of the development of agricultural biotechnology and its impact on agriculture, health, the social structure and the environment.

This and other initiatives promote the exchange of information and focus on: 1) existing national and international efforts at regulating agricultural applications of biotechnology; 2) the interaction between potential risks and potential benefits (including the issue of biodiversity and the environment) of agricultural biotechnology; 3) the state-of-the-art in the development of the technology on one hand and its applications on the other hand; 4) the actual definition of biosafety, food safety and tests that are involved; and 5) sharing public opinions on scientific information among general public/end-users of such biotechnology products.

Perhaps, most important of all is an understanding of the interactions between the issues mentioned above and the ramifications of events of developments in one area into what is often conceived as a totally different area of activity.

A serious delay in any one of these areas can prevent advancement in other areas. The impact of

the regulatory framework on other areas is substantial. It not only affects the timelines of development but also the direction.

### **Technology Transfer**

Various factors could influence the establishment of biotechnological capacity in many developing countries such as policy, technology recipients, societies, infrastructure, capital, investment, resources, educational levels and national will. These factors are discussed in Altman and Watanabe.<sup>36</sup> Efforts to institutionalize agricultural research capacity in developing countries must be intensified and there is a need to establish substantial basic biological research and training programs in tropical developing countries.<sup>27,74-76</sup> However, these efforts may take time since future progress will depend on an international agreement on regulatory issues on GMOs and the Convention on Biological Diversity. A few authors believe that the CBD may function in a negative way; thus, the transfer would not be made easily.<sup>77</sup> Feasibility and applicability studies are now under way in many developing countries to provide comprehensive information on plant biotechnology to end-users or small farmers. Thus, plant biotechnology will meet the needs of small farmers at the grassroot level involved in agricultural and environmental protection.<sup>78,79</sup>

### **USING PLANT GENETIC RESOURCES WITH PLANT BIOTECHNOLOGY: A SUPER HYBRID FOR THE 21ST CENTURY**

The value and management of plant genetic resources are discussed by Rao and Iwanaga in chapter 4. The best matching area of plant biotechnology with plant genetic resources is in vitro preservation and use of molecular markers in monitoring genetic diversity. First, the conservation of precious genetic resources can be facilitated by various means of plant biotechnological tools, including low cost tissue culture, organ and cell culture, cryoconservation and preservation of particular genes isolated from such important germplasm.<sup>80-81</sup> Second, molecular markers facilitate the study of genetic diversity in nature, identification of duplicates, and the establishment of a genetically representative core collection.<sup>81</sup> Through genetic resources utilization, these molecular markers could facilitate breeding processes in areas such as time, labor and resources, as well as in plant quarantine requirements.<sup>53,55</sup>

The use of genetic resources has been widely discussed and has been found to be very valuable.<sup>33-35,82</sup> Many of the plant genetic resources

could also result in the production of deleterious or unnecessary genes for cultivar development; thus, plant breeding has been engineered for introducing valuable traits while simultaneously eliminating the exotic/wild genes. Previously, it used to take decades to make a single cultivar. With advanced new tools in plant biotechnology, this process has been greatly shortened.<sup>34-35,82</sup>

The systematic management and use of plant genetic resources by the application of plant biotechnology tools were identified by Dodds and Watanabe at the beginning of the 1990s,<sup>83</sup> and are now widely valid. Various biotechnology tools are applied to crop genetic improvement and these have helped generate far more improved cultivars in a shorter time with more genetic precision, compared with the decades of time taken for cultivar development during the first three-fourths of this century.<sup>84</sup> Specific examples in commodity plant species are given in later in this book.

The social problems of intellectual property rights of the plants are presented by Lesser in chapter 5. However, this section should re-emphasize this aspect. Because of the useful and precious value of plant genetic resources in commercial development, movements towards privatizing genetic resources and genes derived from them have been rapidly taking place.<sup>85</sup> Social issues, especially international transfer of germplasm and the proprietary rights associated with plant genetic resources, are a major aspect of the Convention on Biological Diversity, with no consensus reached yet.<sup>86</sup> Member countries need to cooperate in the conservation of biodiversity and to use genetic resources sustainably and equitably. Careless exploitation and development of such precious genetic resources especially in situ would destroy environments and restrict the traditional communities which have depended on them for ages.<sup>87-88</sup>

### **CONCLUSIVE SUMMARY**

The future for biotechnology looks bright. Several new products are now starting to appear on the market. These, in conjunction with conventional technology, increase food, feed and fiber production to meet the growing demands of a burgeoning global population that will reach 11 billion by 2050. In this chapter only the main global issues affecting biotechnology and sustainability have been highlighted. Other chapters in this book summarize the different biotechnological opportunities that can be exploited in different food crops.

The big question which still remains to be answered is: Shall we realize gains and benefits from plant biotechnology in international

agricultural research? While a case has been made that the application of plant biotechnology can contribute directly to agricultural and social objectives, national financing and planning decisions are required in order to bring these to fruition. The cost of such development is high. The number of applications adopted for development must be realistic. Without such control, numerous activities will be started and only a few will be developed. This will jeopardize future investment and support for plant biotechnology.

Recently, a global plan of action was developed for the conservation and sustainable utilization of plant genetic resources for food and agriculture.<sup>89</sup> The plan focuses on in situ and ex situ conservation and development, utilization, institution and capacity building. The cost of implementing these plans to save and develop crop germplasm is tentatively estimated between US \$130.6-303.8 million averaged over 10 years, depending on the determination of national and international responsibilities, the extent and duration of efforts undertaken, each country's financial situation and genetic resource capabilities, among many other factors and considerations, all of which are under continuing deliberation.

The range of possibilities that plant biotechnology and genetic resources offer is enormous, but in the near future the most important contribution that biotechnology can make is to increase the quantity and quality of global food, feed and fiber. It is also clear that this new technology alone will not be sufficient to address agricultural growth. In order for developing countries to absorb these technologies, they have to look beyond increasing land and/or cropping intensity for more productive agriculture. In this regard, sustainability can provide a context for biotechnology, as long as both are built on a well-maintained foundation of conventional agricultural research. Facilitating the development of biotechnology by creating capacity in people, technologies and institutions provides an opportunity for catalyzing new knowledge and innovation as well as equal partnerships, which in turn provides a basis for interdependence and sincere international collaboration.<sup>36</sup>

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## CHAPTER 2

# FOOD SECURITY, ENVIRONMENTAL ISSUES AND HUMAN HEALTH: A POTENTIAL WITH BIOTECHNOLOGY AND PLANT GENETIC RESOURCES WITH ECONOMIC, SOCIAL AND POLITICAL VIEWS

E. Pehu and M. Rojas

### INTRODUCTION

The fundamental question on population growth and food supply is: is it fundamentally the earth or humanity that produces food? If the former, we can already see signs of approaching catastrophes, if the latter, judging by history, there is a reason to believe that world farmers will succeed.<sup>1</sup> Today the world is home to 5.7 billion people and produces food for all, even if it is unequally distributed. But the challenge ahead is tremendous: annual gain in world population is 90 million people. Loss of top soil has been estimated at 25 billion metric tons. It has been further estimated that by 2050 the land area supporting the food needs of one inhabitant is 0.14 ha compared to 0.44 ha in 1961.

Crop production increased up until 1985 when it peaked and started to decline, while an increase in livestock production was maintained up until 1992 after which it also declined, especially in Africa and the Near East. Reasons for the decline include depletion of water resources due to imbalances in hydrological cycles, reduction in soil fertility due to overgrazing or 'mining' of nutrients and the fact that crop pests are becoming resistant to pesticides. All of these factors call for urgent attention to reduce pressure on the limited resources needed for food production.

Rapid population growth imposing growing demands on natural resources in food-deficient countries leads to difficulties in sustaining democracy and economic viability. Population growth projections suggest that in about 50 years countries with large populations and fast growth rates like Kenya, Pakistan and Bangladesh will not be able to feed their inhabitants without the methods of modern, intensive agriculture.

Interestingly, the three most quoted recent 'basic scenario' studies on global food supply all agree that an increase in agricultural production will meet the need of increased population pressure up to 2010.<sup>2-4</sup> All of these studies also call for regional and national solutions. One such solution is that the World Bank develops national economies in order to maintain economic growth. IFPRI suggests operationalizing this goal by improving physical and financial infrastructures and by adopting technological

innovations to improve national food production systems. Local adoption is even further stressed in the FAO strategy.

In addition to the basic scenario, there are also three others proposed by IFPRI: 1) low-population-growth scenario; 2) low-investment/slow-growth scenario; and 3) high-investment/rapid-growth scenario. Numbers 2 and 3 are of interest regarding the role of agricultural research. In scenario 2, investment in agricultural research is predicted to dramatically decline; this leads to a reduction in cereal and animal production and subsequent repercussions on non-farm income and investments in public health and education with a negative impact specifically on women's lives. In scenario 3, the assumption is that funds for national and international research institutes increase, especially support for biotechnology and plant breeding programs. Yields increase by 6% compared to the basic scenario. Part of the increased public spending is channeled to improving public health and social programs, which is reflected, for example, in increased education of girls. The education of women, in turn, has been widely recognized to reduce population growth. The most prominent beneficiaries would be China, Latin America, Western Asia and North Africa.

If we believe that agricultural research has such a significant role in reaching food security, what kind of research does this entail? A second round of the Green Revolution or an entirely novel approach? However, before discussing the role of research, it is important to address the question of food security.

## **FOOD SECURITY**

One of the definitions of food security states that it is a condition in which all people at all times have access to the food they need for a healthy, active life. Food security has four dimensions: availability, accessibility, safety and reliability.

Factors contributing to food security include biological, technological, ecological and political factors. Environment, soil, nutrients, varieties and crop management require skills and knowledge in planning and farming. Past increases in cereal production have largely been a result of new varieties, increased farmland and improved crop management. Success, however, depends on the most limiting factor, which can be in any of the spheres mentioned: nutrient depletion, drought, pests and disease outbreak, military conflict, lack of credit, lack of marketing infrastructure, etc. Rapid urbanization increases the challenge for food security: transport, storage, processing, packing and marketing. The mere increase in food production is

not sufficient as road and marketing infrastructures are also required.

There are tremendous regional differences in food security. While the global average in per capita food availability has increased, in Africa, population growth rates of 3% have outpaced food production gains since 1981 and one-third of Africa's population is estimated to be malnourished. Ironically, the natural resource base of the already troubled areas is severely limiting: two-thirds of the most degraded land is in Africa. Depletion in organic matter and micronutrients leads to reduced yields even if macronutrients are supplied as fertilizers.

Therefore, in agreement with Engelman and LeRoy,<sup>1</sup> we argue that the strategy to cope with this situation is three-pronged: it calls for technological innovations to increase agricultural production, the conservation of natural resources and the stabilization of population growth.

## **INCREASED CROP PRODUCTION**

Increase in crop production can come from the extension of arable land or the intensification of cropping on existing farmland. Since the 1960s, the increase in cereal production has mainly been due to increased yield, accounting for 98% and 92% of yield increases in developed and developing countries, respectively. However, yield increase has only contributed 52% of the cereal production increase in Sub-Saharan Africa.<sup>5</sup> The possibilities of extending farmland in the future are diminishing, therefore yield will become the primary factor in the growth of food production.

The Green Revolution doubled cereal yields in 30 years from 1961 to 1990. There are, however, now biological, environmental and socioeconomic constraints reducing yield increases. These include salinization of irrigation water, pest-resistance, depletion of micro-nutrients, lack of funds to purchase inputs, lack of input distribution and product marketing infrastructures, etc. All of these constraints are specifically prominent in zones with low agro-ecological potential such as Sub-Saharan Africa; while yield increases have been to the tune of 2.5% on average in developing countries, they have only been 0.8% in Sub-Saharan Africa. Another issue in Africa is that local food security is largely dependent on secondary cereals (sorghum, millet) and root and tuber crops, while the Green Revolution varieties were developed for major cereals.

It can be concluded that the Green Revolution as implemented in its first generation has limitations. Thus, agricultural research has to meet the global challenge of food production in other ways as well.

## DIRECTIONS FOR AGRICULTURAL RESEARCH

The technology transfer oriented 'Green Revolution' approach still has a role in areas where ecological and social infrastructures can support it. However, in marginal areas with environmental constraints and lack of required infrastructure, it has to be complemented by participatory technology generation approaches.

Examples of the past five years of development assistance have shown that joint natural resource development approaches with participation from rural communities in planning, implementation and evaluation are the key to sustainability. This should be reflected also in any research conducted. In crop production it means that if sustainability is added to yield improvement research, it has to shift its focus from plant breeding and crop management to interface with ecology and socioeconomics.

To arrive at this location, specific solutions for livelihood strategies and risk management prioritizing research questions should be reached together with the people of the concerned communities. This requires that researchers develop skills in communication and sociological aspects of development.

## WHAT CAN BIOTECHNOLOGY OFFER IN THIS CONTEXT? IS "GENE REVOLUTION" THE ANSWER?

"Biotechnology and genetic engineering are among the most effective solutions for achieving food security in low potential zones"<sup>6</sup> through better adaptation to environmental stresses such as drought, salinity, pests and diseases.

Several engineering strategies are being explored to improve crop plants against environmental stresses such as drought, salinity, high temperatures and frost. Similarly, resistance strategies against pests and diseases are the focus of many research groups. The first of these innovations has reached the marketplace and many more are coming in the next 5-10 years.

Biotechnology is also a tool for analyzing and maintaining biodiversity. It is often claimed that genetic engineering would further narrow the genetic basis of crops grown. It is important to note, however, that most varieties can be engineered and that this is one of the potentials of transgenic technology. The original variety accepted by the consumers can be improved by the gene of interest without altering the phenotype otherwise. In conventional breeding, the incorporation of traits and further breeding changes the genetic composition of the breeding line and requires several generations of further selection or backcrossing.

Thus, there is no technical reason why genetic engineering would reduce biodiversity. However, the problems are institutional and socioeconomic. The questions are: are funds available for molecular biology research of food crops internationally? Are there active national programs on food crops which could absorb the engineered lines in multiplication and breeding, or engineer crops themselves?

## RESPONSE OF NATIONAL RESEARCH SYSTEMS TO THE NEW CHALLENGES

As indicated earlier, the CGIAR system was a key player in developing Green Revolution varieties of cereals, while less emphasis was placed on other crops. Now, facing and recognizing the limits of the Green Revolution and also being challenged by the donor community, the objective of the international centers has become to use international research expertise in partnership with national research programs to contribute to sustainable improvement in crop productivity, especially for low-income groups.<sup>7,8</sup>

What is the situation in national research programs? In the recent past, many developing countries have invested in the research of high value export crops at the expense of food crops. Many traditional self-sufficient economies have thereby shifted toward intensive cash crop production. In addition to economical and political implications, this has also affected the role of women as farmers in rural Africa. Farming has shifted from women to men as mechanization has intensified and less food has been planted which requires cash for purchase of food. Moreover, this development has had serious implications to the focus of national research programs, which may neglect research on food crops and feature a commodity-oriented research agenda.

As a large share of the cash crops has been produced in large scale, the suppliers of technical knowledge and its users have only involved a small portion of farmers. Many national research programs are weak and overstaffed with little money for other than salaries. Similarly, national extension services extending information on food crop production have been poorly funded and largely ineffective.<sup>9</sup> It is important that resources be allocated to strengthen both institutional capacity as well as human capacity. It is, however, important that the size of national programs not extend to the point where they are research facilities without programs.<sup>10</sup>

In both the international research centers and the national programs, the need to integrate sustainability into natural resource management

research is being recognized. Furthermore, the importance of public health to productivity has become a central issue.<sup>11</sup>

### NATIONAL CAPACITY IN PLANT BIOTECHNOLOGY?

Significant progress has been made in national research programs to include biotechnology capacity. National Centers for Genetic Engineering and/or Biotechnology have been established in Cuba, Nigeria, Sudan and Thailand and national biotechnology programs have been initiated in Argentina, Brazil, China and Egypt. The Republic of Korea issued a new patent law in 1987 to help encourage the commercial utilization of research results of the Korean Genetic Research Association.<sup>12</sup> The most constraining is the human capacity basis in biotechnology in Sub-Saharan Africa. By 1991, there were only 106 people trained in biotechnology in the whole region.<sup>13</sup> A good illustration of the situation is Nigeria, a country of 90 million having only 10 scientists trained in gene cloning.

### ROLE OF BIOTECHNOLOGY

#### IN SUSTAINABLE AGRICULTURE

Biotechnology has already shown its potential in crop improvement. It is also a powerful tool for developing food production systems at a reduced cost to the environment. A review of field tests of transgenic plants shows that pathogen/pest resistance, herbicide tolerance and food quality account for 80% of the field testing permits issued globally by 1992. The remaining 20% covered topics of mainly non-food plant development.<sup>14</sup>

The first generation of biotechnology developed techniques in tissue culture, which have been widely accepted and adopted in national research and development programs. In India, micropropagation of elite lines of cardamom, banana and forest trees has been well-adopted.<sup>15</sup> For example, micropropagated cardamom plants are grown in an area of 102 ha. The yield advantage of these elite plants is 28% over the conventionally propagated ones. Over 200,000 micropropagated trees of *Anogeissus spp*, *Populus spp*, *Eucalyptus spp* and *Tectoan grandis* have been established in the field as a result of joint efforts by private and public sector research. Biological insect pest control programs based on *Bacillus thuringiensis* preparations have been developed in sugarcane, tobacco, cotton and sunflower. In his commentary, Bhatia states, "Biotechnology is information intensive and not capital intensive. The success will depend on how fast we can develop appropriate biotechnology and extend it to the farming community."

### LINKAGE BETWEEN BIOTECHNOLOGY AND BIODIVERSITY

Success in engineered crops depends on availability of the technology required, knowledge of the underlying biochemistry and genetics of a desired trait and availability of the genes of interest. It could be concluded that biological diversity is crucial to the future of genetic engineering. Much of the diversity is located in developing countries. It is unfortunate that genetic resources have been declared to be the common heritage of mankind, which has led to germplasm being taken from developing countries and used in breeding or genetic engineering in the north. For example, resistance genes against yellow dwarf virus in barley were brought to breeding programs in the U.S.A. from Ethiopia without compensation to Ethiopian farmers. Numerous similar examples have shown that we have to adjust our thinking on genetic resources. To this effect the Biodiversity Convention acknowledged the sovereignty of countries over their genetic resources.<sup>16</sup>

There are also already examples of joint arrangements for commercial benefits of value-added innovations on biodiversity. For instance, the pharmaceutical company, Merck, has a contract with an NGO in Costa Rica, InBio, whereby Merck is offering a payment for biological accessions gathered by parataxonomists.<sup>17</sup> Similarly, another pharmaceutical company, Shaman Pharmaceuticals, has announced a policy whereby a percentage of the profits will be channeled to the communities it has worked with in such a capacity.<sup>18</sup>

### STRENGTHENING NATIONAL PROGRAMS

One way of gaining control over genetic resources nationally is to increase the capacity for its development and exploitation in national programs. Biotechnology can provide the means for making a significant contribution to solving problems in low-input agriculture. However, the national programs need to act now and embark on initiatives that supplement existing crop improvement programs to develop varieties with good yield performance and reduced external inputs. The strategic development plans should be carried out in view of the physical, financial and human capacity available.

### PARTICIPATORY RESEARCH

The role of community involvement in decision-making and setting the research agenda has been stressed earlier. In many parts of the world, women are the chief managers of natural resources. Today global food security depends on 15 major

plant species, while, for example, among the Indians in America it included over 100 species of over 40 genera and 120 families. Much of the selection of resistant maize plants in India is carried out by women. There is remarkable botanical knowledge in rural communities throughout developing countries and there is growing recognition for the role of farm families in *in situ* conservation of land races and cultivars.<sup>19</sup>

Increasingly, we are seeing that formal and informal knowledge systems should be able to work together and that there is no division between knowledge generators and users. Joint strategic research planning with communities and the formal research community has already shown its power and potential (e.g. pharmaceutical innovations). To expand participatory approaches to research, an environment should be created whereby farmers and communities have practical decision-making power on the utilization of natural resources supported by professional, institutional and policy commitment.

## AGRICULTURAL TRAINING IN THE NORTH

Most of the scientists and policy makers in senior positions making critical decisions on agricultural research policy both in developing and developed countries have had strict disciplinary training. Their perceptions are also influenced by their life experience and political, economic and ethical beliefs. Most senior agricultural research scientists are white males. For example, in the U.S.A., a dozen land-grant institutions have trained nearly three-fifths of the public sector agricultural scientists.<sup>20</sup> It seems that the scientific community suffers from 'inbreeding' and lacks experience from other institutional and disciplinary backgrounds and the life experience of female and ethnic minority scientists.

Participatory approaches and multidisciplinarity are also new to most senior agriculture professionals. Therefore, awareness building and retraining is necessary. Moreover, many crop improvement and agronomy curricula still follow structured disciplinary lines and a new challenge is to bring the dimension of sustainability to this context.

Universities and colleges of agriculture have a key role in meeting the challenge of training future professionals with a systems approach to science, high ethical integrity, appreciation for social plurality and cross-cultural communication skills.<sup>21,22</sup>

## CONCLUDING REMARKS

"Contemporary development history has shown that technological change is not deterministic and therefore its evolution can be governed to achieve

certain social goals."<sup>23</sup> It is the scientific community and those setting research priorities that can have a say in what way we apply biotechnology. Initial high investment costs of biotechnology research and, thus, the prominent role of multinational companies coupled with patenting legislation to secure the commercial interest of these companies have had an impact on research priorities. However, the shift in research priorities in the international research centers and in the public domain research of developed and developing countries, as well as the novel arrangements for joint commercial protection and exploitation of biodiversity, is positive. It is a journey to explore the potential of biotechnology in diversifying the economic activities and development of crop improvement for a sustainable agriculture.

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## CHAPTER 3

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# BIOTECHNOLOGY: NEW TECHNIQUES FOR AGRICULTURE AND THE ENVIRONMENT

G. Persley

The World Bank has contributed around US \$140 million since 1985 to biotechnology research and development (R&D) for national and international programs (Table 3.1). New biological techniques allow researchers to conserve genetic resources, tailor the performance of crops and livestock to particular production systems and identify and develop environmentally benign farm inputs and management strategies. These advances promise improvements in agricultural productivity, processing and product quality, and promise to reduce dependence on agro-chemicals. There is considerable potential for biotechnology to contribute to sustainable agricultural growth in developing countries. This prospect needs to be viewed realistically in light of constraints on resources, institutions and policies.

### AGRICULTURAL BIOTECHNOLOGY

Modern biotechnologies range from relatively straightforward and inexpensive procedures of tissue culture to advanced applications of molecular biology, including genetic engineering. Together, these new techniques provide powerful new tools for agricultural research and technology generation.<sup>1</sup>

Global R&D expenditures in agricultural biotechnology are currently estimated to be around US \$4 billion per year.<sup>2</sup> To date, commerce's products in the agriculture sector have been limited to niche markets in micropropagation plant and animal health diagnostics, vaccines and bio-pesticides. Novel products are being marketed, however. The first commercial release of a genetically engineered plant variety, a tomato with improved shelf life, was made in the U.S.A. in 1994; genetically engineered plant crops with improved pest and disease resistance, for example, cotton, are also close to being marketed. Rice varieties with enhanced virus tolerance should be available to farmers within a few years.<sup>3</sup> By the year 2000, annual farm level sales of biotechnology-derived products are estimated at around US \$10 billion, with 70% of this based on seeds and 30% on veterinary and other products.<sup>4,5</sup> In terms of new product release, the medical applications of modern biotechnology have moved far more rapidly, and several novel pharmaceuticals are already on the market.

### IMPORTANT ISSUES

A number of developing countries have established policies and programs in biotechnology.<sup>6</sup> The incentive to do so arises from both anticipated production benefits and the knowledge that competitiveness in agricultural markets will increasingly

**Table 3.1. World Bank's Lending for Biotechnology: Most Active Projects (Figures in US\$ million)**

Year	Country	Project	Total	Loan/ credit	Biotech Component	Cost
1993	India	National Agriculture Research Project	72.1	38.8	Plant and animal biotechnology	2.0
	India	Rubber Project	143.8	92.0	Tissue culture; somatic embryogenesis	0.40
	Rwanda	Second National Agriculture Research Project	36.5	15.0	Tissue culture; potato/sweetpotato	0.40
1992	Mexico	Agriculture Technology Project	300.0	150.0	Biotechnology training M.Sc./Ph.D.	1.15
	Mexico	Science and Technology Infrastructure Project	45.8	31.6	Basic research grants and facilities	20.0
	Turkey	Agriculture Research Project	77.6	55.0	Molecular biology; crop research	4.00
1991	Argentina	Agriculture Services & Infrastructure Development	83.2	33.5	Molecular biology; vaccines/diagnostics	2.70
	Nigeria	National Agriculture Research Project	—	78.0	Tissue culture; yam multiplication	—
	Zaire	National Agriculture Research Project	65.2	16.7	Germplasm conservation; tissue culture	0.56
1990	Korea	Second Technology Advancement Project	45.8	31.6	Support to genetic engineering	5.70
	Niger	National Agriculture Research Project	28.0	19.9	Livestock: microbiology/nitrogen fixation	—
	Pakistan	Agriculture Research II Project	81.9	57.3	Biotech R&D: Post harvest horticulture	3.00
1989	Brazil	Third Agriculture Research Project	97.8	47.0	Plant molecular/cell biology, biocontrol, biofertilizers, animal health	5.30
	Indonesia	Agriculture Research / Management Project	50.4	35.3	Crop & post harvest biotechnology	0.80
	Madagascar	National Agriculture Research Project	70.6	24.0	New sugarcane varieties; tissue culture	—
	Senegal	Agriculture Research Project	38.9	18.5	Tissue culture	0.46
	Congo	Kindarscha Food Crop Development (IFAD)	6.1	4.0	Tissue culture: cassava	0.39
	Kenya	National Agriculture Research	203.8	19.6	Animal health: diagnosis/vaccines	2.50
1987	Malaysia	National Forestry Research	—	—	Tissue culture: tree species	—

depend on the incorporation of advanced technology. National efforts are being supported by international development agencies including the World Bank. There are, however, several factors which limit expectations of widespread benefits in developing countries without substantial public and private sector investment.

Biotechnology R&D is expensive: the Rockefeller Foundation's investments in rice biotechnology are estimated at about US \$50 million over ten years.<sup>7</sup> Support by governments and donors compete directly with other needed investments in the agricultural sector.

Infrastructure and markets: many developing countries have limited scientific infrastructure, technology delivery systems and markets and therefore limited capacity to translate the development or import of biotechnology products into farm-level benefits.

Domination by private sector: private bioscience companies are the principal investors in agricultural biotechnology, but poor market prospects in most developing countries and lack of intellectual property legislation are disincentives to private R&D and to technology transfer agreements (Box 3.1).

## INVESTING IN AGRICULTURAL BIOTECHNOLOGY

Individual countries are developing capacities adapted to their particular needs. The largest and most technologically advanced, for example, Brazil, China, India and Mexico, presently have an opportunity to mount substantial biotechnology research programs and to attract significant invest-

ment. Less technologically advanced countries are often not able to build sophisticated biotechnology capacities and have limited opportunity to recoup such investments in the absence of delivery systems and markets.<sup>3</sup>

Productive investments are most likely where upstream cell and molecular biology groups work closely with applied research scientists in problem-oriented R&D programs linked to effective marketing and extension channels and contact with end-user groups. A division of labor exists between public sector research organizations, universities and the private sector, with the last group bearing much of the costs for technology development. Government agencies need to encourage collaborative linkages, provide investment incentives and directly fund public good elements. Effective and transparent regulatory systems, including public forum, are established to assess and monitor environmental, health and ethical issues in genetic engineering research and the evaluation and release of transgenic organisms.

In many countries, the priority is to strengthen the agricultural research system and help create the enabling conditions outlined above through institutional and policy reforms and improved agricultural research management and services (Box 3.2). In such circumstances, support for biotechnology research and training should emphasize an adaptive capacity such as tissue culture and germplasm conservation facilities. Where the conditions are more favorable for biotechnology R&D, support may be provided for relatively sophisticated research, education, training and associated infrastructure. In this latter context, opportunities should

### ***Box 3.1: Intellectual Property System Stimulates Biotechnology Investment.***

The extension of intellectual property rights (IPR) to living materials (in mostly industrialized but also some developing countries) has been the major spur for the private sector to invest in biotechnology R&D. While there is considerable international debate about the desirability of extending IPR to natural organisms and the benefits and costs for countries with differing strengths in science and technology, a growing number of countries have enacted or are considering changes in legislation, and this trend will be given new impetus under the GATT.

There are two kinds of operational implications. First, a project supporting biotechnology may involve specific negotiation concerning the acquisition of proprietary technological and/or ownership of results from research. There are a number of options ranging from material transfers to specific licensing.<sup>11,12</sup> Second, a project should consider helping to strengthen a borrowing country's capacity and systems to assess options and implement IPR policies. A recent example is the World Bank's assistance to Mexico to support the start-up of the Mexican Institute of Industrial Property and to build institutional capacity for the efficient administration of Mexico's 1991 IPR legislation, which will extend patent rights to biotechnology products.

be sought to facilitate and otherwise support private sector participation, including public-private joint ventures. There is no single blueprint for the design of a project to support biotechnology development. The elements discussed below are likely to be relevant in most cases.

## BIOTECHNOLOGY RESEARCH POLICY

Depending on the existing capacity, policy support should be provided to help national research systems assess biotechnology research priorities, resource allocations and enabling policies in the light of both domestic opportunities and changing international comparative advantages. It is essential to integrate biotechnology resources and institutions with more conventional research and technology components, e.g. plant breeding and seed systems as a whole, and meet the needs of diverse participants including private firms, universities, NGOs, producers and consumers. Specific forums have been established in a number of countries to bring these different interest groups together, with the aim of promoting a widespread uptake of biotechnology products and a broad institutional base to R&D.

## STIMULATING R&D

Many developing countries are considering reforms in intellectual property legislation in response to the recent GATT (Box 3.1). Technical assistance should be provided to advise on this and other incentives to R&D, such as more liberal investment regulation, tax credits for R&D and financing arrangements including venture capital and competitive grant funding.

## REGULATORY ENVIRONMENT

Technical assistance and special facilities may also be provided to strengthen and implement biosafety,<sup>8,9,10</sup> the World Bank should ensure that an efficient and effective regulatory process is operational in countries where it supports the use of molecular biology, or the import and field testing of transgenic products.

## TRAINING, INFRASTRUCTURE AND INFORMATION

Basic biological science skills should be developed through support of relevant university education and research programs. Applied training support can include the supplement of staff from, or internships to, advanced public or private laboratories, short courses or long-term scholarships. Support for education and training, and investments in laboratories and equipment, should be considered in the context of an overall program where the links to productive sectors are clear. Biotechnology R&D demands up-to-date information; an information component should be included in any biotechnology project supported by the World Bank to improve access to current technical and policy-relevant information via journal subscriptions, online database and other information products, including CD-ROMs.

## RESEARCH PROGRAM AND NETWORKS

Biotechnology R&D should target problems where significant time or other resource saving can be achieved through the use of more conventional approaches (e.g. rapid micropropagation of perennials with long sexual cycles). While most products

### **Box 3.2: Biotechnology Skills Complement 'Conventional' Agricultural Disciplines.**

Applying biotechnology to agricultural research and natural resource management requires collaboration between molecular and cell biologists, plant and animal breeders, pathologists, microbiologists, agronomists, social scientists, and other disciplines, as determined by the particular problem to be addressed. Products of such initiatives are used at both the laboratory and farm levels, for example:

- Genetically superior seeds and livestock, and virus-free planting material, for example, with greater biotic or abiotic stress tolerance, or traits preferred by the market.
- Farm inputs based on microbial technologies including environmentally benign biofertilizers, biological control agents and livestock vaccines.
- Diagnostic technologies for characterization of genetic resources, gene mapping as a contribution to breeding strategies and on-farm management of pests and pathogens.
- Other basic and applied research tools including in vitro germplasm conservation techniques for conservation of biodiversity and exchange of breeding materials.

of biotechnology R&D such as seeds and biological control agents will be essentially scale neutral, assessments should consider specific needs in small farm systems and potential bias of costs, management skills, access to extension advice or other factors required.

Research links and networks should be identified and supported in biotechnology projects (Box 3.3A and 3B). Upstream links with advanced

### ***Box 3.3: Some International Networks and Programs***

Investments in developing country biotechnology should be considered in the context of the wider international market place for public and private sector biotechnology research and services in which novel institutional linkages and funding mechanisms are possible. In addition to private firms, there are now several non-traditional actors that can contribute including university groups, biotechnology advisory services and 'biotechnology brokers.'

### ***Box 3.3A: Consultative Group on International Agricultural Research (CGIAR)***

Several of the centers have biotechnology research capability and projects relevant to their particular commodity mandates. The World Bank is a substantial contributor to the CGIAR. Some US \$40 million per year is provided to research activities including applications of biotechnology. These programs include:

CIAT	cassava and beans
IPGRI	plant genetic resource characterization
CIP	potato and sweet potato
CYMMYT	wheat and maize
ICARDA	wheat, barley and legumes
IRRI	rice
ICRISAT	sorghum and groundnuts
ISNAR	biotechnology research management/policy advice
ILRI	animal diseases policy advice

### ***Box 3.3B: Other International Programs<sup>13</sup>***

- The Rockefeller Foundation Rice Biotechnology Program provides around US\$7 million annually for research and training in rice biotechnology designed to meet the needs of developing countries.
- The Cassava Biotechnology Network supports over 30 biotechnology projects sponsored by an international group of donors; activities include multidisciplinary needs assessments and development of pest and disease resistant varieties.
- The International Service for the Acquisition of Agri-Biotech Applications is a not-for-profit international organization with the aim of facilitating the transfer of agricultural biotechnologies to developing countries.
- International Center for Genetic Engineering and Biotechnology is a UN sponsored organization with facilities in India and Italy that undertakes biotechnology research and training for developing countries.
- The Netherlands Directories General for International Cooperation provides recommendations for Dutch government support to biotechnology and administers a biotechnology and development program of about US \$85 million.
- Agricultural Biotechnology for Sustainable Productivity, a U.S. AID program managed by Michigan State University, enhances the U.S.A. and other developing countries' capacities in sustainable biotechnology R&D.
- The Intermediary Biotechnology Service was established at ISNAR by an international group of donors to act as an independent adviser to developing countries on biotechnology research management and policy.
- McKnight Foundation Plant Molecular Biology Collaborative Project donated a total of around US\$60 million to enhance the basic research capacity with collaborations by advanced institutions.

public and private laboratories in OECD countries and the international agricultural research center provide access to technologies, information and training. Horizontal linkages enable countries with limited technical resources and markets to collaborate on strategic research of regional importance, development agencies, and consolidate funding. This approach may have the potential to accelerate biotechnology development in Southern Africa, for example.

## COSTS AND BENEFITS

It follows from the above that the component cost elements of a project to support biotechnology development are as follows: 1) policy and planning; 2) institutional and legal reforms; 3) regulatory processes and public forum; 4) research financing; 5) education, training; and 6) infrastructure development.

The benefits that could be expected from the successful development of biotechnology capacity can be identified at three different levels: National/sectional—the improved capacity to maintain export competitiveness based on price, quality and reduced residues in export commodities, to assess

and conserve biological resources, and to reduce environmental damage from agriculture, possibly with new crops and production strategies, research and technology systems; the development of more choice, greater efficiency and precision in R&D strategy, as well as an increase in private sector participation; and the arm level—the implementation of plant and animal varieties offering improved yields or reduced costs or both, and reduced yield variability from biotic stresses and reduced exposure to health risks from agrochemicals (Box 3.4).

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### **Box 3.4: Small Farmers are producing farm inputs in 'low-tech' biotechnology enterprises.**

Applications of biotechnology are not always expensive, sophisticated or capital-intensive. Farmers and local technicians can be trained in skills required in the handling of biological materials, and these skills can be used in the production of biofertilizers, biopesticides and micropropagated plantlets. During the 1980s, small farmers in the Dalat Hills of Vietnam were helped to establish micro-enterprises, based on intensive use of family labor inputs, to produce high-yielding potato plantlets by micropropagation.<sup>14,15</sup> This local industry is now self-sustaining and has played an important role in generating dramatically increased potato production in the area. In Colombia, farmers are producing biopesticides on farms from insect pathogenic fungi (*Metarhizium* and *Beavaria spp*) grown on rice substrata; the product is formulated and used locally to control plantain, coffee and potato pests.<sup>16</sup>

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## CHAPTER 4

# UTILIZATION OF PLANT GENETIC RESOURCES

V.R. Rao and M. Iwanaga

### INTRODUCTION

Crop genetic resource collections are assemblies of genotypes or populations representing plant cultivars, genetic stocks and related wild and weedy species. They are maintained in the form of plants, seeds, tissue culture, etc.<sup>1</sup> Functionally, plant genetic resources can include landraces, advanced/improved cultivars and wild and weedy relatives of crop plants (either domesticated, semi-domesticated or non-domesticated). Landraces are distinct local types, adapted to the many variations and interactions of natural and cultural environments in different regions of the world to which crop species were gradually introduced. These landraces may contain coadapted gene complexes that have evolved over decades, making them location-specific.<sup>2,3</sup> Such landraces, sometimes referred to as folk varieties, are the most important of the plant genetic resources. They exist alongside the indigenous knowledge of the people/communities that were responsible for their development. Additionally, advanced cultivars, especially those of recent origin, are also important resources. Most of these cultivars have been bred using a fairly large genetic base (locally adapted landraces) and hence may also have been incorporated into co-adapted gene complexes from landraces. These, along with genetic stocks (natural or induced mutants, breeding lines with specific characteristics, accessions with resistances, etc.) also have a part to play in the future improvement of economically important plant species and therefore need to be preserved.<sup>1,4,5</sup>

To appreciate the evolution of the work on plant genetic resources it is necessary to look briefly at the history of plant collecting. Although much exploration, plant collecting and introduction had been done previously, systematic work did not start until early this century. In the late 19th century, de Candolle initiated the conceptualization of the geographic distribution and origins of cultivated plants. The work of Nikolai Vavilov during the 1920s-1940s was a major milestone in the field of plant genetic resources. Vavilov described for the first time the 'centers of origin of domesticated plants' and theorized that one could determine the center of origin by an analysis of patterns of variation in plants in a region.<sup>6,7</sup> This concept, which is more intuitive than empirical, has been much debated and modified<sup>2,8,9</sup> but the basic outline remains the same. The main difference is that presently we think in terms of centers as well as noncenters that can hold genetic diversity of plants.<sup>3</sup> This concept is crucial from the point of view that the distribution of genetic diversity in plants is not random and has both spatial and synclinal patterns. This influences the way we work with plant genetic resources and our understanding of the utilization of the resources. The genetic

diversity existing in various gene pools of plant species has vast potential for current and future uses. To exploit this potential, we need to make every effort to safely and effectively conserve and use plant genetic diversity for the betterment of human life and for the protection of the environment in which we live. The potential could be exploited either through conventional means or through the use of biotechnology. In either case, plant genetic resources are the raw material, without which no progress can be made.

The activities that relate to conservation and use of plant genetic resources include: 1) germplasm acquisition; 2) characterization and evaluation; 3) conservation, assessment of variation and identification of useful genes; and 4) germplasm exchange and genetic enhancement.

Acquisition includes collecting, which refers to gathering seed or propagules of landraces, wild species, etc., from the field and also to the assembly of materials through correspondence and exchange. The collected plant genetic resources have to be studied to understand their genetic structure and to identify useful traits. This is done by a systematic characterization and evaluation of material.

Conservation includes the management and preservation of known plant genetic resources. This takes two approaches, ex situ and in situ. Ex situ conservation maintains plant genetic resources outside the original habitat in facilities that have been specifically created, such as the seed, field and in vitro gene banks or botanical gardens.<sup>1</sup> Plant genetic resources can also be conserved as pollen, DNA libraries, etc., although at present the access time, i.e. the amount of time required to make use of the material, is inconveniently long. The infrastructural facilities required to hold resources with such methods also may not be within everyone's reach. The other approach, in situ conservation, conserves ecosystems and natural habitats so that viable populations of species can be maintained in their natural surroundings. In the case of domesticated or cultivated species, this means the surroundings where they have developed their distinctive properties (as defined by Article 2 of the Convention on Biological Diversity). In either case, the enhanced use of conserved plant genetic resources to improve the standard of human life and achieve a balance between sustainability and productivity becomes critical. To make best use of the conserved material, it needs to be exchanged freely. This involves moving germplasm in the form of either seed or other types of propagules, not only within a country but between countries. Finally, the plant genetic resources that have been col-

lected, studied and conserved have to be used for plant improvement.

Use of plant genetic resources could be achieved through simple selection from the material that has been assembled or may involve the highly complex process of hybridization, testing, selection, etc., depending on the genetic distances between the materials used for the purpose of improvement. This process has been carried forward more recently with biotechnological methods. In this chapter, we attempt to look in some detail at the role that biotechnology can play in conserving genetic diversity and facilitating its use. We have not attempted to make an exhaustive survey. This is impractical because of the speed at which the field is progressing. We have also not tried to provide specific institutional details on any topic as these also may quickly become out-of-date. The emphasis is on the problems and opportunities that biotechnology presents for conservation and utilization of plant genetic resources.

## **CONSERVATION OF PLANT GENETIC RESOURCES—WHY?**

We have just said that the great wealth of genetic diversity existing in the gene pools of economically important or potentially important plants holds vast potential for the current and future uses of humankind. Generally speaking, genetic resources are non-renewable and it is essential that we be concerned with their conservation, be it at the species level, gene pool level or at the ecosystem level. The limitations and dangers inherent in the narrow genetic base of many modern cultivars have been stressed many times.<sup>10-13</sup> Genetic diversity is a defense against genetic vulnerability which has been built into the genetic structure of landraces through selection by farmers over many generations. Such defense mechanisms need to be introgressed into modern cultivars to make them sustainable.<sup>14-16</sup> Countries which still hold significant amounts of genetic and species diversity have a responsibility to themselves and to humanity at large to safeguard such diversity and make it available for their own national development as well as for other countries. At the same time, the countries that are not endowed with rich genetic diversity should support the efforts of other countries for better conservation of plant genetic resources, since they have benefited in the past from plant genetic resources and will continue to receive benefit, directly or indirectly.

Particular attention must be paid to the landraces, which are the result of many years of systematic domestication, selection and improvement by farmers and communities. Due to the

increased pressure on agriculture, they are the most immediately threatened germplasm and face the danger of extinction. The genetic resources that include the wild and weedy species used in agriculture, forestry or horticulture are also in danger because of deforestation, developmental activities (e.g. irrigation, hydroelectric projects, mining, oil exploration, road building and urbanization), expansion of agricultural activities into new areas, etc. Additionally, some forest species, especially in the tropics, are endangered due to the pressure on the land for food production, housing, locating industries and other needs of a growing human population.

Many disasters have occurred as a result of a crop having a narrow genetic base, with consequent minimal resistance to certain biotic and abiotic stresses. An often quoted example is the Irish potato famine of 1840s, when the potato crop was virtually wiped out as the cultivars grown at that time had no resistance to leaf blight disease. The combined effects of typhoon and rice brown spot disease in the Bengal area in 1943 led to serious famine in India.<sup>17</sup> Another example is the impact of southern corn leaf blight in 1970 in the southern states of the U.S.A. which decreased production by about 25%. However, in this case public and private plant breeders had access to genetic diversity and were able to produce resistant material within a relatively short time. In 1993, taro leaf blight destroyed 95% of the taro crop in Western Samoa, where it is the major staple food.

There may be several such major disasters developing at present, together with a number of smaller ones. For example, several important traditional crops of Oceania are highly threatened with genetic vulnerability due to their narrow genetic base.<sup>12</sup> This is threatening their sustained production. The vineyards in California are being invaded by a new biotype of *Phylloxera*, the aphid relative that affects the root system. Since more than 70% of wine grapes in the Napa and Sonoma counties are grafted on to susceptible rootstock, the crop is seriously threatened. The spread of the disease is considered very likely.<sup>18</sup> A review of the situation after 1970 can be found in Council<sup>19</sup> who recommends that a wide range of genetic and agronomic strategies be employed to minimize crop uniformity and consequent susceptibility. From this, it is clear that genetic uniformity is the basis for vulnerability to epidemics and use of genetic diversity (functionally the plant genetic resources) is essential for improving and maintaining agricultural production.

There are a number of studies on the impact of efforts undertaken in the conservation of crop

genetic resources and most of them have shown that efforts have paid significant dividends. For example, rice production in Asia increased 42% from 1968 to 1981 following the use of high-yielding and short-duration cultivars. The increase was about 110 million tons in one year. At the price of US \$250 per ton, a profit of US \$27,500 million per year was generated while the money used for the conservation of rice genetic resources worldwide is less than US \$2 million per year. A conservative estimate is that 50% of the profit is due to rice improvement based on the use of rice genetic resources. Another example is the new hybrid of pigeon pea, called ICPH 8, developed by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). This hybrid requires only 100 days to mature, increases yields by 30-40% and can be cultivated in a wide range of growing conditions. The reduced maturation period may mean savings of up to \$100 million a year to growers because ICPH 8 can escape serious damage by fungal and viral diseases as neither of these can establish themselves sufficiently in 100 days. The discussion here is not to contradict the effect improved cultivars have been having on the genetic diversity in general. There is no doubt that there is a need for increased agricultural production, which can be achieved through the efficient use of available genetic resources. At the same time, there is a need to enhance genetic diversity on the farm, especially where such an enhancement will be useful to the farmers to balance productivity with sustainability.

The world's agriculture is confronted by numerous problems.<sup>20</sup> For example, we do not know what new diseases, insects or other pests, or soil atmospheric problems we will have to face in the future. New strains of pests continue to develop and may attack those crop cultivars that were originally resistant to these pests. We do not know what physiological and morphological changes will be needed for crops to perform well in the future. We have been warned that global warming due to the 'greenhouse effect,' along with many other changes that are occurring around the world, may result in changes in the world's environment. If this happens, there will be a need for new varieties which can adapt well to the new environment. Since it is difficult to predict what will be actually needed in the future, plant genetic resources have to be collected and conserved for future use before they disappear forever.

In 1850, there were 1.1 billion (i.e. thousand million) people on earth. This number had increased to 2.0 billion by 1930 and is currently around 5.5 billion. The world's population is

expected to stabilize between 10.1 and 23 billion sometime between the late 21st and late 22nd centuries,<sup>21</sup> based on the assumption that the current level of fertility declines (annual increase now is 93 million). As the world's population continues to increase, it demands increasingly higher production from agriculture. With static land area, there is a need to increase biological yield levels of most crop species. Recent research in cell biology, molecular genetics, recombinant DNA, plant tissue culture and related fields is opening up new possibilities for progress in agriculture. The development in biotechnology allows scientists to transfer genes for crop improvement in a relatively short time. But the genes for such 'engineering' manipulations must be provided from genetic resources.

## CONTRIBUTION OF PLANT INTRODUCTION TO THE GLOBAL ECONOMY

In this volume, there are case studies on different crops/commodities that look closely at the introduction of a particular crop or commodity. Nevertheless, it is relevant to take a brief look at the history of plant introduction and its role in the spread of agriculture and in crop improvement. The first recorded importation and assembly of plant material appears to have been done by an Assyrian king, Tilgath-Pileser I, on cedar trees, vines, etc. from various parts of the region around 1130 B.C.<sup>22</sup> However, unrecorded introductions must have taken place much earlier because new and interesting plants always attracted the attention of farmers.<sup>23</sup> Movement of plants within the Old World must have occurred with great frequency due to the proximity of the countries in the region and very early contacts, almost on a routine basis. However, the discovery of the New World resulted in a spate of explorations and plant introductions due to interest in new plants/species. These included maize, cassava, beans, squash, peanut, potato, tomato, pepper, sweet potato, tobacco and many other species during 1500-1800. A number of botanists/plant explorers went around the world collecting plant materials. Towering over all these efforts were those of N.I. Vavilov, whose work on exploration and introduction were so systematic that they resulted in agroecological classification and the basis for centers of origin of crop plants and centers of diversity, as mentioned previously.<sup>7,24</sup> During the last few centuries, much exchange of economically useful plants from different regions in the world has taken place.<sup>25</sup> European colonialism took many of these crops to Asia, Africa and other regions of the world. Introduced crops like cotton and sugar brought new prosperity

to many such regions. Based on the area and production levels of all these introduced crops, it can easily be seen that the impact of these crops has been very significant. We will now briefly look at a few examples of such introductions from the New World to the Old and from the Old to the New World, and their impact in general.

Asian rice (*Oryza sativa* L.) has been reported to have been grown in China for over 7000 years and for over 5500 years in Thailand.<sup>26</sup> The distribution of wild relatives and spread of ecogeographic races of *O. sativa* across Asia and Oceania may have occurred from the area where Assam, Bangladesh and Myanmar are now located.<sup>26</sup> The differentiation of the species into the subspecies *japonica* and *indica* is seen as an indicator of possible independent evolution of these two types. Such evolution corresponds to the areas of past and present distribution of wild species in China on one hand, and south and southwest Asia on the other.<sup>27</sup> These conclusions support extensive collecting of germplasm in southern China and southwest Asia. The rich diversity found in cultivated rice in Asia is due to long years of evolution, and adaptation with intense ecogenetic diversification under different hydrological, seasonal and cultural regimes.<sup>28,29</sup> Asian rice was introduced to the Middle East, North Africa and Europe as early as 1000 B.C.<sup>29</sup> However, about 92% of the world's rice is still produced in Asia.<sup>30</sup> During the period of the Green Revolution, rice yields more than doubled, and rice continues to be the most important grain for human consumption in the world. However, over the years, the genetic base of major rice cultivars has become eroded due to the use of selected genetic resources.<sup>14</sup> This situation needs to be corrected. There are ample opportunities to enhance the diversity of rice cultivars developed in the future as the genetic diversity available to breeders is vast and it is well-characterized. Additionally, the linear replacement of traditional technology has been challenged.<sup>31</sup> The multilineal and partial technology theories predict a far more heterogeneous future for agricultural evolution than the proponents of genetic erosion originally envisioned. This would lead to maintenance of genetic diversity on farms.<sup>32</sup> This is probably true for rice as well and there is scope for remedial measures. Rice is an example of a crop that has arisen in Asia and not only continues to benefit producers and consumers in Asia, but also has spread around the world.

*Citrus* L. (Rutaceae) and its relatives arose in South or Southeast Asia, the main center apparently being in northeastern India. The natural distribution of citrus species ranges from India and

southern China to northern Australia and New Caledonia. Records of earliest cultivation in China date back to 2200 B.C.<sup>33</sup> Cultivation of citrus in Southeast Asia is considered to be as old as that of China, although there is no recorded evidence for this. Citrus species were introduced into the Mediterranean following the conquest of Alexander the Great. From the Mediterranean, citrus moved to the New World, probably during the 16th century. The introduction of many Asian cultivars the New World occurred in the 19th and 20th centuries. Citrus is now grown throughout the tropics and subtropics, roughly between 44°N and 35°S.<sup>34</sup> Over the last century, countries in the Mediterranean region and South and Central Americas have become major producers of these fruits, accounting for 86% of commercial plantings.<sup>34</sup> Asia accounts for less than 10% of plantations, and its share in international trade is negligible. Low yields in Asia account for low levels of production. For example, Kusumo<sup>35</sup> reported that citrus yields were 7 t/ha-1 in Indonesia compared with 20-30 t/ha-1 in other countries. Several debilitating diseases appear to be responsible for less than optimum growth and low productivity of citrus in Southeast Asia.<sup>33</sup> The development of resistant or disease-free planting material from citrus selection programs has been successful in the major production areas of the world.<sup>34</sup> However, such attempts have not been undertaken on any significant scale in most Asian countries. Major citrus species in world markets are sweet orange (*C. sinensis*) followed by mandarin (*C. reticulata*), lemon (*C. lemon*), lime (*C. aurantifolia*) and grapefruit (*C. paradisi*). In Southeast Asia, however, orange, grapefruit and lime are of little importance. Mandarin and a large number of minor citrus species are important at regional or local level. The situation is not very different in other parts of Asia. A vast reservoir of citrus diversity exists in the region in both wild and cultivated forms, but erosion of these genetic resources is a cause for concern.<sup>36</sup> Citrus is an example of species that originated in Asia, with most production and benefits from citrus genetic resources occurring elsewhere in the world.

The genus *Musa* is native to Asia where some 45 species abound in the tropical rainforests and open grasslands of a vast region extending from India to the eastern fringes of Papua New Guinea.<sup>37</sup> While seed dispersal for wild species may be extensive, species adaptation delimits their natural distribution. *Musa acuminata* and all its subspecies are typical, second story vegetation in tropical rainforest. Solid stands of *M. balbisiana* are also observed in forested areas but members of this

species tend to dominate open spaces, particularly at the periphery of forests.<sup>38</sup> The edible and seedless bananas and plantains were derived from the wild and seedy *M. acuminata* and *M. balbisiana*. Through the development of sterility, coupled with parthenocarpy, edible diploid and triploid forms of the two wild species evolved. Human selection identified the superior forms which are propagated asexually and disseminated through suckers. From its center of origin in Southeast Asia, bananas and plantains were carried east to the South Pacific through Polynesian migrations. In their travel westward, they reached East Africa and then West Africa where the plantains became a very important staple food. During the 16th century, they must have reached tropical America, where production and export of dessert bananas became the primary agricultural enterprise of many countries. Presently, banana is internationally recognized as the most important fruit exported in fresh form. Asia accounts for about 28% of global production of bananas and plantains, with Africa and the Latin American region producing about 35% each. Only 10% of global production is exported, as most production is consumed in the areas where it is produced.<sup>39</sup> Conservation of *Musa* germplasm in Asia in general, and southeast Asia in particular, is therefore essential for the future improvement of this crop around the world. *Musa* is another example of a species that originated in Asia and spread to most of the tropical regions in Africa and America, where it became important either as staple or as commercial crop. The three continents seem to derive equal benefit from these species, although in monetary terms the share of Asia may be lower than either Africa or South America.

Soybean, *Glycine max* (L.) Merr., was first cultivated in China at least 3000 years ago. It has also long been grown in Southeast Asia and in eastern Siberia adjacent to China. In a region extending from north India, Nepal and Bhutan through northern Pakistan into Afghanistan, small, dark-seeded primitive types of soybean are grown. Presumably this is also an area of ancient cultivation.<sup>40</sup> The range of wild soybean, *Glycine soja* Sieb. and Zucc., is restricted to the countries of China, Japan, the Korean peninsula, and eastern Siberian regions. Soybean reached North America some 200 years ago but arrived even earlier to Europe.<sup>41</sup> Over the years, soybean production developed rapidly, especially after the 1950s, probably due to the increased demand for vegetable oil and protein, and the expansion of the crop on a large scale in Brazil and the U.S.A. The harvested area increased from 15.1 million ha in 1950 to 52.37 ha in 1985, making soybean the most important legume and

oil crop in the world. The total yield increased from 18 million tons in 1950 to 100.8 million tons in 1985.<sup>41</sup> It developed rapidly in North and South America in the last 50 years. Over 90% of the world's soybean is now grown in four countries (U.S.A. 56%, Brazil 17%, China 11% and Argentina 7%). Soybean oil accounts for 60% of the edible oil in the U.S.A. and 95% of that in Brazil.<sup>41</sup> Thus soybean, a crop that originated from Asia, has contributed significantly to other regions of the world.

Similarly, several crops that originated in one part of the world have found a home in another, very different area than the original zone of domestication. For example, a number of species that originated and were domesticated in South and Central America have spread to Asia to become very important food and industrial crops. These include cotton, maize, potato, groundnut, oil palm, rubber, etc.<sup>42</sup> For example, groundnut (peanut), *Arachis hypogaea*, is native to the New World. The early explorers found that it was cultivated extensively in both Mesoamerica and South America. Remnants of groundnut shell tissue recovered from archaeological sites in Peru date its domestication as far back as 3900-3750 years ago.<sup>43</sup> Presently, groundnut is an important oil, food and forage crop widely distributed over tropical, subtropical and warm temperate zones and cultivated in about 80 countries of the world. The exact origin of *Arachis hypogaea* is still shrouded in mystery; it is presumed to have originated at the base of the Andean mountains in southern Bolivia. Peruvian civilization, relics of which provide clues to the domestication of *Arachis*, apparently began along the eastern slopes of the Andes extending into northwestern Argentina.<sup>43-45</sup> There is no evidence for the pre-Colombian introduction of groundnut to the Old World. However, it should have occurred soon after the discovery of the New World by Columbus. It appears that the two-seeded groundnuts were first taken from Brazil to West Africa by the Portuguese and the Peruvian-type to the western Pacific, to China, Indonesia (Java) and to Madagascar. It is also recorded that the Spaniards took groundnut from Mexico to the Philippines in the 16th century. Groundnuts reached China in 1538 followed by their introduction into India. At present the cultivation of groundnuts in the area of origin, Mesoamerica and South America, is very low. India and China together produce about 60% of the world's output, followed by the U.S.A., Nigeria, Senegal, Indonesia and Myanmar. From this distribution, it is apparent that although groundnut originated in South America it is now a very important food and cash crop for the whole world.

From the above examples it becomes clear that plant introduction from times before recorded history has played a significant role in the spread and development of agriculture. Such introductions have had great impact on both agrarian societies and industrialized nations. The movement of plants from one country to another and from one region to another, that resulted in the expression of new forms and the adaptation to new environments, has been a critical factor in the development of plants and forms of plants on which humankind is highly dependent today, both in terms of agricultural produce and environmental stability. This also highlights the fact that no country is, or can be, self-sufficient in the plant genetic resources that are required for its needs. Therefore, among all plant genetic resources activities, exchange (i.e. introduction) becomes crucial for the future needs of conservation of plant genetic resources biodiversity and crop improvement.

## THE ROLE OF BIOTECHNOLOGY IN PLANT GENETIC RESOURCES CONSERVATION

In relation to the utilization of plant genetic resources, it is important to understand how biotechnology could be used to better study and conserve the plant genetic resources. This understanding is essential since the methods used for studying and conserving genetic resources are closely linked to the means of making efficient use of them. The subject area of germplasm conservation, in a broad sense, comprises exploration, acquisition (collecting and assembly), maintenance and conservation, characterization and evaluation (to understand the extent and distribution of genetic variation in a given gene pool and the inter- and intra-specific relationships), exchange and genetic enhancement. For the purposes of this chapter, estimation of genetic diversity and classification of genetic resources are included under characterization and evaluation. This is because the study and classification of germplasm by any method or tool can broadly be described as characterization and evaluation. The role of biotechnology in these areas, in functional terms, constitutes in vitro technology and molecular techniques. The initial discussion below is on the role of technology in different genetic resource activities, then the focus changes to its role in the use of plant genetic resources. This is essential as these activities have a direct bearing on the end use of plant genetic resources conservation, i.e. the use of plant genetic resources in enhancing sustainability and productivity.

One of the major concerns for the current activities on biodiversity (and plant genetic resources) conservation is the costs involved in collecting and conserving the vast range of genetic diversity that is available to us, and making it accessible for use. One major role that biotechnology can play in plant genetic resources conservation and use is to make the whole exercise cost-effective. As we will see later on, moving material from field gene banks to in vitro gene banks, slow growth techniques to cryopreservation, can reduce the costs involved in maintaining and managing plant genetic resources, especially of clonally propagated species. Similarly, as technology advances, making more efficient use of time and funds through marker-assisted germplasm enhancement vis-à-vis traditional backcrossing, it will make utilization of germplasm more cost-effective. As we will see, most of the technology is at a level that can be slowly transferred to developing or gene-rich countries and will be critical in the implementation of any agenda set for these countries under the Convention on Biological Diversity.

#### GERMPLASM COLLECTING

Germplasm collecting, as a part of acquisition, involves exploring areas of known genetic diversity for plant species and collecting seed material from different species or populations. It is normally done by collecting seeds from farmers' fields, market places, wild habitats, etc. However, there are numerous asexual species which do not produce. In such cases, vegetative material such as roots or tubers, or other regenerative materials have to be collected. This makes collecting laborious as well as time-consuming. Many other species may first produce seed that may be difficult to collect for two main reasons: First, the seeds may be too large to make collecting cost-effective and efficient. Additionally, the material collected may be bulky, deteriorate quickly and can be infested with pests. Second, the seed of some species cannot withstand drying to the low seed moisture contents (5-9%) required for transport and storage. Such seeds are called 'recalcitrant,' as opposed to the normal 'orthodox' seed that can be dried to low moisture contents and stored at cool temperatures. Consequently, collecting such seed material is problematic. In both cases described here, there will be a need to send the collected samples to the base frequently so that the viability of the material is not lost, or to have short collecting periods. This makes collecting time-consuming as well as expensive. Novel methods need to be used in such cases, such as in vitro collecting.<sup>5,46</sup> Most methods used for in vitro germplasm collecting have been simplifications and refinements of standard laboratory methods.<sup>47</sup>

In the area of plant genetic resources collecting, biotechnology can help reduce practical impediments to efficient collecting by providing information on the available genetic diversity in a given area, and producing in vitro methods for application in the field to provide new ways of collecting. Due to the advances in tissue culture technology, a range of techniques is now available for in vitro collecting of germplasm of so-called 'problem' species.<sup>46,48</sup> Precise methods to collect several plant species are now available. Some examples are given here.

An example of such early developments was the inoculation of collected cacao (*Theobroma cacao*) budwood in the field on to a medium containing fungicides prepared in advance in the laboratory.<sup>49,50</sup> This method has been successfully used under difficult tropical conditions. In the case of coconuts, successful field inoculation of zygotic embryos on to medium has been achieved. The extracted embryos can be kept in salt solution for transport to a laboratory.<sup>51</sup> Embryo cultures of coconut have been performed to circumvent problems of storage and transport caused by the weight and bulk of the nuts and the absence of dormancy, together with an in vitro technique that can be routinely used for collecting large number of embryos.<sup>52</sup> This technique has been further refined.<sup>51,53,54</sup> Another example is the development of a simple in vitro technique for collecting *Digitaria eriantha* subsp. *pentzii* and *Cynodon dactylon* to obviate deterioration, quarantine and bulk problems of conventional methods of collecting vegetative material.<sup>55</sup> As collecting viable seeds of cotton and its wild relatives is problematic, attempts to use tissue culture technology were made although not very successfully.<sup>56</sup> In vitro techniques are routinely used for collecting root and tuber crop germplasm.<sup>57</sup>

An understanding of the extent and distribution of diversity within a population is essential for effective sampling.<sup>58</sup> The use of molecular techniques in studying genetic diversity (see next section for details) in recent years has contributed to a better understanding of the genetic diversity of some species.<sup>59</sup> Ecogeographic surveys provide information on species distribution as well as infraspecific diversity. Biochemical as well as molecular techniques can be applied during such surveys for a proper assessment of the genetic diversity patterns that would then permit effective sampling of a particular region. For example, using restriction fragment length polymorphisms (RFLPs) demonstrated that the probability of adding new genes to a tomato collection would be about 20 times higher by adding one accession of *Lycopersicon peruvianum*, a wild relative of tomato.<sup>60</sup>

Another example is the genetic diversity of 13 *Juglans* species. Forty-one populations were characterized using RFLPs by hybridizing single locus probes to walnut DNAs digested with restriction endonuclease.<sup>61</sup> There were 10-fold differences in heterozygosity levels between species belonging to different sections. Taxonomic affinities were also indicated, which suggested that *J. cinerea* should be included as a part of section *Cardiocaryon* rather than as a unique section. From the conservation point of view, the studies indicated that additional collections of *J. cathayensis*, *J. mandshurica*, *J. mollis*, *J. neotropica*, *J. olanchana* and wild *J. cinerea* germplasm were needed. The authors suggested the collecting method as the one used for outcrossing species. It was also suggested, based on the genetic diversity distribution among and between populations and species, that collecting should be equally divided between sampling within locations (i.e. populations) and at different locations. A majority of the variation detected was present within a single population, but additional variation existed between populations. Similar studies which can assist in focusing collecting efforts are required in many other plant species.

More recent developments in the area of extraction of DNA from dried specimens such as herbarium material and fossils will help us to better understand the patterns of genetic diversity and phylogenetic relationships.<sup>62-64</sup> Efforts are also under way to develop methods that can be used right in the field to assess within-population diversity to facilitate sampling of maximum diversity. Developments in molecular biology may lead to the development of a practical field protocol for DNA collecting,<sup>65</sup> an additional option for germplasm collectors in the future if DNA libraries become an important component of conservation. For successful exploration and collecting, there is a need for well-coordinated efforts based on sound scientific principles, along with sufficient appropriate financial and staff resources.<sup>57</sup>

## CHARACTERIZATION AND EVALUATION

Systematic characterization and evaluation of plant genetic resources are prerequisites for the efficient use of the material, be it through conventional methods or modern techniques.<sup>5,66</sup> Until recently, most of the characterization and evaluation of plant genetic resources have been based on recordings of either qualitative and/or quantitative morphological characters. During the past decade or so, more and more emphasis has been placed on biochemical characterization and more recently on the use of molecular techniques. The use of mor-

phological phenotypes for genotype characterization has advantages and disadvantages.<sup>5,67</sup> The multilocal nature of most of these characters provides information that is extremely useful to breeders. However, the complex inheritance makes prediction of breeding difficult. The use of gene products (proteins, peptides) or metabolites (terpenes, flavonoids, etc.) partly solved this problem. Mendelian inheritance of isozymes makes genetic analysis easier. However, variation in isozymes is often low. Molecular genetic characterization has several advantages, such as no environmental influence, any plant part from any growth stage can be used, there is no limit on numbers for analysis, only small amounts of material are required, and since DNA is highly stable, even dry samples can be used. There are some practical disadvantages. For instance, the technique is not very suitable for large-scale screening. Experimental data on nucleotide sequence variation usually characterize only very small parts of the whole genome, often not related to economically interesting traits. More work is needed on repeatability and on the use of non-radioactive isotopes.

## AREAS OF CHARACTERIZATION

Four areas of germplasm 'characterization' in which biotechnology can be used have been identified: 1) identification of genotypes, including duplicate accessions; 2) 'fingerprinting' of genotypes; 3) analyzing genetic diversity in collections or in natural stands; and 4) assembling a core collection.<sup>47</sup> Gene banks receive many accessions, significant numbers of them without any relevant passport data. Hence, most gene banks carry an overload of duplicate accessions resulting in increased costs of management of collections. DNA fingerprinting with molecular markers can be very useful in this case.<sup>68</sup> However, identification of accessions, especially commercial cultivars, though possible, is yet to be used on a large scale for identification of duplicates in collections. The value of fingerprinting is more in the area of varietal identification. The determination of the extent of genetic diversity and its maintenance in collections can be assisted by analysis of isozyme variation and molecular genetic variation.<sup>60,69-71</sup> Variation in DNA sequence has been used to examine single copy genes, multigenes and organelle genomes, but relatively few studies of variation in populations have been carried out. Although there is potential here, the available technology is slow and expensive. However, there has been much progress in the last few years. RFLP maps have been produced for some crops, though their use in studying genetic diversity has been

limited. RFLP linkage maps have been constructed for several crop species, including maize, tomato, rice and potato.<sup>72-78</sup>

### Applicability of Molecular Techniques

Molecular analysis is not influenced by external environment, developmental stage or by plant part used.<sup>79</sup> However, the methodologies available have some other problems. One of the major constraints for their employment in plant genetic resources conservation and use is that the researchers are not usually concerned with the large-scale screening that is required for population genetics or germplasm screening. Despite these practical limitations, rapid progress is being made in this area. Below are some examples of application of the techniques described above, either individually or in combination to characterize plant genetic resources.

The genetic diversity of sorghum, as compared to maize, is less well-characterized at the genetic and molecular level despite its worldwide economic importance. Vierling et al<sup>80</sup> investigated the genetic diversity in sorghum for RFLPs and RAPDs. Using oligonucleotide probes, the fingerprinting of plant DNA could have several applications in plant genetic resources conservation and use. These have been highlighted by Kaemmer et al and Weising et al.<sup>81,82</sup> Some of these are used: 1) to characterize the extent of genetic variability within races; 2) to assess the 'purity' of inbred lines; 3) to select the recurrent parents in back-cross breeding programs; 4) to identify crop cultivars; 5) to characterize fusion hybrids; and 6) to evaluate the extent of somaclonal variation at the molecular level. The usefulness of some of these applications has been demonstrated in apple,<sup>83</sup> avocado,<sup>84</sup> and *Brassica oleracea*.<sup>85</sup> Use of molecular markers to study genetic stability of material conserved in gene banks, especially that conserved in vitro, has been attempted. Isozyme analysis, RFLPs and RAPDs are being successfully employed in monitoring somaclonal variation in banana and plantain.<sup>86-88</sup> The range of technology required in the case of potato has been described in detail by Watanabe.<sup>89</sup>

Isozyme markers have been used to identify genotypes, fingerprinting and study of genetic diversity.<sup>90-99</sup> However, in most cases, relatively few loci and alleles have been used in the analysis. Since any method would look at a small part of the genome, there is a need to use a variety of methods.<sup>100</sup> Some of the drawbacks with isozyme analysis may be overcome with the development of molecular techniques. To get a really complete picture, there is need to combine morphological

and agronomic evaluation of germplasm with biochemical and molecular analysis,<sup>101</sup> since these studies provide complementary information. A fairly good example of such complementary study was reported by Zhang et al<sup>102</sup> in comparing isozyme and RFLP analysis in wild barley (*Hordeum vulgare* subsp. *spontaneum*). In this study, isozymes demonstrated a larger amount of within-population diversity, whereas RFLPs resolved a higher proportion of between-population differentiation and detected more heterozygosity than did isozyme analysis. So by using both methods it was possible to determine that the particular set populations of *H. vulgare* were not only divergent, but that each population was highly heterozygous. Similar examples of complementarity of morphological and molecular analyses are yet to be noted.

### Core Collections

One of the major uses of characterization of germplasm using molecular techniques is assembling a 'core collection,' which is a tool to gain access into the large collections and make better use of the available diversity. A core collection has been described as a limited set of a crop species. Its content would represent, with minimum of repetitiveness, the genetic diversity of a crop species and its wild relatives.<sup>103</sup> From the time it was defined, the concept has been much abused and used, and many different kinds of cores have evolved.<sup>104,105</sup> The main purpose of a core collection is to access the larger collection and not for the purpose of conservation. However, within a complementary strategy, especially for vegetatively propagated species and species with recalcitrant seeds, core collections could become a part of the continuum of field collections, in vitro collections and seed collections (as populations rather than genotypes). Core collections can be established by using different kinds of data available on the germplasm conserved, for example, simple morphological characterization and evaluation data could be used.<sup>106</sup> If molecular and biochemical markers, such as isozymes, RFLPs and seed proteins, have been used to characterize genetic diversity in germplasm collections then these data can be used to select a core collection.<sup>107</sup> However, characterizing entire collections using biochemical or molecular markers will present a major disadvantage. Simplification of the methods may reduce this difficulty to some extent. Associating these markers with morphological traits can also help to solve the problem. One of the points to highlight is that the core collections are dynamic, not only due to the addition of new material but also to the use of new technology. This dynamism is expected to help researchers associated with genetic resources

management solve problems and provide opportunities to devise new ways of making more efficient use of the germplasm available to us.<sup>108</sup>

RFLPs and other molecular techniques can help, along with other information such as morphological characterization data and geographic distribution data, to select representative genetic variation from an entire collection. If the core contains, after appropriate validation, the majority of the genetic diversity in the larger collection, it can be used for various crop improvement purposes. Then the total collection can be conserved for the long-term, perhaps by cryopreservation,<sup>47</sup> thus becoming a component of complementary conservation strategy.

In plant genetic resources work, there is a need to study and analyze fairly large numbers of samples. Most of the work to date on analyzing the genetic diversity in collections has been done using small collections. Such analysis of genetic diversity may be used to assemble a core collection. As we shall see, information on genetic diversity in a collection will assist in its maintenance as well as in making use of the genetic diversity for crop improvement. Information on these aspects will follow in later sections. For a detailed analysis of the situation and the development and application of various techniques, their advantages and disadvantages and their complementarity with other techniques, see reference 5. As noted earlier on, all these studies require analyzing fairly large samples and hence there is a need to develop methods that can be used on a practical scale. The analysis of the data generated is another topic that needs to be considered carefully. It is common knowledge that different statistical packages allow different conclusions to be drawn. Giles<sup>109</sup> highlights some of the problems of using, analyzing and interpreting biochemical or molecular techniques to study biodiversity. It was concluded that the most important thing was first to formulate the question that needs an answer, then adopt an appropriate method to solve it. The appropriate choice of molecular method (the non-radioactive method will be more suitable than the one that uses radioactive materials) for screening large numbers of samples and classifying genetic diversity appeared to be difficult. These and other related issues were discussed in a workshop, and more work is needed to make the molecular tools useful for the study of genetic diversity.<sup>110</sup>

## CONSERVATION

Here the term conservation is used in its narrow sense, including only preservation and maintenance of genetic diversity and genetic integrity in gene banks. As indicated earlier, there are two main

approaches to conservation of plant genetic resources: ex situ and in situ. It is important to emphasize that these two approaches are complementary in nature. Conserving a gene pool should employ a combination of methods, from nature reserves to gene banks. The appropriate balance depends on factors such as the biological characteristics of the gene pool, infrastructure and human resources, number of accessions in a given collection and its geographic site and the intended use of the conserved germplasm. As there is a need to strike a balance between methods used, biotechnology can play a significant role, especially for the so-called 'problem' crops (clonally propagated species, plants with recalcitrant seeds and plants with severe seed-production problems). We will now look at what assistance biotechnological tools can provide to better conserve our precious germplasm, and also address some of the problems in using them effectively until more work is carried out.

## Ex Situ Conservation

### *Conservation of Seeds*

It is well-known that under cool and dry conditions orthodox seeds are viable for long periods. It is well-recognized that seed longevity is to some extent directly proportional to the storage temperature, humidity and seed moisture content. If seeds are maintained under such conditions, the life processes in seeds are minimized so that they can be stored for a number of years with little loss in genetic diversity, genetic integrity and viability, reducing the need to regenerate them at frequent intervals.<sup>111,112</sup> Nevertheless, due to the conditions under which most gene banks operate, there is a need for periodic regeneration of accessions and for restocking of seed in cold store. This is either due to loss in seed viability or depletion of seed stocks from use and distribution. While regenerating gene bank accessions, it is necessary to take all the necessary precautions to minimize any change in genetic structure due to genetic drift, genetic shift, selection, outcrossing or through simple mechanical mixture due to human error.<sup>113,114</sup> It has long been known that prolonged storage can cause genetic damage, the extent of the damage being highly variable. Biochemical and/or molecular techniques can be used to study the loss of genetic diversity in the germplasm stored and regenerated over a number of times.

### *Conservation of Vegetatively Propagated Material*

There are a number of important plant species, including important staple food crops and fruits

such as cassava, potato, sweet potato, taro, yam, apple, banana and citrus that cannot be conserved as seeds. Such material presents different problems. Generally, these are conserved in field gene banks. Although the field gene banks provide easy and ready access to conserved material for research as well for use, they run a greater risk of being destroyed due to natural calamities, diseases or changes in the landuse patterns. Field gene banks also require more space, labor and are expensive to maintain.<sup>115</sup> Another group of plant species that are also conserved in field gene banks are those with recalcitrant seeds, as mentioned in the above section. Many tropical fruit species such as avocado, cacao, coconut, jackfruit, mango and a number of forest species produce such recalcitrant seeds, presenting tremendous problems for conservation of genetic diversity in these species. Several techniques to conserve such vegetatively propagated species have recently been developed and some of them are undergoing rigorous testing. In vitro conservation techniques offer several advantages compared to field maintenance of vegetatively propagated crops.<sup>47</sup> Some of these include: 1) reduced cost of labor and space; 2) reduced risk of contamination by pests and diseases; 3) reduced risk of damage by natural calamities; 4) year-round availability of all plant material; and 5) ease of rapid multiplication in large numbers. However, it must be noted that in several developing countries there are still problems, such as an uncertain power supply and the availability of chemicals, in making the technology fully viable in such countries. Three areas of tissue culture techniques that are promising are discussed here.

### *In Vitro Conservation*

Here we consider mainly the tissue culture techniques and slow growth methods aimed at short- to medium-term conservation. Possibilities now exist to conserve plant genetic resources as tissue culture.<sup>116</sup> As noted earlier, for some species, in vitro conservation may be the only option available. Although tissue culture offers great potential to conserve germplasm of vegetatively propagated material and species with recalcitrant seeds, two major constraints have been hindering its extensive use. First, material conserved in tissue culture is genetically unstable due to somaclonal variation at the time of regeneration of the tissue into seedlings. Second, the length of storage as tissue is limited. Significant work is being done on both these aspects and for some species, tissue culture can be used effectively due to improved techniques resulting in low levels of somaclonal variation. Work on cryopreservation of tissue cul-

ture, so that these could be preserved for long periods, is also making rapid progress. It appears that the morphogenetic potential is not affected by freeze preservation of tissue cultures and normal plants could be reproduced.<sup>117</sup> Once these techniques are refined through further research and development, large-scale adoption will be possible and conservation of plant genetic resources could become very cost-effective.<sup>70,118-121</sup>

The full exploitation of in vitro genetic conservation is possible only when a species can be propagated from cultured tissues or cells,<sup>19</sup> i.e. regeneration and propagation are essential to in vitro conservation. This is also one of the most interesting applications of in vitro biotechnology in plant breeding. Although much work has been done and protocols for clonal multiplication are well-established for several species,<sup>121</sup> more work needs to be done for species like coconut for which protocols are not fully developed. Similarly, methods of propagation have to be carefully devised to minimize the somaclonal variation which results in genetic instability as well as loss of genetic integrity of the material conserved, e.g. in *Musa spp.*<sup>46</sup> Additionally, the merit of establishing large-scale production areas using rapid multiplication and clonal propagation has to be carefully studied, as the danger of genetic vulnerability due to the uniform nature of the plantation is real. Therefore, it is very important that any in vitro gene bank establishes collections which adequately represent the range of genetic diversity for a given species. Care should also be taken to distribute sufficiently diverse material to the users, along with information on the dangers of using uniform material.<sup>121</sup> In vitro culture work on rare medicinal plants will have an impact not only from the point of view of ex situ conservation but also on the implementation of effective in situ conservation programs.<sup>122</sup> Through micropropagation and establishment of cultures that produce the chemical needed or by establishment of fields of the plants, the stress on the material in the wild can be reduced and uncontrolled extraction from the wild can be avoided.

Although there is a wealth of information on tissue culture of plants, the most important aspect of in vitro conservation using this technique is the capacity of plants to regenerate in vitro. The physiological and biochemical basis of this process is still poorly understood. A recent study on tomatoes indicated that some progress is being made in this area.<sup>117</sup> In this study, RFLP linkage analysis of the gene that controls regeneration ability in tomato was combined with morphological and physiological analyses. This approach showed that the genetic component associated with regeneration

determines the morphogenetic competence and not the sensitivity to hormones as previously assumed. A locus that exerts a major effect on the tissue culture behavior of tomato has been mapped. The eventual cloning of a tomato regeneration gene may assist in better understanding of the regeneration process. However, it may not solve all problems encountered in regenerating other genotypes or species. Different steps controlled by different genes may have limited effects on others.

For the purposes of conservation of plant genetic resources in vitro, there are basically two approaches: slow growth and cryopreservation.

### **Slow-Growth Technology**

For the purpose of conservation of plant genetic resources, the growth of cultures should be kept to the minimum, if not completely arrested. This is essential to avoid frequent transfer to fresh media, which would require a high level of inputs and will make in vitro conservation expensive. There are several ways to achieve slow growth, such as use of immature zygotic embryos, modification of culture medium by adding osmotic or hormonal inhibitors or other growth retardants, reduction of storage temperature (4–10°C for temperate species and 15–25°C for tropical species), mineral oil overlay, reduced oxygen tension and defoliation of shoots.<sup>48,123</sup>

It is important that any such methods developed or adapted do not interfere with the genetic stability of the material conserved. Usually, organized cultures such as shoots are used for slow growth storage as unorganized tissues such as callus are more vulnerable to somaclonal variation. A good review of tissue culture-induced variation in plants can be found in Phillips et al.<sup>124</sup> With some root and tuber crops, temperate fruits, ornamental and horticultural species and a few forestry species, apparently routine slow growth storage for one to 15 years (what could be called a medium-term storage) is possible using cycles up to two years before subculturing.<sup>48</sup> However, there are not enough data on long-term effects of slow growth. More research on in vitro conservation is in progress so that this biotechnological tool can be used effectively for conserving genetic diversity. Although the medium-term in vitro conservation of *Musa* by slow growth is now routine, somaclonal variation is a major problem for long-term preservation. Research is in progress at the University of Birmingham, UK, to develop early markers to detect variants using RAPDs.<sup>125</sup> Long collaboration between CIAT and IPGRI has resulted in in vitro conservation techniques and management procedures for cassava germplasm. These results

indicate a high potential for using in vitro conservation technology in many other vegetatively propagated species.

### **Cryopreservation**

Theoretically, cryopreservation using liquid nitrogen (either by immersion or in gas) as a storage medium is ideal for long-term storage since it virtually suspends all the metabolic activities in any living tissue, be it seed, cell suspensions, callus, cultured tissue, pollen or a shoot tip. It is a relatively new conservation method. Research on development of protocols for cryopreservation of in vitro plant material started in the early 1970s,<sup>126</sup> almost at the same time as experiments for cryopreservation of seed were initiated.<sup>127,128</sup> Availability or development of plants from cryopreserved cells and meristems is the basic requirements of cryopreservation.<sup>129</sup> Some of the advantages of cryopreservation of in vitro material are: physical and genetic stability for a long period of time, at least theoretically, it is relatively economical<sup>123</sup> and conserved material is easily accessible.<sup>130,131</sup> However, as indicated earlier, there is a need to gather data on a long-term basis to confirm these conclusions. IPGRI is supporting research on potato in Germany to refine a cryopreservation method for potato.<sup>125</sup> IPGRI is supporting research in Costa Rica on developing a cryopreservation method for somatic embryos of *Musa*.

The stages in cryopreservation of in vitro cultured material are: 1) selection; 2) excision of plant tissues or organs and culture of source material; 3) selection of healthy cultures; 4) pregrowth-related treatments; 5) application of cryoprotectants; 6) cooling/freezing; 7) storage; 8) warming/thawing; 9) post-thaw treatment; 10) viability testing; and 11) recovery growth.<sup>123,132,133</sup> Cryopreservation is most successful for cell cultures, since differentiated cultures may be damaged by structural injury. One promising method that counters such effects is ‘vitrification.’ This method involves immersion of the culture in a very high concentration of cryoprotectant solution, followed by rapid cooling. The remaining water in the tissue vitrifies, forming a non-crystalline solid.<sup>46</sup> Cultured cells and somatic embryos derived from the mesophyll tissues of asparagus were cryopreserved by vitrification by Uragami et al<sup>134</sup> and the survival rate, as determined by shoot formation, was 63%. For a review of the methodology and the woody plant species that have been successfully cryopreserved, the reader is referred to the work of Sakai.<sup>129</sup>

Cryopreservation of somatic, pollinic and zygotic embryos has also been successfully done in

some 12 species.<sup>123</sup> Work on cryopreservation of nucellar and somatic embryos of mango is in progress in the University of Florida, U.S.A., with IPGRI's support. RFLPs and RAPDs may be used to monitor genetic stability of cryopreserved material.<sup>135</sup> All the analyses performed so far using phenotypic, isozyme and molecular techniques have not indicated any genetic changes in cryopreserved material in comparison with controls.<sup>21</sup> However, the number of tests and the length of time are not yet sufficient to recommend this method on a large scale. This is another area which requires further research.

Another promising method for the conservation of clonally propagated species with recalcitrant seed, based on cryopreservation is the possibility of producing the so-called 'synthetic' or 'artificial' seeds and conserving them as true seeds.<sup>46</sup> This involves encapsulation of shoot-tips and somatic embryos in semi-solid material that serves as an artificial seed coat and endosperm to produce 'beads.' Beads may also contain nutrients and pesticides. Somatic embryos and shoot tips are active and lack desiccation tolerance. So a developmental switch to induce tolerance can be provided through the inclusion of a hormone 'signal' in the medium. This has been achieved in somatic embryos of alfalfa where 100% of the embryos survived drying to 10% moisture content (also see Dereuddre et al<sup>137</sup> and Schulthies et al<sup>138</sup>).<sup>136</sup> If the current pace of development in this technology continues, and reproducible and widely adaptable results are achieved, production and storage of artificial seeds could become an extremely important technology in plant genetic resources conservation and use.<sup>46</sup> Redenbaugh<sup>139</sup> has reviewed the developments of synthetic seeds, including new methods for encapsulation of somatic embryos and the creation of synthetic endosperm.

Case studies of the application of synthetic seeds are presented for crops such as alfalfa, carrot, celery, grape, lettuce, mango, mulberry, orchard grass, sandalwood, soybean and spruce. Encapsulation of somatic embryos can be an effective method to handle otherwise fragile somatic embryos. Encapsulation of somatic embryos using sodium alginate and calcium chloride solutions was successful, as was growth of plantlets of encapsulated carrot embryos.<sup>140</sup> Work is also in progress at the University of Florida on mango, a prospect for developing artificial seeds for conservation purposes.<sup>125</sup>

Among the biotechnological tools available currently, further development of cryopreservation technology for a large number of plant species, especially for those which need to be maintained in fields and orchards and/or in vitro, using slow

growth methodology, is expected to be extremely cost-effective. This technology will reduce routine gene bank operational costs, space requirements and long-term maintenance costs. If liquid nitrogen is available, this technology could be used in most of the developing countries also. As indicated earlier, there are a few problems that need to be solved before the technology can be used routinely, but the potential is very high.

### *In Vitro Gene Banks*

So far we have discussed different components of an entirely in vitro-based conservation scheme for clonally propagated and other problem crops. We have also seen that there are still a number of details that need to be worked out. While these developments are going on, there is need to put all these components together—protocols for tissue culture, successful regeneration, transfer to soil (the protocols which differ from species to species, sometimes there are genotypic differences), genetic stability, and cryopreservation of cultured material either by vitrification or by encapsulation. When this is achieved we have a viable long-term conservation strategy for plant genetic resources for an in vitro gene bank. To date, only for strawberry have all the stages of such a scheme been well researched and all major problems resolved, though significant progress has been made in potato and cassava.<sup>132</sup> The production of potato and cassava plants capable of undergoing normal tuberization and rooting from meristems cryopreserved for 4 years with no significant changes in viability and genetic composition has been reported.<sup>141</sup> Day-to-day management of in vitro gene banks has also been studied to some extent.<sup>142</sup> This would consist of receiving vegetative material from either a field gene bank or a collecting mission, followed by processing the material for disease indexing, therapy and quarantine, as required. Healthy and clean material will go into the next stage of in vitro culturing. From this stage the material could either go into the cryopreserved base gene bank (long-term) and/or into the in vitro active gene bank in slow-growth conditions. In the later case, subculturing and transferring to new medium may have to be carried out at regular intervals of one to two years, depending on the material. An active bank will be used for supplying material to users and/or to establish field gene banks.<sup>121</sup> Successful implementation of an active in vitro gene bank for 14 yam species, as was proposed by IBPGR,<sup>143</sup> has been recently reported.<sup>144</sup> Similar successes, though partial, have been reported by several workers.<sup>145-148</sup>

From the above discussion it is clear that conservation technology based on tissue culture and

cryopreservation is very promising, and could change the way we look at conservation issues. The methods hold vast promise. In some cases they are operative already since seed- or field-based conservation techniques for the particular species do not work for them.

### **DNA Libraries or Gene Banks**

At the molecular level, storage of DNA may be considered a conservation of genetic resources.<sup>19</sup> The progress in genetic engineering has resulted in breaking down the species or genera for transferring genes. The first successful plant-to-plant gene transfer was in 1983. Since then transgenic plants have been produced with genes transferred from viruses, bacteria, fungi and even mice.<sup>121</sup> Progress in this field has led to the establishment of DNA libraries, containing single useful genes for breeding programs. In addition, suggestions have been made to store total genomic information of germplasm in the form of DNA libraries.<sup>149-153</sup> The University of Queensland, Australia, for example, established a gene library in 1989 to collect and preserve DNA from Australasian species, especially the species that are rare, endangered or have biotechnological value.<sup>153</sup> According to Mattick et al,<sup>153</sup> such libraries may serve the following two functions: 1) a resource for exploring biological diversity and evolutionary history; and 2) a resource of increasing importance for the advancement of biotechnology. Despite the current problems with this approach, rapid progress may make the storage of DNA an additional option for the conservation of plant genetic resources.<sup>121</sup> Such readily-available genetic resources (without going through the process of collecting, extracting, etc.) for scientists working at the molecular level will be an added advantage. The technique may also allow the recovery of genes from apparently extinct taxa by using herbarium and other non-viable materials, as demonstrated by the sequencing of DNA of fossils.<sup>64,154</sup> In a discussion of the prospects of accessing DNA banks for the isolation of genes encoding biologically active proteins, it was concluded that the technology might not have a major limitation for utilization of DNA from banks but the basic knowledge concerning biochemistry, bioactivity and pharmacology of plant chemicals appeared to be limiting.<sup>155</sup>

### **In Situ Conservation**

As defined earlier, in situ conservation involves conservation of diversity in the habitats where it evolved or occurs. In situ conservation can be carried out either in nature or on farm, depending on the material under consideration. This type of

conservation is dynamic as opposed to the semistatic nature of ex situ conservation, providing the species or population with an opportunity to continue to evolve under natural conditions. For some forms of biodiversity, in situ conservation is the only option. The main reason given for choosing in situ conservation over ex situ is the need to maintain the evolutionary potential of species and populations.<sup>1,156,157</sup> This view is not only from the plant breeder's perspective but also, of late, from the conservation biologist's concern for maintaining the variability in small populations and endangered species. The three general research strategies that are needed for in situ conservation are to assay genetic variation represented in specific areas to document its relationship to overall patterns of geographic variation (e.g. studies of isozyme- or DNA-restriction site variation) within and among populations, to conduct genecological studies (reciprocal transplant studies or progeny tests) to compare performance of native-site-derived material within and among seed zones, and to emphasize further study of special features of genetic variation that have been revealed by either previous research or experience with managed populations or plantations.<sup>158</sup>

From the above, it follows that for successful operation of any in situ conservation program for crop landraces we also need information of the following genetic aspects: 1) studies on genetic erosion due to the introduction of new varieties; 2) identification of regions rich in genetic diversity; 3) effects of land fragmentation on genetic diversity; 4) temporal and spatial changes in genetic structure of populations; and 5) biogeographic studies, especially when introgression is involved. In the case of agrobiodiversity, the effects of farmers' practices, cultural preferences and environmental factors are the most important factors. In almost all the studies that provide information on the above, it should be possible to use biotechnological tools to allow precise assessment of extant diversity as well as changes in it over space and time. These can also be used to monitor changes that occur in gene frequencies,<sup>59,72,159-162</sup> which is the core of any program of conservation.

### **Germplasm Exchange**

It is important that all accessions in the gene bank should be available to all those who wish to use them, either for crop improvement or for other studies. Although such free exchange of plant genetic resources is highly desirable, it may pose problems from a germplasm health point of view. This is because pests can move along with the seed/propagules of any plant material and can cause

serious problems in the new environment into which they have been introduced.<sup>163</sup> Biotechnology has played an important role in assisting the safe distribution of plant genetic resources through exchange of plant genetic resources as disease-free cultures.<sup>47,164-167</sup> Meristem culture is a way of cleaning clonally propagated plants of infections of bacteria and/or viruses. This method is extremely useful for making disease-free stock plants for exchange or for starting a cycle of plantation.<sup>168</sup>

Reliable virus detection methods are important and several diagnostic methods are under development.<sup>169</sup> An urgent need for the development of broad spectrum tests for virus detection has been identified since a separate test for each virus, using a specific antiserum or DNA probe<sup>170</sup> can be laborious and expensive. Application of broad spectrum serological methods, such as those developed for potyviruses,<sup>171</sup> may become important tools as they are fairly simple to apply and require less sophisticated equipment than nucleic acid hybridization.<sup>172</sup> Nucleic acid hybridization methods using <sup>32</sup>P, however, have been successfully employed for detection of small quantities of viruses in plants.<sup>163</sup> Some initial results on the use of DNA probes which would recognize coat protein genes or other genes common to a number of different viruses and possibly bacteria have been reported,<sup>173</sup> but are yet to be used routinely.<sup>169</sup> Detection of pathogens has to be followed up by their elimination if a safe exchange or conservation of particular germplasm material is needed. Some of the widely used methods for the elimination of viruses are heat therapy, meristem tip culture alone or combined with chemotherapy.<sup>163,165,174,175</sup> There is a need to expand research on disease transmission through embryos and disease detection methods using in vitro samples.

Simple methods such as thermotherapy and meristem culture can be applied to in vitro materials to eradicate virus and viroid diseases.<sup>47</sup> Many gene banks are able to use such technology, sometimes with help from external sources, so that pathogen-free genetic resources can be supplied to users worldwide without imposing quarantine risks on the recipient program or country.

## Biosystematics and Evolution

At the conceptual level, several authors believe that two plant species isolated by a chromosomal barrier can, via hybridization, give rise to new fertile diploid species that are partially reproductively isolated from both parents. This is termed recombinational speciation.<sup>176,177</sup> The actual extent of this mode of speciation in nature is unclear and detailed genetic information is necessary to

confirm or reject the hypothesis. One solution is to use genetic markers representing both the biparentally inherited nuclear genome and a uniparentally or clonally inherited cytoplasmic genome such as the chloroplast or mitochondrial genome.<sup>178-180</sup> Much work is in progress in many laboratories around the world. This is probably where the molecular genetic tools will have greater use since such information is the basis for conservation of biodiversity.

Research on plant genetic resources, integrating many fields of study, greatly helps in understanding the evolution of cultivated plants and their wild relatives.<sup>181</sup> A good understanding of the evolution of a given species, its relationship to other species belonging to the same gene pool, including the traditionally distinguished primary, secondary or tertiary gene pools, as well as to species more distantly related, is of great importance to plant genetic resources conservation and use. Historically, it was mainly morphological information that was used to study and understand systematics, species relationships and evolution. This was followed by the use of biochemical markers and isozymes to study intraspecific and interspecific relationships. Some examples are the studies on common beans,<sup>182</sup> maize,<sup>183</sup> finger millet,<sup>184</sup> sorghum,<sup>185,186</sup> tomato,<sup>187</sup> potato<sup>188</sup> and on *Musa*<sup>91,189</sup> (for a general review see Buth<sup>190</sup>). Over the last ten years or so, there has been a rapid increase in studies that use biotechnological tools to better understand biosystematics and evolution of plant species.

Biosystematics at both interspecific and intraspecific levels are essential for proper classification of the material conserved to determine breeding behaviors such as incompatibility, etc. for effective conservation and use of plant genetic resources and biodiversity.<sup>191</sup> Although the advent of genetic engineering enabled us to move genes much more easily, even between completely unrelated taxa, we should not forget that recombination DNA technology is not going to free the geneticist from the need to make crosses and to screen segregating populations, etc. Additionally, the plant species that are most amenable to genetic analysis and manipulation via classical Mendelian methods are also where molecular technologies are being most avidly applied, e.g. *Arabidopsis*, tomato, potato and maize,<sup>192</sup> though the situation has changed dramatically over the last few years, as we will see later on. The available techniques need to be applied to more complex taxa so that the techniques can be useful in deriving new relationships rather than just confirming what is already known through the use of other techniques.<sup>193,194</sup>

This will be essential since a good knowledge of species relationships as well as genomic homologies will facilitate the transfer of genes or even of substitutions of chromosomes or chromosome segments from one species to another.<sup>135</sup> A major strength of molecular genetic analysis is that it provides numerous independent molecular characters that can often rigorously define monophyletic lineages. The use of molecular markers can involve two separate steps. The first one is to address questions about phylogenetic relationships. The next step is to address questions about character evolution (where, when and how the character states arose). Through this second application, molecular phylogenetics has a major impact on many aspects of systematics, evolution, genetics and ecology.<sup>193</sup> Keeping this background at the back of our mind, we will now look at a few examples in which biotechnology has assisted in studying better the biosystematics and evolution of plants.

Miller and Tanksley<sup>60</sup> used RFLP analysis to study the phylogenetic relationships and genetic variation in the genus *Lycopersicon* and drew interesting conclusions, including the fact that the amount of genetic variation in the self-incompatible species far exceeded that found in self-compatible species. CpDNA analyses, in combination with morphological data, have been used to investigate relationships between genera, subgenera and species within the *Solanaceae* family, revealing that *Lycopersicon* and *Solanum* are congeneric and both belong to the subgenus *Potatoe*.<sup>194,195</sup> Sugar cane taxonomy, genetics and breeding are made difficult by the extreme genetic complexity of this species.<sup>196,197</sup> Molecular techniques have helped to reduce the confusion.<sup>198-201</sup> Classification of rice in botanical varieties *indica*, *japonica* and *javonica* is important because of the difficulty in hybridization and recombination between these groups. Genetic diversity analysis of rice cultivars using RAPDs provides an immediate practical application as a fast method to classify relatively uncharacterized accessions.<sup>202</sup>

Several such applications have also been reported by using the variable number of tandem repeats (VNTRs) technique, in which a core sequence of only a few base pairs is classed as a 'microsatellite' locus and if larger as 'mini satellite'.<sup>161</sup> Armour et al<sup>203</sup> give a good overview of this technique and its applications. There are numerous examples in the literature, appearing in almost every issue of modern journals. Most of them use a combination of markers and either nuclear or cytoplasmic DNA (chloroplast or mitochondria). Some examples are lentils,<sup>204</sup> potato,<sup>205</sup> rice,<sup>206,207</sup>

maize,<sup>208,209</sup> *Triticum*,<sup>210</sup> Guinea yams,<sup>211</sup> cucurbits,<sup>212</sup> *Musa* spp.,<sup>213-215</sup> *Glycine* spp.,<sup>216</sup> *Papaver* spp.,<sup>217</sup> *Allium* spp.,<sup>218,219</sup> *Cajanus* spp.,<sup>220</sup> and barley.<sup>102,221</sup> There are still a few problems and much work is in progress on many of the biotechnological tools that are being used in clarifying taxonomic problems in many plant species.<sup>5,222,223</sup>

## UTILIZATION OF PLANT GENETIC RESOURCES

One of the major objectives of conservation of plant genetic resources is to make genetic diversity available for immediate or future use. Abundant evidence exists showing that it is necessary to preserve a wide range of diversity in order to meet the crop improvement needs. However, it is also evident that the widest possible range of genetic diversity has to be conserved in order to meet future, as yet unknown, needs.<sup>59</sup> Any plant genetic resources program is expected to promote and facilitate the use of conserved material through the maintenance of healthy and readily accessible and adequately characterized/evaluated material, and proper documentation of the relevant information. Although plant breeders recognize that their working collection is limited, most make little use of extensive genetic variability available in gene banks and other collections. This is because breeders continue to make reasonable progress in most crop species. Broadening the activated genetic base generally dilutes agronomic performance. Yet new germplasm can raise the genetic ceiling of improvement, decrease vulnerability to biotic and abiotic stresses and add new developmental pathways and ecological adaptations to breeding material.<sup>15</sup> Biotechnology can enable breeders to use biodiversity at the gene level without introducing wild characteristics. Biotechnology offers various means of manipulating the fundamental processes of energy flow and biogeochemical (nutrient) cycles.<sup>224</sup>

Besides being a useful technique to analyze genetic diversity, molecular techniques can play a major role in utilizing this diversity in plant improvement programs in the future for enhanced sustainability and productivity. Biotechnology comprises powerful sets of tools which can be used in conjunction with plant breeding to develop improved or new crop cultivars. This technology is mainly based on the component disciplines such as molecular biology, cell biology and plant breeding. These include tissue culture technology (cloning, haploid production, cell fusion, plant regeneration, etc.), DNA diagnostics (such as finger printing, sequencing) and genetic manipulation/genetic engineering methods (gene cloning, gene

transfer, transformation and other recombinant DNA technologies). However, it is essential to keep in mind that most of the emerging technologies tend to disregard the many interactions between genes i.e., primarily looking only at the effects of gene(s) and not the whole effect of the gene(s) in the genomic background of crop plants, the genotype.<sup>225</sup> There is a need to increase efforts to correct this aspect as these methods could be used effectively for improving the productivity of plants on a sustainable basis.

Persley<sup>226</sup> cites the following as areas in which biotechnology has made a significant contribution and fulfilled its early potential: 1) the development of genetic engineering techniques for crops; 2) new tissue culture techniques (e.g., embryo rescue) to enable wide crosses to be made between different crop species; and 3) cost-saving techniques (e.g., refinement of tissue culture techniques to facilitate conservation of plant genetic resources). Considering the work that has gone on in different laboratories, it may be concluded that biotechnology, despite a number of technical problems and social and ethical issues that need to be solved, has a vast potential for increasing the number of plant species that humans have been using for their food, fiber, etc. This may be due to the fact that the genetic engineering techniques may target underexploited plant species to increase the number of crops we depend on and thus diversify agriculture. It can also assist developing nations by focusing on regionally important species for use, including sources of food, fiber, medicine, etc.

#### DNA MARKERS USED FOR CONSERVATION AND USE OF PLANT GENETIC RESOURCES

A brief note on the major developments in the technology that has greatly helped in making rapid advances in genetic engineering of plants will be appropriate. It is not the intention of the authors to describe these tools in great detail but only to review major advances that are particularly useful in using the molecular marker technology in accessing or using the large amounts of variation that are available to plant improvement scientists. An attempt is also made to indicate the level of technology needed so that researchers in developing countries can determine the appropriate technique that may be used in their laboratories. Readers may consult numerous reviews on the topics, as well as some cited here for greater detail.

#### Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis provides information on the number and sizes of DNA fragments following

digestion of DNA by restriction enzymes. The fragment pattern, as interpreted by hybridization with single copy random genomic clones, cDNAs, rRNAs or other repeated sequence probes, depends upon the presence or absence of restriction sites and also upon the restriction enzymes and probes used. RFLPs provide resolution at the genomic or chromosomal level. It is mainly a 'marker' technique.<sup>79</sup> RFLPs are inherited in codominant fashion, they look at a part of the genome, need an intermediate level of technical capabilities, are highly reliable, require relatively large quantities 2-10 µg but crude extracts of DNA and their use of radioisotopes and data comparability is intermediate. RFLPs are considered most useful in genome mapping and marker assisted selection. RFLPs may not have any adverse effect on the overall fitness of the populations (neutral) and, consequently, the transfer of a particular RFLP marker into an adapted cultivar is not expected to cause any damage or reduce the overall fitness of the recipient. However, a substantial proportion of RFLP variation may be the result of insertional events<sup>227,228</sup> and there is the possibility that the insertional mutations are either slightly deleterious or unstable. In either case, it is important to conduct careful population surveys of insertional variation as a first step.<sup>71</sup>

Despite the problem mentioned above, the application of modern methods appears to have contributed to rapid progress in breeding using wide hybridization. RFLPs have been used to fingerprint, to generate genetic maps<sup>229</sup> and to enable the identification of specific genotypes and agronomic traits.<sup>47,230</sup> High density RFLP maps provide an opportunity to resolve complex traits into their individual genetic components. It might then be possible to treat these characters as single gene traits or quantitative trait loci (QTLs<sup>135</sup>). The use of the polymerase chain reaction (PCR) has further improved the application of biotechnology by increasing our understanding of DNA sequences through selective amplification of a specific DNA segment. This facilitates specific transfer of the desired segments.

#### Random Amplified Polymorphic DNA (RAPD)

RAPD marker analysis allows genetic diversity studies in species for which no molecular information exists and permits much more rapid data collection than RFLP. Because of this fast and cost-effective method, analysis of large numbers of accessions is possible. This will contribute to a better exploitation of plant genetic resources collections.<sup>100</sup> This is a PCR-based methodology which uses about 10-mer oligonucleotides as primers with

randomly generated sequences.<sup>231</sup> The pattern of amplification products is visualized through simple agarose electrophoresis. The pattern depends on the number and distribution of recognition sites throughout the genome. Resolution is at the genomic level and this is also a typical marker technology.<sup>79</sup> Without any other modifications, RAPDs are most useful for population genetic studies. RAPDs are inherited in a dominant fashion and allow researchers to look at the whole genome. It is a relatively simple, relatively reliable (lab to lab variation can be significant) technique that uses 10-50 µg of DNA extract, and no radioisotopes; however, data comparability is low.

### **Variable Number of Tandem Repeats (VNTRs)**

This provides a measure of the number of serial repeats of a core DNA sequence through the overall length of the repeat region. A core sequence with only a few base pairs is classed as a microsatellite locus and if it is larger it is classified as a minisatellite. Alleles at the same or different loci may vary significantly in length due to the variation in the number of serial repeats. Length variations are visualized as multilocus fingerprint phenotypes or as single locus genotypes. VNTR are inherited codominantly; the technique is capable of looking at the whole genome; reliability is variable depending on the level of skill and laboratory facilities; the technique requires small quantities (µg) of moderately pure DNA and it may or may not require the use of radioisotopes; and data comparability is low. Depending on their length (micro or mini) VNTRs can be used to study single locus (identification of individuals and pattern analysis) or multilocus (marker-assisted selection, determining relatedness of individuals) problems and in genetic diversity studies.

### **Polymerase Chain Reaction (PCR)**

The information consists of base sequences of known genes. A particular gene is amplified, sequenced and the base composition is revealed by using 20-mer specific primers. This provides the highest level of resolution because the differences are measured at the level of a base. Rapid progress in this area has led to extensive automation of certain steps in the procedure and to large-scale DNA sequencing studies. This method may be used to look at varied lengths of genome, inherited in codominant fashion, it is highly reliable, only small quantities (pg-nl) of crude DNA are required and data comparability is high. PCR/sequencing, by itself, can be used in population studies, taxonomy and phylogenetics.

Besides these basic techniques, it is possible to carry out genetic characterization by combining some of the above techniques with other methods such as allele-specific oligos (ASO) and denaturing/temperature gradient gel electrophoresis (DGGE/TGGE). Such methods can detect differences down to single base substitutions, in the former, by means of allele-specific oligomers and, in the later, through a chemical or temperature gradient in a electrophoretic gel.<sup>79,232</sup> The field of genetic markers is fast growing and there are several combinations possible with acronyms such as SCAR, SPLAT, AFLP, SAP, etc.

### **AVAILABLE TECHNOLOGIES**

Most of the efforts directed toward the use of biotechnology tend to be used in combination; very rarely is a single technique used. These techniques, along with conventional breeding methods, can be viewed as the evolution of plant breeding.<sup>233</sup> Hence we plan here to look at the individual tools available and then look at a few examples where such techniques have been used. There is a need to be cautious, as this is a field in which very rapid progress is being made. By the time this chapter is published there may be several new additions to the list. However, they may all be based on the study of sequencing of DNA and hence the basic ground rules will still hold good. Additionally, no attempt is made here to provide detailed information on each of the methods, we only provide some singularities that may affect the way in which each of them may be used. Broadly speaking, the biotechnology that can be used for plant improvement can be divided into two areas: tissue culture technology and recombinant DNA technology. Each of these constitute several variations. However, as just indicated, it is very rare that any one technique may be used for plant improvement; it is easier to deal with these in the following manner.

### **CELL AND TISSUE CULTURE TECHNIQUES**

#### **Haploid Production**

One of the most useful methods developed for plant breeding is the production of haploid plants from both male and female gametophytic cells through the culture of anthers, microspores, ovaries and ovules. Most species exhibit a positive in vitro response to culture conditions that lead to the development of embryos and/or calli from which plants can be regenerated. Haploid plants can be raised as pure breeding lines for hybrid cultivar development in highly heterozygous cross pollinating crops. Desirable genetic recombinants can

then be selected from the homozygous lines established by chromosome doubling. Cultured microspores can be used in mutant isolation and highly embryogenic isolated microspores offer great potential as recipient cells for the introduction of alien genes. Haploid production technology is not very complicated and the technology can be easily modified and adopted in different conditions. However, this technique is yet to be exploited to its full potential. Rice was the first cereal to be regenerated into complete plants from cultured tissue,<sup>234,235</sup> and particularly anther culture.<sup>236</sup> Selection for herbicide resistance in canola has been greatly simplified by the use of the microspore culture technique.

### Protoplast Technology

The production of somatic hybrids depends on successful integration procedures such as protoplast isolation, protoplast fusion, cell wall regeneration and cell division, the identification of hybrid cells and the regeneration of hybrid plants. The progress in in vitro culture techniques has led to the production of several new hybrid plants over the last 10 years with novel combinations of mitochondria and chloroplasts which encode cytoplasmic male sterility and herbicide resistance. Somatic hybridization has numerous applications as a means of producing new combinations of desired characteristics. Protoplast technology and reliable regeneration procedures should be developed for tropical crops in order to maximize the potential significance of somatic hybridization for crop improvement. The potential of somatic hybridization in crop breeding, including solanaceous crops and brassicas, has recently been reviewed.<sup>237</sup> It was concluded that the somatic hybridization method can be used for combining species with different degrees of genetic divergence and that even species from different tribes can be hybridized. Several hybrids, symmetric or asymmetric, have the potential to be used as a bridge between the alien species and the crop plant. With the development of molecular techniques, better methods to detect and follow the introgression of an alien DNA in the receptor genome can be devised.<sup>238</sup> Protoplast technology provides the breeders with the potential tool for combining sexually incompatible species and enhances the chances of utilizing the diversity available to them.

Wide hybridization, which will be discussed briefly later on, is an important strategy for introgressing traits between cultivars that show sexual incompatibility and other problems, as well as the need to eliminate significant amounts of background genetic material from the donor. In

such cases, the somatic hybridization method has great potential for novel wide crosses. These techniques may combine protoplast isolation, culturing and regeneration. As reported by Waara and Glimelius<sup>237</sup> significant successes have been achieved in solanaceous types and brassicas, but in other species, mainly due to regeneration problems, success has been limited.<sup>239</sup> Some commercially important examples of cybrids and other novel types have been reported in *Brassica* crops<sup>240</sup> and in solanaceous crops.<sup>241</sup>

### Somatic Embryogenesis

The application of tissue culture and genetic engineering techniques for making efficient use of available genetic diversity depends on the ability to regenerate plants from cultures of tissues, cells and protoplasts. Proper protocols to regenerate fully developed plants that grow to maturity from cells and tissues are obviously a prerequisite for making use of most of the biotechnologies for plant improvement. Plant regeneration in vitro occurs via somatic embryo formation or a new individual with bipolar structure arising from a single cell in callus or suspension culture. These individuals can be induced to form in large numbers subsequently and to develop into whole plants. Using this technique it is possible to propagate a selected line (elite line) with a uniform phenotype (clone) and has significant potential for vegetatively propagated root and tuber crops and in forest species. One of the methods described under cryopreservation earlier on, development of artificial seeds using the desiccation tolerant somatic embryos, is also being seriously researched because it allows the inclusion of initially required nutrients and plant protection agents in the beads.<sup>136,139,242</sup>

Efforts are underway to develop protocols for somatic embryogenesis of elite clones for transgenic and mutant plant production in rubber. Recent studies have indicated that the origin of *Hevea* embryoids via anther and ovule culture is a single cell.<sup>243</sup> Increased efforts are underway to develop better protocols for regeneration and propagation of the products of plant transformation.

### Microppropagation

Micropropagation of plants has been the most basic and extensively used tissue culture technology in the clonally propagated plants and in plants that may be difficult to reproduce through sexual means, or in cases where the rate of multiplication is slow or when a highly uniform planting material was desired. Given enough tissue culture knowledge and skills, improved techniques for rapid micropropagation using disease-free cultures (callus,

cell suspensions, protoplast cultures or somatic embryos) can be applied to numerous species, such as oil palm, cacao, aroids, root and tuber crops, ornamentals as well as forest species.<sup>53,54,244,245</sup> The skills and facilities needed are not very sophisticated for exploiting this technique. Micropropagation can play a crucial role in producing the required number of seedlings for either establishment of plantations or for reforestation.<sup>168</sup> It has been widely used in scaling up production of propagules of clonally propagated crops,<sup>47</sup> even in less developed countries. However, there is a need to be cautious when deciding whether this technique has the potential to propagate highly uniform material. Such high uniformity can result in genetic vulnerability of the material. This can potentially be a major problem, especially for perennial species. The example of grapevine in California has already been given. The risk in this case is much greater than that for annual seed-propagated crops, because more time is required for searching and replanting woody species.<sup>19</sup> The Food and Agricultural Organization of the United Nations (FAO) has also cautioned against massive use of crop varieties reproduced through in vitro culture, which contain genetically identical copies of the parent, as this could provoke increasing genetic erosion.<sup>246</sup>

### Embryo Rescue

This method is used to rescue F<sub>1</sub> embryos that result from crossing genetically widely separated (incompatible) plants. The embryo that might abort due to various reasons in such wide crosses is excised and cultured to develop into normal seedlings. Conventional hybridization, tissue culture and DNA analytical techniques to confirm the hybridity are some of the techniques that may be used in this method.<sup>54,247-249</sup> Preliminary studies on embryo rescue in *Hevea* yielded some success and further work is in progress.<sup>243</sup>

### Somaclonal Variation

Somaclonal variation is considered to be a major problem while using tissue culture technology for conservation of genetic resources since it can damage the genetic integrity of the material conserved. Nevertheless, the same may be used with advantage in crop improvement, in that somaclonal variation is in a unique position. This could be termed a method of generating 'new' variation for evaluating, selecting and identifying new plant types.<sup>250</sup> Karp<sup>251</sup> has reviewed this topic in detail. The main factors that influence the variation generated from tissue culture are the level of organization of the tissue, the genotype, growth regulators and tissue source. Karp concludes that, despite the

increased understanding of how these factors work, it is still not possible to predict the outcome of a breeding program based on somaclonal variation. Some new cultivars have been selected based on somaclonal variation. However, it must be noted that, in most cases, new variants could not be selected because the variation observed in general was negative. In addition, the changes observed were not novel and were not stable after selfing or crossing. Karp concludes that somaclonal variation is not a precision tool and only minimal control can be exercised over it during any breeding program based on it. Nevertheless, it can offer a rapid and easily accessible variability for use in breeding programs. Maluszynski et al<sup>252</sup> provide some examples of using mutation breeding in conjunction with somaclonal variation. However, the use of this technology is restricted to a few clonally propagated species and ornamentals.<sup>239</sup>

The somaclonal method is cheaper than other method of genetic manipulation. At the present time, it is also more universally applicable and does not require 'containment' procedures. It has been most successful in crops with limited genetic systems and/or narrow genetic bases, where it can provide a rapid source of variability for crop improvement.<sup>251</sup> However, there is evidence for the lowering of the agronomic potential of transformed plants due to somaclonal variation arising out of the tissue culture associated with transformation.<sup>253</sup> It is also argued that new problems can also arise with somaclonal variation, principally the high frequency of unstable variation resulting from methylation and sequence amplification among other effects.<sup>239</sup>

### GENETIC ENGINEERING TECHNIQUES

This is a term that is used to encompass all the methods for transfer of alien genes which cannot be incorporated through conventional hybridization techniques (or even to transfer genes between compatible species to hasten up the breeding and to avoid transfer of the rest of the genetic background of the donor parent). This technology basically uses several different techniques in conjunction with traditional breeding techniques. Genetic engineering has evolved from an understanding of how cells function naturally, particularly how DNA codes for the production of proteins. Based on this, the recombinant DNA technology (which comprises a series of techniques) developed to allow manipulation of genetic processes that determine the phenotype of the plants. The principle involved in this type of manipulation is that the genetic material can be transferred from a cell of one species to another (thus breaking

all species barriers) and be made to express itself in the recipient cell, which has been transformed as it now has the recombinant DNA. Basically there are three components: identification and isolation of suitable genes for transfer, the delivery system to introduce the desired gene into the recipient cell, and expression of new genetic information in the recipient cells.

### Transformation Technology

Genetic transformation technology has advanced rapidly in the past 10-15 years so that a segment of chromosome or a single gene can now be delivered efficiently and integrated quickly into the recipient genome. Initially the use of *Agrobacterium* limited the technique to dicotyledons as the bacterium could not infect monocots. However, rapid progress has been made and the technology is now very well established. It has been used routinely to produce transgenic plants in most crop species<sup>254</sup> that have been studied for integration mechanisms, gene regulation and the molecular basis of selective expression of genes. The OECD Report (1993)<sup>254</sup> indicates that between 1986 and 1992 there were 1257 applications for field release of genetically engineered plants and several of these are being released for commercial cultivation. The major limiting factor in producing transgenic plants has been the lack of effective means to introduce alien genes into elite germplasm. However, the development of a novel, direct DNA transfer technique, particle bombardment,<sup>255</sup> bypassing the limitations imposed by *Agrobacterium*-host specificity and cell culture constraints, has made genetic manipulation possible in almost all major crops, including formerly recalcitrant cereals, legumes and woody perennials.<sup>256</sup> This technology has broadened the scope of genetic resources as the gene pool of any plant or animal species has been tremendously expanded since genes can be accessed and introgressed from many different sources. This in turn has expanded the scope for utilizing germplasm that was hitherto difficult to handle or neglected. The gene transfer constraints appear to be much less now and the technical problems that still remain appear not too difficult to be solved.<sup>257</sup>

An area of great concern that should not be neglected is the issue of public perception of environmental hazards due to the release of transgenic plants. Genetically engineered plants have been grown in the field since about 1983, and the studies so far indicate that hazards to humans and the environment are negligible.<sup>258-260</sup> However, this should not lull us into complacency and all the biosafety regulations should be followed. Levin

noted that, though the effects of the release of genetically transformed organisms on biodiversity may be no different from the introduction of exotic species or other cultivars, it is essential to note that introduced species/cultivars are one of the main causes of loss of variation in nature/on farm<sup>261</sup> and argued for risk-assessment field experiments, even for the release of material developed by traditional methods. There are some proponents of early release<sup>262</sup> so that the benefits of use of plant genetic resources reach everyone quickly. If one takes the analogy of safety of drugs, then due care needs to be taken in the case of transgenic plants. It is definitely not a question of 'choice between rapid and delayed introduction.' The reader is referred to *Euphytica*, Vol. 85(1-3), 1996, where several examples of extending and expanding this technology for better exploitation of available germplasm can be found.

### Genome Mapping

Geneticists and breeders have depended for a long time on developing linkage maps to hasten the crop improvement process. The use of markers, such as isozymes, allowed researchers in a few cases to analyze and produce linkage maps which could be used in conjunction with cytological and chromosome banding techniques. As molecular biology progresses, new techniques to map random DNA fragments or cloned genomic sequences that produced linkage maps are becoming available. These are the RFLP maps that can be used by germplasm specialists and crop improvement scientists for better understanding the genetic resources and making use of a wide range of genetic materials in plant improvement.

RFLPs have been used to construct genetic maps in several plant species. Detailed RFLP maps make it possible to identify and detect the effects of genes controlling complex traits, often referred to as polygenic or quantitative traits.<sup>263,264</sup> Such maps assist in measuring the effects of genes controlling quantitative traits and hence can be very useful in plant improvement (e.g. Song et al<sup>264</sup>). The utility of RFLP technology may be compromised by the low levels of RFLP encountered in crops with a narrow (molecular) genetic base such as tomato,<sup>60</sup> wheat,<sup>265</sup> groundnut<sup>266</sup> and others. However, these crops do contain substantial variation for yield, disease resistance, quality characters and many other characteristics that plant breeders wish to manipulate. Given the difficulty in identifying informative (low copy) RFLP markers for such species it may be necessary to check other means such as sequencing along with PCR. Such work was initiated in tomato by Phillips et al,<sup>267</sup> who

used simple sequence repeat oligonucleotides to probe the tomato genome for elements displaying variability among commercial cultivars. One subclone was able to distinguish all 27 cultivars tested, demonstrating its utility as a genetic fingerprinting probe for cultivar identification (see below). Amplification from two contiguous tandem repeats by PCR primers might help in assessing the purity of  $F_1$  hybrid seed lots.

Advances in the three major components of genetic engineering technology—identification and isolation of suitable genes, transfer of selected genes and expression in the transgenic organism—have not proceeded at the same speed. The complexity of genetic systems had been the main reason. Engineering for traits like herbicide resistance has been possible since a single enzyme step degrades the chemicals involved. However, traits such as yield or stress resistance may be governed by a myriad of genes, involve numerous enzymatic steps and make genetic manipulations much more difficult. Finding the exact gene to use is the key to successful genetic engineering. Gene mapping using RFLPs, etc. will promote better use of available diversity. However, the ease with which a genetic map can be developed and applied to a target crop depends on the genetic complexity of the species and extent of DNA polymorphism present in the species. Genetic mapping in genetically monomorphic species has usually been achieved by using wide crosses between highly divergent parental genotypes, sometimes even using different species. The low frequency of DNA polymorphism within a species can also limit the utilization of mapped DNA markers in crosses that are of agronomic importance, but involve genetically more monomorphic parents.<sup>268</sup> As more and more species are mapped, the potential of comparative gene mapping, to look at a group of crops, say legumes, and relate the maps with each other will be realized. This new era of comparative plant genetics is expected to have considerable impact on both plant genetics and crop improvement.<sup>254</sup>

#### SOME EXAMPLES OF USES OF BIOTECHNOLOGY TOOLS WITH PLANT GENETIC RESOURCES

We will now look at some specific cases that demonstrate the use of various biotechnological methods, generally in combination, for the efficient use of plant genetic resources for plant improvement.

#### Wide Hybridization

Sharma<sup>269</sup> reviewed wide crosses in wheat in relation to various factors that facilitate wide cross-

ing to show that wide crosses can be as wide as one can make them. Included in this interesting review is a particular reference to wheat-wheatgrass (*Agropyron* complex) crosses and an update on wide crosses of wheat with various genera of the *Agropyron* complex. Hybrid seed set is too variable to predict whether a wide hybrid, where no seed was obtained in one attempt, will not be possible. High crossability genes seem to facilitate not only fertilization but also seed development, enabling embryo rescue using tissue culture technology. It appears that, contrary to conventional thinking, several wide hybrids with wheat can be produced when species with lower chromosome numbers are used as the female parent. Pre- and post-fertilization barriers to wide crosses do not appear to be equally strong, and can be overcome by the development and application of various biotechnologies. In addition, molecular techniques can be used to ascertain the pedigree of the hybrid. Thus the emerging technology can help in using the germplasm within a gene pool that was previously thought to be unusable. There are numerous examples in the literature on this aspect and it is not intended to provide an exhaustive review of the same here but only to provide a couple of examples to emphasize the potential of the technology. For a review of breeding of industrial oil crops combining different biotechnologies, including wide hybridization, see Thierfelder et al.<sup>270</sup>

Recently the production and cytology of the first interspecific hybrids between cultivated alfalfa, *Medicago sativa*, and perennial species, *M. daghestanica* and *M. pironae*, was described.<sup>271</sup> An ovule-embryo culture technique was used to rescue hybrid embryos and all hybrids were diploid. Predominantly bivalent chromosome pairing was observed at meiotic metaphase. All  $F_1$  hybrids were sterile. Trispecies hybrids could be efficiently recovered by crossing diploid  $F_1$  interspecific hybrids of *M. sativa* X *M. rupestris* with either *M. daghestanica* or *M. pironae*. Ovule-embryo culture had to be used to recover these trispecies hybrids, with a recovery efficiency about 10 times greater than for bispecies hybrids. Most chromosomes paired as bivalents in the trispecies hybrids. Importantly, progeny could be recovered from crossing the trispecies hybrids with *M. sativa*. Therefore, the *M. sativa* X *M. rupestris* hybrids provide a bridge cross to potential introgression of *M. daghestanica* or *M. pironae* germplasm. Analysis of RAPDs in the trispecies hybrids indicated that the markers offered considerable potential for assaying genetic introgression following complex hybridizations of this type.

Increase in the genetic accessibility of related species through ovule culture or other means has a major role to play in broadening the ever-narrowing genetic base of our crop plants. There are a number of cases where incompatibility within the genus has hindered the improvement work. For example, sweet potato, *Ipomoea batatas* (L.) Lam., is a widely grown root crop of both subsistence and economic importance. Genome differentiation (A & B) and incompatibility among species of the genus *Ipomoea* have hindered its improvement. The ovule culture method was developed for the cultivated and other related species of sweet potato as a means of overcoming certain postzygotic incompatibility barriers.<sup>272</sup> The possibility of using ovule culture as an approach to obtain A- x B-genome hybrids was explored and fertile hybrids of *I. triloba* x *I. trifida* cross combination were obtained.<sup>273</sup> This technology could be extended to cross the cultivated species *I. batatas*, a B-genome species, with B-genome species introgression of some important traits. Kräuter and Friedt<sup>249</sup> using embryo culture in vitro in a crossing program identified hybrids using different techniques. Following this, RFLP analysis allows rapid and safe characterization in the early developmental stages of hybrids<sup>248</sup> and selection of true hybrids.

As Jones and Cassells<sup>239</sup> point out that improved understanding of factors determining interspecific incompatibility, such as endosperm balance number<sup>274</sup> and the role of late-replicating heterochromatin, has increased the range of interspecific hybridization using more widely applicable in vivo hybridization technologies. More research is needed to make wide hybridization technology that combines conventional and biotechnological methods to exploit the wide range of biodiversity within a gene pool.

### Tagging Desirable and Pyramiding Genes

When a particular trait is controlled by more than two genes, the inheritance becomes complex as the heritability tends to be very low. The established methods for screening become tedious, labor intensive and expensive. The differences between the expression of the trait under the greenhouse conditions where segregating populations are artificially inoculated and under field conditions tend to be highly variable and selection may become ambiguous. If a molecular marker, which can be easily scored, can be tagged to a disease-resistance locus, then the job of screening becomes easier.<sup>254</sup> A good example of such work is on tagging of the gene for eyespot disease resistance in bread wheat using an isozyme marker.<sup>275</sup> Use of gene tagging is expected to increase making the

selection process for polygene-controlled characters much easier.<sup>276</sup> However, in crops where molecular polymorphism is low (e.g. groundnut)<sup>266</sup> tagging may be difficult and other approaches such as inserting an appropriate molecular marker may have to be devised.<sup>254</sup>

Being able to tag genes for disease resistance may be a good method to accumulate two or more genes, referred to as pyramiding genes for resistances. In *Phaseolus* beans, the bean rust, caused by *Uromyces appendiculatus*, is an important disease causing significant yield losses. The *Up2* gene has been identified as an important source of dominant resistance to this fungal pathogen. *Up2*, in combination with other rust-resistance genes, may be used to obtain potentially stable genetic resistance. Combining rust-resistance genes effective against a single race has been found to be difficult due to epistatic interactions. A strategy that employed bulked DNA samples formed separately from the DNA of three BC<sub>6</sub>F<sub>2</sub> individuals with *Up2* and three without *Up2* as contrasting near-isogenic lines (NILs) was used to identify RAPDs tightly linked to the *Up2* locus. Only one of 931 fragments amplified by 167 10-mer primers of an arbitrary sequence in the PCR was polymorphic. The RAPD marker amplified by the 5'-TCTGTGCTGG-3' primer (OA141100) was repeatable and its presence and absence easy to score. No recombination was observed between OA141100 and the dominant *Up2* allele within a segregating BC<sub>6</sub>F<sub>2</sub> population of 84 individuals. This result suggests that OA141100 and *Up2* are tightly linked. It was also found that the marker is of Andean origin. These results suggest that OA141100 will be very useful for pyramiding *Up2* with other genes for rust resistance into germplasm of Mesoamerican origin where the marker does not traditionally exist. The use of bulked DNA samples may have concentrated resources toward the identification of RAPDs that were tightly linked to the target locus. Marker-based selection may provide an alternative to the time-consuming test crosses required to pyramid bean rust resistance genes that exhibit epistasis.<sup>277</sup>

Developing insect- and disease-resistant cultivars has long-term implications for environmentally friendly agriculture, as such systems can avoid spraying of undesirable chemicals to control insects and other pests. Breeding through conventional means can be laborious and time consuming. Use of molecular biological tools, though, will not do away with the conventional breeder, although it can assist the breeder in cutting down the time required and at the same time give greater control over the process.<sup>278</sup>

## Marker-Assisted Selection

As seen above, if there is information on genes tagging desirable genes and if it is easy to follow the tagged genes, selection becomes easier. However, presently all the required information is not available in most cases<sup>254</sup> and more work is needed to do proper tagging. It must be noted that the relative efficiency of marker-assisted selection depends on the number as well as the proximity to the desired genes.<sup>279,280</sup> Moving from a few genes to polygenes (quantitative trait loci, QTL), molecular markers are expected to have the most effect in the area of quantitative characters. To locate QTL, several approaches have been used, including intervarietal substitution lines as the starting point.<sup>281</sup> A number of studies have shown that the phenotypes of the derived, single chromosome recombinant lines have fallen into two distinct classes, indicating the location of single QTLs. This approach is tedious<sup>254</sup> and there is need for more work in this area.<sup>282-285</sup> However, the advances in developing efficient and phenotypically neutral marker systems are changing the speed at which this technology can be used for the identification and manipulation of QTLs.<sup>238</sup>

Marker-assisted germplasm enhancement, which is complementary to transformation technology, can help in selecting complex genotypes, thus making it possible to use not only widely divergent biodiversity but also to develop genotypes with a wide genetic base, which is the major aim of maintaining large germplasm collections. For this purpose, marker-assisted breeding utilizes a range of molecular genetic markers to distinguish between different parental genotypes segregating in a cross and to select for specific recombinations.<sup>238</sup> The available markers, i.e. isozymes, RFLP markers and PCR-based markers, help detect not only natural variation but also variation at a large number of loci. This makes characterizing and manipulating the whole genome possible.

As we have just seen, continuing advances in marker-assisted selection may soon make the selection and manipulation of an entire genetic background possible. This means that transgenes can be transferred to new and often 'untransferrable' cultivars with relative ease, thus avoiding any genotypic effect on transformation. To carry out this process efficiently requires the correct choice of male and female parents, the use of appropriate marker-systems and the concentration of selection of the most appropriate generations. Efficient phenotypically neutral marker-systems have revolutionized the identification and manipulation of QTLs. The loci that modify the expression of transgenes are a form of QTL. Desirable alleles at modifier QTLs can be transferred to new cultivars along with the

transgenes themselves, using marker-assisted breeding. Thus, the strategies for marker-assisted selection are becoming ever more sophisticated. A wide range of complementary marker systems assists the selection of the most desirable genotypes. Additionally, the meiotic process including the re-assortment and recombination that produces new genotypes is becoming better understood. It is expected that the most efficient plant breeding methods and the most powerful genetics will make optimal use of both markers and meiosis.<sup>238</sup>

## Cytoplasmic Male Sterility

Cytoplasmic male sterility (CMS) is widespread in plants and provides a convenient means of producing hybrid seeds. In onion, *Allium cepa* L., CMS was originally reported by Jones and Emsweller in 1936. This report has given rise to nearly all CMS lines used by breeders in Japan and the U.S.A.<sup>286-288</sup> It has been reported that the mitochondrial DNA (mtDNA) of the male fertile (normal) and the Jone's CMS onions gave distinct restriction profiles.<sup>287,288</sup> This allows rapid identification of cytoplasm. If pairs of male sterile/maintainer lines were developed from locally adopted cultivars, they could be directly used as seed parents in a breeding program.<sup>289</sup> This idea is being pursued in Japan with onion breeding.<sup>286</sup> Similar systems could be used in other crops as well.

## Plant Variety Protection

With increased emphasis on plant variety protection and other intellectual property rights-related concerns, there is a need for unambiguous identification of an improved and released cultivar developed either through conventional means or through the use of biotechnology. The concept behind such protection is to offer the owner of a plant cultivar some level of legal protection for the exclusive sale of a protected plant variety. Such protection is offered in many countries by issuing certification for a new cultivar. To obtain such a certificate, the owner must demonstrate that the variety under consideration is unique. Initially such identification was carried out using highly heritable morphological markers. This became more and more difficult with the release of numerous varieties which tended to look similar, as most of the new cultivars developed shared similar parentage. In recent years, isozymes have been used successfully, along with some key morphological traits, though some problems remained in unambiguous identification of the cultivars.

To deal with the problem, RFLPs have been used to develop DNA profiles of the genotypes.<sup>263,290-292</sup> However, in species with a limited

number of alleles per locus (e.g. soybean<sup>293</sup>) the amount of information generated by individual RFLP probes has been observed to be of limited use for clear identification of cultivars. The attention then shifted to an alternative type of DNA marker, the microsatellite or simple-sequence-repeat (SSR), which has been shown to be highly polymorphic and composed of tandemly repeated 2-5-nucleotide DNA core sequences. The DNA sequences flanking SSRs are generally conserved within members of the same species. This allows the use of PCR primers that amplify the intervening microsatellite in all the individuals and the variation in the number of tandem repeats results in PCR product length differences.<sup>294,295</sup> SSRs present in some plant species have been demonstrated to be highly polymorphic,<sup>296</sup> as many as 23 alleles have been reported for one soybean SSR locus.<sup>297</sup> Because of the high level of polymorphism, SSRs can provide much more information than RFLPs in species like soybean. Hence, these markers should be able to assist in developing unique and unambiguous DNA profiles for plant cultivars for identification, which can be used for plant variety protection. Rongwen et al<sup>293</sup> concluded that the set of microsatellite markers used in their study provided a positive assessment of the ability of SSR markers to produce unique DNA profiles of soybean genotypes. A system that combines a carefully selected set of 10-15 highly polymorphic loci, in conjunction with the other conventional identifiers, could become a powerful method for unambiguous identification of cultivars. Some work has been done by using RAPDs in sweet potato through pattern analysis that employed both a classification and ordination method.<sup>298</sup> However, it was also indicated that self-incompatibility problems will hinder the speed of application of molecular markers to sweet potato improvement, as classical mapping populations will not be available. Nevertheless, RAPDs appear to have great potential for use in selecting for difficult traits such as weevil resistance and nutritional quality in sweet potato.

### Analysis of Genetic Relationships

Genetic improvement of crop plants is based on the identification of favorable genes in accessions and the subsequent manipulations to incorporate these genes into adapted cultivars. Recent molecular genetics work mainly focused on the use of molecular techniques to facilitate cloning and introgression of favorable genes. Most often, the lack of knowledge of the organization of the genetic structure of populations in the available plant genetic resources limits the identification of desirable genotypes. Knowledge of relative genetic rela-

tionships among genotypes is useful in a breeding program because it permits the organization of genetic resources.<sup>299</sup> Further, the sampling and utilization of plant genetic resources will become more efficient. At the inception of a breeding program, it would be highly advantageous to complement available morphoagronomic data with information on genetic similarity to maximize the genetic diversity of parents. Information on genetic similarity between genotypes could influence the hybridization program in terms of choice of parents to optimize expression of heterosis.<sup>300</sup> Nienhuis et al<sup>299</sup> studied the genetic relationships among 76 *Phaseolus vulgaris* genotypes and breeding lines based on 80 polymorphic RAPD bands. They concluded that plant breeders can use molecular markers to organize genetic resources into related groups to make better informed decisions regarding choice of parents. However, relationship matrices generated by comparing genotypes with each other for a set of RAPD markers were found to be no more informative than the original data.

A DNA-amplification fingerprinting (DAF) approach was employed to develop specific individual profiles and analyze genetic relationships in sweet potato (*Ipomoea batatas* (L.) Lam.).<sup>301</sup> These studies indicated that the DAF could be usefully employed for characterization and identification of duplicate accessions or even for the development of core subsets. DAF data may also be useful to allow identification of parents for a breeding program to ensure a broad genetic base.

### Application of Biotechnological Tools in Forestry Species and Woody Perennials

Due to the obvious uniqueness of this group of plants, discussion under a separate section is appropriate. This is because, although marker-assisted selection has proven to be a useful tool on many annual crops, the technique has not been used to the same extent in forestry species and woody perennials, despite its advantage in eliminating undesirable genotypes prior to planting. Weeden et al<sup>302</sup> reported the development of extensive linkage maps, consisting of primarily molecular markers for apple, pear and grape cultivars. The intrinsically high heterozygosity present in the outcrossing perennials was used to produce segregating populations directly from a cross between varieties. Linkages conserved between the pear and apple genomes have been identified. The use of molecular linkage maps for improvement of these fruit species has been demonstrated. Based on the comparison of RAPD patterns of a genomic DNA sample from *Malus floribunda* (a small-fruited apple relative), a fragment (*OPD20/600*) was proved

to be linked to the *vfgene* that confers resistance to *Venturia inaequalis* (apple scab). This could be used for screening progenies with *M. floribunda* for scab resistance. Relatively, RAPDs have been used more extensively in forestry species compared to other types of markers, but yet much needs to be done.<sup>303</sup> One of the problems has been that most often the allozyme and nuclear RFLP variation in trees is weak in differentiating populations, even distant ones.<sup>304,305</sup> Available evidence for population differentiation from the RFLP analyses of cytoplasmic genes (mitochondrial and chloroplast) presents a different picture.<sup>306,307</sup> Currently, the wide use of these markers for tree species is believed to be difficult because of lack of probes, low sensitivity of restriction enzymes and complex RFLP technology. However, recent development of primers specific for mitochondrial and chloroplast sequences and mismatch detection of sequence variation offer much simpler and faster means for construction of non-recombinant cytoplasmic markers.<sup>308</sup>

DNA marker techniques such as RFLPs and RAPDs have been established for rubber (*Hevea spp.*). With use of these techniques, DNA polymorphisms between species as well as within species have been demonstrated.<sup>309</sup> Additionally, interspecific polymorphism between two clones which share a common female parent was reported. More recently, marker techniques like sequence-tagged sites (STS) and microsatellites have been used to study genetic variation in *Hevea* species. Low et al<sup>243</sup> reported significant progress in genetic diversity studies in various forest tree species. These studies are important as they will provide information on genetics of the forestry species, a large gap in most tropical forest species. Such genetical information helps in determining the natural boundaries, developing sampling strategies, managing the natural stands, assessing the inbreeding effects, especially in the case of smaller populations and finally in identifying useful genes.

With regard to genetic transformation, most woody perennials, along with cereals, have been considered as recalcitrant as they could not be regenerated from protoplasts. However, particle bombardment technology was used in the case of transformation experiments with poplar (*Populus spp.*). Three different target tissues (callus, nodules and stems) were used to produce transgenic plants through electric discharge particle bombardment.<sup>310</sup> Similar successes have been reported in yellow poplar, *Liriodendron tulipifera*, white spruce, *Picea glauca* and papaya, *Carica papaya* (cited from ref 310). These are encouraging results that will assist in the increased use of genetic resources in forest and tree species.

From the foregoing, it is obvious that the application of modern molecular techniques will enor-

mously help curators to increase significantly the knowledge base of individual accessions of an entire species, and of its gene pool. Gene identification and its transfer into an existing genetic background using recombinant DNA technology and other applications of these new technologies will further increase the importance of ex situ collections and, thus, enhance their value.

## NATIONAL POLICY AND INSTITUTIONAL FRAMEWORK

The Convention on Biological Diversity, which has been ratified by numerous countries, gives much importance to biotechnology and considers it as a means to enhance sustainable use and equitable sharing of benefits from such use. Hence, it will be appropriate to discuss the current situation as well as some future perspectives on this subject. Transfer of technology, including biotechnology, has been a key political issue argued by developing countries. A successful application of biotechnology at practical levels (national programs), an institutional framework and national policies favorable to the practical application of biotechnology are needed. The individual characteristics of the world's flora and fauna are the basis of biotechnology. Individually, they produce the molecules on which biotechnology depends. These molecules have no other source.<sup>311</sup>

It must be recognized that only diversity can allow sustainability. Only diversity can support social and economic systems that allow the poorest to meet their food and nutritional needs and the cultural diversity of various countries of the world.<sup>312</sup> The biological resources of each country are important, but not all countries are equally endowed. The relative values of various resources are different. In general, it is well-known that a few countries lying within the tropics and subtropics account for a very high percentage of the world's biodiversity. The CBD became an international agreement on December 29, 1993 when more than 30 countries ratified it. Issues related to technology transfer, funding mechanisms, property rights and access to genetic material are being discussed at various levels. There are strong proponents of IPR for genetic resources and its products as well as strong opposition to it at the conceptual level.<sup>313,314</sup> The CBD encourages both access to and transfer of technology (including biotechnology) among nations, especially to developing countries. Access to and transfer of any technology shall be consistent with the adequate and effective protection of intellectual property rights. Necessary policy measures shall be taken up to access and to transfer technology on mutually agreed terms, as should

plant genetic resources. Such measures shall assist joint development and transfer of technology for the benefit of both governmental institutions and the private sector organizations in developing countries. The major problems that have been identified for transfer of technology as well as their adoption in the recipient country are access to capital, human resources and support services, intellectual property rights, regulatory issues such as biosafety and exchange of information and knowledge, environmental concerns and transaction costs.<sup>315,316</sup>

The CBD encourages countries to take legislative, administrative or policy measures to handle issues related to biotechnology and the sharing of its benefits on a fair and equitable basis. Countries may be required to set up appropriate procedures (such as code of conduct) in the field of safe transfer, handling and use of any living modified organisms resulting from biotechnology that may have an adverse effect on the conservation and sustainable use of biological diversity. National committees may be set up to discuss and recommend the necessary measures. The need for providing any available information about the use and safety regulations in handling such organisms (biosafety regulations), as well as available information on the potential adverse impact of the specific organisms is recognized.

Broadly speaking, benefits of biodiversity for the biotechnology industry can be two-fold. First, biodiversity significantly lowers the research and development costs of the industry since it serves as a highly productive *in situ* stock of genetic material.<sup>317</sup> The potential uses of biotechnology for conservation are many and one of the most important is in the context of making conservation and use of plant genetic resources cost-effective. For the implementation of the CBD, it is essential that the benefits overtake costs and thus conservation becomes attractive to policy makers. Additionally, biodiversity represents insurance for agriculture because it diminishes the risks of productivity variations as it can rely on many instead of only a few cultivars. The preferential technology, including biotechnology, basically means that the developing countries will at least partially be able to circumvent license fees without risking any sort of retaliation. This arrangement may be looked at as an essential mechanism for donor support to biodiversity conservation. For a fairly detailed discussion of several issues related to technology transfer, national policies, areas of application of biotechnology, etc., see Altman and Watanabe.<sup>316</sup>

## NETWORKING ON PLANT GENETIC RESOURCES AND BIOTECHNOLOGY

So far, we have discussed the role of biotechnology in the conservation of plant genetic resources and their enhanced utilization for crop improvement. We have also seen briefly the various technologies that are available that can either singly or in combination help plant improvement workers to achieve results that have not been possible through conventional techniques, or which used to take long periods. One major theme that can be seen through all the discussion is the multidisciplinary nature of both biotechnology and plant genetic resources. They are both made up of a number of sometimes overlapping disciplines. Such a multidisciplinary nature requires excellent cooperation among all concerned for successful use of the tools for utilization of the resources available. This is especially true when the issue of transfer of technology is concerned. To make the transfer more effective and to ensure equitable sharing of profits, a multilateral collaboration is more desirable. This logically leads us to networking. We will discuss a couple of examples here of how such networking can be taken forward, in relation to plant genetic resources and their utilization. However, it must be noted that the networks under consideration are not those formulated for biotechnology work but for genetic resource management purposes. Within this scope, the efforts are under way to link up the network members in using biotechnological tools for the study and use of genetic resources.

The International Plant Genetic Resources Institute (IPGRI), with financial support from the government of Japan, is an active partner in the International Network on Bamboo and Rattan (INBAR) and is mainly concerned with the plant genetic resources aspects of INBAR through its Working Group on Biodiversity and Genetic Resources. Here we consider rattans or canes, which are a unique group of climbing palms in tropical rain forests. There are more than 600 species in 13 genera, *Calamus* (400 species) and *Daemonorops* (115 species) being the largest.<sup>318</sup> Millions of people in the tropics depend on rattans for their livelihood. As there are numerous species of rattan it is not possible to work on all of them. Some level of prioritization is required to undertake activities on rattan plant genetic resources conservation and use. Initially the species on which work will be carried out is determined<sup>319</sup> through consultation

of the network members, assisted by resource persons. Along with priority species, some activities were identified, including assessment of the status of rattan genetic resources, assessment of the degree of threat of genetic erosion, conservation priorities and the development of a database. There is need for a clear taxonomy and a simple practical key for species of economic importance. Studies focusing on genecological variation and genetic diversity are in progress, with possible links to training. Ethnobotanical studies focusing on the traditional knowledge and management of rattan need to be developed. Assisting in exploration and collecting of rattan and conduct of ecogeographic studies are essential. There is a need to develop technology for long-term conservation and utilization, including in vitro culture methods for ex situ conservation.<sup>320</sup> Development of sustainable in situ conservation strategies for these species is another area that needs urgent consideration. This would require an assessment of in situ diversity using molecular markers before such methods could be used for improvement purposes. Within the network of INBAR, in partnership with national programs such as Thailand and linkages with advanced institutes in Sweden, IPGRI is supporting the study of genetic diversity in rattan.<sup>321</sup>

Another example is the International Coconut Genetic Resources Network (COGENT), under the auspices of IPGRI, which uses the network approach to tackle many problems of coconut, including coconut improvement. Research on conservation and improvement of genetic resources of coconut is being coordinated by COGENT with financial support of the Manila-based Asian Development Bank. The network has 31 coconut-producing countries as members who are committed to sharing germplasm, resources and technology. There are strong socioeconomic and environmental justifications for research investment on coconut at international level. There is significant scope for genetic improvement of coconut in the context of germplasm collecting, evaluation of genetic resources and their utilization through selection and breeding.<sup>322</sup> Traditionally, genetic improvement of coconut is a long-term process and to hasten up the speed of the process involved COGENT/IPGRI will pursue activities involving molecular and cell biology. So the program has a strong component on transfer of biotechnology through collaboration between selected developed and developing countries. It involves adaptation of currently available biotechnological tools to work on coconut, such as coconut zygotic embryo culture, use of isozymes and molecular markers to

study genetic diversity for the purpose of conservation and utilization. This is again a collaborative effort between the developing countries in Asia, Africa and Latin America and agencies in Europe.

## CONCLUDING REMARKS

Given the rapid rate of the world's population increase, coupled with distribution problems, there are increasing demands on agriculture for higher yields and better quality. However, this is not a new problem. The world's agriculture has always been confronted by many problems. We do not know what new diseases, insect or other pests and soil and atmospheric problems we may have to face in the future. We do not know what physiological and morphological characters will be needed for crops to perform well in a possibly changed environment. We have been warned repeatedly that the 'greenhouse effect' may cause temperature changes resulting in global warming and concomitant climate changes in other related phenomena that have a direct influence on agriculture. If this happens, new varieties which can adapt well to the new environment will be required. However, the future of the global environment is largely unknown, and hence we cannot predict what genes will be required in the future and in what combination. Therefore, representative genetic resources have to be collected, studied and conserved for future use before they disappear forever.

There is a greater need to be concerned with conservation of genetic diversity that has accumulated within a species during the long evolutionary processes. Many such species have been useful to us since the dawn of agriculture; they have provided us with food, fiber, etc., to survive and to enhance the quality of life. The current situation is very dynamic—due to the changing pest problems, changing needs in human nutrition, growth in population, the need to extend agriculture to more environmentally stressed and marginal conditions and the increased use of plants as biomass for energy—and hence it is impossible to predict the genetic diversity needs to meet these changing demands. This requires conservation of a large assemblage of diverse genetic resources. To conserve and use such complex gene pools, biotechnological tools can be used with advantage. The rapid progress in in vitro culture technology and molecular biology is expected to assist both conservation and use, as seen in the above discussion. Some of the most important help will be in the areas of plant genetic resources conservation in vitro (use of growth retardants, growth regulation by reduced incubation temperature, vitrification, encapsulation and cryopreservation) and microp propagation,

production of disease-free material and safe exchange, better understanding of genetic diversity, both its extent and structure, gene identification using molecular techniques and transfer of desirable genes from the useful plant genetic resources accessions.

Recent research in cell biology, molecular genetics, recombinant DNA, tissue culture and related fields is opening up new possibilities for progress in agriculture. The development in biotechnology allows scientists to transfer genes for crop improvement in a relatively short time. But the genes for such 'engineering' manipulations have to be provided from genetic resources. Law<sup>254</sup> provides an excellent review of the uses of molecular markers and genetic engineering techniques and at the same time cautions by illustrating the limitations. Initially, for example, marker-assisted selection may appear to be fine, but as the breeding lines become more and more uniform, the extent of polymorphism is reduced and marker-assisted selection then can be a problem.

Additionally, despite all the advantages of having the modern biotechnological tools, there is a great amount of discussion going on about their use, especially in developing countries in terms of access, property protection, etc. There is a growing awareness that the profits generated from the exploitation of plant genetic resources, especially through biotechnology, are not shared equitably. There is some argument regarding whether these could be used more effectively to bring direct benefits to farmers.<sup>262</sup> But these are not exactly for the farmers in developing countries, who make up the bulk of the farming community in the world. These issues are not discussed here but it is important to remember that most of the progress in biotechnology, unlike the Green Revolution, is financed largely by the private sector. Hence, legal issues and issues related to property protection and sharing of profits come to the forefront. Additionally, if appropriate measures are not taken, biotechnology may prove too expensive for the developing countries (thus hindering development), its use may increase the gap between developing and developed countries.<sup>323</sup> There is an urgent need to develop procedures to link commercial benefits from the exploitation of plant genetic resources through biotechnology (and other methods of exploitation) to conservation of plant genetic resources.<sup>5,67</sup> This will assist sustainability of our conservation efforts which will in turn benefit biotechnologists by making the raw material accessible, resulting in increased productivity. It is sometimes argued that except perhaps in some of the few remaining subsistence societies biological produc-

tion (agriculture and allied products) is a business and seeks to maximize returns to producers.<sup>324</sup> Nevertheless, it must be noted that these subsistent societies may be small in number but represent large numbers of population. There are several limitations for the developing countries to quickly adopt and benefit from the new technologies and these limitations include capital, human resources, support services, property rights, etc. Hence, there is need for a spirit of understanding and sharing of ideas as well as technologies that will help us all to do a better job of conservation and use of the most important natural resources available to man, i.e. plant genetic resources, for the present but also for many generations into the future. Prudent conservation of crop genetic resources and their active use is essential.<sup>325</sup> Only then will it be possible to realize the most obvious point that can be made about plants, animals fungi and microorganisms: they are the means to attain enhanced sustainability and productivity.

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## CHAPTER 5

# INTELLECTUAL PROPERTY RIGHTS ASSOCIATED WITH PLANT BIOTECHNOLOGY AND PLANT GENETIC RESOURCES

W. Lesser

### INTRODUCTION

Intellectual property rights (IPR) associated with plants, or indeed with any living organism, are of quite recent origin. In the past century, Pasteur has often been credited (at least in the U.S.A.) with receiving the first patent on a living organism—a yeast especially adapted for fermentation—but it is evident the organism was treated as an industrial product, not living material. The U.S. Plant Patent Act of 1930 (subsequently incorporated into the Patent Act, Sec. 161-62) was an early specific treatment of plants. It provides Plant Breeders' Rights (PBR) like protection for asexually propagated plants.

Until 1960, with the passage of the International Convention for the Protection of New Varieties of Plants, broad-based IPR protection was not available. Additional forms of protection resulted from the 1980 U.S. Supreme Court Chakrabarty decision. While that decision focused specifically on micro-organisms, an extension to plants was made in 1985 on internal Patent Office appeal (*Ex parte Hibberd*). Thus the stage has been set in the U.S.A. for a significant scope of coverage for plant biotechnology. Other countries have been proceeding at a slower rate in allowing similar multiple forms of protection.

These protection mechanisms apply typically to plants for which there has been significant research input, whether it be breeding or genetic manipulation. Largely excluded has been a protection mechanism for "unimproved" materials, be they landraces, wild relatives, or wild materials. Such resources, the basis for all of the subsequent science, have traditionally been considered as a "heritage of mankind," with no direct monetary remuneration for use. Much of that material, which shall categorically be referred to here as plant genetic resources, originates from developing countries, while the bulk of breeding and biotechnology is carried out in the major industrialized nations. Unsurprisingly, representatives of developing countries view this asymmetric IPR system as unfair, sometimes charging that they buy back their own resources. Perceptions of multinational firms enriching themselves on materials acquired at no cost have further strained the relationship in a system that for many years operated solely on a nonprofit basis within the public sector. Earlier efforts, such as the 1983 FAO undertaking for plant

genetic resources to establish a compensation fund have been unsuccessful to date.

Then in 1992, the Convention on Biological Diversity (Article 3) declared all genetic resources to be the “sovereign right to exploit” of the country where they occur, contingent on not damaging the environment of other countries. While, strictly speaking, this article only restated existing rights, the statement nonetheless heralded a new era in which countries would exercise control over the use of their genetic resources through the passage of access legislation. This legislation, or more often the sensitivities associated with it, have already begun to affect access to and use of plant genetic resources for research and commercialization purposes. This rapidly changing environment has caused a wider interest in the operation of existing IPR systems for plant biotechnology and genetic resources, especially as it impacts researchers. This chapter discusses the existing IPR systems and projects the direction of near-term changes. It begins with a general overview of the forms and operations of IPR, and continues in the third and fourth sections to consider the particular applications and attributes for plant biotechnology and plant genetic resources.

## **FORMS AND FUNCTIONS OF IPR ASSOCIATED WITH PLANTS**

Strictly speaking, IPR refers to five forms of law: patents, Plant Breeders’ Rights, trademarks, trade secrets and copyrights. Of these, all but copyrights are potentially applicable to plants. Each form of protection has its own characteristics which must be evaluated to understand how a change would affect inventors and breeders. At the same time, these several forms are partially overlapping and complementary. That is, inventors may substitute one form of protection for another, such as patents for PBR, or may combine several, especially patents and trade secrets. The purpose of this section is to describe, in brief, their forms and functions.

### **FORMS**

#### **Patents**

Patents, like other forms of IPR, operate as a balance between the inventor and society. Society grants a temporary, partial monopoly to the inventor. Temporary refers to the duration of protection (generally 20 years), and partially describes the scope of protection or the degree of difference required before a related development is not covered by a patent. What society receives in exchange is more investment than would otherwise be made and the revealing (disclosure) of the invention.

Disclosure “in such full, clear and concise and exact terms as to enable any person skilled in the art or science to which it appertains ... to make, construct, compound or use it” is one of the patentability requirements. Disclosure not only permits competition soon after a patent lapses but also provides a storehouse of technical knowledge which would not otherwise exist.

Additional patentability requirements include novelty, utility and nonobviousness. Novelty requires that the invention not be previously known, that it be new. Utility mandates at least a single use for the invention to be specified. The identified use need not be practical in an economic efficiency sense—the marketplace ultimately decides on the practical merit of inventions—but it must be specific. Patent applications from the Human Genome Project failed that requirement and were not pursued. Nonobviousness (inventive step) mandates that a patentable invention must not be obvious to one skilled in the art; it must be a notable extension of existing art, not a mere permutation.

As a consequence of these requirements, it is not possible to patent any plant; the requirements are specific and exacting. Moreover, there must be human intervention in the inventive process. The mere identification of something existing in nature (technically known as discovery as opposed to patentable inventions) would not be sufficient for a patent. Examples of human intervention are the purification of a strain of microbes, or the identification of an especially rare rose mutant.

To identify a specific hypothetical situation, it should be noted that a patent would not apply to all tomatoes. Rather, an application would apply to tomatoes with certain characteristics (delayed ripening in the case of the well-known FlavrSavr™ tomato). Because such a characteristic would often be conferred by a transformed gene, the patenting of the gene constructs themselves is an alternative approach to patenting the plant containing the constructs. The choice between the type or types of patents sought depends on the complexity of strategic issues and scientific factors such as the ease of minor modifications in any step.

#### **Plant Breeders’ Rights**

Plant Breeders’ Rights (PBR) are a specialized patent-like system for cultivated plants. PBR were first systematized in 1961 under the International Union for the Protection of New Varieties of Plants (UPOV in its French acronym). Presently there are 28 members. Membership requires, among other steps, that signatories adopt national legisla-

tion along the lines of the Convention. Most members adhere to the 1978 Convention; a modified 1991 Convention has since been drafted with several significant changes, including the requirement to extend protection to all classes of plants rather than to partial groupings.

In place of the novelty, utility and nonobviousness requirements of patent law, PBR use distinctness, uniformity and stability (DUS). Uniformity and stability are measures of reproducibility true-to-form, respectively, among specimens within a planting and intergenerationally. The principal test then is distinctness, that the variety be "clearly distinguishable from all" known varieties. The DUS attributes are measured in growouts of the planting materials in most member states (but not in the U.S.A.).

PBR are further distinguishable from patents by the allowance of so-called "farmers' privilege" and "research exemption." The farmers' privilege is the right to hold materials as a seed source for subsequent seasons (farmer-saved seed or bin competition), something which would generally be an infringement with patented materials. For nonhybrid seeds, commercial farmers buy new seed on the order of every three years. This is done for several reasons, including limiting the "genetic drift" that occurs over time and reduces the quality of the material with each successive generation, and accessing improvements added to new varieties in the interim. There is nothing magical about three years, rather the figure is an average across crops and producers, but it carries great relevance for seed producers. The research exemption refers to the right to use protected materials as the basis for developing a new variety or other research uses. Research or experimentation under patents is permissible but is not as well-defined.

Because of these differences, PBR are generally considered to provide less protection than patents. They also apply to the whole plant or the propagating materials thereof. What they do not protect is the unique characteristic (the distinguishing characteristic) of the variety. For that reason, no real protection is provided for a variety with a bioengineered gene, as the gene can legally be removed and used in another variety or with another distinguishing attribute.

## Trade Secrets

Trade secrets, to describe them in their simplest terms, assist in the maintenance of secrets by imposing penalties (the recovering of costs) when information held as secret is improperly acquired or used. Examples of trade secrets include customer lists and

practices for improving the efficiency of a manufacturing process. An employee going to work for a competitor typically would be enjoined from revealing sensitive information for a specified period. Unlike patents and the like, no formal application procedure is needed for a trade secret; rather, the information must have some commercial value, and an effort must be made to keep it secret. As long as these conditions are met, protection can be permanent.

For plants, F-1 hybrids may be considered a form of trade secrets. As long as the crosses and/or the pure lines are protected, the product is difficult to copy. However, the self-reproducible nature of most living organisms precludes a major role for agricultural products. In other technological areas, trade secrets may substitute for complement patents. When a product or process is difficult to copy, then trade secrets can be substitutes. An example of complementarity with patents would be the insertion of a patented gene in a hybrid.

## Trademarks

Trademarks are the reservation of a word or symbol in association with a product or service. In effect, the trademark name represents the product to consumers, justifying an investment in its identification. From a theoretical economic perspective, trademarks assist customers in identifying products of consistent (and often high) quality. Trademarks are permanent as long as they remain in use, are identified as such and do not acquire a generic connotation. Often a trademark, such as Coca-Cola, is the most valuable asset of a corporation.

Within agriculture, trademarks can be associated with products at the firm level (Pioneer Hi-Bred), or with individual products such as the FlavrSavr™ tomato. Note that the tomato variety is also patented so the two forms of IPR are, in that instance, complementary. At the plant variety level, the role, however, could be more of a substitute than complement. Indeed, because of the farmers' privilege and research exemption under PBR, Lesser<sup>1</sup> has previously argued that in the U.S.A., the PBR law really protects the variety name rather than the germplasm itself. The argument made was that the limited distinctness requirement used in the U.S.A. meant that many firms had functional copies of their competitors' products, but buyers were not always aware of which varieties were the near matches. Hence, what was unique among them was not the genetic material, but the association given by customers—the variety name. This conclusion is most applicable in the U.S.A. where the distinctness requirement is narrower than in many other countries.

## FUNCTIONS OF IPR

### Roles

There are two fundamental justifications for IPR systems, known as the personal property or “natural law” and economic incentive approaches. The personal property approach is based on Locke’s concept of a right to property being conferred by God upon all men in common (see Thompson<sup>2</sup> and Hughes<sup>3</sup>). This is in contradistinction to the absolute power of sovereigns. That concept applies to common (real) property, but what of personal property? Locke handles that matter by introducing the idea of labor, “he that mixed his labor with and joined it to something that is his own, and thereby makes it his property.” Underlying is a view that a free person controls his labor, and a loss of the right to the product of that labor implies a loss of freedom. Property rights, including IPR, are thus a means of protecting freedom.

The economic incentive approach is more pragmatic and less philosophical (the classical explanation is Machlup<sup>4</sup>). It recognizes the inventor assumes time and other costs associated with the creation process such that she/he could never compete on equal terms with copiers whose costs, minus the creation process, are lower. Hence, the creator will always be undersold and has no incentive to invest. IPR legislation redresses the balance, at least in part, by prohibiting direct copying as long as the protection is in effect.

To be more specific, the invention process has been divided into three components: discovery, development and commercialization. The discovery process itself seems to be driven more by the creative drive, or mere luck, and hence is somewhat removed from financial incentives. Development and commercialization, however, are the lengthy and costly processes of turning an idea, an insight, into a marketable product. Work at these stages is very responsive to incentives and can be considered as the real target of IPR systems.<sup>5</sup> Much plant biotechnology fits this description.

Of these competing concepts, which is the operable one for current systems? An insight can be gained from the authorizing legislation in the United States. There in the Constitution (Article 1, Sec. 8, emphasis added) it states, “[T]he Congress shall have the power ... *To promote the progress of science and the useful arts*, by securing for limited times to authors and inventors the exclusive right to their respective writings and discoveries.” This terminology has quite conclusively been identified as fostering economic incentives.<sup>6</sup>

### Incentives

The key function of IPR is therefore to provide incentives for investment in the creative process, and in particular the transformation of basic insights into marketable products. These incentives are most applicable to private entities but have been used increasingly by the public sector as a source for generating research funding. Certainly that has been the experience in the U.S.A., Canada and likely elsewhere.

When considering the incentive effects, it is important to recognize what privileges IPR do and do not provide. They do not assure a return; indeed only up to 15% of patents are ever commercialized.<sup>7</sup> They do not necessarily permit the use/practice of the creation. That is often controlled by regulation (biosafety) or even other patents. All they allow is the right to exclude others from use, which can be called negative rights. All financial rewards come from market sales. Hence, key factors such as the breadth (scope) of protection and enforcement are critical in determining the practical value of IPR.

### Access

A second and generally less recognized aspect of IPR is the ramifications for access to protected creations.<sup>8-10</sup> Since, in the absence of IPR or of effective enforcement, it is often difficult or impossible to prevent copying, creators may choose secrecy as an alternative mechanism. This may mean creations are unavailable in IPR-deficient countries, available only following substantial delays, or are only available to, for instance, large farms which are more cost-efficient to monitor. Those entities for which access is denied or delayed are potentially at a commercial disadvantage, something which could be quite critical in the highly competitive food sector. Indeed, there is a current trend for new bioengineered foods to internalize the entire production and distribution process to the exclusion of independent producers and suppliers. Calgene, owner of the FlavrSavr™ tomato, for example, is said to be producing exclusively under contract or using its own facilities. No open sales of seed are permitted.<sup>11</sup> Access also implies access to regulatory dossiers submitted elsewhere in the world. The cost, often in the millions of dollars for both biological and food safety, and time delay of recreating those data, should be counted as a cost of the patent protection absence.

In agriculture it is difficult to predict how significant the access issue will become. Certainly self-reproducible living organisms, once released

anywhere in the world, are largely noncontrollable. Seeds can easily be picked up and transported into and from anywhere so that it is impractical to deny access altogether. On the other hand, private investors would understandably be reluctant or unwilling to release a variety where little in payments could be expected, and where there is some possibility that illegal sales will enter the home market.

## EVIDENCE ON EFFECTS

### Investment (R&D)

Since a major justification for IPR is the attraction of funds for research and development (R&D), it is a reasonable question to inquire about the evidence indicating actual experiences (this material is drawn principally from Lesser<sup>8</sup>). For patents covering all technologies, what is known is inconclusive. The analytical complication is largely methodological, attempting to determine what would have happened in the absence of the legislation. Additionally, for many technologies, other forms of protection can serve as at least a partial substitute for patents. Indeed, surveys of business leaders typically place a low ranking on patents as a stimulant for R&D investment.<sup>12</sup>

When specific sectors are examined the results become more definitive. In general it is recognized that patent protection is especially important for pharmaceutical products and for living organisms. Both are relatively expensive to develop and easy to copy. A major cost is that of satisfying regulatory requirements. For pharmaceuticals in the U.S.A., human trials are said to use the bulk of the \$250 million per product development cost, while the preparation of a food safety dossier for a genetically engineered food is said to cost in round numbers, \$1 million. One source of information on the role of patents is the implications of the removal of protection. In India, pharmaceutical R&D fell 40% from 1964-1970 to 1980-1981, an occurrence Deolalikar and Evenson<sup>13</sup> attribute to the weakening of patent protection in 1970.

An ancillary point, and one particularly relevant to agricultural applications, is that of adaptive research. Deolalikar and Evenson,<sup>14</sup> again referring to the case of India, conclude, "If anything, the relationship that is often observed is one of complementarity." In Evenson's view,<sup>15</sup> "[I]ndirect transfer does not take place without research capacity in the destination country."

A number of more formal economic studies have been conducted on patent system aspects. Examples are such components as optimal duration and the consequence of the "winner take all" approach (review in Primo-Braga<sup>16</sup>). Overall, as might be expected, these issues are very sector

specific, and general studies lead to inconclusive results with limited policy implications. However, indications are that patenting and R&D are not dominated by major firms. Rather, medium-sized entrepreneurial firms, which are dependent on technological advantages for their market position, are the market leaders.

Overall, PBR are relatively much more recent and sector specific than patents, which eases the methodological problems in evaluating the impacts. A major study was conducted in the U.S.A. in 1980, a decade into the Plant Variety Protection Act.<sup>17</sup> When considering the results, it should be recognized that the U.S.A. interpretation of not requiring objective standards for performance claims means that the scope of protection in the U.S.A. is relatively narrow.<sup>1</sup>

Despite these caveats, it was found that PBR did have a significant impact on private investment and numbers of private breeders, especially for soybeans. Those results have been confirmed by other observers (e.g., Brim<sup>18</sup>: Tables 3 and 5). Butler and Marion<sup>17</sup> added the recommendation that continued public breeding is an important hedge against domination by the private sector.

Recently, limited information has begun to appear on the operation of PBR in other countries. A graphic plot of new variety registrations in South Africa indicates a notable increase following the adoption of PBR in 1976 (van der Walt<sup>19</sup>: Table 1). Similarly, a provisional study shows that the Argentine private sector increased its investments in plant breeding, but only after the law was enforced.<sup>20</sup> Hence, the available information is consistent with the theoretical expectations that increased IPR do indeed lead to greater investment, especially for easily copied products like open pollinated plants. The more relevant, and difficult, question for export is the implications for trade.

## Access

The conclusion that PBR lead to greater internal investment in breeding expenditures leaves some ambiguity regarding their effects on access. Access conceivably could be enhanced, supplanted by recipient country investments, or remain unaffected. Many of these are long-term issues for which a few countries are just approaching the initial stages. Nonetheless, there are some bits of information that do suggest that the presence of PBR does indeed enhance access. Much of that information can be viewed from the perspective of access as discussed above.

A strong motivation for the recent (1990) adoption of PBR by Canada was access to improved, protected potato varieties from Holland. As well,

within Canada there was a reluctance to export varieties to the U.S.A. because of the concern that the varieties would be transported back into Canada.<sup>21</sup> Similarly, cut flower producers experiencing difficulties with accessing new varieties were major proponents of the Colombian national law and subsequent application for succession to UPOV. Uruguay adopted PBR largely to prevent trade disruptions with Argentina, to which its economy is closely tied (Jaffé and van Wijk<sup>20</sup>; p. 20). A different perspective can be gained by examining the percentage of PBR certificates granted to foreign firms, recalling that PBR apply only when national protection has been granted. Foreign ownership ranges from 3% in Japan to 20% in Argentina to 80% in Belgium. In general, the expectation is that the percentage rises under PBR, which is an indirect means of saying that access increases.

## APPLICATIONS TO PLANT BIOTECHNOLOGY

The preceding described the general situation with respect to IPR, with emphasis on living organisms. This section is an exploration of current issues specific to plants, and plant biotechnology applications. Those issues include the geographic scope of protection, deposits, scope and materials exchange.

### GEOGRAPHIC SCOPE OF PROTECTION

As was noted above, IPR apply only where protection is available and has been sought and granted. That is, IPR are national law. A patent holder, for example, may generally prohibit imports of a product into a country where a valid patent exists and often of a product produced by a patented process. But there is no control over the use of a patent in, or trade among, countries where a patent has not been granted. A few systems of multinational patents do exist, the most active of which is the European Patent Convention, but even there where the application is multinational, the grant is a "bundle" of national patents. All this says is that the availability of IPR for plants on a national basis is a key factor in the operation of intellectual property rights systems. Plant biotechnology is particularly limited in this regard.

### Countries Currently Allowing IPR for Plant Biotechnology

At present (1988 data in WIPO<sup>22</sup>; Annex II), some 44 countries specifically exclude plants from patents. Many others are silent on their patentability, but to date no patents have been issued, making the effective patentability status unclear. One

of the complicating factors is the terminology in the European Patent Convention, which is carried forward to many national laws worldwide as indicated in the 44 exclusionary countries. These countries prohibit patents for "plant and animal varieties and essentially biological processes for the production of plants and animals (EPC Article 53(b))." Lawyers and scientists are unclear as to what constitutes a "variety," and what is an "essentially biological process." One approach which has been used in Europe is to define "variety" narrowly as a fixed form, and "essentially biological" as, effectively, a traditional breeding process. With these two stipulations, genetically engineered plants become patentable subject matter. But definitionally the approach is tenuous.<sup>23</sup> Most recently, in March 1995 the European Parliament vetoed the 1988 draft directive which laid out the operational definitions. It is still too recent an action to determine definitively what effect it will have on plant patenting in Europe.

At the same time, only 28 countries are members of UPOV, and of those only three are developing countries (Argentina, South Africa, Uruguay). A number of additional countries, including in part Kenya and recently India, have national PBR legislation, but the form of those laws and particularly their operational status is often poorly documented. There is no listing of countries with trade secret legislation as those laws are national and not subject to any international agreements or conventions. Nonetheless, it is believed that trade secret protection is not widely available in developing countries. Overall then, biotechnologists presently have a limited scope of IPR protection.

### GATT/TRIPs

Developed countries, led by the United States, seeing their role in the evolving world economic order as technology suppliers, began to view limited IPR as a barrier to trade in those products. In short, it was argued that the absence of IPR limited opportunities to export technologies without concern for losing them. Certainly, as is described above, that argument could be applied to plant materials which are self reproducing. This position prevailed in the recently concluded Uruguay Round of the GATT, leading to the inclusion of the so-called Trade-Related Aspects of Intellectual Property Rights (TRIP).

The TRIP agreement requires signature states, including some 70 developing countries, to provide for the following protection (MTN/FA II-A1C). Contracting parties shall provide for the protection of plant varieties by patents and/or by an effective *sui generis* system (Section 5, Article

27[3b]). Patents may be prohibited to protect public order or morality, provided there is a justification exceeding the mere prohibition in domestic law (Section 5, Article 27[2]). Plants and animals other than micro-organisms and “essentially biological processes for the production of plants and animals” may be excluded from protection (Section 5, Article 27[3b]). Compulsory licenses may be issued in limited cases of due diligence to make a licensing agreement, adequate remuneration and subject to judicial review (Section 5, Articles 30 and 31). For process patents, the burden of proof of infringement may in some specified circumstances be shifted to the defendant to prove that the patented process was not used (Section 5, Article 34). Persons shall have the option of preventing others from using without permission information of commercial value so long as reasonable efforts have been made to keep it secret (Section 7, Article 39).

Even with this legislation, restrictions will remain, for example, the five years (and up to 10 years depending on product and level of development of the country, with further delays possible on approval) allowed for developing countries to adopt and implement the changes (Part VI, Articles 65 and 66). Moreover, as noted, similar terminology to “plants and animals and essentially biological processes for the production of plants or animals” exists in the European Patent Convention but there reference is to “plant or animal varieties” (emphasis added). How it will be interpreted with the new terminology is not known for sure at this time, but in all likelihood patents for most life forms (except micro-organisms) will be prohibited in at least some countries, but not biotech processes even when applied to living organisms.

Additionally, note that countries may exclude patents which are contrary to “public order or morality.” This terminology exactly parallels the EPC (Article 53[a]), and the European Patent Office has rejected an animal patent on those grounds. Thus, there is a likelihood some developing countries will exclude classes of inventions, living organisms in particular, based on moral objections. TRIP do require countries to adopt enforcement procedures which are “fair and equitable,” are “reasoned” but “not unnecessarily complicated or costly (Section III.”

TRIP are quite specific in requiring the allowance of either plant patents or a *sui generis* system for plant varieties, or both. *Sui generis* means separate or independent, as in a distinct form of legal protection. This is widely interpreted to mean Plant Breeders’ Rights as in one of the UPOV

conventions. That is, UPOV membership, although no specific interpretation has to date been issued would in all likelihood satisfy the commitment. Presently, only the 1991 Act remains open for membership (although countries already signatories to the 1978 or earlier Acts need not change). For the plant biotech industry, the 1991 text has several major benefits as it allows for the protection of the entire plant as well as the harvested materials and products made directly from harvested materials (subject to national ratification) (Article 14[2]) and 14[3]). Hence, the importation of cut flowers or soybean meal produced from unauthorized planting materials could be barred, according to my interpretation. Additionally, all genera and species must be allowed protection within a ten year period. Overall, however, PBR provides very limited protection for plant biotechnology.

PBR’s insufficiency to provide protection for genetically engineered traits must be considered separately for the 1978 (and earlier) Act, and the 1991 one. Under UPOV 1978 text, any variety which is distinct in one (recognized) characteristic can receive protection. Thus, if a firm bioengineered a rice variety for pest or disease resistance and a different firm bioengineered that rice variety to improve the yield, the improved variety, resistance and all, would be owned by the second firm. The dependence stipulation in the UPOV 1991 Convention text would allow more ownership control by the biotechnology firm. If the disease-resistant variety were accorded “initial variety” status, derivative varieties could not be commercialized without permission. However, nothing would prevent a firm from removing the responsible genes for transfer to another distinct variety. A combination of 1991 UPOV and patents on the genes themselves would seem to provide sufficient control.

The other option for countries is the adoption of a national PBR law, as presently exists in several countries. There would be two major considerations in planning such a step. First, TRIP read “an effective” *sui generis* system. Just who will be interpreting what constitutes “effective” and on what grounds is not clear at this time. A second and more enduring matter is the forgoing of UPOV membership benefits. One of the more significant is the relatively straightforward understanding of what the law allows, based on experiences of multiple other countries. More significant yet is the concept of national treatment, in short the prohibition of discrimination against nonnationals (Article 4).

Under TRIPs, countries have the right to exclude patents for plants (and animals). Considering the controversialness of this matter, it seems many might do so, or more correctly, continue to

do so. Countries, however, are diligently working to fulfill their obligations under TRIP by adopting PBR legislation. India, Pakistan, the Philippines, Chile, India and Mexico, among others, are said to be close to joining UPOV 1991. Many others have established PBR committees but are progressing at different rates.

IPR, as with all laws, is only as effective as its enforcement. Yet this involves complex legal and technical matters. For example, the experience in Argentina was that nothing happened under the law until an enforcement mechanism was implemented.<sup>20</sup> TRIP do have mandates that law enforcement be "fair and equitable." Presently little systematic information exists on the status of enforcement in many countries. The U.S.A. Trade Representative's Office has conducted a survey of firms in this regard. The most common complaints in 54 identified countries were inadequate protection of patent rights, unreasonably slow enforcement and politically motivated decisions (1988 report quoted in Guterman 1993<sup>10</sup>, p. 102). The representativeness on these reports, as well as interpretation of what constitutes "unreasonable," is difficult to evaluate due to the informal nature of the survey. Thus, while the particulars remain unclear, it is nonetheless clear that enforcement of any statutory rights should not be assumed and indeed will be a significant issue for the foreseeable future.

#### DEPOSITS

The disclosure requirement for patenting (see above) directs an invention be described in sufficient detail that it can be recreated by one skilled in the art. When the invention is a living organism not readily available from standard channels, it has been the practice in the U.S.A. since about 1949 to require a sample deposit of that organism.<sup>24,25</sup> When the organism is genetically altered a consideration known as "undue experimentation" comes into play. Undue experimentation applies when a very substantial effort would be required to recreate the invention *de novo*.<sup>26</sup> That is, if a genetically engineered plant could be produced at a frequency of say 0.1% of the attempts, the patent examiner may require that a deposit of the organism be made. Deposits may be made at one of the 13 internationally recognized collections. Under the Budapest Treaty of 1977, a deposit made in one recognized facility will suffice to satisfy the disclosure requirement in all signatory countries.

Once deposited, different rules and regulations apply to access and use. These are of key importance to the patent holder for the deposit is the

invention; no great skill or investment is required to produce it. Under the European system where applications are published 18 months following the initial filing, access and use must be requested through a third party prior to the issuance of the patent, at which point access becomes open.<sup>27</sup> Under the U.S.A. system, applications are secret until granted so that "interim protection" is generally not an issue, even though the deposit, when required, must be made during the application and review process. Subsequent to publication, samples must be made available on request.

PBR legislation typically requires the deposit of a sample in all cases, at least when ex situ storage is a possibility. Samples, however, are held in the national gene bank which assures the material will be preserved in case the owner abandons the variety at a future date. Samples are not made for the purpose of disclosure. In most instances, samples can be purchased on the market, although that would not apply for varieties which are never commercialized, or for pure lines used for breeding and rarely sold.

#### PATENT SCOPE

Patent scope describes the degree of "close copying" which falls within the underlying patent or certificate of PBR. Anything which is included in the scope requires permission of the patent/PBR holder for commercial use. Scope is determined by the novelty search and the claims, the statements of what constitutes the invention. In general, broad patents benefit inventors, while narrow patents are less costly for the public. The optimal balance can always be debated.

Biotechnology patents have sometimes been criticized for being overly broad, especially in the case of a 1992 patent granted to Agracetus for a method of genetically engineering cotton, this covering all genetically engineered cotton varieties. Critics are understandably concerned that such a broad patent would stifle research and allow a firm to control a major agricultural crop, even though its contribution in an overall context is limited.<sup>28</sup> The nature of the patent awarding process contributes to the issuance of broad patents during the early stages of a technology. In simple terms, patent examiners must document —by showing, for example, a prior reference to the work— why a patent should not be granted. The literature on a new technology is limited by definition, providing few grounds for rejection of broad claims.

An example of a second means of achieving a broad patent is to claim a wide range of applications, even when it has not been demonstrated that the

procedure functions in all claimed cases. A fairly extreme example was the Oncomouse, the first patented higher animal, where the claims read to all nonhuman mammals even though the procedure had been successfully completed on mice only.

While such cases are certainly problematic, remedial steps can be taken. Patent offices can and do reevaluate granted patents, as the USPTO is presently doing with the Agrobacter cotton patent. Revocation or reduced scope are possible outcomes. Alternatively, the patent can be infringed openly and, if taken to court, the holder must prove his/her patent is valid for the particulars of the infringement. Such actions are not without their risks for infringement cases are expensive to defend and a patent has a presumption of validity which, in general, would favor the holder. Larger firms, nonetheless, often take early patents to court for a fuller interpretation of the scope.

In most countries (excluding the U.S.A.) where applicant varieties are grown out, the scope is typically established by a crop committee. The committee defines the characteristics for which distinctness may be established (disease resistance, maturation date, storability, etc.), and at times establishes statistical criteria based on a named reference variety.<sup>1</sup> The result is a very systematic process. By contrast, in the U.S.A. no growouts are attempted and distinctness may be established in any characteristic, even a color shade difference which may not be apparent to casual observers. The result is a substantially narrower scope.<sup>1</sup> To date, it has not been possible to determine which is societally more beneficial, but the U.S.A. industry pressed strongly for the 1991 UPOV text as a consequence of perceived inadequate protection under the earlier acts.

## RESEARCH ACCESS

Advances in plant genetics has been dependent on an open exchange of genetic materials. This was assumed in the "common heritage" era, but concerns have been expressed under a broad IPR regime. Clearly, terms of access have been evolving. Whereas previously sharing was open, many improved materials are now accompanied by a Material Transfer Agreement (MTA). MTAs typically, at a minimum, prohibit sharing with third parties and mandate permission if a resultant product is to be commercialized.

The effect which this has had on access is difficult to gauge as few systematic studies have been conducted. Several studies from the 1980s found little overall effect due to PBR.<sup>17,29</sup> A controlling factor seemed to be the recognition of reciprocal dependency between the public and

private sectors. If so, and the arguments are compelling, there is reason to be concerned about smaller entities, public and private, which have nothing to reciprocate with at present. Researchers are also finding the current system increasingly unwieldy and time consuming, with each bit of material tagged with ownership and conditions of use. With so much of this material of limited monetary value, there is an immediate need for a more streamlined system. Scientists should be involved in its development.

## APPLICATIONS TO GENETIC RESOURCES

The preceding section described IPR applications to what can be called the technology and finished products of plant biotechnology. This section addresses available protection for the inputs to much of that work to the genetic resources themselves. In addition to a clear need for completeness, protection mechanisms for genetic resources will control access and commercialization rights to final products in which they are incorporated.

## LIMITATIONS OF TRADITIONAL IPR

### Patents

There is no inherent reason why genetic materials with agricultural uses would not be patentable, at least in concept. The hindrance is rather a practical matter. Patents are not granted for a plant in its entirety, but for a plant (or other product) with unique characteristics, as specified in the patent claims. In the past those plant attributes have been elevated triptophane levels, herbicide resistance and the like among agricultural applications and attributes introduced/induced through technological procedures. It is likely some landraces have such unique attributes - one traditional potato variety for example has hairy leaves which aid in aphid (and hence virus) resistance. For pharmaceutical and industrial applications, generally a genetic sequence is identified and removed from the source organism. Identifying and characterizing such traits at the level required by patent offices is a significant task, costing about US \$20,000 for a U.S.A. application and, due to translation charges, twice that in Europe.<sup>30</sup> Thus, patents are not practical for protecting genetic materials in bulk, although they may be used in certain cases where permitted.

Another category of patents with some useful attributes is petty patents (alternatively called utility models). Petty patents are in effect a weaker form of patent for more modest inventions. They are distinct because the duration is typically up to

10 years as opposed to around 20 years, and the standard for the invention (the inventive step requirement) is typically lower. Thus, applying for and receiving a petty patent is generally less expensive than the procedure for a full patent, although the royalty rate would, as a result, be expected to be lower as well. The Japanese system has the added option of switching from a petty to a regular patent application. That provides additional flexibility. Studies of petty patent systems indicate that they are effective in encouraging investment at the local level in developing countries.<sup>31</sup>

The principal limitation with petty patents is that they are usually designed for and specifically limited to manufacturing products. The Japanese utility model law, for example, reads, “shape or construction of articles or combination of articles so as to contribute to the development of industry (Law No. 123, 1959, Section I.1).” For developing countries, a plow design would be an example. Kenya has established an example of an innovative system where petty patents have recently been allowed for traditional medicinal knowledge.<sup>32</sup> That system should be studied for possible application elsewhere.

### **Plant Breeders’ Rights**

PBR is relatively easy and inexpensive to apply for, costing about one-tenth the amount of a patent.<sup>33</sup> Furthermore, varieties discovered in the wild are protectable with PBR, although some breeding would typically be required to satisfy the homogeneity and stability requirements.<sup>34,35</sup> Hence, PBR would seem to apply to many of the needs of protecting genetic materials for use in agriculture. The UPOV text is not intended to protect plants in general as is made evident by the list of genera to be protected under the 1978 text. Hence, it would not generally be applicable to wild plants.

Where PBR fail even for agricultural uses, or would seem to fail, is in not providing remuneration under either the 1978 (and earlier) text or the 1991 version which introduces “dependence.” Under the earlier versions, a variety which is bred from a protected variety is not infringing (owes no royalties) as long as the new variety is distinct according to the UPOV interpretation. If the protected variety is a landrace which is used (as is permitted under the research exemption) in a breeding program—a general case because landraces seldom are acceptable for commercial-type farming operations—the resultant new variety or varieties would receive the sales with no payments owing to the owner of the landrace.

The 1991 UPOV text rectifies that situation in part by differentiating between initial and essen-

tially derived varieties, with essentially derived varieties requiring marketing permission from the protected variety’s owner. In most cases that permission would be granted for a royalty fee. However, UPOV Article 14(5) establishes two conditions for derived varieties, that they be “predominately derived ... while retaining the expression of the essential characteristics.” As an example, the essential characteristic could be disease resistance found in a landrace. In the breeding process, the remaining (undesirable) genetic material would be bred out so that the genetic composition of the resulting commercial variety would be predominately from another source. That would seem to preclude its being established as an initial variety under the proposed interpretations. Those interpretations also specify the existence of a single initial variety for any derived variety.<sup>36</sup> The interpretations are advisory only, and eventual national applications could be more favorable to PBR use for genetic resources.

### **Trade Secrets and Contracts**

Trade secret legislation allows those whose industrial secrets have been improperly acquired to use the courts to stop further use and/or seek restitution. The community aspect of much genetic material makes secrecy problematic, and indeed secrecy would be contrary to the open exchange considered necessary for maximizing advances with genetic resources. Thus, trade secret legislation is not really applicable.

Contracts refer to a wide range of agreements drawn between two or more parties. They are being used extensively with the transfer of genetic materials, the best publicized example being the Merck/INBio genetic prospecting arrangement in Costa Rica.<sup>37</sup> Contracts, however, are binding only for the signatories. Anyone else who gains access to the materials is free to use them, subject to the trade secret laws discussed above. In short, if genetic materials are to be made openly available, then contracts will not suffice.

### **ALTERNATIVE FORMS**

Intellectual property rights, as was suggested above, are but one means (and not a very applicable means) of claiming control of and remuneration from genetic materials. Other possible approaches to be considered here include “Farmers’ Rights,” treatments of folklore and codes of conduct.

### **Farmers’ Rights**

Farmers’ Rights is the term developed by the FAO under the so-called revised undertaking for plant genetic resources. While not necessarily re-

stricted to plants with agricultural applications, it is quite evident that they are the intended focus of the undertaking. In Resolution 5/89 Farmers' Rights are defined as "rights arising from the past, present and future contributions of farmers in conserving, improving and making available plant genetic resources...." Farmers' Rights are to be "implemented through an international fund on plant genetic resources which will support plant genetic conservation and utilization programs, particularly, but not exclusively, in the developing countries, (FAO Resolution 3/91, Annex 3 to the International Undertaking)." No further details on the implementation and operation of this fund are included.

In concept, Farmers' Rights operate more as a moral obligation than an economic incentive. They are not connected with any specific future action but rather with a general conservation and equity objective. This is said without prejudice but only to note that the objectives and hence the likely results of the system are quite different from IPR.

### Folklore

Many of the issues associated with protecting genetic materials have parallels in protecting expressions of folklore. That is particularly true of landraces which, like folkloric expressions, are the result of long-term community contributions. And again like landraces, there is no system of compensating or even acknowledging those communities for their contributions. Perhaps then attempts to protect folklore will provide some insights for use with genetic materials.

Treatments of IPR for folklore culminated in the joint 1985 "Model Provisions for National Laws" by WIPO and UNESCO.<sup>38</sup> There, the expressions of folklore are defined as "characteristic elements of the traditional artistic heritage developed and maintained by a community ... or by individuals reflecting the traditional artistic expectations of such a community." These expressions may be verbal (folk tales), musical or action (dances) as well as tangible expressions like art, musical instruments and architectural forms (Model Law, Section 2). When used "with gainful intent outside their traditional or customary context" such expressions are "subject to authorization" by the competent authority of the community (Section 3). The expressions may originate from the community or elsewhere, provided they were subsequently further developed, adopted, or maintained through generations (Par. 35).

As can be seen, the issues are indeed similar to those for selected genetic materials such as landraces. However, no helpful detail is included on how to

implement what can only be described as concepts. For example, in the frequent situation where neighboring communities practice slight variants of the same tradition, whose permission would be required—any one of the communities, some or all of them? How or who would determine when an expression is different enough to be a separate form of expression? What competent authorities would be identified to represent a community? And what constitutes an "artistic heritage?"

All IPR systems involve similar types of definitions. A full system, however, includes a definition of who makes the decisions (the national patent office), on what basis (the application, especially the patent claims) and grounds for appeals. The intent is to make the process open and systematic so that it is possible to know within reasonable bounds (questions will always remain in individual cases) what is protected and what would be infringing. It is this kind of specificity which is lacking from this model law and from a system for genetic materials. An evaluation of efforts to develop a system for folklore helps clarify the issues but contributes little to the development of a system for genetic materials.

### Codes of Conduct

Codes of conduct refer to standardized but voluntary agreements specifying obligations. They are similar to a one-sided contract voluntarily entered.<sup>39</sup> The FAO has over several years prepared a "Code of Conduct for Plant Germplasm Collecting and Transfer," still in draft form, that could serve as a model for protecting genetic materials.<sup>40</sup> Kew Gardens also operates under its own voluntary code of conduct.

The FAO Code, which is directed primarily to governments, has the principal objectives of promoting respect for the environment and local traditions and cultures, and establishing mechanisms for compensating local communities and farmers for their conservation and development activities (Article 1). The mechanism for achieving these goals is to require collection permits (Article 8) subjectable to certain conditions, including "financial obligations," restrictions placed on the distribution or use of the germplasm or improved materials derived from it, the use of care in the collection process and provision on request to the country of duplicate sets of the collected materials (Articles 8, 10 and 11). Separate obligations apply to sponsors ("see to degree possible collectors abide by Code," Article 12), curators (provision of further samples, Article 13) and users ("consider providing some form of compensation," Article 14).

This Code is seen as serving temporarily until national legislation is passed, or possibly a legally binding international agreement like a protocol under the Biodiversity Convention. A protocol is a separate agreement binding to those countries which adopt it. However, it and others of its type have the limitation of being strictly voluntary at this time.

### ACCESS LEGISLATION

With Farmers' Rights failing to provide any compensation to date, countries began to claim ownership, culminating in Articles 3 and 15 of the Convention on Biological Diversity.

Article 15(1) from the Biodiversity Convention reads as (emphasis added): "Recognizing the sovereign rights of States over their natural resources, the *authority to determine access to genetic resources rests with the national governments* and is subject to national legislation." The authority of the Biodiversity Convention was not required for claiming these rights. Indeed, the Convention recognizes only existing rights, but it marks a turning point in the treatment of genetic resources as being under national control.

Article 15(3) limits the scope of the Convention to "Parties that have acquired the genetic resources in accordance with this Convention." This clause is generally interpreted to mean that the Convention applies only to materials which are exchanged following the date when Article 15(3) went into effect.<sup>41</sup> As a general rule, convention stipulations do not apply retroactively. Hence, the large mass of materials which were collected prior to December 29, 1993 do not fall directly under the conditions of the Convention.

### Bilateral Approaches

The imposition of national sovereign rights to genetic resources is generally recognized to require some form of access legislation. Some have argued that the wording of Article 15 implies a general prohibition that applies until enabling legislation is adopted nationally. Yet under established legal practices a sovereign right is distinct from a property right over individual resources (FAO<sup>42</sup>: Appendix 3). A national law is required to establish the individual property rights.

Access legislation has, to date, taken two basic forms. The Philippines (Executive Order 247, 1995) have a national proclamation stating the conditions for access and the responsible authorities for granting access. That permission applies only for research purposes; a separate agreement is required for commercialization. To date, besides the Philippines, only Queensland (Australia), Costa Rica and Brazil have specific legislation, although

the Andean Pact has for some months been in the process of drafting terminology and Argentina in 1991 considered a draft law.<sup>43</sup>

The second major form of controlling access is through research permits. These are forms of two-party contractual agreements that have been used for some time in the exchange of research materials, so-called material transfer agreements, or MTAs. A typical agreement would stipulate the following: (1) approved use is for research purposes only; (2) commercialization requires separate approval; and (3) materials may not be shared with third parties without permission.

### Bilateral Agreements

The FAO has long held the position that bilateral agreements would unduly restrict movement of genetic resources for agricultural purposes, much of which is of limited commercial value. Hence, it has supported an open, bilateral system of access. That system must be considered in the framework of the Biodiversity Convention, which roughly divides the ownership issue into the periods before and after the framework went into effect in December 1993. For the pre-Convention period, attention has focused particularly on the major ex situ collections of International Agricultural Research Centers (IARC), which were transferred to FAO control in 1994.

The bulk of the acquisitions in the IARC collections appears to have been made under frequently loose legal agreements with countries of "in trust" protection. In trust does not suggest ownership with its implicit privileges of control over use. Rather a global reciprocal system of contributions and access was employed. Those underlying agreements, both formal and informal, shaped the context of subsequent arrangements for access and use (brief history in Witmeyer 1994<sup>44</sup>).

Current FAO access and use policy stipulates:<sup>45</sup> 1) designated germplasm would be held in trust for the benefit of the international community; 2) intellectual property rights would not be sought over designated germplasm or related information; 3) designated germplasm and related information would be made available without user restriction; 4) the international authority of the FAO Commission on Plant Genetic Resources for setting International Network policy would be recognized; and 5) recipients are ensured of being bound by the same access and intellectual property protection restrictions as are the Centers. Yet the terminology provides sufficient flexibility for alternatives in some instances.

The approach preferred by FAO for materials collected post-Convention (recognizing as a practical matter the distinction will often be difficult to

establish for individual materials) is a multilateral system of open access for participants, which may be nations, institutions, individuals, or corporations, with the possibility of subsequent negotiations for the sharing of value generated by commercialization. The details of the system, including whether it will incorporate a Farmers' Rights-like fund system, have yet to be developed, nor has there been much attention to how the monies in the fund might be specifically used for conservation purposes.

## SUMMARY AND CONCLUSIONS

This section assesses the roles and effectiveness of existing IPR regimes applicable to plant biotechnology and genetic resources with a projection of the effects of expected near term changes.

### IPR AND PLANTS

Intellectual property rights are justified from two distinct perspectives, as a personal right or as a form of economic incentive for investment in creative activities. In general, the economic incentive role is predominant.

IPR provide incentives by prohibiting direct copying without permission. The concept is that the inventor or other creator cannot compete with a copier who shares none of the R&D costs. IPR legislation is national law, applying only in those countries where it is available and has been granted. For this reason, IPR are important for accessing creations made in other countries.

There are four major forms of IPR that can be applied to agricultural biotechnology: patents, PBR, trade secrets and trademarks. Each form has a specific purpose such that a full range of protection would necessitate all forms be available.

Detailed evidence on the actual impacts of IPR is limited and generally inconclusive for skeptics. That position is doubly true when attempting to measure the very recent impacts of protection for living organisms. Nevertheless what evidence is available is in agreement with expectations that IPR do indeed increase private investments in agricultural biotechnology, and that being relatively easy to copy living material is in greater need of IPR than are many areas of technology. IPR also assist in accessing plant technologies which have been developed in other countries.

### APPLICATIONS TO PLANT BIOTECHNOLOGY

Several issues specific to plant biotechnology determine the particular consequences of IPR in that area. These include geographic scope of protection, deposits, patent scope and research access.

Presently, 44 countries specifically exclude patents for plants, and numerous others have yet to act on a plant application. But three of the 28 signato-

ries to UPOV are developing countries so that overall the geographic scope of protection for plants is limited. Trade-related aspects of intellectual property rights (TRIPs) within the GATT mandate certain levels and forms of IPR legislation. These include some form or combination of PBR and plant patents. Numerous countries are presently developing PBR legislation, and Chile, India, the Philippines, among other countries, are believed to be near to joining UPOV (only the 1991 Act is open at present). Most countries are unlikely to adopt plant patents at this time, which is a limitation for biotechnology as PBR do not provide effective protection. None of the TRIPs-mandated changes are required for five to 10 years and depending on product and country, further extensions could be granted.

A common patent requirement for unusual and difficult-to-create living organisms is a deposit of a sample of the material. Thirteen international depositories accept such material, and an international treaty, the Budapest Treaty, describes the system. Following the grant of the patent, access to samples is open. PBR samples are for collection purposes and are not generally made available to the public.

Several early plant biotech patents have been criticized for being overly broad, a matter more likely to occur in the early stages of a technology. Remedial steps exist, either within a patent office or for an individual firm through intentional infringement.; however, the latter is typically limited to large well-financed firms. IPR have changed the conditions of genetic resources access but according to available studies, despite the concerns expressed, the degree of exchange does not appear to have diminished. Smaller and newer entities which cannot reciprocate may, however, experience access difficulties.

### APPLICATIONS TO GENETIC RESOURCES

Those who complain of a double standard regarding IPR genetic materials protection have a legitimate position. Existing IPR legislation is applicable to improved plant varieties but is not really suited for landraces and the like, even though they are technically protectable. Neither are trade secret applicable for they require secrecy in a system based on open exchange. For the short term the best option is the use of simple contracts requiring payment if the materials are commercialized in the future.

Neither are alternative property rights forms, including Farmers' Rights, folklore systems and codes of conduct really applicable, although the final provides a good model for access legislation. As a code, it suffers the limitation of being voluntary.

Countries are currently in the process of adopting access legislation regulating use of genetic resources in association with the Biodiversity Convention. These are bilateral and multilateral. Bilateral systems, as exemplified by the Philippines legislation, are suited to higher valued uses such as pharmaceuticals.

The FAO is presently advocating a multilateral system for agricultural genetic resources. A system has been outlined but details remain to be completed. As presented, the system calls for participants—countries, institutions, individuals, firms—to agree to a form of standardized contract covering research access. Commercialization agreements would be negotiated separately. That system would apply primarily to materials collected after the Biodiversity Convention conditions went into force (December 1993). Previously collected agricultural genetic resources held in the IARC gene banks under FAO auspices since 1994 are regulated by a different system emphasizing continuing open access.

There have in the years following the Biodiversity Convention ratification been numerous calls for new IPR systems applicable to plant genetic resources. A broadly suitable system has yet to emerge, but efforts are underway at the national and international levels. Agricultural biotechnologists must be especially aware of the possible ramifications of those evolving systems for access and exchange of plant genetic resources.

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CHAPTER 6

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# MAJOR CONCERNS ON PLANT BIOTECHNOLOGY APPLICATIONS IN PLANTS: SAFETY ISSUES AND BIOETHICS

D.R.J. Macer

## BIOETHICS AND BIOTECHNOLOGY

Bioethics is a trendy word meaning the assessment and study of ethical issues raised in biology and medicine. The word "biotechnology" means using living organisms, or parts of them, to provide goods or services. The word can apply to agriculture over the past thousands of years, but is often applied to new techniques. Biotechnology started when people first started to plant crops (plant biotechnology), and farm livestock (animal biotechnology). Both share similarly ancient roots. All civilizations were formed in need of 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 genetically engineered foodstuffs made from plants are already being sold in parts of the world.

Bioethics especially includes medical and environmental ethics. The word was mainly applied to issues of medical ethics in the 1970s and 1980s, but the 1960s and 1990s saw much more attention placed on environmental ethics. We must include both. Medical ethics includes any factor affecting health, and ecological and environmental ethics must include human-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 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 worldview.<sup>1</sup> Bioethics is therefore challenged to be a multi-sided and thoughtful approach to decision-making so that it may be relevant to all aspects of human life.

There are two basic approaches in bioethics, descriptive and prescriptive. One describes how people make decisions, and the other suggests the process that can be used to make decisions.<sup>2</sup> When we think of these terms for plant biotechnology, the descriptive side would look at what happens in the world, describing consumer choices, company marketing programs, researchers' plans and intentions. The prescriptive side would look at the regulations covering food safety and formation of public policy. Both aspects will be considered here.

Bioethics is a new word derived from concepts that have been passed down to us through our human heritage for millennia. 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. 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. The ethical issues raised are not fundamentally different to those of the past,<sup>3</sup> and I would reject the use of the word "genethics."

## BENEFICENCE AND BIOTECHNOLOGY

Some people think of the negative side of bioethics, the concept of "do no harm," when they hear the word. However, one of the basic concepts of bioethics is beneficence, an imperative to "do good." This is the reason for publicly supported research in technology and arguably behind the advancement of plant biotechnology in general. Biotechnology has become a popular word and many people hope it will be a solution to the world's ills. Undoubtedly, commercial incentives also play a role in the development of biotechnology, as discussed below.

While all agree that beneficence is good, we do need to consider who benefits most, an issue with many implications for those in developing countries.<sup>4</sup> Biotechnology has already had an effect on developing countries, which have been said to lose \$10 billion annually from their exports due to biotechnology-based product substitutions.<sup>5</sup> International competition implies that there may be some winners and losers in the competition, and it is not yet predictable whether these will be developing or industrialized countries, the producers or users of techniques,<sup>6</sup> the poor or rich within countries, or even how it will change international relations.

Whether countries can use new biotechnological techniques to improve life depends on several major factors. There must be a social acceptance and willingness to use new technology. We can see there is support from opinion survey data presented here. However, there must be sufficient resources to allow its use, and it must be user friendly. There needs to be trained personnel to introduce the technology so that ordinary people can use it effectively, and training for farmers so that they may use the new cultivation systems. The barriers that slow the adoption of better techniques and/or varieties should be removed, and conserva-

tively minded policy makers, dictatorial scientists and village elders should accommodate biotechnology to boost sustainable local production.<sup>7</sup> These are questions of national benefit, but international aid is required to allow research and to introduce new technology in smaller countries. There are international questions such as whether technology is transferred from countries with a high level of research capability to countries that do not and how, if at all, intellectual property rights should be protected as discussed in other chapters of this book.

The desired benefits may be similar in different countries, for example, the desires to raise the quality of life of citizens, and to maintain living standards at a reasonable level. The maintenance of reasonable lifestyles and quality of the environment, consistent with a sustainable way of life in the international community, are the primary goals of many countries. International competition should be adjusted to encourage more sustainable economic policies.<sup>8</sup>

We can hope that trade barriers and protectionism are reduced, but inside most countries the protection of small rural farmers is considered socially important; one must balance the questions of international trade versus national socioeconomic structure. Biotechnology could aid the survival of farmers if more disease-resistant and climate-tolerant varieties are introduced. The production of biomass as renewable energy, and industrial and pharmaceutical products in crops and livestock, will provide additional need for agricultural production. However, multinational petrochemical and pharmaceutical companies may control the seed needed for such crops, and they could produce hybrid seed rather than open-pollinated varieties so as to maintain their control and steady profits. If fees need to be paid for seed, larger farms may succeed more than smaller farms. It is questionable whether biotechnology will support the survival of traditional village structures and small land holders. A free market approach would not do this unless there were strong incentives and disincentives established.

As with every technology, different companies benefit from the sale of their own products. In intensive agriculture with chemical fertilizers, pesticides and multi-application procedures, companies can benefit more if they sell more product to farmers. Considering the long-term benefit to the future generations, farmers and environment, efforts should be made to switch to crop and animal systems less dependent upon intervention. Companies in industrialized countries are continuing much research on applications of biotechnology

that require such inputs. An example is the development of herbicide-tolerant plants, where both seed and herbicide are controlled by the same companies, though they should have environmental advantages when substituted for systems using non-biodegradable herbicides. There should also be attempts to use biological pest control and genetic engineering to insert genes directly into openly pollinated crops, which can be used by farmers in developing countries without dependence upon seed and chemical companies (which are often controlled by the same multinationals).<sup>9</sup> The question is who decides what varieties should be grown in developing countries, and whether it is for local or “international” needs, and for whose benefit?

Within developing countries, applications should attempt to preserve rural structure, so that villages could create small-scale biotechnology “factory” supplies to earn income. In developing countries, the agricultural sector employs over 80% of the active population, but in industrialized countries only 5-10%. Some crops are labor intensive and others are not; for example, oil palm plantations require about one-third of the labor required in banana plantations. Production of new products, such as a single cell protein, may reduce labor. Weeding is one of the most labor-intensive operations, but it will be reduced as herbicide-tolerant crops are introduced and will lead to loss of work for many people, especially women.<sup>10</sup> However, year round crop production may increase labor. The effects depend on the country; for example, the use of bovine somatotropin (BST) to increase milk production in dairy cows is being opposed by many groups in Western countries because it may favor larger farms,<sup>11</sup> but in some developing countries, such as Mexico or Pakistan, its use would be welcomed because it may reduce imports of milk powder.

The arguments about benefits are thus complex ethical and social ones. We need to balance benefits

with the concerns about risks, when we make decisions about policy for plant biotechnology. Most people believe that science brings more benefit than harm, and the results of public opinion surveys shown in Table 6.1 support this. In all countries, there is a positive view of science and technology because it was perceived as increasing the quality of life by the majority in all countries. Less than 10% in all countries saw it as doing more harm than good. One of the intractable policy questions is how much of the policy in a democracy should be decided by public opinion?

## PUBLIC CONCERN ABOUT PLANT BIOTECHNOLOGY

The word “concern” can be used as a verb or a noun. Some linguistic analysis is revealing (from the *American Heritage Dictionary*). The verb includes four meanings: 1) to have to do with or relate to; 2) to be of interest or importance to; 3) to engage the attention of; and 4) to cause anxiety or uneasiness in. The noun also distinguishes several meanings including: 1) a matter that relates to or affects one; 2) regard for or interest in someone or something; and 3) a troubled or anxious state of mind arising from solicitude or interest. It is the fourth meaning of the verb and the third meaning of the noun that I use in this chapter. However, we do need to ask whether plant biotechnology relates to everyone (meaning 1 for both verb and noun), and if people have an interest in it (meanings 2 and 3 for the verb and meaning 2 for the noun)? Plant biotechnology relates to everyone because we all eat plant-derived substances, directly or indirectly. Not all the food in the world could be said to be the result of biotechnology, e.g. simple fishing or hunting of wild animals, but most is.

Do people have an interest in plant biotechnology and a concern about the way food is made? This means an interest in how the food reached them, or what occurred before the supermarket

**Table 6.1. Perceptions of benefits and risks of science and technology in different countries. Responses to the following question: “Overall do you think science and technology do more harm than good, more good than harm, or about the same of each?”**

	NZ	A93	A89	J93	J90	In	Thai	R	Is	UK89	China
More harm	5	4	10	5	7	7	3	5	10	9	2
More good	57	66	56	42	53	53	54	40	66	44	82
Same	34	27	26	45	31	36	42	49	22	37	12
Don't know	5	3	2	8	10	4	1	6	2	10	5

Abbreviations: NZ = New Zealand, A = Australia, J = Japan, In = India, Thai = Thailand, R = Russia, Is = Israel. Data from 1993 International Bioethics Survey (Macer 1994),<sup>1</sup> except 1989 Australia and 1989 UK<sup>12</sup> and Chinese<sup>13</sup> data.

shelves? From consumer patterns we would see that not everyone is concerned about the production, rather people tend to be more concerned about the price. Hoban and Kendall found that more people in the U.S.A. would buy a product because it was 10% cheaper than because it was 10% better quality.<sup>14</sup> This may be different across socioeconomic groups, which can also be reflected by cultures and local availability of food; however, some people do not care what they drink, eat, or smoke. Some people judge by taste and others for perceived health benefits. Ultimately, all must rely on the public health authorities for their food safety. Even though in surveys many may express suspicion in practice most people do not read food labels carefully beyond the expiry date.

Nevertheless, most of what we know of people's concerns comes from opinion surveys. For details of these I refer people to the references. In summary, the major reasons we can see that have been cited in the surveys I have conducted on the unacceptability of plant biotechnology or genetic manipulation can be grouped into five categories: 1) it is unnatural, playing God, unethical, feels wrong; 2) it will cause a disaster; fear of the unknown; bad ecological and environmental effects; 3) fear of human misuse, eugenics, cloning; insufficient controls exist; human society will be changed; 4) health effects, mutations, deformities; and 5) reason not stated. Group 1 concerns may persist with the development of technology, but group 2 and 4 concerns may be lessened by development of technology and by risk assessment for environmental and food safety (discussed below). Group 3 concerns can be lessened by regulations. People who do not cite a reason may feel less strongly about the issue, but there is no real indication of what concerns they had. We should also note that many people expressed reasoning across several of these types of concerns. 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.

Group 1 concerns are related to religious concerns that may not be specific to a particular religion. In agriculture, the major cultural and religious divisions are overuse of animals, and the exclusion of certain animals by religious dietary laws tend to follow cultural boundaries more than use of particular plants, which are diverse within 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

and to multiply and to have dominion over the created order (Genesis 1:28, 2:15). Biotechnologists could consider they are continuing the "good" work of creativity. However, we find interpretations of these scriptures differ within followers of each religion, and rather than stressing one particular

**Table 6.2. Perceptions of benefits and risks from genetic manipulation in Japan in 1991**

Sample:	Public	University Biology Students	High School Biology	Scientists Teachers
Number	485	192	221	518
<b>Human cells</b>				
No Benefit	63	48	46	39
Benefit	38	52	54	61
No risk	17	10	14	29
Risk	83	90	86	71
<b>Plant cells</b>				
No Benefit	21	15	13	12
Benefit	79	85	87	88
No risk	61	40	45	57
Risk	40	60	55	43
<b>Microbes</b>				
No Benefit	31	25	19	13
Benefit	69	75	81	87
No risk	46	26	30	48
Risk	54	74	70	52
<b>Animals</b>				
No Benefit	47	39	29	26
Benefit	53	61	71	74
No risk	39	27	31	46
Risk	61	73	69	54

Nation random mail response surveys conducted in Japan in 1991, except students which were from the University of Tsukuba (Macer 1992).<sup>5</sup>

Responses to the question: "Which of these biological methods could provide benefits for Japan?"

Manipulating genetic material in human cells; microbes; plants; animals.

1 No benefit, 2 Benefit (If a benefit, what benefits do you believe, each one could produce?)

Which, if any, of those biological methods could present serious risks or hazards in Japan?

1 Risk, 2 No risk (If a risk, what serious risks or hazards do you believe each one could present in Japan?)

view the bioethical tradition is that of tolerance for the views of others. Some people interpret biotechnology as playing God and others as serving God, so it is difficult to draw religious boundaries.

People make decisions about plant biotechnology applications based on balancing of the perceived benefits and risks of research goals. The results of an International Bioethics Survey which I conducted with collaborators in 1993 in several countries<sup>1</sup> show that there are variations in the way benefits and risks are balanced (Fig. 6.1). There are variations in the number of people who said they "don't know" in response to the question, "Do you personally believe biotechnology is a worthwhile area for scientific research?" Many in New Zealand and Australia responded yes. However, there is a general correlation between "yes" to a benefit and having less worries.

It is an interesting question to ask which is more important belief in a benefit or concern about a risk. The general question does not differentiate between animals, plants, microbes or humans. In 1991, surveys examined this question with a series of different questions.<sup>15</sup> Plant biotechnology fares well compared to applications on animals, mi-

crobes or human cells as shown in Table 6.2. Similar results have been found in surveys in New Zealand<sup>16</sup> and the U.S.A.<sup>17</sup> We see that the general public perceived most benefit from plants and saw them as having the least risk, as did scientists. Interestingly, scientists in New Zealand saw both animals and plants as presenting a similar degree of risk but, disproportionately, more thought there would be benefits from plant biotechnology applications. In these questions a wide variety of benefits were cited in open response to both questions, and a variety of concerns can be seen.<sup>18</sup>

Therefore we could conclude that from the descriptive viewpoint the answer from surveys about whether risk or benefit is more important appears to be ambivalent. However, from the prescriptive side, regulatory authorities appear to put more emphasis on risk assessment and prevention than they do on the potential benefits of research. The relative benefits of different applications may be promoted by budgetary decisions, though budgetary decisions can also stop public funding of risky areas. This will be discussed more below. This emphasis is also reflected in the nature of the subtitles in this chapter, most deal with

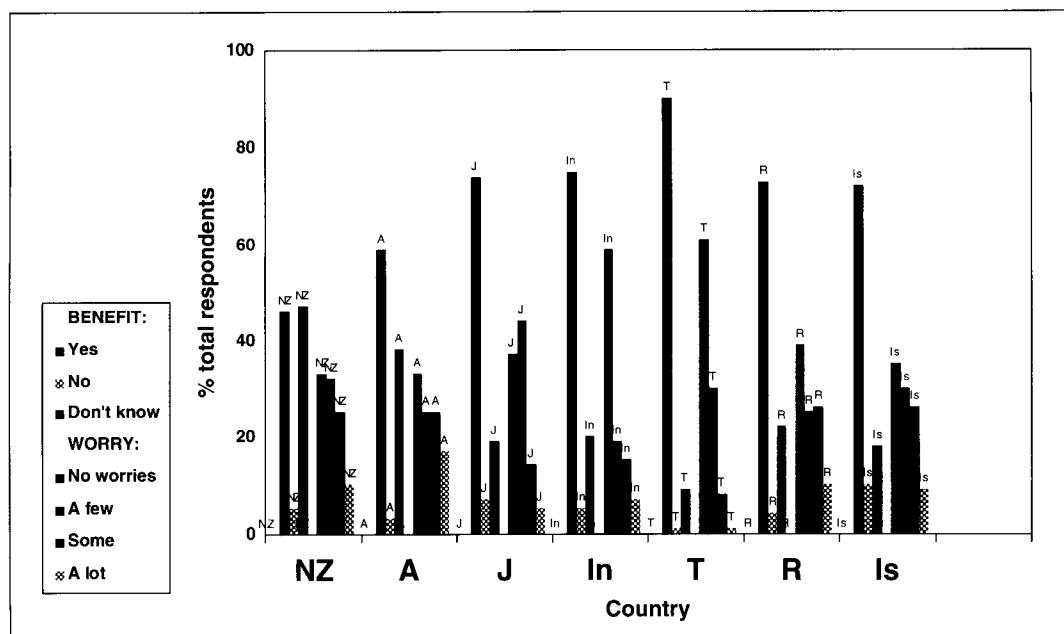


Fig. 6.1. Scattergram of perceived benefits and risks of biotechnology by the public in 1993. Data from International Bioethics Survey (Macer 1994).<sup>1</sup> NZ = New Zealand, A = Australia, J = Japan, In = India, T = Thailand, R = Russia, Is = Israel. Questions were:

"Do you personally believe **biotechnology** is a worthwhile area for scientific research? Why?..."

**Yes      No      Don't Know**

"Do you have any worries about the impact of research or applications of **biotechnology**? How much? Why?..." **No worries      A few      Some      A lot**

safety and concerns; however, we do need to consider the benefits and risks of applications.

There may be particular uses of plant biotechnology that not everyone agrees with, but the distinction that is seen between luxury (e.g. making game fish bigger) and utility (e.g. meat with less fat) among animals may be seen less with plants. In plant biotechnology, there are major industries based on ornamental plants, in addition to food and oil production. If less people perceive risks from plant applications, there will be less objections. We can see a case-by-case approach in these responses to questions on the acceptability of different specific applications of genetic engineering with the highest level of support seen for disease-resistant crops or bacteria to clean oil spills, but also

for tomatoes with a better taste also being supported by about two-thirds of people (Table 6.3). The approval of the Calgene Flavr Savr™ modified tomato that has delayed ripening for general cultivation in the U.S.A. was given by the USDA in 1993, approved for general commercial food consumption by the FDA in 1994 and sold in the summer 1994 in some parts of the U.S.A. The results show that it would be generally supported around the world.

The case for cows that make more milk 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 U.S.A. conducted by Hoban, it was found that consumers gained

**Table 6.3. Approval of environmental release of GMOs**

%	Public							Medical or biology students							
	NZ	A	J	In	T	R	Is	NZ	A	J	In	T	P	S	HK
<b>Tomatoes with better taste</b>															
Yes	49	54	69	73	83	35	40	54	53	71	77	88	68	74	58
No	35	35	20	20	10	45	44	21	36	15	17	5	27	17	32
DK	16	11	11	7	7	20	16	15	11	14	6	7	5	9	10
<b>Healthier meat (e.g. less fat)</b>															
Yes	54	60	57	66	84	35	44	74	71	65	68	88	75	72	62
No	30	31	26	22	9	43	42	20	23	18	18	4	21	17	27
DK	16	9	17	12	7	21	14	6	6	17	14	8	4	11	11
<b>Larger sport fish</b>															
Yes	22	19	22	48	58	13	20	28	23	24	50	64	54	44	42
No	61	65	54	27	25	61	58	63	65	52	31	20	40	39	37
DK	17	16	24	25	17	26	22	9	12	24	19	16	6	17	21
<b>Bacteria to clean up oil spills</b>															
Yes	75	82	71	74	87	63	70	92	89	76	74	85	78	86	70
No	11	11	13	14	5	20	12	1	4	10	13	6	19	6	23
DK	14	8	16	12	8	17	18	7	7	14	13	9	3	8	7
<b>Disease resistant crops</b>															
Yes	70	78	66	78	91	54	50	81	81	67	81	91	82	83	72
No	16	13	17	13	4	25	28	7	13	13	11	5	15	8	14
DK	14	9	17	9	5	21	22	12	6	20	8	4	3	9	14
<b>Cows which produce more milk</b>															
Yes	36	39	44	75	84	23	38	55	44	49	72	86	70	57	54
No	45	42	32	19	7	38	40	31	35	29	19	5	26	25	34
DK	19	19	24	6	9	39	20	14	21	22	9	9	4	18	12

Responses to the question: "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

Abbreviations: NZ = New Zealand, A = Australia, J = Japan, In = India, T = Thailand, R = Russia, Is = Israel, P = Philippines; S = Singapore, HK = Hong Kong. Data from the 1993 International Bioethics Survey (Macer 1994).<sup>1</sup>

confidence about consuming milk produced from cows treated with BST after receiving scientific facts attributed to respected agencies (e.g. AMA, FDA, NIH).<sup>19</sup> Further discussion of food safety will be made below. Larger game fish are rejected by more than half of the people in most countries.

A further concern that some people may have is cross species gene transfer. Four specific questions were used to explore the acceptance of food products made from cross species gene transfer. In all the countries in this survey plant-plant gene transfers (Q9) were most acceptable, with animal-animal (Q11) next, and animal-plant (Q10) or human-animal gene transfers (Q12) least acceptable (Table 6.4). In the U.S.A.,<sup>14</sup> the proportion accepting these were 66% (Q9), 39% (Q11), 25% (Q10) and 10% (Q12), and the trend was also seen in Canada.<sup>20</sup> In the International Bioethics Survey, the question "why?" was

added to each option, and a variety of reasons were given. The ideas expressed in the comments were placed into up to two categories and the results of this analysis are shown in Table 6.5. The range of concerns is as discussed above and illustrates that there are still ethical concerns with plant biotechnology, which increase with genetic transfer from animals.

## ENVIRONMENTAL SAFETY

The first concern that scientists had with modern plant biotechnology was that of environmental safety, and these concerns are reflected in the regulations for field testing of genetically modified organisms (GMOs) found in many countries discussed below. We can also see that a number of persons in the opinion surveys had environmental concerns (Table 6.5).

**Table 6.4. Public and student acceptance of genetic engineering and cross species gene transfer**

% NZ	Public							Students							
	A	J	In	T	R	Is	NZ	A	J	In	T	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?															
+	56	56	39	56	82	45	50	86	75	51	58	78	65	79	76
-	27	23	25	21	4	24	24	9	9	18	18	7	17	8	11
?	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?															
+	19	23	11	29	48	16	22	49	42	16	27	48	17	25	25
-	60	54	40	42	19	42	52	32	24	37	39	19	58	48	48
?	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?															
+	29	40	20	40	68	32	26	50	42	30	42	68	42	41	42
-	46	40	41	27	10	35	46	25	27	35	24	13	36	27	31
?	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?															
+	10	16	6	16	29	10	14	20	20	11	18	30	7	14	19
-	78	66	53	52	44	66	64	65	53	52	41	44	81	65	70
?	12	18	41	32	27	24	22	15	27	37	41	26	12	21	11

+ = Acceptable

- = Unacceptable

? = Don't know

Responses to the questions indicated. Abbreviations: NZ = New Zealand, A = Australia, J = Japan, In = India, T = Thailand, R = Russia, Is = Israel, P = Philippines, S = Singapore, HK = Hong Kong. Data from the 1993 International Bioethics Survey (Macer 1994).<sup>1</sup>

**Table 6.5. Reasoning about genetic engineering and cross species gene transfer**

%'s	Public							Students								
	Q?	NZ	A	J	In	Th	R	Is	NZ	A	J	In	T	P	S	HK
Not stated	9	24	24	35	41	21	52	66	25	17	32	40	15	40	40	31
	10	23	21	40	49	23	62	66	25	20	37	44	14	43	52	42
	11	24	21	39	49	29	61	66	22	22	34	49	18	44	54	37
	12	22	24	41	50	30	60	60	19	26	40	53	22	41	41	39
Unethical	9	0.6	0	0.6	0	0	0	0	0	0	1	0.6	0	0.6	0	0
	10	12	12	3	6	1	2	4	14	26	2	9	2	8	6	3
	11	13	11	6	8	0.6	2	2	23	25	6	10	2	7	11	9
	12	6	7	9	6	5	6	4	14	17	6	5	5	7	8	12
Unnatural	9	16	11	13	11	1	5	6	4	4	8	8	4	2	4	7
	10	32	28	21	18	5	9	14	19	12	16	14	6	20	14	20
	11	24	16	18	11	2	6	14	17	12	12	5	2	7	4	11
	12	23	22	18	9	6	6	8	12	11	16	6	3	8	12	11
Playing God,	9	3	3	3	4	1	2	0	2	1	2	4	0	2	2	1
	10	3	3	2	2	2	0	0	2	2	1	3	1	3	4	4
	11	2	2	3	2	1	3	0	1	0	1	3	1	0	2	1
	12	25	12	7	17	12	7	16	31	18	9	12	14	27	19	19
Cross bad	9	3	6	8	2	2	5	6	2	1	6	2	2	4	1	5
	10	10	9	13	3	6	5	6	2	0	15	4	7	10	6	11
	11	7	6	10	2	4	3	4	2	2	8	2	4	7	3	8
	12	13	10	20	4	10	8	6	4	3	16	7	10	11	7	10
Product bad	9	0	0.5	0	0.2	0	0.2	0	0	0	0	0	0	0	0	0
	10	2	0	0	0	0	0	0	0	0	0	0.3	0.4	0	0	0
	11	0	0	1	0	0	0	0	0	0	0	0	0.4	0	0	0
	12	0.3	0	1	0.4	0	0	0	0	2	1	0	0	0	1	0
Human's special,	9	2	3	3	2	1	0.2	4	3	6	5	2	3	4	3	2
	10	3	2	4	2	3	1	0	2	6	4	4	6	7	4	1
	11	3	2	4	2	3	1	0	1	5	4	0.6	5	6	5	1
	12	3	3	2	3	3	0	0	1	3	2	2	4	3	4	1
Cannibalism	9	1	3	4	0.4	0.4	0.4	0	2	6	3	1	0	0	1	0
	10	0.6	3	2	0.2	0.3	0	0	2	3	1	0.3	0	0	0	1
	11	1	1	2	0.4	0.3	2	0	0	3	2	0.3	0	3	0	0
	12	0	0	1	0	0	0	0	0	1	2	0.3	0	0	0	0
Fear of unknown,	9	2	3	3	2	1	0.2	4	3	6	5	2	3	4	3	2
	10	9	13	3	6	5	5	6	2	0	15	4	7	10	6	11
	11	7	6	10	2	4	3	4	2	2	8	2	4	7	3	8
	12	13	10	20	4	10	8	6	4	3	16	7	10	11	7	10
Feels risky	9	0	0.5	0	0.2	0	0.2	0	0	0	0	0	0	0	0	0
	10	2	0	0	0	0	0	0	0	0	0	0.3	0.4	0	0	0
	11	0	0	1	0	0	0	0	0	0	0	0	0.4	0	0	0
	12	0.3	0	1	0.4	0	0	0	0	2	1	0	0	0	1	0
Dangers	9	2	0	5	0.6	0	1	0	3	0	4	0	0	0	0	0
	10	3	2	5	0	0.3	1	0	2	0	4	0	0.4	0.6	0	0
	11	4	2	6	0.2	0.3	2	0	3	1	9	0	0	0	0	0
	12	5	2	7	0	0	1	0	7	3	5	0	0.4	0.6	0	1
Social affects	9	2	3	3	2	1	0.2	4	3	6	5	2	3	4	3	2
	10	3	2	4	2	3	1	0	2	6	4	4	6	7	4	1
	11	3	2	4	2	3	1	0	1	5	4	0.6	5	6	5	1
	12	3	3	2	3	3	0	0	1	3	2	2	4	3	4	1
Eugenics	9	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	10	2	0	0	0	0	0	0	0	0	0	0.3	0.4	0	0	0
	11	0	0	1	0	0	0	0	0	0	0	0	0.4	0	0	0
	12	0.3	0	1	0.4	0	0	0	0	2	1	0	0	0	1	0
Harm health	9	2	3	3	2	1	0.2	4	3	6	5	2	3	4	3	2
	10	3	2	4	2	3	1	0	2	6	4	4	6	7	4	1
	11	3	2	4	2	3	1	0	1	5	4	0.6	5	6	5	1
	12	3	3	2	3	3	0	0	1	3	2	2	4	3	4	1
Deformities	9	1	3	4	0.4	0.4	0.4	0	2	6	3	1	0	0	1	0
	10	0.6	3	2	0.2	0.3	0	0	2	3	1	0.3	0	0	0	1
	11	1	1	2	0.4	0.3	2	0	0	3	2	0.3	0	3	0	0
	12	0	0	1	0	0	0	0	0	1	2	0.3	0	0	0	0
Environment or Ecology concerns	9	1	3	4	0.4	0.4	0.4	0	2	6	3	1	0	0	1	0
	10	0.6	3	2	0.2	0.3	0	0	2	3	1	0.3	0	0	0	1
	11	1	1	2	0.4	0.3	2	0	0	3	2	0.3	0	3	0	0
	12	0	0	1	0	0	0	0	0	1	2	0.3	0	0	0	0
Insufficient Controls, Misuse	9	2	0	5	0.6	0	1	0	3	0	4	0	0	0	0	0
	10	3	2	5	0	0.3	1	0	2	0	4	0	0.4	0.6	0	0
	11	4	2	6	0.2	0.3	2	0	3	1	9	0	0	0	0	0
	12	5	2	7	0	0	1	0	7	3	5	0	0.4	0.6	0	1
Don't need	9	10	9	7	1	1	2	0	6	6	8	1	2	2	0	2
	10	9	9	6	1	2	4	4	5	6	5	1	3	3	0	0
	11	13	15	11	2	1	3	2	11	8	11	3	1	7	2	5
	12	7	10	5	1	3	2	4	8	6	4	0.6	4	1	2	2
Conditional Benefit, Don't know	9	10	10	10	7	5	12	6	11	12	14	6	4	11	7	5
	10	9	11	13	8	11	12	6	13	22	17	12	13	10	11	8
	11	13	12	8	7	6	9	10	15	26	11	8	7	7	10	6
	12	6	12	8	5	7	6	2	6	17	12	9	10	3	7	3
Medicine	9	8	8	3	9	4	1	2	19	26	5	11	4	10	11	3
	10	3	3	2	3	4	0	0	11	5	0.5	3	4	0.6	1	0
	11	7	11	8	3	7	1.6	4	11	6	4	18	9	16	10	17
	12	7	1	1.8	2	2	0	2	3	0	2	3	1	0.6	1	1
Agriculture Food, Variety	9	13	13	17	13	9	5	2	12	6	11	18	10	12	12	19
	10	2	7	2	3	3	2	0	5	3	2	4	6	3	3	5
	11	2	5	4	2	2	2	2	3	3	4	2	3	4	2	3
	12	0	2	0.6	0	0.7	0.4	0	0	2	0	0	1	1	0.8	2
Humanity, Better, Medicine	9	17	14	12	11	19	6	4	19	20	11	14	28	16	10	28
	10	4	4	2	5	9	1	2	13	7	4	4	15	3	3	5
	11	5	8	5	3	11	3	0	10	5	7	5	20	3	2	3
	12	2	2	1	0.6	3	0.4	0	2	2	1	1	6	3	2	5
Same genes; No problem	9	14	11	4	10	31	5	12	24	16	5	7	20	8	24	11
	10	6	9	1	8	21	3	4	12	15	2	7	18	3	7	5
	11	7	13	1	6	24	3	2	13	11	2	5	25	5	5	9
	12	4	2	2	7	14	9	0	7	7	2	6	16	7	1	5
Number	9	328	199	335	528	686	452	50	95	109	421	316	230	154	250	104
	10	325	199	338	532	687	451	50	95	109	421	315	229	153	250	103
	11	325	199	337	530	685	451	50	95	109	422	315	230	153	250	100
	12	322	199	341	532	684	451	50	95	110	428	314	229	155	250	103

Responses to the questions indicated in Table 6.4. Abbreviations: NZ = New Zealand, A = Australia, J = Japan, In = India, Th = Thailand, R = Russia, Is = Israel, P = Philippines; S = Singapore, HK = Hong Kong. Data from 1993 International Bioethics Survey (Macer 1994).<sup>1</sup>

There are different components of the risks to environment. The probability of each component occurring must be multiplied together to give the likelihood of harm. The components include:<sup>21</sup> 1) incorporation of gene for hazardous trait into an organism; 2) chance of release into natural environment; 3) survival of the organism there; 4) multiplication of the organism in the environment; 5) gene exchange or dissemination; and 6) chance that this will be harmful.

There have been different schemes proposed for assessment of the risks,<sup>22</sup> and some of the criteria that are used are discussed below.

There have now been over a thousand field trials of GMOs,<sup>23</sup> and a dozen varieties are deregulated in the U.S.A., meaning they can be grown unrestricted. Other countries lack any regulation and have been encouraging large scale field trials for a few years, for example, China. From the results of controlled field trials we can obtain estimates of the actual risks of gene transfer, which are finite risks. A Scottish Crop Research Institute (Dundee) using oil-seed rape in a 4 hectare area found the density of airborne pollen from the GMOs was 69% 100m away, and they found significant pollen at 2.5 km.<sup>24</sup> As GMOs are grown over larger areas there will be gene transfer, which makes the final step in the list above "chance that this will be harmful" the most important question to evaluate. A careful choice of genes should be made.

There is an additional concern with the use of biopesticides, plants containing genes or proteins that will selectively kill certain insect pests. Like all pesticides, insects will develop resistance. Strategies to lower chances of resistance to *Bacillus thuringiensis* insecticidal protein include the patchwork farming of treated and untreated fields, and methods to reduce the amount of untreated fields (that may suffer more insect attack) by computer simulation.<sup>25</sup> There is no assurance that all farmers will use new products in a wise way, thus the fear of unknown human use complicates risk assessment.

There is a fundamental ethical question, "Why would we be concerned about gene transfer, 'genetic pollution?'" Human health does depend on the environment, and the easiest way to argue for the protection of the environment is to appeal to the human dependence upon it. There are also human benefits that come from products we find in nature, from a variety of species we obtain food, clothing, housing, fuel and medicine. The variety of uses also supports the preservation of the diversity of living organisms, biodiversity. As we have learned, the ecosystem is delicately balanced, and the danger of introducing new organisms into the

environment that may upset this balance is another key issue raised by genetic engineering. However, we have been using agricultural selection for 10,000 years, so the introduction and selection of improved and useful microorganisms, plants and animals is nothing new, and we should learn from mistakes of the past.

The above arguments should convince people of the value of the environment, and that is the first stage. However, it appeals to our sense of values based on human utility. There is a further way to argue for the protection of nature and the environment, and it is a more worthy paradigm. It is that nature has value for itself because it is there. We should not damage other species unless it is absolutely necessary for the survival of human beings (not the luxury of human life). Nature has life, thus it has value. Another paradigm for looking at the world is a religious view that God made the world so the world has value and we are stewards of the planet, not owners. This paradigm can make people live in a better way than if they look at the world only with the paradigm of human benefit. We need to know what these perceived limits of changing nature are before we grossly change the characters of individual organisms, or make irreversible changes to the ecosystem and human society.

Biodiversity may have some value in itself, though it is yet to be defined in nonreligious terms. If we want to preserve biodiversity, it is essential that we separate parts of nature on land and ocean as nature reserves or parks, away from the parts of nature which are agricultural areas. However, while we separate these areas physically we should not separate them psychologically as areas that we can abuse and areas that we protect. This applies both in terms of sustainable environmental protection and animal rights. In fact, agricultural biodiversity is of direct human utility and we should attempt to stop its continued loss.<sup>26</sup>

## FREEDOM OF RESEARCH AND CONCERN OF SCIENTISTS

Scientific freedom and freedom of expression are admirable goals, but not always absolute if they infringe on other human rights and safety. Scientists are called upon to take responsibility for the social consequences of their research. Recently we can see the growth of ELSI (ethical, legal and social impact) grants from genetics and biotechnology research programs. We can also see the emergence of movements such as the Universal Movement for Scientific Responsibility (MURS). Such moves represent important steps in the growing maturity of scientists. These may illustrate a paradigm shift among scientists to concentrate more attention on

the social impacts of their research, especially in areas such as biotechnology and genetics.

Scientists will win more public support for research by involving the public in decision-making and being open. The public has a high level of suspicion of safety statements made by scientists, especially those involving commercial decisions. In surveys conducted in Japan,<sup>15</sup> New Zealand<sup>16</sup> and the U.S.A.<sup>17</sup> high school biology teachers and government scientists were even more suspicious of statements than the public. Even company scientists did not trust themselves. Committee meetings involved in the regulation of biotechnology and genetic engineering should be open to the public. Such open decision-making would gain more public support than closed meetings, and openness would improve public confidence in regulators. It may also result in better safety than regulations that put industry on the defensive and result in closed-door discussions. Moreover, an open approach may be better at winning public support than the current approach of spending money on advertisement campaigns that could be seen as pro-biotechnology "propaganda" campaigns. Most people are already aware of the benefits of biotechnology, but they will remain concerned about decision-making that is hidden.

There was more support for specific applications of genetic engineering than there was for general research, suggesting that the public will better support worthy applications of technology if they are told the details of them. When people were asked whether they would use gene therapy to cure serious genetic diseases, the majority in all countries surveyed do accept the use of human genetic manipulation for curing serious genetic diseases.<sup>27</sup> A similar effect was seen regarding the approval for environmental release of GMOs (Table 6.3).

There has been an information campaign supporting biotechnology by Bioindustry Associations and specific companies, such as Monsanto. Recently, following a survey of scientists in the U.S.A. engaged in recombinant DNA research that found that more saw public attention on genetic engineering research as beneficial than harmful to their research, public education programs to stress the benefits of biotechnology have been started.<sup>28</sup> The results of the survey discussed above question the effectiveness of such programs, and also whether their goal is desirable. Rather than attempting to dismiss feelings of concern, society should value and debate these concerns to improve the bioethical maturity of society.<sup>29</sup> However, media responsibility is crucial.

## FOOD AND PRODUCT SAFETY

There are already products being consumed from GMOs in many countries. In the UK, those on the market include chymosin from *Aspergillus awamori* and from *Kluyveromyces lactis* (Gist Brocades), from *E.coli* (Pfizer), a tomato paste, oil from oil-seed rape and processed products from soybean. The 1990 approval of a baker's yeast was the first foodstuff from a GMO but it has yet to be marketed. Sainsbury and Safeway supermarket stores in the UK label tomato paste made from genetically modified tomatoes. However, the widest controversy has been seen in the U.S.A. where there is a campaign against foods made from GMOs.

We can see some of the public concerns with foods from attitudes to products such as "tomatoes with better taste" (Table 6.3), and we find that many say they approve. In separate questions on the acceptability of foodstuffs made from GMOs in the International Bioethics Survey, plant products were the ones with the least concern;<sup>1</sup> however, people did not differentiate as much as with the plant-animal distinctions seen in other questions (e.g. Table 6.5). There has been a range of national studies on the perception of risks using surveys, including in Europe, the UK,<sup>30</sup> Holland, New Zealand<sup>16</sup> and the U.S.A.,<sup>17,31</sup> but the real test is whether consumers buy the products when they are sold. There have been reasonable sales of the Calgene Flavr Savr™ tomato, trade name, MacGregor, in the U.S.A. since 1994 when it was released.<sup>32</sup> Similar tomatoes are also being sold in the UK.

The more time spent on testing the safety of a new product, or the environmental safety of a new organism, the higher the financial investment. Ethically, we may say doing no harm has priority, and require long periods for testing of new products. However, this means that the average costs for the development of new drugs are so high that only large companies can afford to take a product through to the market after safety approval.

Nevertheless, society does impose safety standards to protect human and environmental health. Another method of attempting to ensure safety is to allow liability suits in courts, which is an additional protection. However, there also need to be limits on liability claims, otherwise research into such areas as contraceptives or vaccines may be inhibited, due to company fears of future litigation for unrealistic monetary sums in such sensitive areas.

In early 1991, the US government attempted to restrict regulations on biotechnology products such

as foodstuffs<sup>33</sup> as an incentive to encourage further industrial investment. We will not know whether this compromised human or environmental health unless future mishaps occur. Large industry may be cautious about liability suits and better ensure safety of products but it has been suggested that allowing industry the option of not asking for independent review of product safety risks exposing the public to untested products marketed by small companies trying to make a quick profit.

Labeling is the most contentious issue. The opposition from Denmark, Sweden, Germany and Austria over the U.K. and U.S. positions not to label foods from genetically engineered soybeans is delaying the introduction of herbicide-tolerant soybeans into the whole of the EU in 1996.<sup>34</sup> A report of the European group of Advisors on Ethical Implications of Biotechnology has announced its guidelines on the labeling of food from genetically engineered foods recommending that when the product is significantly changed in composition, nutritional value or intended use, it should be labeled.<sup>35</sup> Generally, they focus on the product rather than the process.

## COMMERCIALIZATION AND SHARING OF BENEFITS

Although we hope that biotechnology can improve life for every person in the world, and allow more sustainable living, the crucial decisions may be dictated by commercial decisions, and by the socioeconomic goals that society considers to be the most important. Human, plant and animal breeding is associated with commerce. International trade for many countries has long been based on biological products. International competition to export products to gain foreign exchange has become intense. It is in this framework that the further use of biotechnology must be viewed, and there could be both positive and negative effects for different countries. Biotechnology will affect every area of a country's economy.<sup>36</sup>

Developing countries are currently economic losers in international competition, so many would say that the situation can only get better. However, if commercial forces are left to operate unconstrained by morality, and trade barriers to the import of foodstuffs continue to exist, in terms of international competition, the situation will clearly get worse for developing countries. This is principally because of product substitution, and because of the increasing ability of industrialized countries to produce enough foodstuffs to become self-sufficient. Products such as sugar, shikonin, coffee, cocoa, vanilla and cotton are just some potential cases. Agricultural producers already have very

difficult times, especially with protectionism. If trade barriers were removed, the future would be brighter for developing countries if they could produce cheaper foodstuffs, industrial raw materials and products in transgenic plants and animals, and especially so if the storage life of foods were increased so that they did not spoil during transport.

The situation in terms of food production and life quality in developing countries may improve, nevertheless, because developing countries will become more self-sufficient and have better quality foodstuffs and increased energy production from biomass. For example, if a pest resistance gene saved 1% of the total rice crop in India from disease, it would save US \$300 million a year.<sup>37</sup> However, self-sustainability for most developing countries is several decades away, and we need to think of different solutions to this trend that harms developing countries.

Research has for many decades also been viewed in terms of the business opportunities, both internationally and nationally.<sup>38</sup> As national budgets become more stretched with other needs, many are encouraging more research by industry, either by industry cooperation with government researchers, or independent facilities. If research were performed in publicly funded laboratories, and were published freely, there may be fewer problems with international technology transfer. National governments may transfer technology to other countries as part of development aid. Nonprofit private organizations are also very important in biomedical research in some countries, and they usually allow export of technology. For example, one of the world's largest gene-mapping laboratories in France, the Genethon, funded by charity, has used automatic DNA sequencing to map the human genome.<sup>39</sup> However, the largest genomic research center is The Institute of Genomic Research (TIGR) of Human Genome Sciences Incorporated (HGS), and there has been much controversy over the conditions for data access.<sup>40</sup> HGS has begun sequencing plant genomes and the same issues will be seen with plant biotechnology for the coming decade.

Another issue concerns prospecting agreements. In 1991, the company Merck & Co. made an agreement with Costa Rica giving it exclusive rights to new potential "products" it might find in an area of tropical forests until the year 2000.<sup>41</sup> It is like a hunting license for useful compounds. If successful, a share of the profits will be paid to Costa Rica. This also should encourage other countries to preserve large areas of their forests. It is important to encourage in situ conservation, and if no other group will put up the finances then it

will be left to large companies who will benefit from the new substances found. This is not such a new phenomenon; industrialized countries have been gathering seeds and genetic resources from other countries for centuries for the development of new crops and products.<sup>42</sup> In June 1992, at the World Environment and Development Conference in Rio de Janeiro, Brazil, a Biodiversity Treaty was signed, which has important implications for the protection of biodiversity by all countries and may preserve the intellectual property rights of products derived from the diverse species. Intellectual property rights are discussed elsewhere in this book.

## **REGULATION OF PLANT BIOTECHNOLOGY**

There have been a variety of laws and regulations made in different countries around the world. Some countries have chosen to have specific laws; The European Union,<sup>43</sup> Russia,<sup>44</sup> and others have achieved control through government regulations, for example, the U.S.A. and Japan. The European Parliament set minimum legal standards for European Community countries, though regulations vary between strict, as in Germany, to nonexistent in other countries that rely on the default European regulations. In Japan, each of the major ministries has its own regulations.<sup>15</sup>

The country with the widest experience of GMO release is the U.S.A., with most field releases regulated by the Department of Agriculture, except those for microorganisms and pesticide genes which are regulated by the Environmental Protection Agency.<sup>45</sup> The USDA amended the regulations on genetically engineered plants introduced under the USDA's notification and petition regulatory processes in 1996<sup>46</sup> to allow most genetically modified plants that are considered regulated articles to be introduced into the environment under the notification process as long as they meet certain eligibility criteria and performance standards. In addition, under the notification process, the amendment would allow a reduction in the field test reporting requirements when no unexpected or adverse effects are observed. Under the petition process, the proposed amendments would enable USDA scientists to extend an existing determination for nonregulated status to certain additional regulated articles that closely resemble an organism for which a determination has already been made.

There are many countries that do not have sufficient resources to enact their own regulations, so a Global Biosafety protocol was discussed in the Jakarta meeting of signatories to the Biodiversity Convention which ended November 17, 1995.

The decision was postponed to be made by 1998, and the developing countries wanted to include internal guidelines as well as international movement of GMOs, whereas the EU wanted to only regulate the latter.<sup>47</sup> There are 168 signatories to the Convention now, and there is debate over how strict and when a biosafety protocol under article 19 of the convention would be enacted. In September, Argentina adopted the UNEP guidelines that were developed by the UK and Netherlands as an alternative. In the absence of specific laws, researchers may follow guidance suggested by various academics<sup>48</sup> or international bodies.

Islands may develop particularly different regulations and enforce them, but regions, such as Europe, need common minimum regulations as neighboring countries are at risk. Conversely, any country that imposes extra regulations must suffer the lower industrial development of their neighbors without a significant reduction in risk. We must also gather information from past releases of new organisms and their ecological consequences. We can hope that the information is shared globally to prevent others from making the same mistakes, and to ensure all countries have a similar minimum standard of protection. It is clear that the authorities and committees that have the most experience with releases should have developed the most skills in assessing the ecological risk. Review should, of course, be independent to avoid conflict of interest.

Independent clinical review of drug safety is already standard in most countries, and to be ethical, we must ensure that all people of the world share its protection. Such protection should be standardized, but it is a more difficult question when a country wants to impose stricter standards. A government has a duty to allow beneficial products and technologies to be used by its citizens. There are various laws concerning the food and product safety in different countries.<sup>49</sup> There are guidelines released for foodstuffs in Europe, as mentioned above, and in the U.S.A. by the FDA<sup>50</sup> and in Japan.<sup>51</sup> Generally, foods made using GMOs do not need very exhaustive safety examination, unless novel components are included,<sup>52</sup> as discussed above. There are however, differences in the labeling requirements, with some foods requiring labeling and others not. Some companies voluntarily label products, and others do not, and supermarket chains have different policies as discussed above. In a rapidly moving and new area, an independent committee approach to regulation is the only way to efficiently and safely examine food safety.

Guidelines also differ on what is included as a GMO. Some exclude organisms that have gene deletions, only including organisms which contain

"recombinant DNA" sequences or parts of vectors. In some democracies, the public has a clear role in the process of regulation and clear opportunities to voice concerns. This opportunity to voice concerns is important to gain public trust, especially when considering the lack of trust (see above). In some countries, hearings are conducted in public, as in the RAC committee hearings on human gene therapy in the U.S.A. The above mentioned survey responses suggest that the public can make well-reasoned arguments concerning biotechnology risk and benefit. The public should be involved more in committees making science policy and regulating applications of science. This requires more public willingness to be involved, and the scientists and bureaucrats should allow third party and public entry to committees. As a minimum standard for ensuring ethical biotechnology, decisions should be made in forums open to public knowledge.

## THE NEED TO ADDRESS HOPES AND CONCERNs

Perceptions of the impact of technology are more complex than a simple perception of benefit or risk,<sup>23</sup> as they should be. The capacity to balance benefit and risk of alternative technologies, while respecting human autonomy, justice and the environment and simultaneously being under the continual influence of commercial advertisements and media stories of varying quality and persuasion, may prove to be an important indicator of the social and bioethical maturity of a society.<sup>29</sup> In addition, to develop the bioethical maturity of society, global human rights need to be increasingly respected so that we achieve social progress as well as scientific progress. All people should equally share both the benefits of new technology and the risks of its development.

There will be future conflicts in determining what is ethical biotechnology. Our concepts will change, and there is no guarantee that unethical applications will be made and even supported by future public majorities. We need to remember history, and also may need to introduce some international laws which make it more difficult for future unethical uses to occur. However, we need to be flexible and as we gather experience, we may need less stringent regulations.

We can think of some summary criteria which may be useful in determining whether any given application of biotechnology is ethical:<sup>23</sup>

1. What is the benefit? To whom? Is it life-saving? Human benefit is greater than monetary benefit.
2. Do no harm to humans. What is an acceptable level of risk?

3. Do not cause pain.
4. Do no harm to the environment. Use the technology that is most environmentally sustainable over the long term. Minimize consumption.
5. Protect biodiversity. Protect endangered species. Allow farmers affordable or free access to breeding stock, and encourage the planting of diverse crops.
6. Justice to all people and future generations. Share benefits and risks.
7. Independent open decision-making on safety questions, consider ethical and social impact.
8. Inform and educate the public and scientists about all dimensions of the projects, scientific, social, economic and ethical, using third party media.

In conclusion we need to think of key concepts of education, progress, responsibility and sustainability. People have hopes in the future of plant biotechnology, and the food problem is the most widely cited hope that people express for "biotechnology" in the surveys that have been conducted.<sup>1</sup> They also have hopes for medical advances. However, among the fears that people have, environmental concerns and human misuse make us aware of the need for responsible science, and to look before we leap. This is essential for the future well-being of the world.

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## CHAPTER 7

# FRONTIER OF RICE BREEDING BY UTILIZATION OF GENETIC RESOURCES AND BIOTECHNOLOGY

R. Ikeda and K. Wakasa

## INTRODUCTION

Advances in the evaluation of rice genetic resources and applications of biotechnologies have been facilitating R&D on rice production, especially in cultivar development. Recent approaches to elucidating questions and avoiding pitfalls in rice breeding are: 1) transgenic rice production; 2) rice genome analysis; and 3) gene cloning. These new biotechnologies and information from them could contribute to the improvement in generating novel genetic variants, which is the primary task of breeders, and to reduce the resources and labor in selecting improved lines. In this chapter we will discuss three topics: 1) the present status of rice genetic resources; 2) the use of molecular markers; and 3) the present status of R&D in transgenic rice plants. A systematic approach using these major components is discussed in the summary of this chapter as their development is tightly connected to each other.

## PRESENT STATUS ON UTILIZATION AND POTENTIAL OF RICE GENETIC RESOURCES

### GENETIC RESOURCES

Rice has a richer germplasm base than other major food crops.<sup>1</sup> Genetic diversity carried by landraces and wild relatives of rice involves valuable and productive resources, since it has enabled rice plants to evolve and differentiate various cultivars to meet with diverse environments. According to Chang,<sup>2</sup> the total number of rice cultivars grown by rice farmers in Asia before the 1950s might have exceeded 100,000, with specific environmental adaptation such as irrigated and nonirrigated conditions. The rich genetic diversity has enabled rice to keep pace with the ever-growing rice consumers and even expand into unfavorable environments, though it remains a subsistence crop in many adverse environments.

Since the late 1960s and the Green Revolution in rice, however, the diversity of rice cultivars in irrigated areas has been drastically reduced. The high-yielding semi-dwarf cultivars now occupy more than 50% of the irrigated fields in tropical Asia. Similarly, associated wild relatives, especially *Oryza rufipogon* and *O. nivara*, were at one time

distributed extensively in South and Southeast Asia, including south China. However, during the past two decades, the wild species and their derivatives have been disappearing rapidly as the result of environmental destruction by development projects, human neglect and lack of in situ conservation.<sup>3</sup>

#### LANDRACES AND WILD RELATIVES OF RICE AS SOURCES OF USEFUL GENES

Efficient approaches to identify resistant or tolerant sources to biotic and abiotic stresses are necessary to utilize the conserved germplasm. They use of landraces as a source of resistance or tolerance to biotic and abiotic stresses is more practical than that of wild relatives because the introgression of target gene(s) from landraces to improved cultivars is much easier than that from wild relatives. This is attributed to adaptation and less likely to affect deleterious (wild) genes in landraces. However, it is very difficult to identify specifically important traits in landraces. In such cases, we must test diverse accessions of wild relatives for accessing target trait(s). Among globally important target traits,<sup>1,2</sup> we present specific examples on the importance of rice genetic sources for crop improvement, showing the distribution of resistance to brown planthopper, bacterial blight and tungro in landraces and wild relatives of rice.

#### Resistance to Brown Planthopper

The brown planthopper (BPH) is one of the most devastating rice insect pests in tropical and

temperate zones in Asia. BPH is distributed in Asia, the Pacific islands and North Australia. Three components are involved in the resistance to BPH; antixenosis (former, nonpreference), antibiosis and tolerance, but only the genetics of total resistance to BPH was clarified. The resistance to BPH could be tested at a young seedling stage of rice by using a mass-seedling testing method. A large number of accessions in rice landraces from the world have been screened for BPH resistance in Japan and at the International Rice Research Institute (IRRI) in the Philippines. The results obtained in the two programs showed that most of the resistant landraces had originated only from South India and Sri Lanka.<sup>4,5</sup>

On the other hand, based on the IRRI data (Table 7.1), frequencies of resistance to each BPH biotype in wild species is 30 times more than in landraces. The frequency of accessions on the reaction pattern of RRR or broad resistance to BPH biotypes is roughly 38% in wild relatives, but is less than 1% in the landraces of rice.

Heinrichs et al<sup>5</sup> listed 12 *Oryza* species and one natural interspecific hybrid group as resistant to BPH (Table 7.2). Four species, *O. nivara*, *O. ridleyi*, *O. officinalis* and *O. minuta* and the natural interspecific hybrid group are distributed in Asia, whereas *O. australiensis* is in tropical Australia. This distribution may be considered to correspond with sympatric resistance since these species are found in the distribution area of the BPH. However, the other species from Africa, *O. brachyantha*, *O. barthii* and

**Table 7.1. Number of accessions in landraces and wild relatives showing resistance to brown plant hopper (BPH)**

	<b>Tested</b>	<b>Landraces Resistant</b>	<b>(%)</b>	<b>Wild relatives</b>		
				<b>Tested</b>	<b>Resistant</b>	<b>(%)</b>
<b>Biotype<sup>a</sup></b>						
1	44,335	682	1.5	723	302	41.8
2	10,053	187	1.9	724	242	33.4
3	13,021	236	1.8	730	272	37.3
<b>Reaction patterns<sup>b</sup></b>						
				7,022	579	
				121	1.7	
RSR <sup>c</sup>		83	1.1		14	2.4
RRS		48	0.7		3	0.5
RRR					219	37.8

a) Reaction of accessions to each biotype,

b) Reactions to 3 biotypes (biotype 1, 2 and 3),

c) For resistance genes, RSR, *Bph-1*; RRS, *bph-2*; and RRR, *Bph-3*, *bph-4*, *bph-8*, *Bph-9* or two or more genes.  
(Modified from database for GEU program at IRRI, 1991)

*O. punctata* African strains of *O. eichingeri* and from tropical America, *O. glumaepatula*, *O. latifolia* and *O. alta* exhibit allopatric resistance. Wild relatives represented a worldwide distribution of BPH resistance genes. Some species display a sympatric resistance to BPH, while others are allopatric.

### Resistance to Bacterial Blight

More than 20,000 rice cultivars from Asian countries have been tested by the author's group for resistance to bacterial blight (BB) since 1986. From their reactions to infection by six BB races from the Philippines, resistant varieties were classified broadly into five groups: Java 14(*Xa-3*), TKM6(*Xa-4*), DZ192(*Xa-5*), CAS209(*Xa-10*) and T N1(*Xa-14*). Varieties belonging to the Java 14 group were found in the majority of Asian countries, but the frequency of resistant cultivars in each country varied from 17.2% in Indonesia to 0.3% in India. Varieties with the *Xa-4* gene were found in all Asian countries. Distribution of the *Xa-5* gene showed regional specificity; the frequencies of occurrence in rice germplasm from Bangladesh

and Nepal were 25.9% and 13.3%, respectively, but in seven countries (for example, Thailand and Indonesia), the *Xa-5* gene occurred in less than 1% of the accessions. Distribution of the *Xa-10* and *Xa-14* genes was even lower; less than 5% of the accessions from any country.

Accessions of wild species were also screened for BB resistance to compare the distribution of resistance genes in wild species with those in cultivated varieties. We tested 198 accessions comprising 10 wild species and 22 natural hybrids from International Rice Germplasm Center (IRGC) at IRRI for BB resistance, using six races. More than half the tested accessions showed resistance to all six races.<sup>6</sup> This is in contrast with other findings since only a few cases in *O. sativa* cultivars have been found as resistant to all six races from the Philippines. On the other hand, *Xa-4*, one of the most common resistance genes in *O. sativa* varieties, was not found in any of the wild rices. Of the 198 accessions tested, 101 originated from Thailand; all were AA genome species. About 70% of the accessions from Thailand showed resistance to all six races; 10 showed a reaction pattern similar to that of germplasm having the *Xa-3* gene. The other known genes were not found in the wild species, but *Xa-3*, *Xa-4*, *Xa-5*, *Xa-10* and *Xa-14* have been found in cultivated varieties from Thailand. The differences in distribution of BB resistance genes between cultivated varieties and wild species constitute an intriguing phenomenon.

A strain of *Oryza longistaminata* from Mali, which had been maintained at CRRI of India, was resistant to all the races of BB in India. The strain showed resistance to all six races of BB in the Philippines as well. Thereafter, the resistance gene was designated as *Xa-21*.<sup>7</sup> Recently, the *Xa-21* gene was cloned by positional cloning (see below).

### Resistance to Tungro

Tungro occurs in South and Southeast Asia. Since the late 1960s, it has caused serious damage to rice production in Bangladesh, India, Indonesia, Malaysia, Philippines and Thailand.<sup>8</sup> Tungro is a disease complex associated with rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV).<sup>9,10</sup> The tungro virus complex is transmitted by six leafhopper species, of which the green leafhopper (GLH) *Nephrotettix virescens* (Distant) is the major vector of tungro. RTBV depends on RTSV for its acquisition and transmission by GLH. It is transmitted only when the vector has been exposed to RTSV infected plants before feeding on RTBV infected plants.<sup>10</sup>

During the last two decades, more than 20,000 accessions of landraces from the IRGC at IRRI

**Table 7.2. Number of accessions in wild relatives of rice resistant to BPH**

Taxa	Genome group	Reaction patterns to BPH <sup>1</sup>		
		RSR	RRS	RRR
<i>O. brachyantha</i>	FF	0	0	2
<i>O. sativa</i> complex				
<i>O. nivara</i>	AA	1	0	9
Natural hybrids	AA	0	1	3
<i>O. barthii</i>	AA	1	0	2
<i>O. glumaepatula</i>	AA	0	0	1
<i>O. ridleyi</i> complex				
<i>O. ridleyi</i>	Tetraploid	0	0	2
<i>O. officinalis</i> complex				
<i>O. officinalis</i>	CC	2	0	37
<i>O. eichingeri</i>	CC	0	2	5
<i>O. minuta</i>	BBCC	0	0	28
<i>O. punctata</i>	BB, BBCC	0	5	7
<i>O. latifolia</i>	CCDD	4	4	5
<i>O. alta</i>	CCDD	0	0	1
<i>O. australiensis</i>	EE	0	0	4

(Data from Heinrichs et al.<sup>5</sup>)

See the footnotes in Table 1 for the resistant genes.

were screened for tungro resistance. Some landraces have been found to be resistant or tolerant to tungro at IRRI. No variety with complete resistance to RTBV has been identified. However, low infection with RTBV and RTSV, low or no infection with RTSV and tolerance for RTBV were observed.<sup>11</sup> A few accessions of landraces resistant to this virus originated from northeast India, Bangladesh and Indonesia, where the virus occurs.<sup>12</sup>

The resistance to tungro has also been evaluated in wild relatives and the African cultivated race, *O. glaberrima*.<sup>13</sup> Representing the genetic diversity in the genus *Oryza*, 211 accessions were tested for resistance to RTBV and RTSV infection. Of these, 53 and 15 accessions were not infected with RTSV and RTBV, respectively, when plants were inoculated with viruliferous GLH. Three accessions of *O. rufipogon* were not infected with either RTSV or

RTBV (Table 7.3). Two accessions of *O. officinalis* and one each of *O. rhizomatis* and *O. brachyantha* were not infected. Although a large number of accessions in landraces have been evaluated, no accession has been found as resistant to RTBV. The wild species identified as highly resistant to tungro are useful donors in developing tungro-resistant rice germplasm. Fifteen RTBV resistant accessions derived from eight wild species were reexamined to determine whether the resistance was attributed to vector resistance and/or virus resistance.<sup>14</sup> Among 15 accessions, three of *O. rufipogon* showed a low or moderate level of antibiosis to the major vector *N. virescens*; they were also resistant to infection with tungro virus particles regardless of vector resistance. Three accessions of *O. officinalis* showed a high level of antibiosis to *N. virescens*, but they showed low levels of antibiosis to *N. nigropictus*. These *O. officinalis* accessions were not infected with RTBV in the virus inoculation test using *N. nigropictus*. One *O. ridleyi* accession showed a moderate level of antibiosis of *N. nigropictus* and no infection was observed with RTBV in the inoculation test. These results suggest that the resistance to RTBV infection of these seven accessions does not relate to the vector resistance but to resistance to virus infection. These wild rice accessions could be useful in developing rice cultivars with high resistance to tungro. However, further investigation in identifying mechanisms of the resistance to virus and genetics of the resistance is essential to make efficient use of the precious resistant genetic resources.

**Table 7.3. Number of accessions in wild relatives of rice resistant to tungro virus**

<b>Taxa</b>	<b>Tested</b>	<b>No. of accessions</b>	
		<b>RTBV</b>	<b>RTSV</b>
<i>O. brachyantha</i>	5	1	5
<i>O. sativa</i> complex			
<i>O. nivara</i>	56	0	5
<i>O. rufipogon</i>	20	3	10
Natural hybrids	35	0	6
<i>O. glaberrima</i>	4	0	0
<i>O. barthii</i>	9	0	3
<i>O. meridionalis</i>	2	0	0
<i>O. langistaminata</i>	9	0	3
<i>O. ridleyi</i> complex			
<i>O. longiglumis</i>	3	1	0
<i>O. ridleyi</i>	5	2	0
<i>O. officinalis</i> complex			
<i>O. officinalis</i>	15	4	6
<i>O. rhizomatis</i>	6	1	1
<i>O. eichingeri</i>	5	0	2
<i>O. malampuzhaensis</i>	3	0	1
<i>O. minuta</i>	13	0	6
<i>O. punctata</i>	7	0	2
<i>O. latifolia</i>	5	1	1
<i>O. alta</i>	3	2	0
<i>O. glandiglumis</i>	2	0	0
<i>O. australiensis</i>	4	0	2
Total	211	15	53
(%)		(7)	(25)

(Data modified from Ikeda et al.<sup>12</sup>)

Virus detection was made with ELISA.

### Search for Sympatric and Allopatric Resistances

Examples given in this section showed that limiting the search for sources of resistance to germplasm where stress is found may not always be appropriate. When sources of resistance to a stress can be found both where stress is present and where it is absent, it may be worthwhile to analyze genes different from both sources. Genes arising from coevolution in a gene-for-gene manner can be overcome by a pathogen. Stress tolerance which arises independently of the stress may be more difficult to break down.<sup>15</sup>

### INTERNATIONAL NETWORK FOR GENETIC EVALUATION OF RICE

Under the Genetic Evaluation and Utilization (GEU) program of IRRI, which ran from 1973 to the late 1980s, a large portion of the germplasm collection was systematically evaluated by teams of multidisciplinary scientists for varietal reactions to various biotic and abiotic stresses. The numerous

accessions were identified as promising sources for resistance/tolerance to the diverse array of stresses and many of these were immediately used in breeding. The former International Rice Testing Program, IRTP (later renamed the International Network for Genetic Evaluation of Rice; INGER), organized and distributed as many as 29 uniform nurseries over 120 sites in 50 countries. The INGER nurseries included both unimproved and improved germplasm. Under GEU and IRTP/INGER evaluation programs, numerous sources of resistance/tolerance to a full array of important biotic (mainly diseases and insects) and abiotic (edaphic, hydrologic, climatic) stresses were thus found.<sup>16</sup> The biological and ecological data obtained from multiplication tests also proved to be useful and valuable in elucidating host-pest interactions in different environments, improving the selection of test sites and characterizing the environment of test site.<sup>16</sup> Moreover, rice research on many fronts was enhanced by the rich diversity and genetic plasticity in the huge germplasm collection. Many advances in rice research owe their impetus to the novel sources that were hitherto unavailable.

#### UTILIZATION OF RICE GERMPLASM

The wild relatives have also yielded novel or unusual sources of desired tolerances. A large proportion of accessions of the wild species have resistance to one or more pests.<sup>5,17</sup> A strain of *O. nivara* from northern India has provided the only source of tolerance to grassy stunt virus biotype 1, but another biotype of the virus has later appeared and rendered the gsv gene from *O. nivara* ineffective. Other novel sources found are salinity tolerant *Porteresia coarctata*, brown planthopper resistant in *O. officinalis*, blast resistant in *O. minuta*, thrip resistant in *O. officinalis*, tungro virus resistant in *O. officinalis*, *O. rufipogon* and *O. barachyantha*.<sup>13,14,18,19</sup> The greatest contribution from a wild relative has come from a sterile wild-weedy plant (*O. sativa f. spontanea*) found on Hainan Island, China. The wild abortive cytoplasmic male-sterility has led to 18 million ha of top-class and high-yielding hybrid rices in mainland China.<sup>20</sup>

In today's Asia, more than 73 million ha of rice land are planted with the high-yielding varieties (HYVs), which are semi-dwarf, nitrogen-responsive and early maturing varieties derived from a Taiwanese variety, Dee-geo-woo-gen.<sup>21</sup>

The next variety in tropical rice that followed the high-yield base was improved or newly resistant to diseases and insects, had shorter growth duration and a small increase in the harvest index on a daily growth basis.<sup>22,23</sup> Future improvements in the favored areas will hinge on an assortment of more complex traits: durable resistance to pests,

tolerance to the little understood abiotic stresses, more efficient use of nitrogen and water, improved photosynthetic efficiency and reduced plant respiration.

Recent advances in rice research employing innovations of biotechnology promise additional avenues to incorporate such traits from distant parents, but developments to date remain at varying stages of experiment or development.<sup>19,24</sup> At present, wide hybridization has provided the actual method for transferring genes from wild species to cultivated rice. Using embryo rescue techniques, hybrids have been produced in elite breeding lines and varieties of rice and several accessions of 11 wild species.<sup>25</sup> Useful genes for disease and insect resistance have been transferred from *O. officinalis* and *O. australiensis* into rice.<sup>26,27</sup>

Molecular markers could help in evaluating and monitoring introgression of unique genes from rice genetic resources for breeding. Moreover, once target genes derived from such precious genetic resources are cloned, genetic engineering could help to utilize such cloned genes from the rice germplasm. In the following sections, we will discuss the importance of molecular markers, marker-assisted selection, positional cloning and transgenic approaches.

#### IMPORTANCE OF MOLECULAR MARKERS

The permeation of DNA manipulation technologies, including restriction endonuclease and polymerase chain reaction (PCR), has developed molecular genome mapping in several plant species. Intensive researches using molecular markers also resulted in highly saturated molecular linkage maps of rice.<sup>28,29</sup> Recent results of mapping permit the generation of a new selection method based on molecular markers and to allow positional cloning.

DNA markers such as RFLPs (restriction fragment length polymorphism) and RAPDs (random amplified polymorphic DNA) are well developed in rice, and two independent groups have generated saturated molecular linkage maps.<sup>28,29</sup> Kurata et al<sup>28</sup> constructed a high resolution rice genetic map which contains 1,383 DNA markers at an average interval of 300 kb. Their gene expression map consisted of 883 cDNAs, 265 genomic DNAs, 147 RAPDs and 88 other DNAs. Some were sequenced to generate sequenced-tagged-sites (STSs). Causse et al<sup>29</sup> constructed the map containing 726 markers which were mainly comprised of RFLPs. In this case, the mapping population was derived from a backcross between *Oryza sativa* and *O. longistaminata*.

## MARKER-ASSISTED SELECTION (MAS) IN RICE BREEDING

Genes for disease and insect resistance if tagged with molecular markers can be pyramided in a varietal background.<sup>30</sup> If we detect a DNA marker linked to a target gene, we will be able to infer the presence of the phenotypic trait by screening for the linked DNA markers in all individual plants of a breeding population. This indirect selection of targeted traits by DNA markers could be very effective in backcross breeding, where undesirable chromosome segments, i.e. generally most of the donor chromosomes except for the gene(s) to be introgressed, must be removed within several back-cross generations.<sup>31</sup>

Several thousand individuals are generally used in breeding. However, in backcross breeding of rice, a typical conventional breeding strategy, a small number of progenies (less than 50 individuals) are used. Under these conditions, it may be possible to employ DNA markers to survey the whole genome of individuals in the breeding population. Thus, a combination of backcross breeding and MAS could facilitate a rapid development of new rice varieties with improved traits.

Although molecular markers are useful for selection, the detection of RFLP markers requires Southern hybridization technique, and RAPD markers based on PCR amplification are very sensitive to several reaction parameters, which could result in confusion.<sup>32</sup> In order to handle a number of samples, a simple, precise and efficient way of screening is required. Once the genomic clones are identified to be linked to target traits, sequences of the clones are used to design pairs of specific primers for a secondary PCR assay.<sup>32</sup> The amplified products will be available for selection markers as shown in linkage analysis of a gall midge resistance gene.<sup>33</sup>

Two kinds of DNA markers tightly linked to important resistance genes were developed in rice. Hittalmani et al<sup>34</sup> identified specific amplified polymorphism between the resistant and the susceptible genotypes to rice blast, which was generated by cleavage of STS because STSs were not polymorphic between the three varieties. Using their marker, they determined the genotypes of the F2 individuals at this locus. The progeny test for the disease response in a F3 generation indicated more than 95% of probability in identifying the resistant plants. Complete success using the markers was achieved when the markers flanking the genes were considered simultaneously. Other resistance genes for rice blast, *Pi-2(t)* and *Pi-4(t)*, were also tagged using RFLP markers.<sup>35</sup>

Reliable linkage of the two RAPD fragments to a gall midge resistance gene (*gm2*) was also identified by Nair et al.<sup>33</sup> Two sets of primers were used together in a single PCR to amplify specific bands, 1.7 kb and 0.6 kb fragments in the susceptible and resistant parents. The work pointed out another advantage of MAS in breeding for insect resistance instead of phenotypic evaluation in which the availability of the insect was the key factor in proceeding screening.

## QUANTITATIVE TRAIT LOCI (QTL) ANALYSIS

Many agronomically important traits in rice are quantitatively segregated.<sup>36</sup> They often exhibit continuous distribution of phenotypes resulting from segregation of multiple genes and modification of environmental effects. Recent progress in DNA markers and their linkage maps enabled us to analyze these individual quantitative trait loci (QTL).<sup>36</sup> QTL analysis provides a way to distinguish individual minor genetic components that are sometimes masked by the interaction of major genes and by the environment. DNA markers that are linked to QTL, which plant breeders want to select in breeding populations, are valuable for genetic diagnostics as the breeding processes in quantitative traits require an enormous time frame and logistics.

Several quantitative traits such as disease resistance, drought resistance, flowering time and yield were mapped using molecular markers and sometimes recombinant inbred lines (RIL) as reviewed by McCouch and Doerge.<sup>37</sup> QTL analysis revealed the components of complex traits and interaction of alleles at different loci. For example, several QTL including *Pi-zh*, partial resistance genes to rice blast, days to flowering and days to heading were found to be linked at the marker loci on chromosome 8.<sup>37</sup> This reflects the complexity of response to stress, where physiological and morphological traits are associated to stress tolerance.

Obtaining durable resistance against rice blast is valuable to dissecting the complex trait of it in a Japonica cultivar, Moroberekan. Wang et al<sup>38</sup> reported RFLP mapping of resistance genes of Moroberekan using 281 F7 recombinant inbred lines and 127 RFLP markers. They identified two dominant loci of resistance genes, tentatively named *Pi-5(t)* and *Pi-7(t)*, and 10 chromosomal segments to be associated with effects on the lesion number of the fungus.

Heterosis or hybrid vigor is an important trait for the base of hybrid rice technology. However, the genetic basis of heterosis is still not resolved. But gradual progress has been made via QTL analysis. QTL analysis of yield components associ-

ated with heterosis was reported in rice and it revealed dominance complementation as the major basis of heterosis.<sup>39</sup> It also suggested the possibility of pyramiding of QTL for heterosis.

#### POSITIONAL CLONING

Another goal of research in rice genome analysis with molecular linkage map would be positional cloning. When the gene products are not identified and a physical genetic map is available, genes can be cloned according to their positions. The first success of positional cloning in rice was reported for the cloning of the rice *Xa-21* gene, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6.<sup>40</sup> A RFLP locus was RG103 identified as linked to the *Xa-21* gene, and it was hypothesized that RG103 might include the *Xa-21* gene. To test this, transgenic rice plants were generated by particle bombardment, using 16 subclones which were partially overlapping and isolated from the RG103 clone. Transgenic plants were inoculated with the pathogen and the subclone responsible for the resistance was identified. Sequencing of the target subclone revealed a single open reading frame (ORF) that encoded a receptor kinase-like protein. Originally, *Xa-21* gene derived from wild rice, *O. longistaminata*.

As shown by Song et al.,<sup>40</sup> a new approach in breeding could be made using the molecular marker tools to identify and facilitate introgression of the valuable genes from precise rice germplasm. Valuable genes derived from rice genetic resources could be cloned according to its position on the physical map. As shown in this experimental process, the identification of the DNA marker to link the target trait is the first step of marker-based cloning. The second step is to obtain physical clones including this marker DNA. Sequence analysis of clones may reveal the presence of ORF(s). However, a genetic linkage between obtained sequence and target trait is not sufficient to prove that it is a causal gene. To prove it, function analysis with transgenic plants is essential, in that overproduction by introduction of additional genes, repression by anti-sense RNAs or destruction of target gene by homologous recombination will be observed.

#### IMPORTANCE OF TRANSGENIC RICE PLANTS

Genetic engineering is now becoming a realistic approach to generate new cultivars by transferring genes from unrelated sources. As mentioned in the previous section, rice genetic resources are very rich in diversity in many breeding traits. However, only a few genes have been cloned from rice and related species, which contain many valuable traits. At present, the genes cloned from microorganisms or viruses are the novel sources for genetic transfor-

mation as shown in the following section. Transgenic rice plants resistant either to herbicides, insects or viruses were generated through the use of cloned genes from microorganisms as shown in the following. In contrast to the overexpression of genes, the use of anti-sense RNAs is based on the inhibition of the action of de novo genes. This is useful to inhibit an expression of an undesirable trait because anti-sense RNAs hybridize the target mRNA and inhibit translation of a corresponding target gene. A well-known example of this strategy is the improved tomato in post-harvest character.<sup>41</sup> This was also applied to rice plants for repressing allergen gene and waxy gene, both of which demonstrated the repression of target genes.<sup>42</sup>

#### METHODS TO GENERATE TRANSGENIC RICE

Rice is the most advanced crop in the gene transfer system among cereals due to the efficient cell culture system available for this crop. Following the first success of transgenic rice generated by the method of protoplast culture and electroporation,<sup>43</sup> several transgenic plants having agronomically important genes were generated by the same method. However, protoplast culture requires an individualized manual skill unlike other culture methods. Moreover, somaclonal mutation such as sterility and abnormal morphology in regenerated plants could be inevitable in protoplast culture. Although it had been thought that Agrobacterium could not infect monocotyledonous species except in a few cases, this was fortunately overcome by Hiei et al.<sup>44</sup> Their method has an advantage in time frame and less personalized skills to generate transgenic rice plants. In contrast to use of the Agrobacterium-mediated gene transfer, a direct gene transfer of exogenous DNA to intact rice cells could be achieved by microprojectile bombardment.<sup>45</sup> This method also the advantage with regard to skill and period to obtain transgenic plants.

While incorporation of the target genes can be achieved by the gene transfer methods, the following processes in selecting the most desirable transgenic plants would require a decent-sized population of regenerates for selecting elite lines among them. In this respect, less variation in transgenic plants should be made when modifying transformation and regeneration systems.

Besides the culture technique, the genetic transformation system requires a proper vector containing the structural gene for the target trait, a selection marker gene and an expression regulatory region (promoter) for both a selection marker and the target gene. Ideally, the target gene should be expressed only at a specific tissue/organ at a

particular period of a developmental stage. This is because a gene resistant to a virus that is vectored by an insect is not required to have an expression in roots and maturation period. To accomplish this regulation, several promoters were cloned and their functions were analyzed precisely, as reviewed by Shimamoto.<sup>46</sup> Excluding constitutive promoters such as CaMV 35S promoter and a maize ubiquitin promoter, various promoters such as light or hormone-inducible and leaf or seed-specific promoters are available for rice.

#### TRANSGENIC RICE PLANTS WITH IMPROVED TRAITS

The novel nature of genetic engineering is to permit the introduction of genes into rice without being hindered by concerning barriers in sexual hybridizations. It means that any genes from any organisms could be utilized, providing that the genes could be modified to make an expression in the heterologous genetic system in rice. Several unique examples of the use of exotic genes are given in the following.

#### Virus Resistance

Since no resistant genes to viruses have been cloned from rice cultivars and their related species, a resistance mediated by viral genes encoding coat protein (CP) has been explored as novel gene source.<sup>47</sup> Rice stripe disease, one of the major virus diseases in Japan, is caused by rice stripe virus (RSV). The CP gene was introduced into two Japonica rice varieties, and the consequent transgenic rice plants exhibited a significant level of resistance to RSV infection.<sup>48</sup> These transgenic rice plants have been carefully field-tested. Another set of transgenic rice plants with the RSV-CP gene has been established by an alternative group including our institute (NARC),<sup>49</sup> and progeny plants have been tested for resistance and general agronomic traits in a field. However, it appears that the resistance in these transgenic rice plants would not be sufficient for confronting the practical production level (unpublished data); the transgenic plants exhibited a high level of protein expression, however, resistance was not proportional to it. The CP-mediated resistance might not be a useful case for RSV, or more transgenic individuals might be needed for identifying highly resistant lines with appropriate agronomic traits.

Alternative challenges to employ viral gene sequences in rice would be satellites such as shown in cucumber mosaic virus,<sup>50</sup> tobacco ringspot virus,<sup>51</sup> and viral replicase genes.<sup>52</sup> One of the new strategies for virus resistance mediated by RNAs

was based on the observations that virus resistance was related to homology-dependent gene silencing<sup>53</sup> and expression of an untranslatable mRNA.<sup>54</sup>

#### Insect and Disease Resistance

Durable resistance to insect pests and diseases are the major theme of crop genetic improvement in an integrated pest/disease management program. Several important genes for resistance were successfully transferred to cultivated rice from wild rice by widecrosses as mentioned above. However, since many genes found in wild rice genetic resources are not immediately ready to use for transformation since no cloning of these genes has been done, two alternative approaches using genetic engineering have been carried out to alleviate the constraints. One approach is to use the genes from other organisms, for example, Bt and proteinase inhibitor, and another is to increase the natural defense system of crops, for example, chitinase.

The insect toxins that are termed Bt d-endotoxins and produced by *Bacillus thuringiensis* (Bt) have been used for more than 30 years as a biological insecticide against insects in genera *Lepidoptera* including leaffolders and stemborers, *Diptera* and *Coleoptera*. CryIA(b) gene, one of the genes encoding d-endotoxins, derived from *B. thuringiensis* var. *kurstaki* HD-1, was introduced to rice protoplasts after modifying the codon usage to fit for rice.<sup>55</sup> The progeny of transgenic rice plants expressing modified CryIA(b) gene were more resistant than original untransformed plants to two major rice insect pests, striped stemborer and leaffolder. Transgenic indica rice with CryIA(b) gene was also generated by particle bombardment and showed resistance to the yellow stem borer, the stripe stem borer and the two leaffolder species.<sup>56</sup>

In the case of hybrid maize plants, which were established between transgenic maize expressing the same CryIA(b) gene and commercial inbred lines, resistance to European corn borer was shown under field conditions.<sup>57</sup> Plants expressing high levels of the toxin exhibited excellent resistance. These successful results and other examples clearly showed the effectiveness of Bt toxins as an insecticide. However, microbial insecticides induce resistance in general. Resistant insects capable of adapting to Bt toxins were already discovered and crossresistance occurs in some insect species.<sup>58</sup> Extensive use of Bt toxins by either conventional or transgenic systems must increase the appearance of resistant insects. Insect control considering durability should be done.

Certain plants induce the systematic synthesis of protein protease inhibitors to inhibit insect and microbial proteases when they are wounded me-

chanically or by insect chewing. Corn cystatin is one such inhibitor and has a wide inhibitory spectrum against various cysteine proteases. This gene was introduced into rice to obtain a rice plant with insecticidal activity to many insect pests that have cysteine proteases.<sup>59</sup> Transgenic rice plants contained high levels of mRNA and proteins of corn cystatin in seeds and leaves. It was confirmed that crude extracts inhibited cysteine protease activity of *Saccosyndye zeamais*. The potato proteinase inhibitor II gene (*pin2*) was also introduced into rice varieties and many transgenic plants were generated.<sup>60</sup> High-level accumulation of the protein and increased resistance to the pink stem borer were observed.

Another strategy for utilizing natural resistance is by employing the gene of pathogenesis-related (PR) proteins, which are synthesized by plants in response to pathogen attack presumably as a defense mechanism.

Chitinases are one of the PR proteins, degrading the cell wall of invading fungi. A first success was reported in transgenic tobacco seedlings which showed resistance to the fungal pathogen, *Rhizoctonia solani*.<sup>61</sup> Transgenic rice plants containing a chitinase gene were also obtained and tested for the resistance to the sheath blight pathogen, *Rhizoctonia solani*.<sup>62</sup> The degree of resistance in the progeny of transgenic rice correlated with the level of chitinase expression, and high level expression resulted in no infection in the upper half of the plant. If they will exhibit enough resistance in field trials, these genes will be useful in the management of sheath blight. If they will not be enough, the additional effect of two PR genes is expected to increase resistance. Transgenic tobacco plants containing the constitutive co-expression of two genes, chitinase and  $\beta$ -1,3-glucanase genes were established to enhance protection against fungal attack.<sup>63</sup> The double homozygous plants for two PR-protein genes showed a high degree of resistance to *Cercospora nicotianae*, a causal agent of frogeye. Several successes of an engineering enhancing natural resistance by constitutive expression of normally inducible defenses suggest the value of this strategy. However, disease control to sheath blight could be achieved by the use of cloned resistance gene, *Xa-21*, through precise characterization of this gene.<sup>40</sup>

Resistance to sheath blight was observed in herbicide resistant rice containing a *bar* gene that creates resistance to bialaphos. Transgenic plants sprayed with bialaphos showed resistance to pathogens.<sup>64</sup> However, the availability of this resistance seems less important when a decreased application of chemicals is expected.

## Tolerance to Abiotic Stress

Tolerances to abiotic stress are one of the major constraints on crops. Stresses by drought, high salinity and low temperature have the common effect of decreasing water potential in any crop plants. In general, organisms under these stress conditions accumulate low-molecular-weight osmolytes, such as sugars and amino acids. Stresses also transcriptionally activate a number of genes.<sup>65</sup> Although several hypotheses based on the nature of responses to water deficit are set, the complexity of response requires more biochemical and molecular biological analyses. Transgenic plants which have genes associated with tolerance are also useful in obtaining better insight into the tolerance mechanism.

There is only one example of transgenic rice plants that contained the gene induced by stress. Moons et al<sup>66</sup> discussed late embryogenesis abundant (LEA) proteins, which belong to the proteins induced by drought stress, although they are highly accumulated in the embryos during the late stage of seed development and their expression is abscisic acid (ABA)-dependent. They showed that the level of group 2 and 3 LEA proteins were significantly higher in salt-tolerant varieties, *Pokkali* and *Nona Bokra*, than in salt-sensitive variety, *Taichung Native 1*. To test the role of LEA proteins against stress, the gene of a barley group 3 LEA protein, HVA1, was introduced into indica rice cells. Transgenic rice plants conferred increased tolerance to water deficit and salt stress, and the extent of tolerance correlated with the level of HVA1 protein accumulation.<sup>67</sup>

Other successes of engineering stress resistance are reported in tobacco plants. Because it proved the usefulness of some genes for stress resistance, the same approach will be successful for generating transgenic rice resistant to stress. Several kinds of genes correlated with osmolyte production were introduced. The transgenic tobacco plants having fructan-producing gene,<sup>68</sup> overproducing proline<sup>69</sup> and trehalose,<sup>70</sup> and accumulating sugar alcohol such as mannitol<sup>71</sup> were reported to protect against environmental stress. These examples demonstrated that they act as an osmoprotectant and contribute to tolerance against water stress.

The last example of a tolerant plant is based on the change of membrane composition. The composition of fatty acids of the chloroplast membrane affects the chilling sensitivity of the plant. Transgenic tobacco plants containing the *Arabidopsis* gene to manipulate fatty acid unsaturation showed decreased sensitivity to chilling.<sup>72</sup>

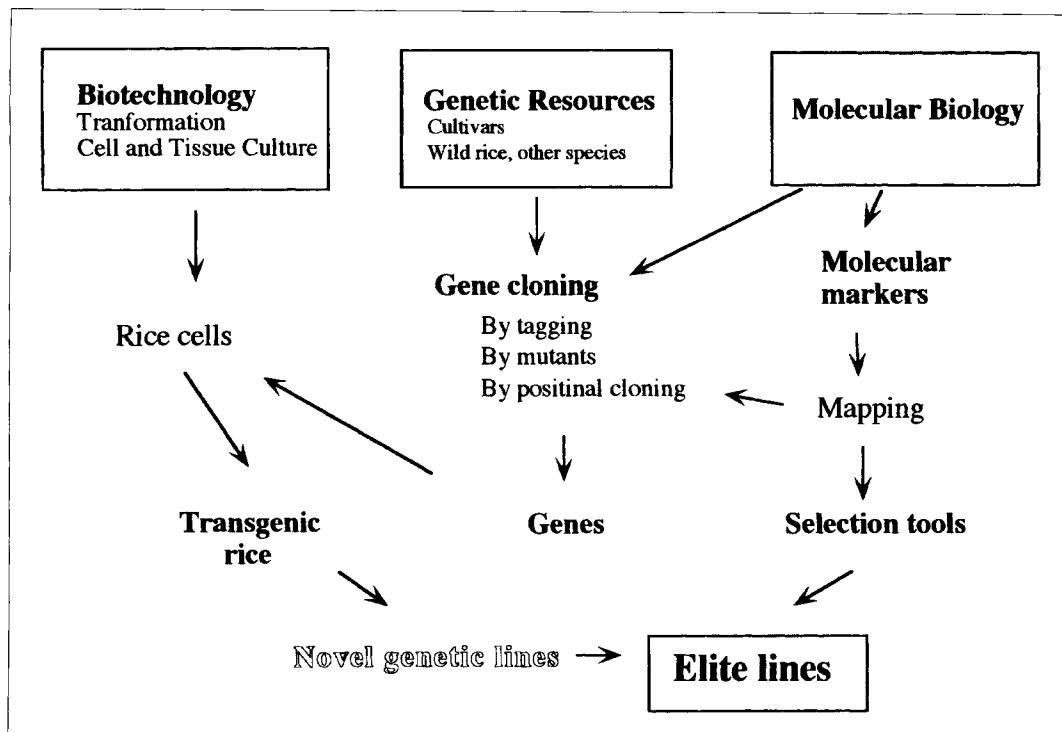


Fig. 7.1.

## CONCLUSION

An unprecedented number of novel sources of rice resistant/tolerant to biotic and abiotic stresses have been identified by the intensive activities of multi-disciplinary and international evaluations as Chang reported.<sup>21</sup> These findings and associated researches have set the stage for rice researchers and molecular biologists to exploit the full genetic potentials in *Oryza* and its wild relatives for further crop improvement.

Plant breeders have been selecting desirable individuals based on phenotypic expression that are influenced by the effects of interaction between genotype and the environment. However, recent progresses in molecular genetics, as well as biotechnology, have enabled us to elucidate questions in the highly complex mechanisms of genotype-environment interactions and have been making it possible to directly restructure genotypes. Hopefully, the analysis of the rice genome structure and its functions will successfully progress and lead to the construction of complete physical maps and to the sequencing of important genomic DNA, including useful genes. It is hoped that these studies will facilitate the isolation, transfer and expression of useful genes. Then, the rice genome will be

drastically restructured to enhance or to improve the organic functions of rice.<sup>73</sup>

In the future, we may be able to create a new kind of paddy rice crop by practically using the rich genetic resources and by making full use of the advanced biotechnology. The perspective process of new breeding applying these advances is presented in Figure 7.1. The new crop will be as productive as the C4 plant and will have multiple resistance to various diseases and insect pests, besides it will be highly tolerant to any abiotic stresses. It will no longer be a dream to create rice that will be able to produce various sorts of useful substances such as protein, oils, vitamins and so forth.

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## CHAPTER 8

# BIOTECHNOLOGY AND GENETIC RESOURCES ON GRAIN LEGUMES: LENTIL AND FABA BEANS

M. Baum, W. Erskine and G. Ramsay

## INTRODUCTION: IMPORTANCE AND USES OF LENTILS AND FABA BEANS

In the period 1979-81, the area sown with faba bean was 3.7 million ha whereas the lentil (*Lens culinaris*) area was 40% less at 2.2 million ha (Table 8.1). In the last decade, the world lentil area has overtaken that of faba bean to become 3.4 million ha while the faba bean area has declined to 2.9 million ha.<sup>1</sup> However, in terms of production globally there is more faba bean (*Vicia faba*) than lentil because of the considerably higher yield potential of the faba bean. Both crops are mostly widely grown in Asia, which accounts for 60% of the world area of faba bean and for 80% of the world lentil area (Fig. 8.1). China is the largest faba bean producing country. After Asia, the most important region for faba bean production is Africa, where the largest areas are in Egypt, Ethiopia and Morocco. Average yields in these countries differ radically from 2.2 t/ha from irrigated Egyptian production to 0.6 t/ha from rainfed fields in Morocco. Other major faba producing countries are Australia, Brazil, Italy and the United Kingdom.<sup>2</sup> For lentil, India and Turkey are the largest world producers and the other significant Asian producing countries are Bangladesh, Iran, Nepal and Syria. Outside Asia, the most important lentil producer is Canada, which has increased its area 10-fold from 1979-81 to a mean of 327,000 ha annually. The major increase in world lentil area over the last decade is attributable to this Canadian expansion, a gradual increase in India and major increases in Iran and Nepal, together with a tripling of lentil area in Turkey through fallow replacement (Table 8.1).

### USES

Excellent reviews of chemical composition and nutritive quality are to be found for lentil in Savage<sup>3</sup> and for faba bean in Hulse.<sup>4</sup> The protein contents of lentil and faba bean are similar ranging from 20-36 % and 19-39 %, respectively. Lentil has a lower fiber concentration than faba bean, largely within the seed testa, so the fiber in lentil meal can be reduced if it is dehulled before grinding. The amino acid composition of both pulses is complementary to that of cereals. Diets containing both cereals and pulses are balanced for simple-stomached animals. With the exceptions of arginine, lysine and leucine, both faba bean and lentil are deficient in essential amino acids.<sup>5</sup>

Lentil contains a number of antinutritive factors, but these are unimportant in human diets because of the cooking and processing which occurs prior to eating. Additionally, its small seeds ensure quick cooking. In these two factors, faba bean contrasts with lentil, being both slower to cook and richer in antinutritive factors, which affect its consumption. The most important antinutritive factors in faba bean are the glycosides, convicine and vicine.<sup>6</sup> The glycosides can cause a hemolytic anemia, which is sex-linked and is often fatal when young males first eat faba bean. Convivine and vicine are also deleterious in the diet of laying hens. The main cause of the disease has been traced to a deficiency in glucose-6-phosphate dehydrogenase.<sup>7</sup> For human consumption, faba beans are commonly consumed as a green salad and vegetable, in addition to preparations of dry seed. Lentil is predominantly prepared from dry seed, either entire or dehulled.<sup>8</sup> The dry seeds of faba beans are used whole in dishes such as *couscous* (a North African dish based on durum wheat flour) and *foul medames* (a faba bean preparation often for breakfast in the Nile valley) or as a flour in *falafel* (crushed faba bean preparation of the Nile valley). In China, faba bean seeds are important for processing and used in many food and industrial products. Lentil is predominantly eaten in India as boiled or fried *dhal*. *Khichri* (Egypt) is made from a mixture of dry *masur dhal*

and cracked wheat. The most common lentil and faba bean-based foods have been summarized in Hawtin and Sears.<sup>9</sup>

Most of the countries producing faba beans do so for domestic consumption. In most years production in the major consuming countries is adequate for domestic needs, but in some years the failure of normal levels of production leads to substantial international trade for human consumption. In these years, EU countries, for example, may have substantial exports to the Middle East. International trade in faba beans for animal feed in industrialized countries is a normal feature of the market.

Lentil seed is used solely for human consumption with most of the production consumed locally contributing widely to national food security. However, the major exporters of lentil are Canada and Turkey. The straw of lentil is important as a livestock feed, particularly for sheep, in the Middle East. The protein content of lentil straw varies from 5 to 7% and its digestible dry matter from 43 to 46%, but there is limited genetic variation in straw quality.<sup>10</sup>

#### INTERNATIONAL AND NATIONAL RESEARCH ACTIVITIES IN FABA BEANS AND LENTILS

Throughout its range, the faba bean suffers from relatively little research effort in comparison to some other crops as a result of low economic value and a generally declining area. However, the impor-

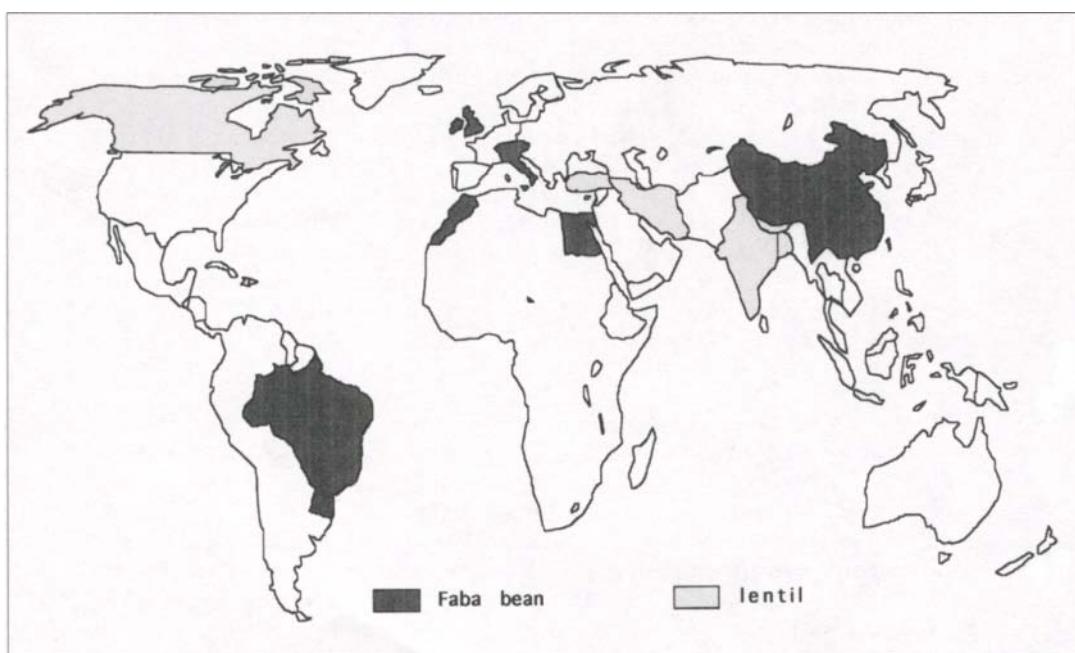


Fig. 8.1. World distribution of lentil and faba bean. Countries with more than 100,000 ha of sown area are highlighted (see Table 8.1 for details).

tance of faba beans in China is reflected by a wide range of research activities in that country, including research on breeding and genetics, agronomy, pathology and utilization. In the EU, the resources devoted to faba bean research have been declining, but a recent rise in interest in the crop in Australia has stimulated new research programs there.

Lentil is also relatively neglected in research because of its low economic value. However, the production area is increasing. In the developed world, programs in Canada, the USA and recently Australia fuel development. In the developing world, India has the longest history of research. The International Center for Agricultural Research in the Dry Areas (ICARDA) was accorded a world mandate for research on lentil and faba beans. The improvement program on the faba bean was scaled down to a genetic resources role in 1990, but is being reinstated in a pre-breeding form. The international effort on food legume improvement has

catalyzed the development of many national programs on these crops in the last 15 years.

## CONSTRAINTS TO PRODUCTION SOLVABLE BY PLANT IMPROVEMENT

The requirement for adequate water supply restricts the faba bean to dry rain-fed areas, but makes it particularly suited to temperate regions, cool and rainy seasons in warmer areas, high altitudes in sub-tropical regions or irrigated production systems.<sup>11</sup> The effects of water stress are well-documented for faba bean, affecting yield<sup>12</sup> canopy, leaf area<sup>13</sup> and flower and pod drop.<sup>14</sup> In contrast, the lentil with its lower water requirement is adapted to drier rainfed areas. However, within such areas, yields are limited by low moisture availability.

Faba beans are among the most cold-tolerant of the major grain legumes, permitting their cultivation as far north as Finland and at altitudes up to

**Table 8.1. Average area (1000 ha) and yield (kg/ha) (three year means for 1979-81 and 1992-94) of faba bean and lentil in the world, regions and major producing countries (those with sown area > 100,000 ha) (empty cells indicate < 100,000 ha sown and not zero production).<sup>1,2</sup>**

Region/ country	Faba bean			Lentil		
	Area 1979-81	Area 1992-94	Yield 1992-94	Area 1979-81	Area 1992-94	Yield 1992-94
World	3688	3051	1336	2218	3379	824
Africa	740	667	1136	109	107	648
Egypt	103	115	2277			
Eth. PDR	327	242	1165			
Morocco	165	186	606			
N C America	63	63	918	131	400	1259
Canada				38	327	1254
S America	200	146	548	95	66	742
Brazil	146	93	257			
Asia	2318	1755	1444	1779	2712	775
Bangladesh				290	207	514
China	2267	1700	1429			
India				934	1174	690
Iran				58	217	593
Nepal				98	152	629
Syria				82	102	882
Turkey				206	691	953
Europe	357	329	1907	105	44	659
Italy	161	80	1475			
UK		146	3480			
Australasia	10	85	1123	1	10	500

3200 m in Ethiopia.<sup>11</sup> Despite this wide adaptation, frost resistance is a limiting factor to overwintering faba bean crops in many regions. Among the major abiotic limiting factors to lentil production are high temperature stress in the spring, and, at high elevations, cold temperatures in the winter.<sup>15</sup> Salinity is an increasing problem and extensively employed irrigation for faba bean production in the Nile Valley and in parts of Central Asia may increase the problem.

Among biotic stresses to lentil, the disease rust, vascular wilt and Ascochyta blight, caused by *Uromyces fabae*, *Fusarium oxysporum* f.sp. *lentis* and *Ascochyta fabae* f.sp. *lentis*, respectively, are the key fungal pathogens. In faba bean, a similar suite of diseases, namely Ascochyta blight (*Ascochyta fabae*), rust (*Uromyces fabae*) and root rot (*Fusarium* and *Phoma* spp) limit yields. One of the most important and serious pathogens to faba bean is *Botrytis fabae*, which, together with *B. cinerea*, causes chocolate spot. A wide range of viruses may cause significant yield loss in faba bean production.<sup>16</sup> Many are spread by aphids (especially by *Aphis craccivora* and *Acyrthosiphon pisum*) though *Aphis fabae* causes more damage by direct feeding. In lentil, the key pest is Sitona weevil (*S. crinitus*), the larvae of which feed on the root nodules. The parasitic angiosperm broomrape (*Orobanche* spp., particularly *O. crenata*) causes serious losses and restricts the cultivation of faba bean, and to a lesser extent lentil, in many Mediterranean countries.<sup>17</sup>

Faba bean is a partially out-crossing species, with cross pollination reported to range from 8–94%, with an average of 35%.<sup>18</sup> Lentil is self-pollinated with cross pollination usually < 1% with a maximum of 6.6% outcrossing recorded.<sup>19</sup> In addition to the above abiotic and biotic stresses, most faba bean genotypes require insect visits to effect maximum level of pollination and pod set. This may limit production or delay maturity in some circumstances. The partially allogamous nature of the crop also has implications for breeding methods and the use of biotechnology. Many cultivars and landraces are interbreeding genetic mixtures, leading to difficulties exploiting methods such as marker-assisted selection or transformation.

## AVAILABLE GENETIC DIVERSITY TO OVERCOME CONSTRAINTS

The overlap of the distribution of early archaeological remains of lentil with the current distribution of the crop's progenitor *L. culinaris* ssp *orientalis* indicates that the crop was domesticated in the Near East Arc.<sup>20</sup> Lentils were associated with the start of the 'agricultural revolution' in the Old World that was initiated by the domestication of

einkorn and emmer wheats, barley, pea, flax and lentil. By contrast, evidence for the timing and location of the domestication of faba bean is not conclusive. It is likely that faba beans were domesticated after the earliest Old World crops, perhaps around 5000 BC, somewhere between the eastern Mediterranean and Afghanistan.<sup>21</sup> However, the lack of any convincing wild progenitor and the paucity of early archeological samples leaves the origin of faba bean open to doubt. Centers of diversity, presumably secondary, occur in Afghanistan, northern India and Ethiopia.

The world collection of lentil currently comprises 7477 accessions of the cultigen and 429 accessions of wild lentil and is maintained at ICARDA.<sup>22</sup> ICARDA also maintains the world collection of faba bean. These are held as both the self-pollinated pure lines (5248 accessions) and the open-pollinated lines (44530 accessions) from which they were derived.<sup>23</sup> These ICARDA collections are under the auspices of the Food and Agriculture Organization of the United Nations System and a safety duplicate set is maintained at the National Bureau of Plant Genetic Resources, New Delhi, India for lentil and at the Agrobotanical Institute, Linz, Austria for faba bean.

Lentil is predominantly grown in semi-arid regions where water is a major limiting factor.<sup>24</sup> Drought avoidance through early flowering and maturity has been identified as a key trait under drought conditions and for this trait there is adequate variability. Increases in water-use efficiency (WUE, the amount of dry matter produced per unit water transpired) may be a way to increase lentil production and to extend lentil growing areas. Most yield improvements have come by increasing the transpirational component through management and breeding, and by increasing the harvest index in certain crops. Little or no increase in WUE has been documented in crop plants. Measurements of  $\Delta$  (carbon isotope discrimination) appear to provide a method for estimating WUE of C3 crop species. In a recent study in Canada, significant genetic variation was observed for  $\Delta$  in lentil and  $\Delta$  had a lower genotype-environment interaction than yield. However, despite a low positive correlation with yield (inconsistent across locations and years), the broad sense heritability of  $\Delta$  was similar or lower than that of yield. These results suggest that  $\Delta$  could not be used effectively to indirectly select for yield in lentil under these conditions.<sup>25</sup> In another study in the USA,  $\Delta$  was not significantly correlated with grain weight, confirming that the potential to improve yield through selection for WUE may be limited.<sup>26</sup>

Few studies have been made on sources of drought tolerance in faba bean.<sup>27</sup> A screening system was developed using osmotic stress in seedlings that correlates with the response to salinity.<sup>28</sup> However, little fundamental research has been done on the mechanisms and genetics of this important stress factor.

Frost tolerance varies widely in faba bean germplasm, reaching a maximum in the French population Cote D'Or, which can tolerate -25°C without snow protection.<sup>29</sup> In the cultivated lentil wide variability in cold tolerance also exists,<sup>24</sup> which is being exploited in the selection of cultivars for winter sowing in the Anatolian plateau, where spring planting is traditional. Improved levels of winter hardiness have been identified in *L. culinaris* ssp *orientalis*.<sup>30</sup>

The few studies on salt tolerance in faba bean (reviewed by Bond et al<sup>27</sup>) and lentil<sup>24</sup> suggest that some variation exists for this trait and that more extensive evaluations of germplasm may be worthwhile. Resistance to the major pathogens of lentil was reviewed by Erskine et al<sup>24</sup> and Robertson et al.<sup>32</sup> Resistance to vascular wilt and *Ascochyta* blight has been found in both the wild and cultivated species of *Lens* and resistance to rust in the cultivated lentil.

Resistance to the major pathogens in faba bean was reviewed by Porta-Puglia et al<sup>33</sup> The evaluation of ICARDA's germplasm collection led to the discovery of accessions from South America carrying resistance to *Botrytis*.<sup>34</sup> These sources of resistance are now widely used by breeders. Their effectiveness in Europe is somewhat less than in the Middle East, due to either stronger pathogen pressures or differences in pathogen genotype in different regions.<sup>33</sup> Although the polygenic nature of the resistance renders breeding difficult, there is optimism that this resistance will be durable.

A number of sources of resistance to *Ascochyta fabae* have been reported, though most of them are of race-specific type<sup>35</sup> and therefore of uncertain durability. Race-specific resistance has also been reported for rust, though some field resistance is also known for this pathogen. Resistance has also been reported for the fungal pathogens *Fusarium*, *Uromyces* and *Peronospora*, the plant parasite *Orobanche crenata* and the viruses BYMV and BLRV. Resistance is also known to aphids. Cultivar Rastatt and derived lines, for example, carry partial resistance to *Aphis fabae*.<sup>36</sup> There are adequate existing sources of variation for the anti-nutritional factors that limit the use of faba beans in some circumstances. Alleles at either of the two different loci prevent tannin synthesis.<sup>37</sup> Similarly, single recessive alleles greatly reduce the levels of vicine and convicine in the seed.<sup>38,39</sup>

In lentil, several other important traits such as biomass yield, pod shedding, nitrogen fixation and resistance to pea leaf weevil (*Sitona sp.*) and the parasitic weed broomrape (*Orobanche sp.*) are not currently addressable by breeding because of insufficient genetic variation. The role of biotechnology in overcoming these constraints could be two-fold: 1) providing tools for the understanding and more efficient handling of sources of resistance or other useful traits; and 2) through genetic transformation introducing novel resistances especially where existing sources are inadequate or are of a race-specific type and vulnerable to breakdown.

In contrast to lentils, faba beans are genetically isolated from all other taxa. Although related species contain a range of useful characters to supplement those present in existing germplasm within *V. faba* (*V. narbonensis*, for example, is both strongly resistant to *Aphis fabae* and drought), this variation cannot be accessed through sexual crosses.

## USE OF BIOTECHNOLOGICAL TOOLS FOR BIODIVERSITY EVALUATION

Biochemical and molecular techniques have been used for biodiversity evaluation, assessment of the genetic structure of natural populations and plant systematics and evolution in the genus *Lens*, as summarized by Ferguson and Robertson.<sup>40</sup> Major investigations were carried out with allozymes,<sup>40,41</sup> seed protein,<sup>42</sup> cDNA and genomic DNA RFLPs,<sup>43,44</sup> chloroplast DNA RFLPs<sup>45</sup> and RAPD analysis of genomic DNA.<sup>46,47</sup> Even though discrimination between lines is possible, seed proteins have not been extensively used for genetic diversity studies.

An extensive study using isozymes was carried out by Ferguson and Robertson<sup>40</sup> using 439 accessions of cultivated and wild lentils that assayed for 11 polymorphic loci. A high mean genetic diversity in *L. nigricans* indicated an evenness of genetic variation distribution as opposed to an allelic richness. This is in contrast to *L. ervoides* and *L. odemensis*, which harbored more alleles of lower frequency. Their phenetical analysis revealed that *L. odemensis* is more closely related to *L. culinaris* ssp *orientalis* than to *L. ervoides* but is more distantly related to *L. culinaris* ssp *culinaris* and *L. culinaris* ssp *orientalis* than ssp *culinaris* and ssp *orientalis* are related to each other. The result of this phenetical analysis is supported by the findings of the phenetic analysis of others by isozyme analysis, by genomic RFLP analysis and by conserved chloroplast DNA RFLPs.

The phylogeny of *Lens* taxa based on restriction site analysis of chloroplast DNA by Mayer and

Soltis<sup>45</sup> differs from the aforementioned analysis. Mayer and Soltis<sup>45</sup> analyzed 399 restriction sites of 125 accessions, but only 11 accessions of the wild species were represented. Their phylogenetic analysis which is based on a cladistic analysis of the restriction data related *ssp culinaris* and *ssp orientalis* closely in *L. culinaris*, and *L. ervoides* and *L. nigricans* were closely related as well. Three mutations in restrictions sites placed *L. odemensis* as the sister taxon to *L. nigricans*.

Random amplified polymorphic DNA (RAPD) markers were used by Abo-elwafa et al<sup>46</sup> to study genetic relationships within the genus *Lens* in 20 cultivars and 4 accessions each of the wild species. Fifty reproducible fragments were amplified, of which 90% showed polymorphism. The wild lentil *L. culinaris* *ssp orientalis*, *L. odemensis* and *L. nigricans* showed wide intraspecific variation, only *L. ervoides* revealed a lower intraspecific variation than *L. culinaris* *ssp culinaris*. Their cluster analysis also clustered *ssp culinaris* and *ssp orientalis* closely together.

Sharma et al<sup>47</sup> analyzed 54 accessions of *Lens* using 24 arbitrary sequence 10-mer primers. These primers generated a total of 88 polymorphic fragments. Cluster analysis revealed that *ssp orientalis* is most similar to cultivated lentil. *L. ervoides* was the most divergent wild taxon followed by *L. nigricans*. The results correspond well with previous isozyme and RFLP studies. However RAPD appears to provide a greater degree of resolution at the sub-species level.

Similar methods have been used to assess and sort the biodiversity found within *Vicia* and within *V. faba*. The origin of *V. faba* and its relationships to other species has always been controversial, even to the extent of the identity of the genus to which the species belongs.<sup>48</sup> In detailed traditional taxonomic studies,<sup>49</sup> *V. faba* has been placed in section *Faba* along with *V. narbonensis* and related species, although more recently Maxted<sup>50</sup> has raised a new section, *Narbonensis*, for the group of species related to *V. narbonensis*. Ladizinsky<sup>51</sup> analyzed seed proteins within section *Faba* *sensu latu* and claimed support for the view that a progenitor for *V. faba* could be found within it. However, van de Ven et al<sup>52</sup> published a detailed molecular study of 55 accessions of species from 3 sections using RFLP and PCR fragments, of which 255 were polymorphic and 178 were informative in that they were present in more than one accession. The result confirmed that the wild species now placed in section *Narbonensis*, including some recently described taxa, formed a natural group. Similarly, accessions in two other sections of *Vicia* and a set of *Lathyrus* accessions formed natural groups. The

position of the cluster of *V. faba* accessions was distinct from section *Narbonensis*, thus supporting Maxted's revision. Raina and Ogihara<sup>53</sup> investigated cpDNA in *V. faba* and species of the group now called section *Narbonensis*. They concluded that the startling differences between *V. faba* and the species now placed in section *Narbonensis* ruled out the possibility of a single lineage. On the basis of their data, which did not include species of *Vicia* from other sections, they suggested that a new genus for *V. faba* may be warranted.

The biodiversity within *V. faba* has also been investigated using biochemical and molecular markers. Kaser and Steiner<sup>54</sup> used protein and isozyme electrophoresis to generate 466 informative bands to characterize 71 faba bean accessions from a wide range of geographical backgrounds. Cluster analysis grouped all German cultivars together and within this cluster, two groups comprising *V. faba minor* and *major* cultivars were found. Along with these German cultivars, no groupings were obvious according to botanical types of geographical background. The presence of rare alleles in accessions from the Near East-Iran-Afghanistan region and from South America indicated diversity in these regions. Link et al<sup>55</sup> explored diversity in European large-seeded lines and Mediterranean lines using RAPD bands, 282 of which exhibited polymorphism. All lines were SSD-derived inbreds and so the complications of diversity within heterogeneous populations were removed. Estimates of genetic distance were subjected to a principal component analysis, which placed the three sets of inbred lines into three slightly overlapping groups. The European *minor* gene pool contained the greatest diversity whereas the European *major* and Mediterranean (predominantly large seeded *equina* and *major* types) overlapped with each other. The data were consistent with the view that minor types of faba bean are long established in Europe and elsewhere and contain the greatest biodiversity, whereas *major* types, now the dominant type in some regions, are of recent origin.

## MARKER-ASSISTED SELECTION

The aim of establishing linkage maps for agricultural crops is to localize within the genome the position of important agronomic traits and to develop tightly linked markers to enable indirect selection by marker-assisted selection. Breeding has received less attention in faba bean and lentil than in other major legumes, such as dry bean and pea,<sup>56</sup> and also their linkage maps are relatively less saturated.<sup>43,44,57-59</sup>

In lentil, Havey and Muehlbauer<sup>44</sup> constructed a genetic linkage map of lentil spanning 333 cM

from 20 RFLP, 8 isozymes and 6 morphological markers in an interspecific cross. Weeden et al<sup>43</sup> using the interspecific cross *L. culinaris* x *L. ervoides* developed a 560 cM linkage map consisting of 64 morphological, isozyme and DNA markers and also mapping the translocation breakpoint between *L. culinaris* and *L. ervoides*. Tahir et al<sup>60</sup> reviewing all mapping efforts in lentil produced a compiled linkage map based on combined linkage data and regions of homology shared with pea *Pisum sativum* L., including seven morphological, 25 isozymes, 38 RFLP and six other loci combining the mapping efforts of several laboratories. Eujayl et al<sup>61</sup> used RAPD markers to establish a linkage map in an *F*<sub>2</sub> population of a partially interspecific cross between *L. culinaris* ssp *culinaris* x ssp *orientalis*. At a LOD score > 3.0, 28 RAPD markers (together with one RFLP, one morphological marker and three oligonucleotide markers) were assigned to nine linkage groups spacing a total of 206.1 cM. In recombinant inbred lines from the *F*<sub>2</sub> population, 88 RAPD primers were linked in 14 linkage groups spanning more than 800 cM (Fig. 8.2)(Eujayl et al, unpublished).

Qualitative traits in lentil such as epicotyl color, seed coat pattern or spotting, pod indehiscence,

etc. have been localized and linked DNA markers have been identified.<sup>60,62</sup> However, agronomically important traits such as resistance to Ascochyta blight, Fusarium wilt and rust have so far not been mapped but their localization is under investigation (Washington State University, ICARDA). Quantitative traits in recombinant inbred lines of interspecific crosses have been localized with isozyme markers.<sup>63</sup> Detected quantitative trait loci (QTLs) were found to be located in six of the seven chromosomes. Abbo et al<sup>64</sup> studied the genetics and linkage of seed weight in lentil in crosses of *L. culinaris* with *L. culinaris* ssp *orientalis* and *L. ervoides*. They found that seed weight was under polygenic control with additive gene action and partial dominance of the low seed weight alleles. QTLs affecting seed weight were associated with morphological and RAPD-markers that were distributed over several linkage groups. An additional cytoplasmic effect on seed weight was observed.

Genetic mapping in faba bean was initiated in the 1930s,<sup>65</sup> placing 19 morphological traits in 4 groups. Picard<sup>66</sup> and Cabrera and Martin<sup>67</sup> discovered other linkages among loci for morphological characters. In molecular mapping, interspecific crosses are often used to maximize the

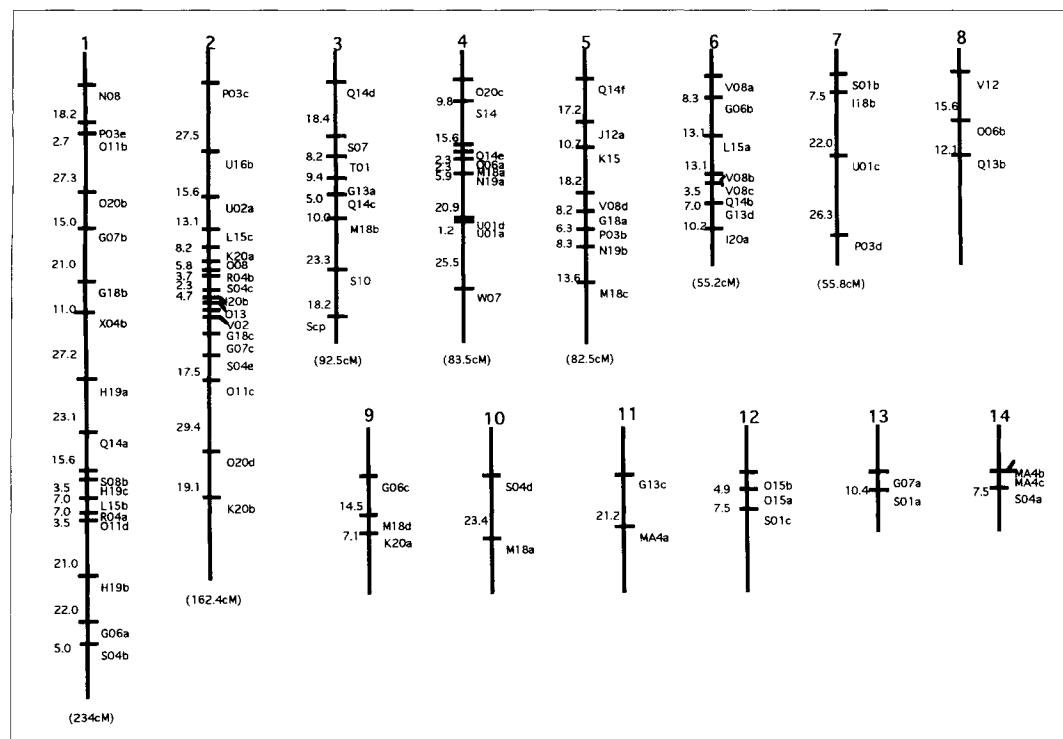


Fig. 8.2. Linkage map of lentil. Eighty-five single seed descent derived (*F*<sub>8</sub>) lines of a partially interspecific cross (*Lens orientalis*) were mapped with RAPD-markers. A LOD score of >3.0 and a recombination value of 0.3 were used to establish the linkage groups.

polymorphism in a cross. As such crosses are not possible in faba bean, unlike in lentil, parents from opposite sides of the spectrum of variation within the species have been used in the crosses used to generate populations for constructing faba bean maps. Van de Ven et al<sup>68</sup> scored 34 polymorphic isozyme, morphological marker, RFLP and RAPD loci and created a map with 17 markers on 7 linkage groups. The inclusion of a number of morphological markers will help align this map with others in future studies. This map was extended to 23 loci<sup>59</sup> and is currently being greatly increased using anchored simple sequence repeat primer PCR and AFLPs in conjunction with other markers on a set of RI lines. Torres et al<sup>57</sup> published a map of 11 linkage groups consisting of 35 isozyme and RAPD markers. The relatively large number of unlinked loci in this study confirms that this map, as well as the map of van de Ven et al,<sup>68</sup> is incomplete, but progress by both of these groups will soon lead to saturated maps.

The process of assigning markers to chromosomes began with Sjödin,<sup>69</sup> mapping three loci using translocation lines. More recently, Cabrera et al<sup>70</sup> and Torres et al<sup>58</sup> have used trisomics to extend these results. One of the linkage groups in the map reported by Torres et al<sup>58</sup> contained the 45rDNA array and was, therefore, assigned to the short arm region of chromosome 1. The 45S rDNA probe and the isozyme markers used by Torres et al<sup>58</sup> were particularly useful for seeking synteny with the maps of other legumes. The 45rDNA-Tpi-p linkage is shared by pea and faba bean but not by lentil. The pea linkage between Prx-1, Acp-1 and Nag appears conserved in faba bean (Prx-1 - Acp-1) and lentil (Prx-1 - Nag).

The genetics of seed weight has also been investigated in faba bean with dominance found for small-seededness.<sup>71,59</sup> At least four loci appeared to be influencing seed size. For this trait and eight other continuous characters, quantitative trait loci (QTLs) were found.<sup>59</sup> Despite the incomplete nature of the map, the most important QTLs were located for many traits. The number of QTLs for each character varied from one (for vicine/convicine ratio) to 5 for leaf width. As in lentil, some regions on certain linkage groups affected a greater number of traits than others. Some sites appeared to affect several related traits (eg. leaf and pod dimensions) simultaneously, though whether this was pleiotropy or clustering of similar loci cannot be ascertained.

In crops with a relatively low breeding input, including lentil and faba bean, there is some doubt whether the resources required to conduct marker-assisted selection in a breeding program will be

cost-effective. More efficient molecular methods may change this, but at present only easily scored markers are likely to be adopted by breeders. The inheritance of levels of vicine and convicine in the seeds of faba beans is one case where marker-assisted selection is likely to make an impact. Among the two QTLs located for this character,<sup>59</sup> the QTL with the greatest effect, possibly coding for a biosynthetic enzyme, lies close to the hilum color gene *n*. This marker gene has been found to be linked to a locus coding for very low levels of vicine and convicine (presumably extreme alleles of the QTL above) independently by two laboratories (Duc, Ramsay et al, in preparation). In appropriate crosses, hilum color will provide a rapid means of pre-selecting for low vicine content.

## LEGUME-RHIZOBIUM SYMBIOSIS

Lentil, through its association with the bacteria *Rhizobium leguminosarum*, fixes nitrogen, reducing the need for the application of inorganic nitrogen fertilizer to the cropping system. With good symbiotic association more than 85% of the total nitrogen need of the crop is met by symbiotic N2 fixation. In West Asia and North Africa, there is no consistent response to inoculation with *Rhizobium*. However, in some soils in other areas, particularly poor sandy soils without a long history of lentil cultivation, a response to inoculation with *Rhizobium* may be found and the application of a starter dose of nitrogen fertilizer up to 20 kg/ha may be economic.<sup>72</sup>

Faba bean contrasts with lentil in accumulating large amounts of dry matter (up to 20 t/ha<sup>-1</sup>) and therefore has a high demand for nitrogen.<sup>73</sup> Nitrogen fixation in faba bean is regarded as being completely effective because the application of nitrogen fertilizer generally has little influence on yield.

The value of the legumes, faba bean and lentil in agricultural systems is limited, in part by *Sitona spp*, nodule feeding insects that disrupt nitrogen fixation and reduce crop yields. *Sitona spp* frequently cause high yield losses of faba bean and lentil in the Middle East.<sup>74</sup> *S. crinitus* has been recorded to destroy 46% to 77% of nodules on lentil. It is of some interest that *Sitona lineatus*, in addition to damaging faba bean and pea nodules, is an efficient vector of BBMV in faba bean, lentil and pea.<sup>75</sup> Currently, insecticides are the only option for controlling damage from nodule feeding insects. Neither host-plant resistance nor significant biological control agents are known in lentil and faba bean.

One approach to control nodule-feeding insects is the genetic engineering of rhizobia to make root nodules insecticidal. Because root nodules are

an essential food resource for larvae of *Sitona spp.*, nodule specific toxins may be used to reduce nodule damage and disrupt the insect's life cycle. To this end, the *cryIII* insecticidal toxin gene from *Bacillus thuringiensis* ssp *tenebrionis* (Btt) was cloned into strains of *R. leguminosarum*. Strains were constructed that used a constitutive *LacZ* promoter or a conditional *nifH* promoter.<sup>76</sup> *CryIII* toxin expression in nodules resulted in significant reductions in nodule feeding damage by *Sitona lineatus* on *Pisum sativum*. Results from a greenhouse experiment indicated that the genetically engineered *Rhizobium* was competitive with the parent wild-type strain of *R. leguminosarum*. Prior to the release of any genetically engineered micro-organism several factors must be considered such as the potential for horizontal gene transfer, alteration of competitive abilities, alteration of plant growth and physiology, detrimental interactions with other organisms and problems with transport and dispersal. In view of the above, the release of transgenic *Rhizobium* is not now being considered.

Another approach to the control of nodule-feeding insects is the genetic engineering of the host plant to make root nodules insecticidal or target the leaf tissues on which adults feed before oviposition. The above project suggests that the *cryIII* insecticidal toxin gene from Btt is a suitable toxin and there is now need for a viable system to produce lentil and faba transgenic plants.

## WIDENING THE GENE POOL AVAILABLE TO BREEDERS

### INTERSPECIFIC HYBRIDIZATION

Based on crossability within the genus *Lens*, *L. culinaris* ssp *culinaris* and ssp *orientalis* and *L. odemensis* belong to the primary gene pool, *L. nigricans* and *L. ervoides* to the secondary gene pool (for a review see Ladizinsky).<sup>77</sup> *L. culinaris* ssp *orientalis* is considered the wild progenitor of the cultivated *L. culinaris*.<sup>78,79</sup> Within *L. nigricans*, two accessions of a differentiated cytotype exist. The accessions are interfertile with each other, but are reproductively isolated from *L. nigricans*. Each cytotype produces partially fertile hybrids with *L. ervoides*. Species within each gene pool are usually readily crossable with each other, excluding a few populations within *L. culinaris* ssp *orientalis*. Within the genus *Lens*, *L. orientalis* displays the biggest karyotype variability. However, embryo rescue can overcome the embryo abortion of the hybrids. Usually, the fertility of hybrids between different species or species and subspecies within *Lens* depends on the extent of the reproductive barriers that led to the species differentiation, abortion of hybrid

embryos, chlorophyll deficiencies such as albinos and complete or partial sterility because of meiotic irregularities (translocations, inversion).

Within *L. culinaris*, ssp *culinaris* is readily crossable with ssp *orientalis*, but the fertility of the hybrids depends on whether the chromosome arrangement of the wild parent, *L. culinaris* ssp *orientalis* is readily crossable with *L. odemensis* (18% seed set). The hybrids are partially sterile due to meiotic irregularities resulting from three chromosome rearrangements. Restoration of meiotic regularity and fertility can be observed in F2, with about 20% of the progeny being as fertile as their parents.<sup>80</sup> Under normal conditions, *L. culinaris* is not crossable with *L. nigricans* or *L. ervoides* due to very early embryo abortion before pod formation. With the help of ovule/embryo rescue, partially fertile *L. culinaris* x *L. ervoides* hybrids could be rescued.<sup>81,82</sup> In some crosses between *L. ervoides* and *L. culinaris*, the embryo abortion takes place at a late stage, sometimes after root and shoot primordia are formed, which may allow a successful embryo rescue. As gene flow from *L. nigricans* to *L. culinaris* is limited but *L. ervoides* is readily crossed with *L. nigricans*, *L. ervoides* can be used as a bridge to enable the gene flow from *L. nigricans* to the cultivated *L. culinaris*.

Hybrid embryo abortion is the most effective crossing barrier in the genus *Lens*.<sup>83</sup> It divides the genus into two crossing groups, the Culinaris and the Nigricans group and it is also evolving within the Culinaris group. The abortion of F<sub>1</sub> embryos in *L. culinaris* x *L. ervoides* and *L. orientalis* x *L. culinaris* cross was strongly affected by dominant gene action. A fixed pod abortion rate between 10-90 in F<sub>2</sub> and the following generations of the *L. culinaris* x *L. ervoides* cross suggests a quantitative inheritance of the character with dominant and additive effects. Embryo abortion in the *L. culinaris* ssp *orientalis* x *L. culinaris* cross declined in advanced generations suggesting that a different genetic control system of embryo abortion occurs in this cross. Despite an *L. culinaris* ssp *orientalis* cross being heterozygous for two reciprocal translocations the F<sub>1</sub> hybrids were free of embryo abortion indicating the control system being independent from the chromosomal aberrations.

*L. culinaris* is readily crossable with *L. odemensis*. Linkage studies using isozyme and morphological markers have revealed that the same chromosomal rearrangement occurs in a *L. culinaris* x *L. odemensis* cross as between *L. ervoides* and *L. culinaris*<sup>84</sup> involving linkage groups I and II of the map of Weeden et al.<sup>43</sup> The translocation breakpoint was confirmed by mapping with RFLP markers.<sup>56</sup>

The successful interspecific hybridization protocol used by Cohen et al<sup>81</sup> for the cross of *L. culinaris* with *L. ervoides* used an embryo rescue technique in which 14-day-old fertilized ovules were cultured on MS medium containing 10% sucrose, supplemented with 0.5 mg/l zeatin. Embryos were rescued after seven to ten days and placed on low (3%) sucrose MS medium that was free of gibberellic acid (GA) and contained 0.3 mg/l zeatin. Interesting to note is that embryos of these interspecific crosses could survive 14 days on the plant before ovule culture, which would allow a good hybrid embryo development.

Ahmad et al<sup>85</sup> were successful in obtaining fertile interspecific hybrids of crosses of *L. culinaris* ssp *orientalis*, *L. odemensis*, *L. ervoides* and *L. nigricans* with *L. culinaris* and *L. ervoides* with *L. nigricans*. The application of GA<sub>3</sub> after pollination led to viable hybrids even without any ovule- or embryo-rescue technique.

The results of Ahmad et al<sup>85</sup> are very encouraging for interspecific hybridization in *Lens*. However, for several important traits such as biomass, resistance to pea leaf weevil (*Sitona* spp), etc., genetic variation is not even found in the wild *Lens* species. Therefore, intergeneric crosses or the transformation of lentil with known function genes of species even outside of the plant kingdom have been sought to introduce sufficient genetic variation.

Unlike *Lens culinaris*, all *Vicia faba* types are cultivated and, as no plants have ever been produced on crossing with other taxa, *V. faba* is reproductively isolated. However, some advances toward the creation of interspecific hybrids have been made. Fertilization following interspecific pollination occurs more frequently with some faba bean genotypes and some wild species than others. Cytological studies suggest that interspecific embryos cease growth at the globular stage.<sup>86,87</sup> Although severe endosperm abnormalities are frequent and precede embryo morbidity, in some crosses endosperm growth appeared relatively normal yet embryo abortion still took place, demonstrating that the link between these two features is not necessarily causal.<sup>86</sup> Lazaridou and Roupakias<sup>87</sup> attempted to match *V. faba* and *V. narbonensis* parents with smaller differences in endosperm nuclear doubling time, but this did not yield more or larger embryos. Improvement to embryo and ovule culture media and procedures have been made<sup>88</sup> but interspecific hybrids by this route are still lacking. A recent advance in the protoplast culture of faba beans<sup>89</sup> opens a new route to interspecific hybrids, but some doubt must remain as to the prospects for the further use of any interspecific hybrids as the genetic distance between *V. faba* and related species is large.

## TRANSFORMATION

The interest in genetic transformation is driven by the aim to introduce a gene of choice into crops. Lentil has been successfully regenerated via organogenesis.<sup>90</sup> Macerated shoot meristems and epicotyls from germinating seedlings were incubated on MS medium supplemented with kinetin and gibberellic acid. Callus tissue was cultured in the dark for four weeks and then under a 16 h photoperiod at 21°C. A small number of explants produced shoots which could be induced to regenerate plants in relatively large numbers even after several subcultures. Mature fertile plants could be recovered from this system.

Polanco et al<sup>91</sup> were able to regenerate plants from nodal explants. On MS or MS with B5 vitamins, supplemented with 2,4-D, calli were induced. Supplementation of BAP and NAA was able to induce shoot formation and lead to plantlet regeneration. Root formation from explants, usually difficult to obtain in legumes, was obtained in media with NAA or IAA.

Malik and Saxena<sup>92</sup> induced a high frequency of shoot regeneration in intact seedlings of lentil by culturing mature seeds on MS medium supplemented with thiadiazuron (TDZ). De novo differentiation of shoot buds occurred from nodal and adjacent areas in cultures exposed to TDZ for four to six weeks. On modified MS medium supplemented with NAA shoots with roots could be developed into whole plants.

Warkentin and McHughen<sup>93</sup> have shown via tumor induction, opine assays and Southern analysis that lentil is susceptible to virulent strains of *Agrobacterium tumefaciens*. Cells of shoot apex, epicotyl and root apex were capable of expressing an intron containing β-glucuronidase (GUS) gene after inoculation with the disarmed *Agrobacterium* strain GV2260:p35SGUSINT. Also, high amounts of transient transformation using agronomical lentil lines, *Agrobacterium tumefaciens*, disarmed Ti plasmids and the GUS assay have been achieved in CLIMA (J. Barton, personal communication). Shoot explants inoculated with disarmed Ti plasmids carrying a herbicide resistance gene could be maintained on tissue culture medium containing the herbicide.

Faba bean tissue culture is widely acknowledged as being particularly difficult. Some authors have reported regeneration from explants of *V. faba*, but in all cases the growth and proliferation of preexisting buds seem a probable route to regeneration. De novo regeneration systems such as that using immature cotyledon explants in pea<sup>94</sup> have worked in faba bean, but at an extremely low frequency. The development of a protoplast regen-

eration system for faba bean<sup>89</sup> is a major advance and may provide a route to transformation, though the method is currently restricted to one cultivar.

As in lentil, *Agrobacterium* will transform faba bean efficiently. Hairy roots expressing marker genes are easily established following infection with *A. rhizogenes*.<sup>95</sup> Hairy roots have given rise to inviable shoot-like growths in *V. faba*<sup>96</sup> so there is a possibility of transgenic faba beans being created by this route. Different methods have been used to achieve pea transformation but recent progress has centered on the use of methods that appear to transform meristem-containing regions of seedling explants,<sup>97</sup> first described in a patent application made in 1991.<sup>98</sup> This clearly has implications for transformation in the related genera *Vicia* and *Lens*. Where seedling explant culture is inefficient, as in faba bean, enhancing the efficiency of the *Agrobacterium*-plant interaction to maximize the numbers of stable transformed cells will improve the prospects for success. Ramsay and Middlefell-Williams<sup>99</sup> reported alteration to basic protocols that resulted in a large increase in the number of cells with the expression of a GUS gene (possessing an altered bacterial ribozyme binding site to restrict expression to eukaryotes) at infection sites on seedling plumules.

In the absence of a transformation system for *V. faba*, *V. narbonensis* has been used as a model to test applications of transformation technology. Brazil nut and sunflower 2S globulins are methionine-rich storage proteins suitable for transformation to improve the amino acid balance in legumes deficient for this amino acid. An efficient transformation system based on *Agrobacterium*-mediated transformation of epicotyl explants was used to introduce the brazil nut 2S globulin into *V. narbonensis*.<sup>100</sup> The 2S globulin under the control of the *V. faba* legumin B4 promoter elicited seed-specific expression which varied from 1% to 4.8% of SDS-soluble seed protein in different transgenic plants.<sup>101</sup> This approach is now being taken in pea, with the first report made of plants expressing the sunflower 2S protein.<sup>102</sup>

Once efficient transformation systems have been developed for lentil and faba bean, there are good prospects for the application of this technology. Protein quality is already being addressed. A diversity of approaches is becoming available that could be used to counter fungal, viral and insect attacks in these legumes. In the case of the control of parasitic weeds such as *Orobanche*, the use of resistance genes as a systemic herbicide may be environmentally justifiable. Insects such as *Sitona* spp on lentil might be controlled by *Bt*-genes. Alternative uses of the technology could be novel sources of male sterility to create F<sub>1</sub> hybrids

in faba beans, where heterosis is very marked, or the production of novel industrial products. Prospects for the use of biotechnology in the amelioration of the major abiotic stresses for the next few years lie more with marker-assisted selection than genetic transformation.

## CONCLUSIONS

Lentil and faba bean have an important function as protein sources for animal and human consumption, especially in the developing world. In the last decade the world lentil area has overtaken that of faba bean; however, there are more faba beans produced than lentil. Breeding has received less attention in faba bean and lentil than in other major legumes and no comprehensive genetic linkage maps have been developed. Therefore, marker-assisted breeding has had little practical application in breeding programs. However, further development of marker technology will lead to easy-to-use markers, linked to important traits, which will allow marker-assisted breeding. Hybridization of the wild species with the cultivated species in *Lens* is possible, but faba bean remains reproductively isolated. However, for some agronomically important traits no variation is found in *V. faba* and the genus *Lens*. Therefore, intergeneric crosses and/or transformation have to be used to overcome these constraints to production. Future improvement of both crops requires an appropriate blend of classical and novel tools.

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CHAPTER 9

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# CONTRIBUTIONS OF GENETIC RESOURCES AND BIOTECHNOLOGY TO SUSTAINABLE PRODUCTIVITY INCREASES IN MAIZE

M.E. Smith and R.L. Paliwal

## INTRODUCTION

**A**t first glance, it might seem that plant genetic resources and plant biotechnology fall at different ends of the spectrum of inputs and tools available to plant breeders. However, there are clear benefits to be gained from integrating across this “spectrum,” and in this chapter we will attempt to highlight some of those benefits for the case of maize (*Zea mays* L.) improvement. We will discuss three general topics: 1) the ways in which maize is unique, both in evolutionary and agronomic terms; 2) the challenges to sustainable maize productivity improvements; 3) and the areas of maize improvement which will most clearly benefit from combined plant genetic resources and biotechnology contributions.

## THE UNIQUE SITUATION OF MAIZE

### EVOLUTIONARY HISTORY

Unlike most domesticated crops, the origin of maize continues to be a subject of controversy. Maize appeared approximately 8,000-10,000 years ago in Mesoamerica (an area including present-day Mexico and Guatemala), and this same area is occupied today by the closest relatives of maize: the annual and perennial teosintes (*Zea spp*) and the tripsacums (*Tripsacum spp*).<sup>1</sup> Most researchers agree that annual teosintes are the closest relatives of maize, but the relationship between the two is not clear. Three general theories to explain the origin of maize have been proposed: 1) maize is the domesticated form of its wild ancestor teosinte; 2) an ancestral form of maize (archeological evidence for which has never been found) gave rise to both maize and the annual teosintes; and 3) that maize is the product of an ancient hybridization between teosinte and another unknown grass species.<sup>1</sup> Most researchers agree with recent molecular evidence showing that a fairly small number of loci with large phenotypic effects account for the key traits that differentiate maize and teosinte.<sup>2</sup> However, proponents of each evolutionary theory interpret this evidence differently, and it can be viewed as consistent with at least the first two theories noted above.

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Maize is in the Maydeae tribe of the grass family (Gramineae). There are seven genera in the Maydeae tribe, including two genera with species of relatively minor economic importance (the genus *Coix*, including *Coix lacryma-jobi* L. or Job's tears, which is used for forage and for popping the seeds as a snack food in Southeast Asia, and the genus *Tripsacum*, including *Tripsacum dactyloides* (L.) L. or gamagrass, used primarily as a forage crop). Maize is the only cultivated species of major economic importance in the entire Maydeae tribe.

The potential value of maize was recognized by early explorers, and its spread from Mesoamerica to other parts of the world was remarkably rapid. When Europeans arrived in the Americas in the late 15th century, maize had already spread from its center of origin in Mesoamerica as far as Canada in the north and Chile in the south. Maize was carried back to Europe by these travelers, and during the 16th century maize spread through southern Europe and into Africa, South Asia, and the Northwest Himalayas. By the mid-16th century, maize was established as a food crop in Africa and South Asia, by the mid-17th century in Indonesia, the Philippines, and Thailand and by the mid-18th century it was grown widely in southern China. In less than 300 years, maize had moved around the world and had become an important food crop in many countries in temperate, subtropical and tropical regions of the world.<sup>3</sup>

The spread of maize into many diverse environments resulted in diverse selection pressures. Farmers in each region selected the crop for adaptation to their environments and cultivation systems, and to meet their specific food uses and taste preferences. Since maize is a naturally open-pollinated crop, the varieties that were moved from place to place carried considerable genetic diversity, allowing farmer selection pressure to result in the development of a vast array of maize races. These races have been relatively well-studied within the Americas, and are much less well-studied but surely quite diverse and interesting elsewhere in the world.<sup>4</sup>

Despite the existence of cross-compatible wild relatives of maize (both teosinte and tripsacum can be sexually crossed with maize), essentially no use has been made of these species in maize improvement to date. Most breeders would agree that this is due to the tremendous diversity present within the maize genome itself, combined with the lack of obvious desirable traits from the wild relatives. Work underway at the International Maize and Wheat Improvement Center (CIMMYT) to transfer a gene for apomixis from tripsacum to maize, if successful, will represent the first significant use of a trait from a wild relative for maize improvement.<sup>5</sup>

This is in contrast to the situation of most other crops of economic importance in the world today, where traits from wild relatives have played an important role in crop improvement.

#### AGRONOMIC SITUATION

Maize is cultivated in diverse growing environments—a diversity that is unmatched by that of any other crop. It is grown from the equator to 50° latitude, from below sea level (in the Caspian Plain) to 4,000 m altitude, and from high rainfall areas of the low humid tropics and fully irrigated conditions to semi-arid areas. The crop growth cycle ranges from three to 12 months.<sup>6</sup> Maize cropping systems include the entire farming spectrum, from intensively managed monocultures with complete mechanization and high levels of fertilizer, water and pesticide inputs grown on fields that are hundreds of hectares in size, to plots that are half a hectare or less in size, where the crop is grown in multi-crop mixtures using only hand labor and naturally available fertility and water resources.

Maize is the top ranking cereal in terms of grain yield per hectare, and is second only to wheat in total world production. It is a C4 plant with a high rate of photosynthetic activity and very high potential carbohydrate production per unit area per day. It is grown on 130 million hectares with annual production of 500 million tons, for an average global yield of 3.8 t/ha. Maize productivity is quite different between temperate and tropical areas, however. Temperate yields are 7.0 t/ha on average, while tropical yields average only 1.8 t/ha.<sup>7</sup> These differences relate in part to climatic factors, such as shorter day lengths, higher night temperatures and more severe biotic and abiotic stresses in tropical as opposed to temperate areas. They are also a function of the intensity of maize breeding effort devoted to the crop in these different regions, and of crop management differences.

Maize varieties being used today range from those that have been highly tailored via scientifically based plant breeding to local varieties that have been selected and maintained by farmers. In countries classified by CIMMYT as "developed," 98% of the maize area is planted with hybrid seed, and the remaining 2% with farmers' own seed (Fig. 9.1). In much of Europe and North America, hybrids are used on 100% of the crop acreage. In those countries classified as "developing," however, and excluding Argentina, Brazil, and China (where hybrids are grown on 72% of the maize area), only 16% of the maize area is sown with hybrids, 11% with commercial seed of improved varieties and 73% with farmers' own seed.<sup>8</sup> Thus,

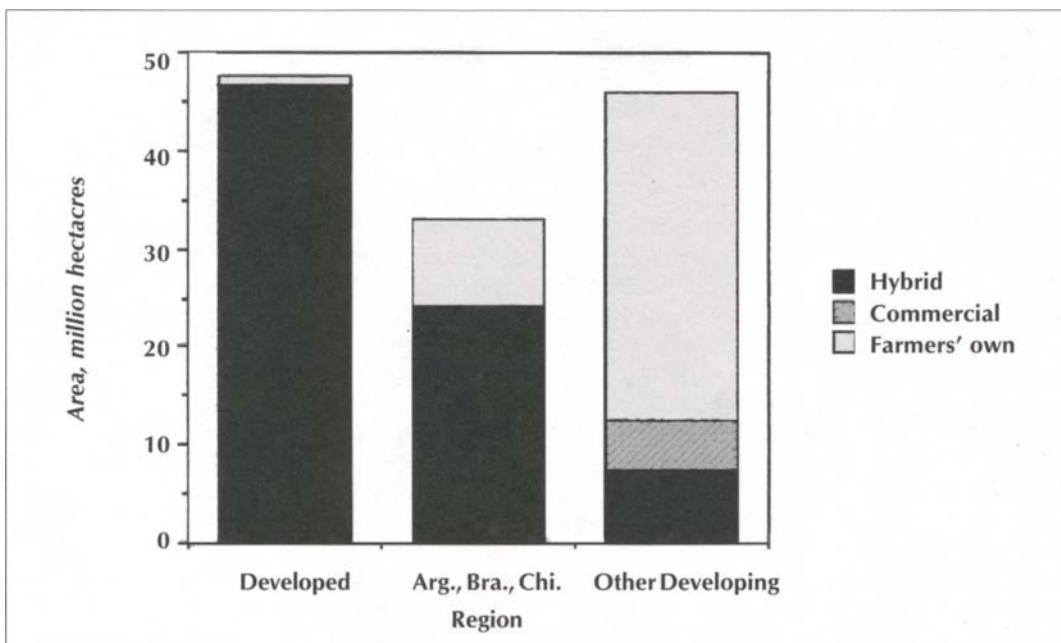


Fig. 9.1. Source of seed for maize plantings in different regions of the world. "Developed" and "developing" countries are according to the definition of CIMMYT.<sup>8</sup> Argentina, Brazil and China are considered separately from the remainder of the "developing" world (designated as "other developing"). "Hybrid" is purchased seed of maize hybrids, "commercial" seed is purchased seed of open-pollinated varieties and "farmers' own" seed is seed grown by farmers for their own use or for trade with other farmers.

the genetic nature of varieties being used differs dramatically between regions of the world.

Maize hybrids are genetically highly tailored, but have an extremely narrow germplasm base. Even among different hybrids, diversity may be quite limited due to breeders' natural tendency to select new inbreds from among the progeny of elite x elite inbred crosses.<sup>9</sup> Smith et al<sup>10</sup> noted that among U.S.A. maize hybrids in 1992, diversity appeared to be relatively constant compared to that measured five years previously. However, the initial diversity measurement had revealed that 60% of the privately released hybrids studied involved three inbreds (or their close relatives) as major germplasm contributors.<sup>11</sup> As recently as the late 1980s, parents of the majority of U.S.A. maize hybrids were derivatives of Iowa stiff stalk synthetic or Lancaster surecropper populations.<sup>12</sup>

At the opposite end of the spectrum in terms of varietal diversity are those varieties being grown and developed, even today, by farmer breeders in those areas where maize has been a traditional crop for centuries. These open-pollinated varieties tend to be genetically diverse, and often possess unique adaptations to the environments and crop management systems in which they are grown, and the uses to which they are put. Wherever local varieties

outperform introduced "improved" ones according to farmers' criteria, they are still grown.<sup>13</sup> The extensive use of farmer varieties noted above makes it apparent that in much of the "developing" world this is still the case.

In addition to diversity in growing environments and varietal types, maize is also a crop with tremendously diverse uses. It is the only cereal that can be used as food at many different plant developmental stages. Young female inflorescences are harvested at flowering and eaten as "baby corn." Immature green ears of sweet maize are eaten as a vegetable. At the dough stage, ears of non-sweet maize are consumed either roasted or boiled as an energy food. Mature grain is harvested and prepared in hundreds of different ways around the world.<sup>6</sup> Additionally, either the grain, the stover, or both can be used as animal feed. Finally, there are many industrial uses of maize grain after processing via wet milling, dry milling, or fermentation.<sup>14</sup> Recent work on genetically engineered bacteria that can hydrolyze cellulose and hemicellulose may make maize cobs or stover more viable feedstocks for ethanol production, opening up yet another industrial use of maize.<sup>15</sup>

Both in terms of its evolutionary history and its agronomic situation, maize is unique among the

cultivated crops. Its true evolutionary origin is unknown. The genetic diversity among cultivated types is immense, with adaptation covering a range greater than that of any other cereal. Maize is highly productive and serves a tremendous diversity of uses, for food, feed and industrial products. Varieties being used range from highly tailored and genetically narrow hybrids to farmer-developed and genetically diverse open-pollinated varieties. Essentially no use has been made of wild relatives in maize breeding. Maize is a crop with tremendous potential to continue filling a vital role in the world's agriculture.

### **CHALLENGES TO SUSTAINABLE MAIZE PRODUCTIVITY IMPROVEMENTS**

Although maize is a crop with tremendous potential for all the reasons noted above, there are significant challenges in meeting the needs for improved maize varieties in the future. Indications are that demand for cereals (both for food and feed) will continue to grow in the future. However, demand for maize is projected to grow more rapidly (3.8% per year) than demand for the other major cereal grains (wheat—2.4% per year, rice—2.1% per year), such that maize will constitute over one quarter of the cereal grain consumed in the "developing" world by the year 2,000.<sup>6</sup> Maize is a staple food in much of Latin America and Africa—areas where population growth rates are still relatively high and food shortages are not uncommon even now. With increasing standards of living, demand for feed maize is likely to grow, contributing to the growth in demand for maize relative to other major cereals.<sup>7</sup> Meeting the need for increased maize production in a sustainable fashion, given that little arable land remains for expansion of agricultural production, represents a significant challenge for maize breeders and agronomists.

This challenge is exacerbated by the variation in maize production environments discussed above. For well-endowed environments, maize yields are very good already and highly tailored hybrids are being used. Maize production in temperate areas is generally of this sort, typified by large-scale, highly mechanized cultivation with high levels of purchased inputs and increasing use of computer-aided management.<sup>6</sup> Limited potential remains for improving crop management in these areas, as it is generally excellent already. There is no evidence at present to suggest that progress from genetic improvement will not continue. In the U.S.A., breeding efforts have increased genetic yield potential at a relatively constant rate of 1.5% per year (70–90 kg/ha/yr) since the 1930s, and there is every indi-

cation that this progress will continue.<sup>16</sup> However, this rate will not meet an increase in demand for maize of 3.8% per year.

For marginal areas, maize productivity at present is low. The tropics, although encompassing a great diversity of maize production environments, include many of the marginal production environments. These areas are typified by small landholdings, and limited use of mechanization or purchased inputs.<sup>6</sup> Low productivity in these areas results from the combination of environmental limitations and genotypes not well-adapted to those environmental stresses. There is undoubtedly scope for productivity improvement in marginal areas, but the need for sustainable productivity growth will mediate against basing that improvement on energy-intensive or environmentally damaging inputs. Thus, genetic improvement for marginal areas may play an even more important role in productivity gains than is generally thought.<sup>17</sup> Limited work has been done on breeding for marginal production environments, so the rates of genetic gain in yield potential for such environments are difficult to assess. The demand for varieties with improved potential in marginal areas will likely increase, since any expansion in cropped area to meet growing food demands will necessarily be onto more marginal lands.<sup>18</sup>

What becomes clear is that improved productivity must be achieved in environmentally sound ways across the entire spectrum of environments in which maize production occurs. Breeding will play a key role in this improvement, both in well-endowed environments (where crop management is already quite good) and in marginal environments (where sustainable crop management improvement options are limited). The rapidly growing demand for maize will require breeders to take creative and novel approaches to this challenge. Plant genetic resources and biotechnology will both figure prominently in those approaches.

Maize plant genetic resources will become an increasingly important source of useful alleles for future maize breeding efforts. There is ample evidence that germplasm collections of maize have not been extensively used in commercial variety development to date. A survey of maize and soybean breeders in Brazil revealed that soybean breeders use germplasm banks regularly, but they were little used by maize breeders. Genetic variability for the maize breeding programs came from breeders' own working collections and from exchange with other breeders because the variability in such agronomically elite improved germplasm provided for acceptable progress from selection.<sup>19</sup> This evidence is even more clear-cut for the U.S.A. case, where Goodman<sup>20</sup> documented that only about

4% of the U.S.A. maize acreage is planted with varieties containing some (10-25%) non-U.S.A. germplasm. This limited use of exotic germplasm in U.S.A. variety development can be attributed to the extent of genetic variability persisting in elite Corn Belt Dent germplasm even after years of intense selection, the poor adaptation of exotics, which makes them difficult and time consuming to work with, and the pressure on maize breeders to turn out improved hybrids regularly.<sup>21,22</sup> However, exotic germplasm has the potential to provide novel alleles that will increase desirable genetic variation and heterosis for yield, alleles for specific traits that are lacking in elite germplasm (such as pest resistance or quality factors), and the chance for major gains in productivity above and beyond that of Corn Belt Dent (which itself represents only about 2% of the available genetic variation in maize).<sup>22</sup>

Although genetic resources of maize have been extensively collected, it is clear that numerous locally used varieties and landraces remain outside of the formal germplasm bank system. Studies of Hopi maize varieties have shown that both genetic shift and genetic drift occurred in populations conserved *ex situ*, and these changes differed from those that occurred in populations maintained *in situ*. Crop breeders seek allelic diversity from germplasm banks, but farmers seek both diversity and a population structure that provides for local adaptation from their landraces. This suggests that local varieties and landraces that remain outside of the formal germplasm bank system may provide diversity that is more appropriate to farmers' needs.<sup>23</sup>

Loss of valuable genetic diversity contained in these varieties may occur as locally adapted and variable varieties are replaced by less diverse "improved" varieties, as changes in crops grown or land use patterns occur, as management changes requiring that certain varietal traits are adopted (such as mechanical harvesting) and as marginal areas become unproductive due to increasing salinity, overgrazing, soil erosion, desertification and similar phenomena.<sup>17,24</sup> In the center of origin of maize, some of these processes also endanger wild relatives that may harbor alleles or allele complexes important to future maize improvement.<sup>17</sup> Human population growth and the trend toward larger, more homogeneous production systems exacerbate the processes contributing to loss of diversity.<sup>13</sup> Since "improved" varieties are most likely to offer a clear advantage under relatively better growing conditions,<sup>6</sup> ironically, local varieties and landraces that are adapted to more optimal growing conditions are especially vulnerable to loss.<sup>13</sup>

Genetic erosion in maize is not likely to occur as rapidly as it might in the naturally self-fertilizing species. Farmers will often grow both "improved" and local varieties adjacent to each other, thus allowing hybridization to occur between them. Frequently they will manage the "improved" variety as they have managed landraces, saving seed of the mixture that resulted from hybridization.<sup>13</sup> This combines some of the best of both varieties in a new farmer variety. Approaches such as this typify the real challenge of carrying out *in situ* genetic resource conservation: maintaining genetic variation within economically viable and socially acceptable land uses.<sup>25</sup> If farm communities in marginal areas do not continue to be viable socioeconomic entities, the crop varieties uniquely adapted to those areas will surely disappear together with farming in the communities themselves.

The new and evolving tools of biotechnology will improve maize breeders' abilities to identify useful alleles and transfer them into adapted elite varieties, as well as farmers' varieties and landraces. Duvick is quick to point out that breeders still depend more on experience and art than on genetics, and this will continue to be the case as biotechnological techniques are refined and improved. He adds that maize breeders can expect relatively little assistance from biotechnology in the next few years because the techniques at hand are as yet too slow and expensive to be of significant use in variety development.<sup>26</sup> In time, however, biotechnological approaches will allow more rapid progress in certain breeding efforts, and will provide the means to bypass barriers of sexual compatibility that limit conventional breeding.<sup>6</sup> Predicting the impact of biotechnology is not readily done, as there is no track record on which to base such a prediction. However, one attempt to quantify this impact predicts continually rising maize yields, with increases of 1.5% per year growing to 2.0% per year during the 1990s.<sup>6</sup> This is more optimistic than Duvick's<sup>16</sup> prediction of continued growth in genetic yield potential at 1.5% per year, which assumes that advances in breeding tools (including biotechnology) will be needed to maintain this rate of increase.

Targets of maize biotechnology at present include stress tolerance (for both biotic and abiotic stresses) and quality enhancement (such as increased content or modified quality of protein, starch, or oil). The quality traits are of greater relevance in countries with strongly developed markets that have need for product differentiation within those markets (i.e. the "developed" countries). To date, less effort has been devoted to enhancing yield and yield stability, which are of

primary interest in the “developing” countries. In time, alleles assembled through the combined efforts of maize breeders and biotechnologists may substitute for certain technologies such as chemical fertilizers and pesticides.<sup>6</sup> Advances of this sort will be necessary to achieve needed gains in maize productivity in marginal environments.

To achieve productivity gains across the spectrum of maize growing environments, maize breeders will need to learn how to take better advantage of genotype by environmental interactions. Breeders have often spoken of seeking “wide adaptability,” and indeed the wide adaptation of dwarf varieties of wheat and rice contributed to impressive gains in production that constituted the Green Revolution. However, wheat and paddy rice growing environments where Green Revolution varieties were grown, although geographically dispersed, are relatively homogeneous environments. The term “wide adaptation,” as Ceccarelli<sup>27</sup> so aptly points out, is used more in the geographical than the environmental sense. To contribute to sustainable productivity increases across the range of maize growing conditions, varieties will need location-specific adaptation to the ecological conditions and stresses of each target environment.<sup>18</sup> In some regions, the variability in both micro-environments and farmers’ selection criteria may be too great to be dealt with through centralized crop breeding efforts.<sup>13</sup> In these areas, farmer-participatory breeding, which combines farmers’ local crop and environmental knowledge with breeders’ genetic knowledge, may allow for the necessary genotype by environment tailoring.<sup>28</sup>

## COMBINING PLANT GENETIC RESOURCES AND BIOTECHNOLOGY FOR MAIZE IMPROVEMENT

In recent years, plant breeders have acquired a heightened awareness of the importance of plant genetic resources. Certain biotechnology techniques (e.g. genome mapping, tissue culture applications) have stimulated interactions between biotechnologists and genetic resources programs. However, most genetic resource programs need to forge even closer links with both breeders and biotechnologists, particularly in the “developing” world, which is lagging behind the “developed” world in this respect.<sup>29</sup> In this section, we highlight several areas where maize plant genetic resources combined with biotechnological approaches have the potential to provide major improvements in sustainable productivity of maize. The areas discussed by no means constitute an exhaustive list, but rather represent those that appear to have the

most immediate promise at this point in time. With progress in biotechnological research occurring at a rapid pace, the opportunities will surely be different a few years from now.

### IDENTIFICATION OF NOVEL ALLELES FROM EXOTIC MAIZE, GENE BANKS AND WILD RELATIVES TO IMPROVE PRODUCTIVITY OF MAIZE IN WELL-ENDOWED ENVIRONMENTS

The genetic base of elite maize varieties is generally limited, and although reasonable progress continues to be made by deriving new varieties from elite by elite crosses, this process cannot continue indefinitely. At some point, minimal residual genetic variability will make selection ineffective. Breeders generally agree that introducing novel alleles for productivity from outside of the elite maize germplasm base would be highly desirable. Studies have documented alleles from exotic germplasm that contribute to yield both tropical and temperate varieties,<sup>30</sup> yield and agronomic quality of temperate germplasm,<sup>31-33</sup> and insect and disease resistance.<sup>34-36</sup> Some potential has been shown for maize landrace contributions as well.<sup>37,38</sup> Published results on novel alleles of agronomic utility from wild relatives of maize are limited at present to preliminary documentation about the potential of the apomixis allele from *tripsacum* for generating apomictic maize.<sup>5,39</sup> Work in progress at Cornell University indicates that alleles with positive effects on yield, grain moisture and stalk quality can be derived from Chalco teosinte (SR Painter, SR McCouch, SD Tanksley et al, unpublished).

The first challenge of using such materials, whether they are exotics, land races, or wild relatives, is identifying those materials that carry useful alleles.<sup>22</sup> Research on exotics has shown that traditional breeding techniques, such as per se or test-cross evaluation, are unreliable indicators of the potential contributions of exotic germplasm to improvement of local varieties.<sup>9,35,40,41</sup> Molecular markers can facilitate this process by allowing the breeder to identify quantitative trait loci (QTLs) associated with desirable genetic variation in the progeny of crosses between elite and non-elite germplasm, and by determining whether a given QTL from the non-elite germplasm is contributing positively or negatively to the trait of interest.<sup>31</sup> Work with elite by wild relative crosses in species other than maize has documented transgressive segregation for numerous traits, and a number of QTLs from the wild relative showed positive effects for traits where the wild relative itself was clearly inferior.<sup>42</sup> Biotechnological advances such as this have stimulated some researchers to focus increased attention on wild crop relatives.<sup>17</sup>

Molecular genetic markers have proven to be a powerful tool for identification of useful alleles from non-elite germplasm, but the populations generally used in such studies ( $F_2$  or advanced selfing generations from crosses of elite by non-elite) comprise numerous variations of agronomically unacceptable types. To recover the desired QTLs in agronomically acceptable germplasm, extensive conventional breeding efforts are required after the molecular analysis is complete. Recently developed techniques combine molecular marker analysis with variety development by searching for useful QTLs in advanced backcross progenies of elite by non-elite crosses. This approach is uniquely suited to discovery and transfer of desirable QTLs from donor germplasm into established elite inbred lines, and thus should prove very useful in maize.<sup>43</sup> It has several distinct advantages. First, selection in early backcross generations can be used to eliminate major negative QTLs, which otherwise would interfere with later yield and quality measurements. Second, QTLs are identified in a largely inbred background, thus avoiding selection of alleles that rely on epistatic interactions with other donor alleles for their positive effects. Next, agronomic evaluations can be done using testcross progeny, thus automatically selecting for dominant or partially dominant alleles that will contribute to hybrid performance. Finally, desirable QTLs identified are in nearly fully elite inbred backgrounds already, and thus only a few breeding generations away from being tested as finished potentially commercial inbreds.<sup>43</sup> Techniques such as this promise to greatly facilitate the utilization of non-elite germplasm for improvement of elite maize varieties.

#### IDENTIFICATION OF ALLELES THAT IMPROVE YIELD AND YIELD STABILITY UNDER MARGINAL CONDITIONS

Data on use of improved seed vs. farmers' own seed in the "developing" world suggest that scientifically based maize breeding has had limited impact in marginal production environments of the tropics. Farmers value their own local varieties over "improved" varieties for traits such as yield stability, short maturation period, suitability for intercropping, storability and taste or cooking traits. The "improved" varieties generally are selected for high yield potential and uniformity—traits that are less important in marginal production situations and where neither crop management techniques nor markets demand uniformity.<sup>44</sup> Farmers in eastern Africa have at least nine possible end uses for their maize crop, and may plant different varieties to provide both different

maturities and varieties tailored to these diverse end uses.<sup>44</sup> In Chiapas, Mexico, farmers chose local varieties over "improved" varieties based on soil pH and organic matter content. At pH values above 7.3 and low soil organic matter (levels at or below 1.7%), the landrace 'Olotillo' was preferred over "improved" varieties.<sup>45</sup>

In the tropics, selection in well-managed conditions and good production environments has not been particularly effective at developing improved varieties for marginal growing conditions, particularly when genotype by environment interactions of a crossover type occur within the spectrum of environments considered. Experimental data have shown that high yield potential under good conditions does not necessarily carry over into marginal environments.<sup>27</sup> Under extremely adverse conditions, barley selections made in unfavorable environments out-yielded those made in favorable environments, with or without fertilizer additions.<sup>17</sup>

Maize selection under managed stress has produced some impressive gains in productivity under stress conditions. Selection under managed drought or nitrogen stress conditions has resulted in development of experimental varieties with increased and stabilized grain yield under these stress conditions, and equal or even increased yield under optimal conditions.<sup>46,47</sup> Even after only three cycles of selection under low nitrogen conditions, dramatic changes in ear growth characteristics were observed in comparison to parallel selections made under optimal nitrogen supply, indicating that low nitrogen conditions subject maize to different selection pressures than do optimal nitrogen conditions. Similar results have been noted for selections made under drought stress.<sup>47</sup> A further study compared selection at one location under managed drought with selection based on performance in multi-location tests in geographically dispersed but environmentally similar locations throughout the tropics. Yield gains over a broad range of conditions (from optimal to stressed) were better in the drought-stress selection, and other agronomic traits were identical or slightly improved in this selection as well. The authors suggest using managed stress environments to select for needed traits, followed by multi-location testing to achieve site-specific adaptation as a final step in the breeding process.<sup>48</sup> There are clearly examples of outstanding plant breeding efforts, where germplasm selected and developed in optimal conditions has performed quite well across a range of environments including stress environments. However, there is a growing number of cases where varieties with excellent performance in stress environments have resulted from selection under stress conditions.

The preceding discussion argues for the importance of selecting maize varieties for stress tolerance, if maize productivity is to be improved in marginal growing environments. Maize landraces and local varieties that have evolved under such marginal conditions surely have useful alleles to contribute to improving stress tolerance, and wild relatives may have such alleles as well. Little application of biotechnology to these problems has occurred to date, but there is every reason to expect that the same types of approaches cited in the previous section should be effective for stress tolerance traits. Such work would combine the power of current scientific techniques with the unique genetics that have been assembled through farmers' talent and wisdom over generations to create maize varieties with the potential to improve sustainable productivity in some of the environments where such improvements are most needed.

As Brush points out, the variability in both micro-environments and farmers' selection criteria in some areas may be too great to be dealt with through centralized crop breeding efforts.<sup>13</sup> Farmer-participatory breeding approaches may be the only reasonable way to approach variety development for such areas.<sup>28</sup> The strength of biotechnology in contributing to this process would be in accurately identifying those specific alleles that can contribute to improved and more stable production under marginal conditions. Reasonably well-adapted and productive genotypes carrying the desired alleles could be developed relatively rapidly using molecular markers to speed up the process. Moving the alleles into a generally acceptable genetic background would avoid introducing deleterious traits into the target material. These alleles could then be introduced into farmers' local varieties by crossing them onto a range of different varieties in a managed breeding nursery or by providing the agriculturally acceptable source directly to farmers to plant adjacent to their own maize plantings. In either case, farmers themselves could carry out the needed subsequent selection work. Such an approach would allow relatively easy introduction of specific alleles into a large number of uniquely adapted farmer varieties, while avoiding introduction of alleles that were significantly deleterious.

#### USING MOLECULAR MARKERS TO TAG UNIQUE ALLELES FOR STRESS TOLERANCE, ADAPTATION AND QUALITY FROM UNDER-UTILIZED PLANT GENETIC RESOURCES

Evidence from study of U.S.A. maize varieties over the past 50-70 years indicates that current varieties, although not selected directly for stress tolerance, are indeed more stress tolerant than

their predecessors.<sup>49-51</sup> However, as Francis and Chang<sup>18</sup> point out, this does not mean that there is no need to search for additional alleles from non-elite germplasm for tolerance to specific stresses. Such alleles, added to elite germplasm, will be a further source for the breeding progress needed to meet future maize demand. Whether for stress tolerance, unique adaptation, or special quality factors, research has shown that useful alleles are present in both relatively elite and non-elite genetic materials.<sup>34-36,52,53</sup> Their use to date has been limited by a combination of the difficulty of identifying the best sources of useful alleles and the presence of unfavorable linked alleles in the source germplasm.<sup>20</sup> Molecular marker approaches can help minimize both of these difficulties, and genetic transformation ultimately will broaden even farther the scope of "non-elite genetic materials" that breeders can tap.

Biotechnology can further facilitate use of unique alleles for stress tolerance, adaptation and quality by contributing to our understanding of how complex traits function. The following section of this chapter, which concerns heterosis, addresses one such contribution. In addition, information derived through biotechnology is contributing to our understanding of quantitatively inherited traits in general. Since most agronomically important traits in maize are quantitative, and such traits present some of the greatest challenges to classical plant breeders, any knowledge gained about the genetics underlying them will surely facilitate breeders' efforts to improve them. Recent studies have identified QTLs for classic quantitative traits such as plant height, and found that many of them are close to known qualitative genetic loci affecting that same trait.<sup>54</sup> Quantitative traits may result from the combined action of numerous alleles with small phenotypic effects at the same loci that have been identified as "qualitative" due to the presence of alleles with large phenotypic effects. The more breeders can learn from biotechnologists about the genetics underlying quantitative traits like adaptation, stress tolerance and quality, the more effective their breeding efforts will become.

Molecular genetic studies have also begun to allow us to dissect phenotypically correlated traits. Kernel oil concentration and kernel size in maize are known to be negatively correlated. One molecular marker study identified 11 chromosomal regions that affect oil concentration, and 11 that affect kernel weight in a segregating population. Most QTLs for increased oil concentration were also associated with decreased kernel weight. However, some loci within these regions were associated with one trait but not the other, and two regions

contributed to increases in both oil concentration and kernel weight.<sup>55,56</sup> Information such as this should allow maize breeders to selectively introgress desirable alleles that do not carry expected undesirable associated effects.

Increased molecular genetic understanding of traits and identification of novel desirable alleles through molecular marker approaches will prove useful to breeders beyond the bounds of the specific species studied. Considerable evidence has been amassed recently documenting the extensive genetic similarity between species. Of particular interest are studies of grasses. Molecular genetic analyses have revealed conserved linkages between maize and sorghum,<sup>57,58</sup> maize and rice,<sup>59</sup> and maize and wheat.<sup>60</sup> A comparative study has detected extensive homology among maize, wheat and rice in a number of genomic regions.<sup>61</sup> As Tanksley et al<sup>62</sup> point out, a high degree of linkage conservation between species means that genetic information and probes developed in one species can be utilized with the other(s). Thus, comparative molecular genetic maps will facilitate identification of QTLs affecting traits of agricultural importance across all the major cereal grains.<sup>63</sup>

Molecular cloning of "R" genes, which enable plants to resist a diverse range of pathogens, has revealed that the proteins encoded by these genes have several features in common. It appears that different plant species may have evolved common signal transduction mechanisms for the expression of resistance to a wide range of unrelated pathogens. Characterization of the molecular signals involved in pathogenicity and of the molecular events that result in the expression of resistance may lead to novel strategies for multiple disease control that would be effective across many crop species.<sup>64</sup>

#### PROBING THE NATURE OF HETEROSESIS, AND DEVISING NOVEL WAYS TO UTILIZE IT

Whether in highly tailored maize hybrids or in farmer selected open-pollinated varieties, heterosis plays an important role in maize productivity. Yet, it remains a mysterious phenomenon. Duvick<sup>26</sup> said it well when he pointed out that, "With all our advances in maize genetics and technology, we still do not know what causes hybrid vigor..." Studies analyzing why newer maize hybrids outperform older ones have found that newer hybrids have more total above-ground biomass, a longer grain filling period (as they are less prone to stress-induced premature plant death), higher rates of dry matter accumulation during grain filling and a greater inherent tendency towards prolificacy.<sup>16,65</sup> This may suggest appropriate directions for selec-

tion in maize breeding programs, but it does not provide an explanation for heterosis.

Molecular genetic analyses have attempted to link heterosis (measured in terms of yield) to marker diversity between parents or within single cross hybrids. Although molecular markers are able to detect inbred associations that correlate well with known pedigree data, they have been ineffective in predicting specific combining ability effects, except under very limited conditions.<sup>66</sup> For distantly related inbreds, measures of relationship based on marker diversity and based on yield are significantly correlated, but this relationship does not hold up for more closely related inbreds.<sup>67</sup> Bernardo<sup>68</sup> notes that effective prediction of hybrid performance based on molecular marker heterozygosity requires that a relatively restrictive set of conditions apply, including that there are strong dominance effects, that allele frequencies at individual loci in the parents be negatively correlated, that trait heritability be high, that at least 30-50% of the QTLs be linked to molecular markers and that no more than 20-30% of the markers be unlinked to QTLs. These conditions seem unlikely to be met in many situations. Although molecular marker techniques are still not effective at predicting heterosis between parents, these studies have certainly shed light on the complexity of the phenomenon.

Contributions of individual locus behavior to heterosis also have been examined. Studies by Stuber et al<sup>69</sup> have detected considerable overdominant allelic behavior in a highly heterotic hybrid, and little evidence for epistatic contributions to heterosis. On the other hand, work by Dudley et al<sup>67</sup> found that the genetic distance between parents (measured as modified Roger's distances) was not correlated with hybrid yields, but that the number of marker loci of the higher yielding genotype present in a cross was correlated with hybrid yield. This suggests that simple dominance may account for a large share of heterosis. In work with a tomato x wild relative cross, evidence of overdominant QTLs was detected.<sup>42</sup> Although it is not yet clear whether dominance or overdominance is the primary cause of heterosis, studies such as these will, in time, shed light on this critical question. A deeper understanding of the genetic basis of heterosis should allow more precisely targeted breeding efforts to improve it.

Classical wide cross breeding combined with molecular marker-facilitated backcrossing may provide a truly novel means of capitalizing on heterosis. Work underway at CIMMYT to transfer the apomixis gene from *tripsacum* to maize could open the door to true breeding hybrid maize.<sup>5</sup> The

possible benefits of apomictic maize for resource-poor farmers, who cannot afford to purchase seed on a yearly basis, could be significant. However, the capability to make hybrids inexpensively available to these farmers will not obviate their need for locally adapted, stress-tolerant varieties that fit their microenvironments, cropping patterns, soil and water constraints and crop end uses. Although offering the promise of truly novel developments, apomictic maize clearly will not replace the challenging breeding work needed to develop these varieties.

## SUMMARY

Maize is a unique crop, both in its evolution and its current agronomic use. It is grown in a wider diversity of environments and put to a wider diversity of uses than any other cereal grain. Plant genetic resources of maize encompass tremendous genetic diversity, and the wild relatives teosinte and *tripsacum* provide additional sources of potentially useful alleles. Maize breeders will need to make efficient use of all the diversity available to them to meet the growing demand for maize as food and feed. Meeting this demand will require increasing the sustainable productivity of maize across the entire spectrum of production environments, from well-endowed to marginal growing conditions. Maize breeding will play an increasingly important role in this effort, as there is limited scope for management improvement in the well-endowed environments (where crop management is generally excellent already), and the need for sustainable productivity increases in marginal environments implies limitations to the use of energy-intensive or environmentally damaging management inputs.

Combining the tremendous genetic diversity of maize and its wild relatives with the scientific knowledge gained through biotechnology will be essential in striving to meet the challenge of improving maize genotypes for the many diverse maize production environments. Areas of significant potential include identifying novel alleles from non-elite germplasm for improvement of elite hybrids, identifying alleles that contribute to yield stability and productivity under marginal conditions, using molecular markers to tag unique alleles for key quality and stress tolerance traits and probing the nature of heterosis to better take advantage of its benefits in improved varieties for all production environments. Surely as research in biotechnology advances, additional possibilities will reveal themselves. However, to meet the tremendous challenge now at hand—that of increasing maize productivity in a sustainable fashion,

without the option of significantly increasing the area under cultivation—maize breeders will have to make creative use of all the genetic diversity and the breeding tools available to them. If this can be done creatively without ever losing sight of farmers' realities and constraints, the needed gains in sustainable maize productivity may be achieved.

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## =====CHAPTER 10=====

# USE OF BIOTECHNOLOGY TOOLS IN POTATO GENETIC RESOURCES MANAGEMENT AND BREEDING

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Potato is a globally important food crop both in developed and developing countries. It is also used as animal feed and as a cash crop for the food processing industry for snack foods, starch and alcoholic products.<sup>1</sup> Potato is widely used as a model plant species in basic plant molecular and cellular biology and in associated higher educational programs in plant science, as well as practical agricultural R&D. The relevant results of such efforts are of great value for associated plant species such as tomato.<sup>2</sup>

A prototype of this chapter was presented by Watanabe et al (1995);<sup>3</sup> however, plant biotechnology and its conceptual applications for strategic uses in potatoes have advanced rapidly since then. This chapter overviews: 1) the power of plant biotechnology tools for enhanced potato germplasm management; 2) uses of potato genetic resources in breeding for sustainable and productive agriculture; 3) acceleration of the breeding process by molecular markers; 4) use of cellular biology techniques in assisting breeding; 5) alternative breeding approaches using genetic engineering; 6) diagnostics in potato crop protection; 7) in vitro techniques for seed production; and 8) a summary on the use of the potato as a model crop in germplasm enhancement and its combination/complementation with biotechnology tools.

### GENETIC RESOURCES MANAGEMENT WITH BIOTECHNOLOGY TOOLS

#### ESTIMATION OF DIVERSITY AND CLASSIFICATION OF GENETIC RESOURCES

Potatoes include about 200 species of wild and cultivated relatives, many of whose accessions are maintained at national or three principle international gene banks.<sup>4,5</sup> Managing the diverse accessions of so many relative species and cultivated taxa is cumbersome, and plant biotechnology tools are useful in the management of these genetic resources.<sup>6</sup> The major tasks in managing such large collections are: 1) identifying duplicates in clonal accessions; 2) estimating genetic diversity and making core collections; 3) monitoring the shift in specific allelic frequencies and diversity in rejuvenation of true seed generations; and 4) measuring the diversity available in situ. All of these aspects are facilitated by the use of molecular markers.<sup>3</sup> For conservation, in vitro collections with pathogen-free status have become well-established, and long-term preservation by cryo-conservation is gradually occurring.<sup>3,7</sup> While the details of

application of molecular markers and in vitro technology to genetic resources management are discussed by Rao and Iwanaga in chapter 4, an emphasis specific to potatoes should be made on some of the advances in biosystematics and genetic diversity in the tuber-bearing *Solanum* taxa using molecular tools.

With the advancement and application of molecular markers, potato systematics have become more comprehensive.<sup>8,9</sup> The findings have not only been of academic interest but they also have assisted in the better organization of potato collections.<sup>10</sup> By studying cytoplasmic and nuclear diversity,<sup>11</sup> new findings on the origin of the cultivated tetraploid potatoes were obtained. Additionally, core collections of potato species can be made more genetically diverse by utilizing information on cytoplasmic and nuclear genome diversity.

More precise genetic information can be obtained using various molecular markers derived from the recent adoption of polymerase chain reaction (PCR) to plant molecular genetics.<sup>10</sup> Cost efficiency and simplification of the technology will provide wide adaptability to various resource conditions, and especially resource-poor gene banks/research programs in developing countries.<sup>3</sup>

## GENETIC RESOURCES TO ENHANCE SUSTAINABLE BREEDING EFFORTS

While many old cultivars such as Russet Burbank in the U.S.A. and Bintje in many potato seed-importing countries are still used in both industrialized and developing countries, developing new cultivars is important to meet emerging needs in enhancing productivity and sustainability. This change is occurring because alternative approaches are needed to replace the use of agro-chemicals, which are being rapidly restricted by regulation due to public concerns about the environment and health. Furthermore, the use of improved cultivars with resistance/tolerance to biotic and abiotic stresses is of great value in sustaining potato production and enhancing economic efficiency.<sup>12</sup>

Wild relatives of cultivated potatoes provide many valuable genes that confer resistances to diseases and insect pests that do not exist in the cultivated gene pool, as well as a diverse genetic background.<sup>13</sup> However, due to their wild nature, the time they take for introgression<sup>14</sup> and their possibly deleterious traits such as high glycoalkaloid content,<sup>15</sup> these valuable genetic resources have been avoided as primary sources for potato breeding.

While there are many artificial genes available that can be integrated into the cultivated potato

genome by modern gene transfer techniques, as discussed in a later section of this chapter, wild potato genetic resources often have very high levels of resistance, such as that conferred by the extreme resistance genes to potato Y potyvirus (*Ry*) and potato X potexvirus (*Rx*), and by a gene conferring immunity to race R01 of golden nematodes (*Hi*),<sup>16</sup> which cannot be accommodated by present genetic engineering methods using synthetic genes. Considering public concerns about the effects of genetically engineered organisms on environmental and food safety, natural genes derived from wild relatives of potato would be much better accepted for generating new cultivars since they are considered to be environmentally friendly, often reduce the use of agro-chemicals and make production practice much easier.<sup>3</sup> Also, due to the diversity of the sources of resistance (e.g. on golden nematodes, *Globodera rostochiensis*<sup>17</sup>), a genetic flexibility could be incorporated using various wild species as sources of resistance; this could be more durable than single-sourced artificial genes available via genetic engineering.

Conventional potato breeding methods have taken decades to achieve the goal of cultivar development—incorporating valuable traits from genetic resources.<sup>13</sup> Advancing generations in potato breeding are dependent on information obtained from screening and selection processes. Screening procedures often require multiple repeated trials to confirm results. This requirement may delay the enhancement practices because it usually takes several years to put valuable genes from wild species into cultivated potatoes.<sup>18</sup> However, modern tools are now becoming available to alleviate pitfalls in conventional germplasm enhancement with wild genetic resources.

## ACCELERATION OF BREEDING PROCESSES BY MOLECULAR MARKERS

Several potato molecular maps were generated using RFLP markers derived from tomato and potato genomic DNA and their cDNA libraries,<sup>19,20</sup> followed by PCR-based markers such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP).<sup>10,21</sup> These potato maps were integrated to achieve a comprehensive analysis of the potato genome with applications to potato germplasm enhancement and breeding.<sup>22</sup> The major areas of R&D for use of molecular markers on potatoes include: 1) monitoring introgression; 2) identifying markers associated with simply inherited traits and marker-assisted selection (MAS) for the

traits; 3) fundamental studies on quantitative trait loci and identification of markers for MAS; and 4) map-based cloning of useful potato genes.

An advantage in the study and use of molecular markers for potatoes is the high synteny of molecular markers available for the tomato genome. Map positions of the tomato markers on the potato genome are well-conserved compared to the alignment of corresponding markers on the tomato molecular map, except for five major inversions on different chromosomes.<sup>19,20</sup> Furthermore, many molecular genetic materials associated with resistance genes of the tomato, which are attributed to the same pathogens/pests that affect potatoes, could have high applicability to potato genetics as orthologous loci (Watanabe et al, unpublished data). Thus, the tomato molecular map and associated specific gene information can be very useful to the ongoing R&D on potato molecular genetics and breeding.

#### MONITORING INTROGRESSION

Historically, problems in the utilization of valuable wild species have included difficulties with: 1) the identification of target hybrids; 2) the accuracy in detecting introgression of the target genes of interest; and 3) the simultaneous selection for elimination of deleterious exotic genes to derive elite genotypes.

Hybrid identification can be made rapidly by the use of parental species-specific markers, microsatellites and single markers.<sup>9,10,23</sup> Rapid progress has been demonstrated by many groups using marker-assisted introgression of some wild *Solanum* species, a process which requires only three to five years compared to 10-20 years using conventional methods. Examples are given in Watanabe (1994)<sup>16</sup> and Watanabe et al (1995).<sup>22</sup> This methodology is not only applicable in developed countries but is also gradually being employed as a feasible technique for developing countries with limited resources.<sup>3</sup>

#### SIMPLY INHERITED TRAITS FOR MAS

Several major important resistance traits have been mapped on the potato genome. These resistance traits are due to single dominant genes that confer resistances to pathogen/pest such as: 1) extreme resistance gene (*H1*, *Gro1* and *GroVI*) to golden cyst nematode;<sup>17,24,25</sup> 2) race-specific major gene to late blight;<sup>26</sup> 3) extreme resistance genes *Rxacl* and *Rxadg* to PVX;<sup>27</sup> and 4) extreme resistance gene *Ryadg* to PVY.<sup>28</sup>

An advantage of using markers for selection could be their capacity to avoid the pitfalls of conventional breeding, including enormous labor,

time and logistic.<sup>14</sup> For example, conventional screening for a pest requires several weeks to months; on the other hand, molecular markers can provide more precise information within a week for a large-scale breeding population. Although this is an example of a short-term application of molecular markers in plant breeding as a selection tool, utilization of these markers requires further technique simplification such as PCR-based assays, which could be combined with immunoassays to reduce the number of steps and costs.<sup>29</sup> Marker technology has not yet reached its full capacity of comprehensive uses and, thus, needs further digestion for end-users.<sup>30</sup> Streamlined application using simple inherited traits must be achieved in order to challenge more complex quantitative traits and to disseminate cost-effective breeding operations to any location, especially resources to poor developing-country programs.

#### UNDERSTANDING QUANTITATIVE TRAIT LOCI (QTL) FOR BREEDING

The tetraploid nature of potatoes and the fact that many important traits in potato breeding are quantitatively inherited make potato breeding processes tedious.<sup>14,16,18</sup> There are several questions to be addressed in the use of QTL in potato breeding: 1) genotype x environment interactions (GxE) on QTL; 2) magnitude of effects of each chromosomal region/locus; 3) types of gene actions; 4) epistasis; and 5) heterosis.<sup>31</sup> To answer these questions, important advances have been made to identify chromosomal regions responsible for quantitative traits that are important for potato improvement (reviewed in Watanabe 1994<sup>16</sup> and Watanabe et al 1995).<sup>22</sup> Three representative examples are given below:

First, naturally occurring glandular trichomes exhibit high levels of resistance to many harmful insect species and are quantitatively inherited, and are representative of QTL which have complex gene actions.<sup>32</sup> It should be noted that the use of such host-plant resistance is very valuable in enhancing sustainability and productivity.

As an alternative or a complement to pesticide usage, use of host-plant resistance such as glandular trichomes would be an asset in an integrated pest management program. This resistance mechanism can reduce technical problems associated with adoption of IPM schemes for small farmers in the developing countries who require simpler implementation procedures. In addition, applicator hazards from pesticides and cost constraints can be avoided

with genetic modifications such as glandular trichomes.<sup>3</sup>

QTL information obtained at the diploid level<sup>32</sup> has been tested both at the diploid and tetraploid level using common genetic background derived from *S. berthaultii*.<sup>33</sup> Under the same genetic background at both ploidy levels, the QTL information corresponded well for selecting high-trichome phenotypes that are resistant either to Colorado potato beetle (Type A trichomes) or to aphids/red spider mites (Types A and B trichomes). QTL information, however, varied among different locations in different countries, therefore further elaboration on G X E was essential in order to practically apply the molecular marker information in MAS. Another requirement is to establish a scheme to simplify the use of QTL information in MAS by simulating a few specific loci instead of using all of QTL in MAS result in drastic labor and cost reductions in these laboratory processes.

Second, horizontal resistance to late blight caused by *Phytophthora infestans* is also polygenically controlled and breeding for the resistance to this pest has been the most difficult target in potato breeding history.<sup>14</sup> Furthermore, the involvement of race specific major genes masks the quantitative horizontal resistance that has made for very slow progress in gaining desirable potato clones. However, molecular marker information on the race-specific major genes and QTL on horizontal resistance will lead to progress in understanding the genetics of host resistance to late blight.<sup>34</sup> Although molecular marker information is still preliminary as far as utilization in breeding applications is concerned, the molecular genetic information is of great help in establishing systematic germplasm enhancement methods for late blight resistances.

Third, cyst nematodes are common problems in developed and developing countries, especially in Japan, Europe and South America. Since the use of nematicides has been becoming more restricted due to environmental and health protections, alternative pest control approaches will be created using resistant cultivars. The most common one is *Ro1* of *G. rostochiensis*, which can be controlled by single dominant *H1* or *Gro1* genes and MAS has gradually come to be used in selection.<sup>17,24,25</sup> Quantitative resistance is also available to both *G. rostochiensis*<sup>35</sup> and *G. pallida*<sup>36</sup> which could provide more genetic plasticity in the changing ecology of the cyst nematode pathotypes and in the mutation of the pest, which could overcome the single resistant gene(s) and thus provide general resistance. QTL information of such loci has been gradually obtained and is now being tested for practical use in breeding.

## USE OF CELLULAR TECHNIQUES IN ASSISTING BREEDING

Specific examples of the use of cellular biology techniques will be given in this section on germplasm enhancement using distantly related species which provide valuable traits that do not exist in the cultivated potato gene pool. General reviews on cell fusion and somaclonal variation in potato may be referred to in Thach et al.,<sup>37</sup> Kumar,<sup>38</sup> Wenzel<sup>39</sup> and Millam et al.<sup>40</sup>

### SOMACLONAL VARIATION

Somaclonal variation was a unique topic in the late 1970s to 1980s. It provided the basis for handling cells in protoplast fusion and *Agrobacterium*-mediated transformation.<sup>38,39</sup> Direct impact on cultivar development using the technique has, however, been modest, though many cultivars have been generated for specific needs in markets in industrialized countries.<sup>39,41</sup>

### SOMATIC FUSION IN TRANSFER OF USEFUL GENES FROM SEXUALLY CROSS-INCOMPATIBLE SPECIES/GENUS

Identification of hybrids and elimination of unnecessary chromosomes from donors are the essential components of successful somatic fusion based gene transfer. Phenotypic observation in hybrid vigor on calli and plants used to be the major methods, together with isozymes.<sup>39</sup>

Now, not only hybrid identification is made but also introgression can be monitored by molecular markers especially via species-specific repetitive markers.<sup>42,43</sup> The use of somatic fusion is now accelerated by the use of molecular markers to monitor the introgression of specific allele(s) of interest and the removal of deleterious genes.<sup>44</sup>

In order to minimize the introgression of the genome from donors, asymmetric fusions can be made by various methods that reduce the incorporation of deleterious genes, especially from distantly related wild species.<sup>41,45</sup>

Two decades of experience in *Solanum-Lycopersicum* and tuber-bearing and non-tuber-bearing *Solanum* somatic fusions now make it possible to transfer mutually valuable genes from one genus to another, and this makes available for potato breeding more diverse genetic resources that could not be utilized conventionally.<sup>46,47</sup> The potential of this technique is enormous since it promotes underutilized non-host resistance from an associated genus, which has not been utilized in potato breeding. For example, non-host late blight resistance can be obtained from eggplant-related species,<sup>41</sup> which could assist poorer sources of resistance in controlling the fungal disease by the presently available germplasm.

## ALTERNATIVE BREEDING USING GENETIC ENGINEERING

Genetic engineering with potatoes has been well-established using *Agrobacterium*-mediated transformation methods.<sup>48</sup> Various entities of transgenic potatoes have been tested for commercialization of specifically improved cultivars from commonly used cultivars.<sup>49</sup> Also, because of its ease in genetic engineering as well as the need for genetic improvement, potatoes have been employed as model crops for studying safety issues.<sup>49,50</sup>

Major areas of transgenic R&D on potatoes are listed in Watanabe et al (1995)<sup>22</sup> and Frederick et al (1995).<sup>49</sup> The listed constraint that natural resistance is not available or too difficult to incorporate into potato cultivars has been rapidly lifted by the employment of transgenic potatoes. One of the genes employed has insect resistance based on bacterial crystallization protein gene *Bt*<sup>51</sup> and another, through the use of a series of membrane-function or pathogenesis-related protein genes from different plant species, controls late blight fungi.<sup>52,53</sup> Both genes are incorporated into potato cultivars and are now being tested for safety aspects.

Also, the transfer of technology goes hand in hand with progress in industrialized countries. Several major organizations have conducted proprietary plant biotechnology transfer; examples include the chimeric gene constructs made from *Bacillus thuringiensis* (*Bt*) δ-endotoxin genes from Plant Genetic Systems (PGS) of Belgium, which is facilitated by the International Potato Center,<sup>3,22</sup> and various transgenic potatoes resistant to viruses, insects and late blight and their associated genes which have been transferred by different international initiatives.<sup>49,54</sup>

## DIAGNOSTIC TOOLS IN PLANT PROTECTION

Diagnostic tools used in plant quarantine are well-discussed in common plant pathology text books and are therefore only briefly highlighted in this section, based on reviews by Watanabe et al<sup>3</sup> and De Boer et al.<sup>55</sup>

There are various potato diseases, as well as insect and nematode pests.<sup>56</sup> The pests associated with international quarantine and seed production are of concern. Major quarantine diseases include: potato spindle tuber viroid (PSTVd),<sup>57</sup> potato virus T (PVT)<sup>58</sup> and bacterial ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Speck and Koth).<sup>59</sup> Since both PSTVd and PVT can be transmitted via true seeds and no major resistance has been identified in potato genetics resources

(unless genetic engineering could be applied to generate resistant germplasm, which could take several years to reach users), all plant materials must be tested for the pathogens in order to protect potato crops. Similarly, potato cultivars have zero tolerance to bacterial ring rot; thus, the quarantine protection is a "must" against the bacteria.

Many fine diagnostic tools are available to promote plant quarantine against such deleterious pests, especially for potato viruses and viroids.<sup>60</sup> Enzyme linked immunosorbent assays (ELISA) have been common and effective for testing many samples for potato viruses. ELISA testing is well-established for many potato viruses and several other pests such as ring rot RR.<sup>61</sup>

More specific testing methods are available with nucleic acid detection methods such as nucleic acid spot hybridization (NASH),<sup>62</sup> which is very valuable in identifying viroids that cannot be identified by ELISA.<sup>63</sup> The advantages of these diagnostic tools are not only their effectiveness, but also their ability to be disseminated to end-users with minimal laboratory facilities. Thus, these tools can be easily adapted to any user through the support of an advanced laboratory, even over international distances.

For well-equipped diagnostic laboratories, PCR-based detection methods can be applied to various pathogens such as potato leaf roll virus<sup>64</sup> and bacterial ring rot.<sup>65</sup> PCR-based techniques for plant diagnosis utilize DNA primers designed to amplify pathogen-specific DNA sequences in sample extracts up to a visual level, and this can be detected either by electrophoresis or combined with ELISA. An advantage of PCR over other DNA-based techniques is that no probes are needed for detection of the amplified product. With viruses and PSTVd, a preceding reverse-transcription step is required to obtain a DNA copy of the RNA genome.<sup>3</sup> PCR-based techniques are qualitative and extremely sensitive. Therefore, they will probably become increasingly important in plant quarantine for the detection of pathogens with low tolerance levels. Furthermore, PCR methods require simpler procedures, equipment and supplies, and these technologies can be transferred rapidly to programs in developing countries for research and plant quarantine. PCR also has applications for other biological constraints such as the detection of toxic substances for food safety and low levels of insect infestations. Also, even a detection of evolving viruses can be achieved by modern techniques, and a prediction could be made for the change of a virus genome.<sup>66</sup>

## IN VITRO TECHNIQUES IN SEED PRODUCTION

In vitro techniques on potatoes are of great value in germplasm conservation,<sup>6</sup> pathogen irradiation<sup>67</sup> and seed production. In this section, the seed production via micro-propagation is discussed for industrial development in many developing countries.

Many emerging regions such as Southeast Asia, Latin America, East Europe and CIS are now

demanding more seed potatoes.<sup>68</sup> The acreage varies from thousands of hectares to one million hectares depending on the countries, however, all countries need high quality seeds to sustain production and to make the potatoes more competitive in markets.

Modestly developed Latin American countries have become successful in producing clean nuclear seed potatoes by in vitro microtuber production, and privatization has followed (Table 10.1). Due

**Table 10.1. Potato Production and seed statistics in selected newly emerging regions.**

Data\Country	Mexico	Argentina	Brazil	Poland	Russia	Hungary
Area (ha)	72,000	111,000	165,000	1,750,000	3,383,000	69,000
Yield (ton/ha)	16.8	18	14	17	11	16
Production (million ton)	1.2	2.0	2.4	29.6	36.9	1.132
Availability of public seed system	Yes	Yes	No/depends on local programs	Yes	Yes, not functional	No
Availability of quarantine system	Yes, but slow	Yes	Yes, but slow	Yes	Yes, not functional	No
Availability of commercial in vitro lab for micro-tubers	15-20 labs saturating the market	Small labs incapable of covering all demands	Small labs incapable of covering all demands	Subsidized by government/ planned for privatization	Subsidized by government/ planned for privatization	No/Entry of Europeans/ North Americans?
Average production cost (US\$)	5,000	3,000	5,000	Subsidized	Subsidized	Subsidized
In vitro microtuber (\$/unit)	0.5-1.1	0.2-0.5	0.5-1.0	Variable	1.0	Variable
Mini tuber (\$/unit)	0.2-0.5 (10-30g)	0.3-0.5 (10-30g)	0.3-0.7 Very variable	Variable	2/kg	Variable
Basic seed (US\$/ 50 kg/bag)	15-50	25	15-? Very variable	Variable	Variable	Variable
Certified seed (US\$/ 50 kg/bag)	20	12	15-70	Variable	Variable	Variable
Ware potato (US\$/ 50 kg/bag)	8-25	6-10	10-50	Variable	Variable	Variable
Uses (%) fresh/feed/ seeds/ processing/ loss	79/0/4/8/9	84/0/5/1/10	79/0/13/1/8	20/44/15/8/12	34/27/25/6/8	54/20/16/3/6
Export (000 t)	1	0	6	464	0	0
Import (000 t)	29	8	1	29	50	Some
Major cultivars	Alpha (70%) Atlantic Kennebec	Bintje Spunta D'Arcy	Spunta Kennebec Araucana	Own	Own	Own
Sources	INIFAP/ PICTIPAPA	INTA-Balcarce	CNPH/ EMBRAPA	Potato Res. Inst. Molochow	Pushochino State Univ., Russia /USAID	National Potato Program

The figures were also verified with CIP (1995)<sup>1</sup>

to instability in the economy, however, such a privatized seed program could disappear, as it did in Cotia Cooperatives in Brazil. Constant support from the public and from federal/national programs is a key factor in their long-term continuity. In many Latin American countries, especially at the South cone, the purchasing power of the growers increased for materials in production and the demand for high-quality seeds became more significant. Economic strength will therefore be provided to the seed industry at a more subsistent level.

Eastern Europeans and CIS have large production areas that could be two to ten times as wide as the average of other regions (Table 10.1).<sup>1</sup> The yield is about half to one-third (10-15 t/ha) of the well-advanced countries (35-40 t/ha), which could be improved by supplying high-quality seeds, together with appropriate integrated pest management, general field practice and post-harvest control. Since these countries have started privatizing industries, seed businesses would be welcomed and made feasible by favorable factors in policy, economics and national will. The seed prices of the Eastern European countries are variable due to changes in the prices of ware potatoes, which are frequently also used as seed potatoes. An average of 50 bags (50 kg x 50= 2.5 ton) of seed potatoes is required to plant one hectare, therefore making high-quality seed potatoes would provide a large amount of cash profit and also benefit local industry as well as food supplies. Growers are becoming educated and wish to buy high-quality seeds, but due to high prices and lack of availability, access to high-quality seeds is difficult.

The price of the seed potato (US \$3000-5000/ha) is an important factor in production/management in many regions, and reduction in cost and time spent on seed production is another important factor in alleviating present problems in potato production. Generally speaking, these factors are required to increase in vitro microtuber production for reducing costs and for meeting emerging demands. Micropropagation is widely used for the clean production of propagules of vegetatively propagated crops. Small-scale micropropagation of potatoes for commercial seed production already has been adapted for use by many lesser-developed countries such as the ASEAN nations, Vietnam, China, Mexico and southern cone nations in South America and North African countries.<sup>3,69-71</sup> A large-scale operation to produce millions of propagules requires a high level of quality control to prevent unacceptable contamination that could require specific and expensive facilities. Commercial production of vegetative propagules,

however, will be spread and adapted quickly to low-profile operation at the subsistence farmer level.

Large-scale as well as low-profile technology in clean seed production photoautotrophy, which does not require major carbon supplies could be suited for scaling up clean microtuber production with low contamination.<sup>72,73</sup> Since a low-profile facility using common industrial materials rather than expensive laboratory-specific commodities can be adapted to in vitro seed production under the photoautotrophic system, this system could lead to low costs regarding facility and lower requirements for operation in seed production. Applications of photoautotrophy based on in vitro systems have been conducted in developing countries and achievements have been demonstrated (see section II of Altman and Watanabe 1995).<sup>74</sup>

## CONCLUSION: THE POTATO AS A MODEL CROP

The potato is widely used as a research material in plant sciences, and is discussed in detail in many textbooks. Use of the potato in biotechnology and as genetic resource for industrial development could also be a good model for many other plant species.

The use of plant genetic resources and biotechnology in cultivar development and seed production helps tremendously in potato production. Besides avoiding devastating biological constraints such as late blight, requires a huge amount of fungicide applications over the production period,<sup>12</sup> improved cultivars help lower production costs and agronomic practices, and thus are more suitable for subsistence farmers. Also, improved seed quality could boost production and benefit industrial users as well as small farmers. In R&D on transgenic potatoes, cases in testing safety issues would be good models for many nations/programs, and the proprietary technology transfer associated with genetic engineering could also function as prototypes for other cases in different commodities.

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## CHAPTER 11

# POTENTIAL OF PLANT BIOTECHNOLOGY APPLICATIONS IN GENETIC RESOURCES MANAGEMENT AND UTILIZATION OF ANDEAN LANDRACE CROP GENETIC RESOURCES

K. Watanabe and C. Arbizu

## INTRODUCTION

There are many locally important plant species in the Andean region that must be protected and improved in the region, and that could be employed in other parts of the world. Four areas of biotechnology could assist in genetic resources management and utilization of underexploited Andean domestic crops: 1) in vitro germplasm conservation with tissue culture and cryoconservation; 2) genetic fingerprinting of collections and monitoring genetic diversity with molecular markers; 3) diagnosis of plant diseases and clean-seed propagation; and 4) potentially, the application of genetic engineering. Among the diverse crops of the Andean region, Andean root and tuber crops (ARTCs) are the topic of this chapter; other important plant species are also briefly discussed.

## ANDEAN ROOT AND TUBER CROPS (ARTCs)

### FEATURES AND CONSTRAINTS OF ARTCs

Plant biotechnology tools assist in the management and utilization of potato and sweet potato genetic resources.<sup>1</sup> These biotechnological tools and strategies for their applications could also be useful in managing at least nine species of Andean root and tuber crops, listed according to their economic importance in the Andean region (Table 11.1). These crops have balanced nutritional values comparable to potatoes,<sup>2</sup> however, due to their social status as poor man's food, their use has been limited.<sup>3</sup>

Ulluco, oca and mashua have been important components of potato-based farming systems for more than 5,000 years in the tuber-growing areas of the Andes.<sup>3-5</sup> Arracacha, yacón, achira, mauka and ahipa have also been grown for thousands of years in the Quechua zone of the warm Andean valleys as one of the main components of maize-

based farming systems.<sup>4</sup> Along with bitter potatoes, maca has played a major role in the herding-based economy of farmers living in the Puna zone of Peru above 4,000 m. Whereas maca and ahipa are seed-propagated crops, the others are vegetatively propagated.<sup>3,4</sup>

#### BIOTECHNOLOGY APPLICATIONS TO ARTCs

#### Genetic Resources Conservation and Micropagation on ARTCs

Ex situ conservation of the genetic resources of the nine species mentioned above has been attempted by 10 scientific institutes working in South America. In July 1995, some 2,034 acces-

sions of ulluco, 3,282 of oca, 725 of mashua, 921 of arracacha, 105 of yacón, 108 of achira, 48 of maca, 2 of mauka and 2 of ahipa were being maintained by germplasm banks working with Andean root and tuber crops (Arbizu and Holle, unpublished data).

In vitro conservation of ulluco, oca and mashua tubers has been carried out for about 10 years by several Andean gene banks and, as a result, more than 60% of the ullucos, ocas and mashuas are being maintained in vitro. In the past three years, evidence has shown that more than 90% of maukas and 50% of yacóns have also been maintained in vitro. The entire collection of arracacha and achira, however, along with about 50% of yacóns, 40% of

**Table 11.1. Main features of Andean root and tuber crops**

Crops	Botanical name	Family	Edible part	Altitude grown (m)	Current uses	Potential uses	Pitfalls
Ulluco	<i>Ullucus tuberosus</i>	Basellaceae	Tuber	3,000-4,000	Staple (soup, stew) Medicine	Cash crop Medicine	Short shelf life
Oca	<i>Oxalis</i> <i>tuberosa</i>	Oxalidaceae	Tuber	3,000-4,000	Staple (boiled and baked)	Industrial starch	Short shelf life Oxalic acid bitterness
Mashua	<i>Tropaeolum</i> <i>tuberosum</i>	Tropaeolaceae	Tuber	3,000-4,000	Staple (boiled and baked)	Industrial starch Medicine	Short shelf life Glucosinolate toxins
Arracacha	<i>Arracacia</i> <i>xanthorrhiza</i>	Apocynaceae	Root	1,000-3,200	Staple (soup and pudding) Staple (baked, industrial starch)	Industrial snack food	Short shelf life
Achira	<i>Canna edulis</i>	Cannaceae	Rhizome	2,000-2,700	Staple (baked, industrial starch)	Noodles, bakery products	Low yield Competition with grain crops
Yacón	<i>Polymnia sonchifolia</i>	Asteraceae	Root	1,300-3,200	Snacks	Industrial starch (diet and diabetic food)	Late maturity
Maca	<i>Lepidium meyenii</i>	Brassicaceae	Hypocotyl	3,900-4,500	Salad porridge, juice, cocktail mix	Medicine	Limited adaptation Glucosinolate toxins?
Mauka	<i>Mirabilis</i> <i>expansa</i>	Nyctaginaceae	Root	2,300-3,200	Salty and sweet preserves	Industrial starch	
Ahipa	<i>Pachyrhizus</i> <i>ahipa</i>	Leguminosae	Root	1,500-3,000	Green and fruit salads	Cash crop, Bio-insecticide	
Potato	<i>Solanum</i> <i>tuberosum</i>	Solanaceae	Tuber	3,000-4,000	Staple	Global hunger solution	

ullucos, ocas and mashuas and 10% of maukas, appears not to have been introduced in vitro yet. They have been maintained as field collections. Insufficient funding and biotic and abiotic stresses are the main constraints to their conservation under field conditions. The International Potato Center (CIP) has played a major role in the conservation of the genetic resources of Andean root and tuber crops, in cooperation with nine South American National Agriculture Research Systems (NARS) working with this material. However, more logistic support is still needed to optimize their conservation and reach their full economic potentials.

Cryoconservation is an alternative to in vitro conservation of clonal germplasm. Technically speaking, many root and tuber crops, including common potatoes, can be maintained by cryoconservation, but no major gene bank has employed this system as the principal conservation system. Cost would be key issues for accepting this technology. For example, in the case of potatoes, an initial introduction of 5,000 in vitro clonal accessions into cryoconservation could cost US \$20,000, with annual maintenance costs of US \$3,000.<sup>6</sup> In comparison, a tissue culture-based sub-culturing system could cost US \$30,000 annually for the conservation of the same number of accessions and further subcultures could cause mislabeling of accessions and contamination. Cryoconservation significantly reduces these risks. The current recovery rate of shoot tips after cryoconservation is very high in potato—more than 90% (Steponkus, personal communication)—but no long-term or large-scale testing has been done for root and tuber crops in general; this could hamper the use of cryoconservation technology.

### Monitoring Genetic Variation and Fingerprinting in ARTCs

Organizing a germplasm collection is often cumbersome, as the clonal accessions have to be evaluated for duplicate identification, as well as genetic diversity. Molecular markers can assist in these activities to more efficiently establish core collections. Furthermore, these same tools can be used for biosystematic studies and in situ management of germplasm.

The genetic diversity of Andean root and tuber crops is not well-known, although distinct phenotypic groups are recognized including different tuber colors and shapes. Because Andean root and tuber crops are underexploited, a rapid assessment of genetic variation in nature, farmers' fields and known collections and varieties is needed. The goal is: 1) to maintain genetic diversity; 2) to identify cultivars; and 3) to establish a comprehensive core

collection. In many national genetic resources programs, in collaboration with international gene banks, Evaluations are conducted using isozymes and protein-gel electrophoresis. The use of DNA marker-based tools such as RFLP and PCR-based RAPD, STS, DAF and SSR is widely recommended for many crop general. However, simple tools could be applied initially with these Andean root and tuber crops for cost-effectiveness and because fewer skills are required for their operation in resource-limited Andean gene banks.<sup>7</sup>

### Plant Quarantine and Seeds

Although pests and diseases of Andean root and tuber crops have not been well surveyed, some virus-like diseases are seen in crops such as ulluco, oca and arracacha.<sup>8</sup> A general assessment of pests is also essential to maintain and improve crop productivity and genetic resources. In order to enhance productivity, we need to diagnose plant diseases and produce clean propagules efficiently.

For example, for diseases caused by viruses and viroids, an ELISA (enzyme-linked immunosorbent assay) or NASH (nucleic acid spot hybridization) test should be effective, while identification and characterization of virus-caused diseases should be enhanced and a service system should be established to provide antisera for ELISA and labeled probing for NASH, as widely demonstrated with common potatoes by CIP.<sup>6</sup> A PCR-based detection system for fungal and bacterial diseases could also be an effective tool. However, basic technology for detecting diseases should previously be well-established and widely distributed.

Seed production of the vegetatively propagated species using micropropagation can be enhanced and following production by the assistance of plant disease diagnostics.<sup>4,8</sup> The accumulated knowledge shows that virus detection and irradiation are important for these vegetatively propagated species; for example, oca yield can be improved up to several folds by using virus-free seeds and secondary infection to other crops at the same farms or in rotation could be avoided.

### Potential for Genetic Engineering on ARTCs

Although the main constraints in cultivating of Andean root and tuber crops have been pointed out,<sup>4,5</sup> the traits to be improved have not yet been indicated in detail. Therefore, more surveys on biotic and abiotic constraints should be conducted to see whether a plant quarantine and seed program in conjunction with efforts on integrated pest management and crop management could improve crop production or whether the genetic

improvement of crops is essential for increasing productivity and sustainability.

Potential areas for genetic studies include: 1) improvement of the quantity and quality of nutritional traits, and a reduction in toxic substances; 2) day-length adaptation; and 3) dormancy. The conventional breeding approach may be inappropriate as the basic reproductive biology of these root and tuber crops is not well understood and a true seed production system has not been established for many of them. Furthermore, many of these root and tuber crops are polyploids with an outcrossing nature, so a complicated segregation of the trait of interest could occur. This would be a disadvantage in conventional breeding, as demonstrated in potatoes and sweet potatoes. However, wild relatives and closely related crop genera are available for Andean root and tuber crops and molecular marker-assisted germplasm enhancement may be an approach to improve these crops and make them available to farmers.

Genetic engineering could facilitate the improvement of Andean root and tuber crops. Two specific areas are given below. First, glucosinolate content appears to be high in mashua and may cause goiter; on the other hand, glucosinulates and their biologically active metabolites may be responsible for medicinal uses of this particular Andean tuber.<sup>9</sup> These compounds and others may also provide variation in resistances to insects, nematodes and diseases, and genetic engineering could provide a change that would get rid of the goitrogenic effects of glucosinulates. Now, using genetic engineering, the manipulation of specific biochemical pathways is possible, so that, simultaneously, specific fractions of toxic substances are controlled while useful components are increased.<sup>10</sup> This can also be applied to other crops such as oca, to reduce unpleasant tastes that may come from oxalic acids and glycoalkaloids. Yacón has been studied for use of its fructose in foods for diabetics. Also, high molecular weight carbohydrates could meet specific industrial needs.<sup>11</sup> Because of their special characteristics, these crops have been adopted by non-conventional regions such as New Zealand, Japan and Italy.

## OTHER ANDEAN CROPS OF IMPORTANCE

ARTCs are important among various crops grown in Andean regions in terms of their sustainability and productivity under the harsh conditions of the highland Andes. Andean farmers have been cultivating diverse crops using hillsides and enormous altitude difference of 2,000–3,000 m.<sup>12,13</sup> Botany books like *Lost Crops of Incas*<sup>3</sup>

give general ethnobotanical information on these crops. Various references are also available in Spanish such as Cárdenes (1969) and Léon (1968)(listed in reference 3). Other potentially important crops besides ARTCs in conventional and non-conventional regions follow: 1) grains and quinoa (*Chenopodium quinoa*) and kiwicha (grain amaranth, *Amaranthus caudatus*); 2) Grain legumes: nuñas (Popping beans, *Phaseolus vulgaris*) and tarwi (*Lupinus mutabilis*); 3) fruits: cherimoya (*Annona cherimola*), passionfruits (*Passiflora spp.*), and pepino dulce (*Solanum muricatum*).

These Andean endemic crops are now being employed in non-conventional regions, as previously discussed. Many of them are grown in the developed world for specific commercial needs or to meet the demand of specific ethnic groups.<sup>14</sup> Indeed, some of these locally important crops have been exported to non-conventional regions such as popping beans for snack food, tarwi for diet food, quinoa for diet food, especially for infants, and passionfruits for table consumption and juice processing. Also, some species have been widely employed in research on agro-biotechnology, enhancing valuable traits from these species to commonly grow to related crop species such as use of pepino and other solanaceous fruit species for comparative molecular genetic study with tomato (Alpert and Tanksley, personal communication). Quinoa could be a good example of a species used for R&D and then subsequently commercialized.<sup>15</sup>

## NATURAL SPECIES FOR MEDICINAL USES

Intriguing domestic plant species available in the Andean region could attract increasing interest of the pharmaceutical industry and consumers of herbal medicines.<sup>16</sup> Many plant species used in traditional medicine are not fully understood; very little is known about the chemistry of the specific substance responsible for the medicinal value.<sup>17</sup> General information is presented in chapter 16 and specific species of the Andean region are presented in this chapter.

As most of these species from the Andean region are wild, it is important to enhance conservation together with general environmental protection and to exploit alternate industrial production methods in order to avoid the destruction of natural resources. Three examples from the Andean region follow:

First, uña de gato (*Uncaria tomentosa*) exists on the hillside of selva and is regarded as ginseng of the Andes. While the roots of ginseng are used as a remedy, the bark of this tree species is used for making remedies. Since the slash-and-burn method

is the only available way to harvest the material, protection of the species and a rapid production system of the seedlings should be urgently implemented. Otherwise, environmental destruction could rapidly advance thereby endangering the species. Micropropagation of seedlings could provide an opportunity to make a specific nursery production for commercial uses. However, the biochemical components of the medicinal values have not been well-identified. In order to address present biological questions and to avoid constraints on its uses, north and south as well as intersectorial partnerships for exploiting such valuable plant genetic resources should be enhanced equitably.

Second, chanca piedra (*Phianthus niuri*) are common weedy plants in the mid-highlands and are used as traditional medicine for kidney disease. It is also known to be effective against cancers and AIDS. Since these weedy plants are true-seed propagated, farming them is possible in both conventional and non-conventional regions. However, a pathological study on possible pests and the tools to detect them is essential for appropriate transfer and use of these genetic resources in non-conventional regions.

Third, ARTCs can also affect human fertility. While mashua, for example, supposedly decreases male fertility,<sup>9</sup> maca, rich in vitamins, is believed to increase female fertility. Many commercial products such as tablets and capsules are available in the local markets of Peru.

## SUMMARY

These Andean crops could be of value for non-conventional production regions, as well as the original production areas. However, it should be noted that these crops are endangered in general due to the development and strong commercialization of industrialized crops.<sup>18,19</sup> Many plant species have been exported from their centers of origin and diversity to non-conventional regions,<sup>20,21</sup> including potatoes, tomatoes, corns, beans, peppers and squashes from the Andean region. These "hidden" plant species, as discussed in this chapter, shall be recognized for complementing regional needs or adding uniqueness to the uses in non-conventional areas. Such uses have been introduced in a number of developed countries.<sup>16</sup>

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## CHAPTER 12

# TOBACCO GENETIC RESOURCES AND BIOTECHNOLOGY

T. Kubo

### INTRODUCTION

Tobacco is one of the most economically important non-food crops in world agriculture.<sup>1</sup> The world production of cured tobacco leaves in 1991 was 7.7 million tons in 4.8 million ha.<sup>2</sup> According to 1992 statistics, the major producers of tobacco are China (3,121,000 ton); U.S.A. (753,000 tons), India (560,000 tons), Brazil (414,000 tons) and Turkey (247,000 tons). Tobacco cultivars are divided into groups according to the method of post-harvest handling, called curing. The major groups of cultivars are flue-cured tobacco, burley tobacco, cigar and Turkish tobacco.<sup>3</sup> Harvested leaves of flue-cured tobacco varieties are contained for several days during the curing process in a facility with artificial heating and forced ventilation at the predetermined temperature and moisture provided in the standardized curing program. Other varieties are cured with natural ventilation. Within each group, cultivars have common genetic components and display group-specific characteristics required as raw materials, which contribute to the aroma, taste or flavor of tobacco products.<sup>4</sup> The market price of tobacco leaves varies with a large deviation and the shipping price of the leaves ranged from US \$1,120 to \$6,110/ton in 1985.

According to botanical classification (Table 12.1), tobacco belongs to the Solanaceae family and to the genus *Nicotiana*. Among the 66 recognized species of the genus *Nicotiana*, most of which are found in the Americas and Australia, *N. tabacum*, called tobacco, is the most widely grown species for commercial production, although its wild specimen has not been found yet.<sup>5</sup> The wild species in the genus *Nicotiana* have served as genetic resources to provide useful traits, such as disease resistance, in tobacco genetic improvement. A number of attempts have been made to introduce disease resistance into tobacco by inter-specific hybridization.<sup>5</sup>

Tobacco is an excellent material for model experiments on plant genetics.<sup>6</sup> It is easy to control pollination and to obtain a large quantity of seeds and progeny without difficulty. Also in recent studies of plant biotechnology, investigators have demonstrated that tobacco is suitable for tissue culture and genetic transformation.<sup>5</sup> It has a superior ability to regenerate from callus to differentiated shoots and then into entire plants, with great reliability.<sup>7</sup> Haploid plants can be obtained with high efficiency in tobacco anther culture. Protoplasts, isolated from tobacco leaves or cell suspension culture, readily start cell division in vitro and result in the formation of regenerated plants through the tissue culture process. Somatic hybrids, through protoplast fusion, have been obtained in tobacco in a number of combinations with different species. Genetic transformation in tobacco has been extensively performed by the *Agrobacterium*-mediated system.<sup>5,8</sup>

**Table 12.1. Species in the genus Nicotiana and their resistance to pests<sup>65</sup>**

Subgenus	Section	Species	Chromosome number (2n)	Source of resistance to: <sup>a</sup>	
Rustica	Paniculata	<i>glaуca</i>	24		
		<i>paniculata</i>	24		
		<i>knightiana</i>	24	BM	
		<i>solanifolia</i>	24		
		<i>benavidesii</i>	24		
		<i>cordifolia</i>	24		
		<i>raimondii</i>	24	PVY	
	Thysiflorae	<i>thysiflora</i>	24		
		<i>rustica</i>	48	WF, BRR, BM	
Tabacum	Tomentosae	<i>tomentosa</i>	24	RKN, PVY	
		<i>tomentosiformis</i>	24	BM	
		<i>otophora</i>	24	RKN, PVY	
		<i>kawakamii</i>	24	PVY	
		<i>setchellii</i>	24		
		<i>glutinosa</i>	24	TMV, PM	
	Genuinae	<i>tabacum</i>	48	BS, BRR, PM, PVY	
Petunioides	Undulatae	<i>undulata</i>	24		
		<i>noctiflora</i>	24		
		<i>petunitoides</i>	24		
		<i>acaulis</i>	24		
		<i>ameghinoi</i>	24		
	Acuminatae	<i>acuminata</i>	24		
		<i>pauciflora</i>	24		
		<i>attenuata</i>	24		
		<i>longibracteata</i>	24		
		<i>miersii</i>	24		
		<i>corymbosa</i>	24		
		<i>linearis</i>	24		
	Bigelovianae	<i>spegazzinii</i>	24		
		<i>bigelovii</i>	48		
		<i>clevelandii</i>	48		
	Nudicaules	<i>nudicaulis</i>	48	WF, BRR, AR	
		<i>bethamiana</i>	38		
		<i>umbratica</i>	46		
		<i>cavicola</i>	40		
		<i>debneyi</i>	48	BM, BRR, PM GP, CW	
		<i>gossei</i>	36		
	Suaveolentes	<i>amplexicaulis</i>	36		
		<i>maritima</i>	32		
		<i>arentsii</i>	48		
		<i>wigandiodoides</i>	24		
	Trygonophyllae	<i>trygonophylla</i>	24		

**Table 12.1. (continued)**

Subgenus	Section	Species	Chromosome number (2n)	Source of resistance to: <sup>a</sup>
Petunioides (continued)	Alatae	<i>sylvestris</i>	24	
		<i>langsdorffii</i>	18	
		<i>alata</i>	18	
		<i>forgetiana</i>	18	
		<i>bonariensis</i>	18	
		<i>longiflora</i>	20	BS, WF, TMV
		<i>plumbaginiflora</i>	20	BS, RKN
	Repandae	<i>repanda</i>	48	TMV, RKN, FE, ALS, WF
		<i>stockonii</i>	48	
		<i>nesophilla</i>	48	
		<i>velutina</i>	32	BM
		<i>hesperis</i>	46	
		<i>occidentalis</i>	42	
		<i>simulans</i>	40	
		<i>megalosiphon</i>	40	BM
		<i>rotundifolia</i>	44	
		<i>excelsior</i>	38	BM
		<i>suaveolens</i>	32	
		<i>ingulba</i>	40	
		<i>exigua</i>	32	BM
		<i>goodspeedii</i>	40	BM
		<i>rosulata</i>	40	
		<i>fragrans</i>	48	
		<i>africana</i>	46	

a)ALS, Angular leaf spot, *Pseudomonas syringae* pv. *tabaci*; AR, Anthracnose, *Colletotrichum tabacum*; BS, Black shank, *Phytophthora parasitica* var. *nicotianae*; BM, Blue mold, *Peronospora tabacina*; BRR, Black root rot, *Thielaviopsis basicola*; FE, Frog-eye, *Cercospora nicotianae*; PM, Powdery mildew, *Erysiphe cichoracearum*; WF, Wildfire, *Pseudomonas syringae* pv. *tabaci*; RKN, Root knot nematode, *Meloidogyne* species; GP, Green peach aphid, *Myzus persicae*; CW, Cutworm, *Spodoptera litura*; PVY, Potato virus Y; TMV, Tobacco mosaic virus.

As mentioned above, tobacco is not only of agricultural interest, but also of interest as model experimental material in plant genetics. In many cases, tobacco has been used as a model crop in the development of new technologies. It is, therefore, very helpful to review the research in tobacco, in order to comprehend the developments that have occurred in the area of plant breeding and genetics. In this chapter, a description of tobacco germplasm available in conventional breeding and tobacco research in genetic engineering is presented. Future status of tobacco breeding is also discussed.

## GERMPLASM AND CONVENTIONAL BREEDING OF TOBACCO

### BREEDING FOR PEST RESISTANCE

The most important traits of tobacco cultivars are disease resistance and leaf quality. The history

of tobacco breeding, therefore, is to develop superior varieties with useful resistance to disease, in order to attain better performance in the field, while maintaining the desirable quality of leaves as the source material for cigarettes. There are a number of diseases that can sometimes cause devastating damages to tobacco.<sup>9,10</sup> The major viruses infecting tobacco are tobacco mosaic virus (TMV), cucumber mosaic virus (CMV) and potato virus Y (PVY). Among the fungal diseases black shank caused by *Phytophthora parasitica* var. *nicotianae*, blue mold by *Peronospora tabacina*, and black root rot by *Thielaviopsis basicola* result in serious damage. In addition, powdery mildew caused by *Erysiphe cichoracearum* and brown spot by *Alternaria longipes* often cause significant losses in some areas. A bacterial disease called bacterial wilt, caused by *Pseudomonas solanacearum*, also often leads to serious damage in the areas where the temperature during growing season is relatively high. Wildfire

caused by *P. syringae* pv. *tabaci* is another bacterial disease to be cautiously noted. Root knot nematodes, *Meloidogyne* species and cyst nematodes, *Heterodera* species, also attack tobacco plants resulting in considerable losses.

Breeding for resistance to these diseases has been extensively performed during this century by using resistant germplasm within tobacco. Breeders also surveyed for resistance in the wild *Nicotiana* species and attempted to transfer it to tobacco by inter-specific hybridization followed by successive backcrosses.<sup>10</sup>

There are some examples of resistance found in tobacco and utilized for practical breeding.<sup>9,10</sup> The resistance to PVY, governed by a single recessive factor, was found in a European variety, Virgin A Mutant, and served as the resistance source in Europe and Japan. A high resistance to powdery mildew, controlled by double recessive factors, was found in a Japanese air-cured variety, Kuo-fam. Florida 301 was the source of resistance to black shank disease for most of the current cultivars, though the resistance is quantitatively inherited and hence the breeding process was not simple. From a Canadian cultivar called Little Dutch, a number of present cultivars resistant to black root rot have been developed. The resistance was governed by polygenic factors. Bacterial wilt is one of the most serious diseases in tobacco. It infects the vascular tissue of plants and causes their death. As yet, no clear-cut resistant germplasm has been found; however, breeders have utilized polygenic resistance derived from TI 448A.

The first attempt to transfer the resistance of the wild *Nicotiana* species to tobacco was carried out by Holmes.<sup>10</sup> He backcrossed the amphidiploid of two species, *N. tabacum* and *N. glutinosa*, to tobacco to transfer the TMV resistance that *N. glutinosa* possesses. After a long series of successive backcrossing, he finally succeeded in obtaining a line of tobacco having a very short chromosomal segment of *N. glutinosa*, responsible for the hypersensitive reaction and the resistance to TMV. The resistance gene, called the *N* gene, has been widely used in current commercial varieties of burley tobaccos.

Following the success of the TMV resistance, the resistance to blue mold and to black root rot was respectively transferred from *N. debneyi* to tobacco by the similar breeding method, and the resistance to black shank was transferred from *N. plumbaginifolia*.<sup>10</sup> The resistance to wildfire, transferred from *N. longiflora*, is also currently used in a wide range of commercial varieties. The resistance to one species of root knot nematode, *Meloidogyne incognita*, was introduced from *N.*

*tomentosiformis* by crossing tobacco with the amphidiploid of the putative progenitor species, *N. sylvestris* and *N. tomentosiformis*.<sup>9</sup> The resistance mentioned above is mostly governed by single dominant factors and easily obtained introgression into a new genetic background by simple repeated backcrossing. Besides, sometimes such resistance of wild species origin showed advantage of a higher level of resistance than that found in tobacco. In some cases, however, unfavorable characteristics have been observed to be associated with the resistance, probably due to the alien genes closely linked and introduced with the resistance.

Thus far, no effective germplasm has been found as a source of resistance to CMV. Considerable effort has been exerted to find such a germplasm in the genus *Nicotiana*, but only a slight reduction in the development of mosaic symptoms was shown by some tobacco plants. Researchers in Taiwan reported that they have developed a variety that shows some degree of resistance to CMV. This was achieved by using Holmes' line whose resistance is controlled by five factors, the N factor from *N. glutinosa*, two factors from Ambalema and another two factors from TI 245.<sup>11</sup>

There are a number of insect pests on tobacco, but there are few germplasms known to be sources of resistance to insects.<sup>12</sup> TI 1112 which has non-glandular trichomes and thus little exudate on its leaf surface was found to be resistant to green peach aphid and tobacco budworm, due to non-preference of the insects. Another breeding line, called I-35, showed resistance to tobacco hornworm. The origin of this resistance has not yet been elucidated.<sup>13</sup> No other germplasm in tobacco has been reported as a source of resistance to insects. However, Rao et al made an extensive screening of the wild *Nicotiana* species and found that *N. gossei* provides toxic effects to aphids, *Myzus persicae* and caterpillars, *Spodoptera litura*. They attempted to introduce the resistance of *N. gossei* to tobacco through an inter-specific cross followed by backcrosses.<sup>14</sup>

As mentioned above, a certain number of disease resistances have been made available by conventional breeding, and some of them are used extensively in the commercial varieties of tobacco. Two problems should be noted to improve breeding for disease resistance. First, there are still a number of diseases and pests for which no resistance source is available, especially insect pests. Moreover, considering the fact that there are variations among the races differentiated by the areas of cultivation, we need more sources of resistance to control diseases and pests. Secondly, even though a resistance may be available, there are limitations

in some cases, due to the association with undesirable traits. To avoid such circumstances, only the segment of DNA responsible for resistance should be incorporated into tobacco. DNA marker technology is believed to alleviate the latter problem.<sup>15</sup>

### Other Traits of Interest in Tobacco Breeding

There are some other traits that are relatively specific to tobacco plants. Leaf surface lipids contribute to the smoke quality of tobacco. Two major diterpenes have been found in tobacco varieties, namely *cis*-abienol and duvatriene-diol.<sup>16</sup> Alkaloid composition is another important character in tobacco because it greatly affects the quality of the smoke. One germplasm, called LA Burley 21, is showing a low alkaloid content, which is about one-fifth of that of the ordinary varieties. This characteristic was analyzed and known to be controlled by two recessive factors. Hibi et al<sup>17</sup> analyzed these genes responsible for low alkaloids, and isolated a gene associated with low nicotine production. Cytoplasmic male sterility (CMS) was observed in tobacco with an alien cytoplasm from an inter-specific cross between *Nicotiana* wild species and tobacco followed by successive back-crosses to tobacco.<sup>18</sup> CMS is used for the seed production of F1 hybrid varieties, mainly of burley tobacco. Asymmetric protoplast fusion provides an immediate method to substitute the cytoplasmic genome and to produce CMS lines.<sup>19</sup>

## BIOTECHNOLOGY IN TOBACCO FOR PEST RESISTANCE

A number of useful genes to control disease and pests have been introduced into the tobacco genome. It is common to engineer tobacco with alien genes by *Agrobacterium*-mediated genetic transformation. The examples of genetic engineering are summarized in the following to produce new traits ready to be used for the improvement of tobacco varieties (Table 12.2).

### RESISTANCE TO VIRUS DISEASES

Virus resistances have been successfully obtained through numerous strategies in plant biotechnology.<sup>20</sup> In tobacco, transgenic plants showing practical levels of resistance to some of the major virus pathogens have been produced. They are expected to be the new genetic resources in breeding for commercial varieties. Some of them are particularly important because they have characteristics that are not currently available among the conventional germplasm. It is of great interest to evaluate the ability of such transgenic plants under field conditions.

The first success in conferring disease resistance to plant was demonstrated in tobacco.<sup>21</sup> A cDNA fragment encoding the coat protein of TMV was introduced and expressed in tobacco plants under the control of a constitutive promoter. The transgenic plants showed a considerable delay in developing disease symptoms after TMV inoculation. Another strategy for virus resistance is the use of an antisense gene.<sup>22</sup> Antisense RNA genes, with and without ribozyme sequences, complementary to the 5' region of the viral genome, were constructed and introduced into tobacco. Some of the transgenic plants in a homozygous state stayed symptomless, even 21 days after inoculation of TMV. The effectiveness of the antisense RNA, in providing protection, was not enhanced by ribozyme sequences.

Efforts to explain the mechanism of the natural resistance of the *N* gene have been made for a long time, but until recently the gene product which triggers a hypersensitive reaction in a resistant host had remained unknown. Whitham et al<sup>23</sup> succeeded in isolating the *N* gene from a TMV resistant tobacco through transposon tagging, using the maize activator transposon. The isolated DNA fragment containing the *N* gene was confirmed to afford TMV resistance to a susceptible tobacco. The *N* gene is considered to have a function in a signal transduction pathway.<sup>24</sup>

The gene complementary to a CMV satellite RNA that attenuates the symptoms induced by CMV was transferred to tobacco.<sup>25</sup> In the transgenic plants, the replication of CMV after inoculation of CMV was greatly decreased and symptom development was suppressed to a large extent. The level of resistance observed depended on the dosage of such satellite RNA genes because plants homozygous for the transgene showed virus multiplication lower than their heterozygous counterparts.<sup>26</sup> The following strategies for CMV resistance were also taken: the CMV coat protein gene, or its antisense gene, was expressed in transgenic tobacco plants.<sup>27,28</sup> The coat protein gene significantly reduced the symptom development after CMV infection, but the antisense gene did not give clear evidence of protection.<sup>27</sup> In addition, transformation of tobacco, with a gene encoding a CMV replicase protein, also conferred resistance on the transgenic plants.<sup>29,30</sup> Recently, broad resistance to viruses was obtained by expressing an antisense RNA for S-adenosyl-homocysteine hydrolase (SAHH), an enzyme responsible for trans-methylation reactions. The transgenic tobacco plants in which the level of SAHH mRNA was considerably reduced exhibited lower susceptibility to CMV and other viruses.<sup>31</sup>

**Table 12.2. List of traits in tobacco made by genetic engineering**

Trait	Gene engineered
Virus resistance	
TMV(Tobacco mosaic virus)	TMV coat protein gene Anti-sense gene for a 5' region of TMV genome <i>N</i> gene from <i>N. glutinosa</i>
CMV(Cucumber mosaic virus)	CMV satellite RNA gene CMV coat protein gene CMV replicase gene Anti-sense gene of tobacco for S-adenosylhomocysteine hydrolase
PVY(Potato virus Y)	PVY coat protein gene PVY protease gene
Fungal resistance	
Sore shin ( <i>Rhizoctonia solani</i> )	Pea chitinase gene Ribosome-inactivating protein gene from barley Chitinase, glucanase and ribosome- inactivating protein genes from barley
Frog-eye ( <i>Cercospora nicotianae</i> )	Rice chitinase and alfalfa glucanase genes
Blue mold ( <i>Peronospora tabacina</i> )	PR 1a gene of tobacco
Black shank ( <i>Phytophthora parasitica</i> var. <i>nicotianae</i> )	PR 1a gene of tobacco
Brown spot ( <i>Alternaria longipes</i> )	Radish gene of antifungal protein, RS-AFP2
Gray mold ( <i>Botrytis cinerea</i> )	Stilbene synthase gene of grapevine
Bacterial resistance	
Wildfire ( <i>Pseudomonas syringae</i> pv. <i>tabaci</i> )	Tabtoxin resistance gene of the wildfire pathogen Thionin gene of barley <i>Pto</i> gene of tomato
Bacterial wilt ( <i>Pseudomonas solanacearum</i> )	Analog of cecropin B gene of giant silk moth
Nematode resistance	
Root knot nematode ( <i>Meloidogyne</i> species)	Anti-sense gene of tobacco root specific gene
Insect resistance	
Hornworm ( <i>Manduca sexta</i> )	<i>Bacillus thuringiensis</i> toxin gene
Cutworm ( <i>Spodoptera exigua</i> )	<i>Bacillus thuringiensis</i> toxin gene
Budworm ( <i>Heliothis virescens</i> )	<i>Bacillus thuringiensis</i> toxin gene Cowpea trypsin inhibitor gene
Peach aphid ( <i>Myzus persicae</i> )	Lectin gene of snowdrop
Herbicide resistance	Phosphinothricin acetyltransferase gene of <i>Streptomyces hygroscopicus</i>
	Bromoxynil-specific nitrilase gene of <i>Klebsiella ozaenae</i>
Male sterility	Ribonuclease gene of <i>Bacillus</i> under the control of the tobacco anther specific promoter
Chilling tolerance	Glycerol-3-phosphate acyltransferase of <i>Arabidopsis</i>
Drought tolerance	Bacterial mannitol-1-phosphate dehydrogenase gene Pyrroline-5-carboxylate synthetase of mothbean Trehalose-6-phosphate synthase subunit 1 of yeast

PVY is one of the potyviruses that expresses a large primary polyprotein and produces mature virus proteins by virus-encoded proteases, cleaving proteolytic sites of the polyprotein. Expression of one of such proteases, NIa, resulted in resistance to PVY in the transgenic tobacco plants.<sup>32</sup> The resistant plants showed no disease symptoms over 50 days after inoculation of PVY. Tobacco plants transformed with a gene to express the PVY coat protein gene also exhibited a significant resistance to the virus.<sup>33,34</sup> Even the untranslatable mRNA of the coat protein gene proved to be highly effective in suppressing disease symptoms.

### RESISTANCE TO FUNGAL DISEASES

Introducing a gene encoding an anti-fungal peptide into tobacco by genetic transformation has provided a chance to produce fungus-resistant plants. Several anti-fungal proteins have been reported to be effective in conferring resistance on plants.

A success in one such attempt was made by expressing a chitinase gene in tobacco. Broglie et al<sup>35</sup> transformed tobacco with the gene encoding pea chitinase and observed that the transgenic plants exhibited elevated levels of chitinase activity by nearly two times in both shoots and roots and also resistance to a soil-born fungus, *Rhizoctonia solani*. The average mortality of resistant plants was less than half of that of the control in seedling inoculation tests. Logemann et al<sup>36</sup> isolated from barley seeds, a gene encoding a ribosome-inactivating protein (RIP) that inhibits fungal growth in vitro. Tobacco plants were transformed with the gene under the control of a wound-inducible promoter. Protection was demonstrated by better growth of the transgenic plants than the control in soil inoculated with the fungus.

The effect of the chitinase gene, in combination with genes encoding other anti-fungal peptides, was examined. Zhu et al<sup>37</sup> observed the synergistic effect of rice chitinase with alfalfa  $\beta$ -1,3-glucanase against *Cercospora nicotiana*. Jach et al<sup>38</sup> reported that a high level of expression of each of the barley genes, encoding class II chitinase (CHI),  $\beta$ -1,3-glucanase (GLU) and ribosome-inactivating protein (RIP), in transgenic tobacco plants resulted in an increased protection against *R. solani*. They also observed that co-expressing the transgenes with GLU/CHI or CHI/RIP revealed enhanced protection against fungal attack.

Pathogenesis-related (PR) proteins are known to be induced to high levels in tobacco, in response to the infection of a pathogen causing necrosis or the application of certain chemicals, such as salicylic acid. Plants chemically induced to express PR

proteins show a significant level of resistance to diseases. Alexander et al<sup>39</sup> introduced the gene of one of the PR proteins, PR 1a, and expressed it in transgenic tobacco. They observed that such plants showed tolerance to infection by two pathogens, *P. tabacina* and *P. parasitica* var. *nicotianae*.

A small cysteine-rich anti-fungal protein isolated from radish seeds, Rs-AFP2, exhibits anti-fungal activity in vitro.<sup>40</sup> The gene of Rs-AFP2 constitutively expressed in a homozygous state conferred enhanced resistance in transgenic tobacco to the pathogen, *A. longipes*, which causes a foliar disease, brown spot. Since there is no germplasm available to control the fungus, this result shall draw a great deal of attention.

Besides these anti-fungal proteins, phytoalexin has been considered to play an important role in the defense mechanism of plants. Hain et al<sup>41</sup> isolated stilbene synthase genes from grapevine and introduced them into the tobacco genome. Transgenic plants expressing these genes produced a high amount of stilbene-type phytoalexin resveratrol and showed resistance to *Botrytis cinerea*.

### RESISTANCE TO BACTERIAL DISEASES

The pathogen of wildfire, *P. syringae* pv. *tabaci*, produces a toxin called tabtoxin that causes the chlorotic symptom associated with the disease. It also has the detoxifying enzyme to protect itself from the toxin. Anzai et al<sup>42</sup> isolated, from the pathogen, the gene called tabtoxin resistance gene (*ttr*) which encodes an acetyl-transferase and introduced it into the tobacco genome. Transgenic tobacco plants showed high expression of the *ttr* gene and exhibited no disease symptoms after the infection of the pathogen. This approach to obtain disease resistance using detoxifying genes is considered effective when the pathogenic toxin is the causal element of the disease symptom.

Antibacterial peptides have been considered an effective source of resistance to bacteria. Cecropin B, a lytic peptide found in *Hyalophora cecropia*, the giant silk moth, is one such peptide, but its level of expression in transgenic plants is quite low possibly due to instability of this peptide in plant cells.<sup>43</sup> Instead, the gene encoding an analog of cecropin B was synthesized and incorporated into tobacco.<sup>44</sup> The transgenic plants expressing the analog were inoculated with the bacterial pathogen *P. solanacearum*, a causal agent for bacterial wilt, and exhibited delayed disease symptoms and reduced disease severity as compared to the control. Thionins are a group of proteins found in cereal endosperm that have a defense function. They were used to obtain a resistance to bacterial pathogens. Carmona et al<sup>45</sup> transferred the gene

encoding  $\alpha$ -thionin from barley endosperm to tobacco and showed that the transgenic plants exhibited an enhanced level of resistance to *P. syringae* pv. *tabaci*.

Cloning of the gene of a natural resistance to *P. syringae* in tomato has been successfully carried out by map-based positional cloning.<sup>46</sup> The gene called *Pto* was then transferred to tobacco. The transgenic plants showed a hypersensitive response to the pathogen and hence exhibited resistance.<sup>47</sup>

#### RESISTANCE TO NEMATODES

Recently, attempts to confer resistance to nematodes by engineering tobacco plants have been made. Opperman et al<sup>48</sup> observed that a root specific gene of tobacco, *TobRB7*, was induced to express during the development of a feeding site, which occurs upon infection of root knot nematodes. They identified the cis-acting sequences that mediate induction by the nematode. An anti-sense gene of *TobRB7* driven by the nematode-responsive promoter showed a considerable reduction of nematode infection in transgenic plants.<sup>49</sup> Since the promoter is responsive to all races of the three *Meloidogyne* species tested, this system is considered to be effective in controlling a wide range of nematodes that cause the same result in the feeding site.

#### RESISTANCE TO INSECTS

The gene encoding for the  $\delta$ -endotoxin of *Bacillus thuringiensis* (*Bt*) has been successfully expressed in transgenic tobacco plants and proved to be effective in controlling lepidopteran insect pests such as the tobacco hornworm, *Manduca sexta*.<sup>50</sup> Efforts have been made to obtain a higher expression level in order to increase the effectiveness of this technology. It includes the truncation of the 3' end of the gene,<sup>50</sup> the change of the nucleotide sequences to plant preferred codons (with the same amino acid sequences)<sup>51</sup> and the elimination of the potential polyadenylation signal sequences and instability motifs.<sup>52</sup> Such modifications are sufficient to obtain resistance against *S. exigua* and *Heliothis virescens*, which are less sensitive to the common strain of *B. thuringiensis*. Another strategy for high expression has been to incorporate the unmodified protoxin gene into the chloroplast genome of tobacco.<sup>53</sup> An extremely high level of accumulation in leaves, 3-5% of the soluble protein as protoxin, has been observed.

Several insecticidal proteins of plant origin such as proteinase inhibitor and lectins have been known to cause retardation of insect growth. Strategies have been taken to incorporate the genes encoding such non-*Bt* proteins into tobacco. Transgenic plants expressing a gene for a cowpea trypsin

inhibitor showed resistance to a lepidopteran insect, *H. virescens*.<sup>54</sup> Lectins isolated from the snowdrop, *Galanthus nivalis*, have also been reported as toxic to insects, particularly to homopterans such as aphids and plant hoppers.<sup>55</sup> Expressing the lectin gene in transgenic tobacco significantly reduced the build-up of aphid populations on the plants.<sup>56</sup> These insecticidal proteins are considered to have great potential in conferring insect resistance in combination with the *Bt* gene.

#### OTHER TRAITS GENETICALLY ENGINEERED IN TOBACCO

There are several other important traits engineered in tobacco in addition to pest resistances. They are herbicide resistance, male sterility and tolerance to environmental stresses. Herbicide resistance genes have been considered effective in weed control with broad spectrum of herbicides. Transformed plants, with genes encoding enzymes to detoxify respective herbicides, resulted in herbicide resistant tobacco and they were ready to be exposed to herbicide application after transplanting.<sup>57,58</sup> In hybrid seed production, male sterility is advantageous to facilitate cross pollination in many crops. Nuclear male sterile plants were obtained by transforming tobacco with a construct of a ribonuclease gene under the control of a promoter specific to anthers.<sup>59</sup> The tapetum cells, which feed pollen in the anthers, were destroyed by the gene expressed in the transgenic plants and the pollen formation was completely hindered to such an extent as to result in male sterility. Environmental stresses, such as chilling and drought, are obstacles in crop production. Transgenic tobacco plants tolerant to chilling injury have been obtained by engineering genes encoding enzymes in lipid metabolism to increase the rate of unsaturated fatty acids.<sup>60,61</sup> Drought tolerance was conferred upon tobacco by incorporating genes responsible for the accumulation of the osmoprotectants such as mannitol, proline or trehalose.<sup>62-64</sup>

#### CONTRIBUTION OF BIOTECHNOLOGY TO THE FUTURE OF TOBACCO BREEDING

As presented in this chapter, numerous kinds of transgenic tobacco plants with valuable traits have been created. This type of research aims to utilize such novel characteristics in combination with those of elite breeding lines developed by conventional breeding. The final goal is to combine all of the useful genes, regardless of natural or engineered origins, into a variety that maximizes the capacity of breeding. Such an ideotype variety will greatly contribute to the more efficient production

of tobacco with less application of agricultural chemicals, cultivation practice and processing processes. So far, transgenic plants have been presented as having an enormous potential to provide breeders with additional advantages, and soon it will be realized in actual breeding programs.

Since people consume tobacco to enjoy the aroma, taste and flavor, we put more emphasis on the quality of cured leaves than the quantity. But the essence of chemical basis determining the smoke quality has not been elucidated yet. It is very difficult to control such traits by engineering a few genes because these traits are considered to be controlled by a number of genes in a very complicated manner. In this sense, the use of traditional germplasm with superior quality will play an important role in tobacco breeding. However, as mentioned previously, in tobacco there are a number of cases where a single gene could improve a trait such as disease or pest resistance. On such a case, genetic engineering will offer a powerful tool for developing new varieties. When no appropriate germplasm is available, it is very valuable to engineer a new trait. For example, effective resistance to CMV or bacterial wilt has not been obtained yet, and the resistance to TMV or PVY is associated with other undesirable characteristics. Genetic engineering can alter only a part of the tobacco genome in order to keep the deleterious side effects of a new gene at minimum.

Plants respond to environmental stress or pathogens. Newly developed superior varieties should respond only when the expression of genes is needed. Gene expression can be controlled by promoters. It is essential to find out an appropriate promoter responsive to the signals from pathogens or abiotic stresses, so it will be able to express the gene as needed. Consequently, a combination of two technologies, conventional breeding for leaf quality and genetic engineering for pest resistances, will give us a greater chance to develop varieties of superior tobacco that previously did not exist.

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## CHAPTER 13

# FLOWER PRODUCTION IN JAPAN AND AGRIBIO BUSINESS AND TECHNOLOGY OF KIRIN: A CASE IN PRIVATE SECTOR APPROACH

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This chapter presents a specific case in a private sector approach on R&D in the flower business. A domestic strategy and also international approaches are provided by an interdisciplinary team in the private sector.

### FLOWER PRODUCTION IN JAPAN

#### TRENDS OF FLOWER PRODUCTION IN JAPAN

Total production value of agriculture has increased 2% in Japan during 1983-1993; in contrast, the production value of flowers has almost doubled over the last ten years. With a rapid improvement in living conditions and more demand on enjoying life, Japanese consumers have come to take more interest in flowers and green plants looking for comfort and amenities in their lives this resulted in a rapid and constant rise in flower production in Japan.

For cut flowers and potted plants, the building of greenhouses has been increasing constantly and a year-round production system has been introduced. The proportion of building of such facilities to the total production of cut flowers has risen to 40%; however, the figure is relatively low when compared with 70% in Holland.

Chrysanthemums occupy 35% of the total cut flower production, followed by roses and carnations. These crops make so-called big three of cut flowers in Japan (Table 13.1).

#### TRENDS OF CONSUMPTION IN JAPAN

A breakdown of consumption of cut flowers and potted plants in Japan is as follows: 40% in gift-use; 25% for commercial facilities, such as hotels and various types of events; 25% in home-use, including religious decorations for Buddhist practices; and 10% for educational purposes in teaching flower arrangement.

Recent tendencies in gift and commercial uses are the result of a strong demand for high-grade flowers. In contrast, for home use, end-consumers would like to purchase modest quality flowers for decent prices. In order to satisfy the different demands, production systems and supply channels for flowers have been re-examined and now, gradually, they are able to meet the demands of the market.

### TRENDS OF FLOWER IMPORT TO JAPAN

As flower consumption in Japan increases, the importation of flowers is rapidly growing; for example, 8% of cut flowers and 40% of bulb flowers were imported in 1993. Major exporting countries of cut flowers to Japan are Holland, Thailand, Taiwan, New Zealand and Singapore. Holland provides more than 90% of bulbflowers of the imported total to Japan. However, countries in Central and South America such as Mexico and Columbia, Kenya and South Africa in Africa, and the ASEANs are new exporters to Japan in recent years.

### AGRIBIO BUSINESS OF KIRIN

#### DIVERSIFICATION OF KIRIN

Formally established in 1907, but with its origin dating back to over a century ago, Kirin Brewery Company, Limited, is Japan's foremost beer company and the fourth largest in the world by sales volume. Moving into the future, Kirin puts its long-term strategy of diversification in five domains

of business activities with 15 business areas (Table 13.2). Applying the expertise and original technologies developed in the production and marketing on beer to other fields, Kirin has diversified its activities in the closely related fields of soft drinks, liquors, wines and foods. Besides beverages and foods, the Kirin group is also diversifying into other domains where it can apply the technological and management skills acquired through its beer-related activities. These domains include biotechnology, services, engineering and information systems.<sup>1</sup>

In biotechnology, Kirin puts considerable expertise and original technologies on fermentation, mass micro-propagation and selective breeding to the best uses in the fields of pharmaceuticals, agribio business and yeast-related business.

### AGRIBIO BUSINESS OF KIRIN

Kirin has built up its agribio business on a global scale, constructing a network of 14 affiliated companies worldwide (Table 13.3). In order to

**Table 13.1. Flower production in Japan<sup>a</sup>**

Crop / Division	Production Under glass	area (ha) Outdoors	Sub-total	Quantity (million)	Value (billion yen)	No. growers (thousand)
Total	9,836	37,753	47,589		614	150
Cut flowers	7,973	10,432	18,406	5,594	295	88
chrysanthemum	2,648	3,310	5,958	1,987	102	
carnation	612	4	615	672	30	
rose	583	2	585	474	31	
gentiana	27	603	630	99	5	
gypsophila	552	4	556	116	10	
Pot plants	1,535	291	1,826	211	101	11
cyclamen	237		237	20	14	
Bedding plants	327	213	540	228	12	
Ornamental trees		15,101	15,101	169	174	36
Bulbs		1,440	1,440	417	8	5
Lawn and ground cover plants		10,276	10,276	8	24	9

a) Source: Statistics in 1993 of the Japanese Ministry of Agriculture, Forestry and Fisheries.

**Table 13.2. Five domains and 15 areas of business activities in Kirin**

Five business domains	Fifteen business areas
Beverages and Foods	Domestic beer, International beer, Domestic Soft Drinks, International Soft Drinks, Hard Liquor, Foods
Biotechnology	Pharmaceuticals, Agribio, Biochemicals (yeast-related)
Services	Restaurants, Real Estate, Transportation, Other Services
Engineering	Engineering
Information Systems	Information Systems

support the business of these companies, Plant Laboratory of Agribio Business Division and Central Laboratories for Key Technology and Applied Bio-research Center of Corporate R&D Division strive to develop and commercialize our own competitive technology. The main activities of the Agribio Business Division are directed toward business in floriculture (chrysanthemums, carnations and others), flower trading, and potatoes, developing synergistic effects among the group companies to work toward expanding the Agribio group as a whole.

In the chrysanthemum business, we promote competitive plant breeding on a global scale, combining the conventional breeding technologies of Fides Beheer BV and the Southern Glasshouse Produce group with Kirin's cell biology and genetic engineering techniques. In Holland, the Kirin Agribio group companies -Straathof and Fides- together control an approximately 35% share of the Dutch market. In Japan, we expand the sales of our proprietary varieties under the brand name of "Kirin Mum."

In the carnation business, we entered the market by acquiring Barberet & Blanc, S.A. of Spain, a major breeder, producer and marketer of carnation cuttings. We also have commercialized carnation cuttings of Kirin-bred pot varieties produced by the Plantlets Production Reactor (PPR) system.<sup>TM</sup>

In the nursery and micropropagation business, our business has been developed by Twyford International, Inc. of the U.S.A., and Verde Co., Ltd. of

Japan and Wintech Inc. of Japan. Twyford specializes in tissue culture production of more than 30 million starter plants annually in ornamental, vegetable, fruit and forest species for the international horticultural and agricultural marketplace.

In the flower trading business, sales have been expanding overseas mainly by Hiljo BV of Holland, and domestically by Flower Gate, Inc. and Plants Partner Inc. Making use of our overseas network of group companies, we focus our efforts on imports of cut flowers.

Increasing its business opportunities, Kirin continues to develop new proprietary varieties. Sales of Fortunia (a brand name of Kirin-bred petunias) series, "Bornfree," "Full Bloom" and "Purple Wave," developed at Plant Laboratory, increased to approximately US \$20 million in the worldwide market in 1995. With enhanced cooperation among group companies, we promote the creation of new plant varieties and an efficient production system and strive to strengthen our marketing network on a global scale.

#### OUTLINES OF INSTITUTES AND AFFILIATES OF THE KIRIN AGRIBIO GROUP

##### Plant Laboratory

Development of new varieties and mass propagation technology to be commercialized in Agribio Group.

**Table 13.3. Affiliated Companies in Kirin Agribio (1995)**

Division / Region	Japan	U.S.A.	E.C.	Other Areas
Chrysanthemum Business	Kirin		SGP (UK) Fides (Holland) Straathof(Holland)	
Carnation Business	Kirin		Barberet & Blanc (Spain)	
Seedling / TCplants <sup>a</sup> Business	Verde Wintech	Twyford Inter. Inc.		
Flower Trading Business	Flower Gate (Retailer) Plants Partner (Wholesaler)		Hiljo (Holland) SGP (UK)	
Potato Business	Kirin	Plant Genetics- Kirin Partnership		
Other Business	Tokita Seed			Ging Dao Inter. Seed (China)

a) Tissue Cultured Plants

### **Chrysanthemum Business**

Fides Beheer BV (Holland) acquired in 1993. The world's largest company engaged in breeding/ cuttings production and sales of cut chrysanthemum varieties. Together with SGP and Straathof, Kirin established a leading global presence in the chrysanthemum business.

Southern Glasshouse Produce Group (UK) acquired in 1992. Integrated business of chrysanthemum from breeding new varieties to production and sales of cuttings and cut flowers.

Straathof (Holland) acquired in 1995. Production and sales of chrysanthemum cuttings.

### **Carnation Business**

Barberet & Blanc, S.A. (Spain); Acquired in 1994. This is one of the major companies in breeding, propagation and sales of carnation cuttings.

### **Seedlings/Micropagation Propagules Business**

Twyford International, Inc. (U.S.A.); acquired in 1991. A world leader in the production and sales of micropagation propagules. A total of our production sites are located in California, Florida and Costa Rica.

Verde Co., Ltd. (Japan); capital investment in 1987. Production and sales of tissue cultured young plants of ornamentals and vegetables.

Wintech Inc. (Japan); founded jointly with Tokita Seeds Co.,Ltd., Kyowa Seed Co.,Ltd. and Fukukaen Nursery & Bulb Co.,Ltd. in 1991. Production and sales of plug seedlings of ornamentals and vegetables.

### **Flower Trading Business**

Hiljo BV (Holland); capital investment in 1993 and acquired whole shares in 1995. An exporter of cut flowers and potted plants based in Aalsmeer, Holland. In addition to the EC market, it is exporting cut flowers to Japan.

Flower Gate, Inc. (Japan); established in 1986. Retailing and mail ordering business in ornamentals, and sales of seedlings. Also engaged in flower schooling and amenity business.

Plants Partner, Inc. (Japan); established in 1991 by Flower Gate, Inc. A wholesaler at Ohta flower auction.

### **Other Business**

Plant Genetics-Kirin Partnership (U.S.A.); established jointly with Calgene, Inc. in 1990. Production and sales of seed potatoes.

Tokita Seed Co., Ltd.(Japan); capital investment in 1989. Development of new vegetable varieties such as tomato, bunching onion and Chinese cabbage. Production and sales of vegetable seed and seedlings.

Ging Dao International Seed Co., Ltd. (China); established in 1990. Production and sales of vegetable seeds.

### **RESEARCH AND DEVELOPMENT IN AGRIBIO RELATED AREAS**

Through active R&D cooperation between the affiliated companies of the Agribio Business group, we aim to make use of tissue culture, cell biology and genetic engineering to develop new production and propagation methods, and varieties and products. Recent product developments include the "Royal Wedding" chrysanthemum, the "Mother Red" carnation, new varieties of "Fortunia" (a brand of petunia), "Nebari Gachi" (a new type of rice) and "Jaga Kids" potato.<sup>2</sup>

Agribio-related R&D structure is in Table 13.4. Plant Laboratory engages in research and development in fields close to the line of Agribio business. Corporate R&D division is in charge of developing the technology that will form the cornerstone of the future Kirin Group, and of building a system wherein such technologies can be fully utilized. Central laboratories for Key Technology play the role of developing the basic technology, and in the Agribio area, molecular breeding using recombinant DNA technique is carried out. the Applied Bio-Research Center engages in the development of technology for commercial applications at the divisional level, such as scaling up technology for efficient seedling production and research on transgenic plants in the Agribio area.

In the Plant Laboratory, conventional breeding such as sexual hybridization, in vitro breeding such as embryo and anther culture and cellular breeding such as cell fusion and utilization of somaclonal variation have been applied according to the breeding objectives in flowers and field crops.<sup>3</sup> In propagation of clonal plants, the application of tissue culture techniques such as shoot organogenesis and somatic embryogenesis have been exploited,<sup>4</sup> and Kirin's new and efficient propagation system called "Plantlets Production Reactor (PPR) system" that uses liquid medium is commercialized in producing mother plants of Kirin-bred pot carnation. For the evaluation and spread of commodities, Plant Laboratory engages in R&D on cultivation technology, quality control such as disease diagnosis, chemical analysis, and technology for safety assessment of genetically engineered plants.

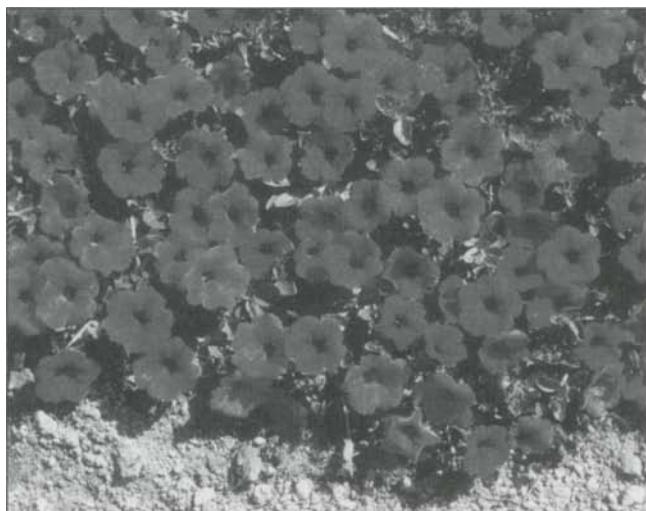


Fig. 13.1. "Purple Wave" petunia, generating "waves" of magenta purple flowers due to its trailing habit, which is unique for a seed propagated petunia. "Purple Wave" was awarded AAS winner for 1995.

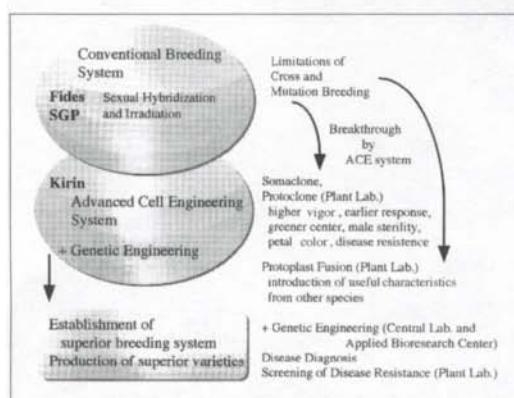


Fig. 13.2. Breeding system of chrysanthemum in Kirin Agribio group: Conventional breeding system of Fides and SGP group; Kirin's biotechnology "ACE (Advanced Cell Engineering) system"; and genetic engineering.

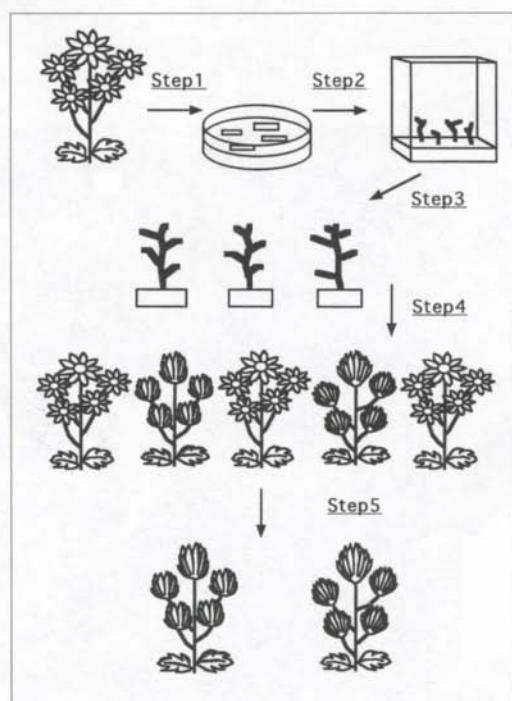


Fig. 13.3. Schematic presentation of "ACE somaclonal variation system." Step 1: Selection of suitable cell or tissues. Culture on medium. Step 2: Shoot formation. Step 3: Selection of superior plants in house level. Step 4: Selection of superior plants in flowering. Step 5: Selection of superior plants with stability. Production of new varieties.

Fig. 13.4. "Royal Wedding" chrysanthemum, pink and white bi-coloured flowers, produced by "ACE somaclone system." See color figure in insert.

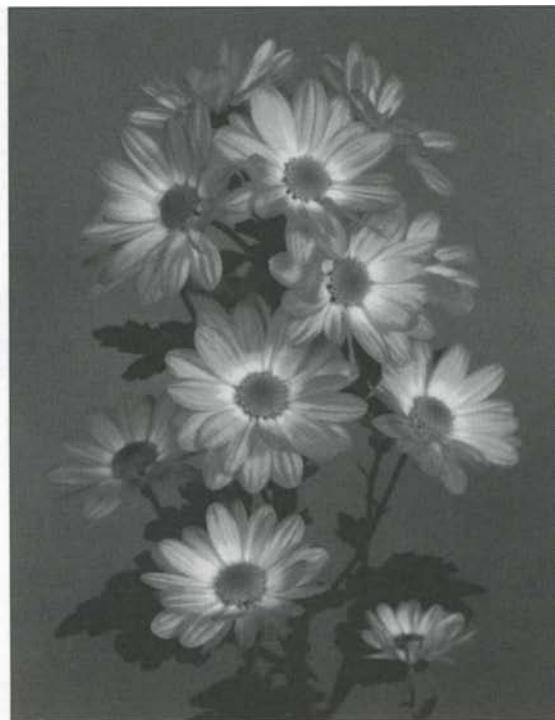


Fig. 13.5. Somaclonal variation in flowers produced by the application of "ACE somaclone system" to cv. Lineker (arrow) chrysanthemum. Variation is found in shape, color and number of petals, male sterility, flower type, etc. See color figure in insert.

**Table 13.4. Kirin technology (Agribio-related)**

<b>Division</b>	<b>Breeding</b>	<b>Propagation</b>	<b>Others</b>
Plant Laboratory (Agribio Business Div.)	Field Breeding Hybridization Mutation In vitro Breeding Embryo Culture Ovule Culture Anther Culture Cellular Breeding Cell Fusion Somaclone Protoclone	Tissue Culture Somatic Embryo Adventitious Shoot Plantlet Production Reactor System Microtuber Nursery/Greenhouse Acclimatization Seedling Production	Cultivation Seed Production Quality Control Disease Diagnosis Chemical Analysis
Central Lab. for Key Technology (Corporate R&D Div.)	Molecular Breeding Genetic Engineering		Technology for Product Safety and Assessment of Genetically Engineered Plants
Applied Bioresearch Center (Corporate R&D Div.)	Transformation and Evaluation of Transformed Plants	Large Scale Culture	
Plant Laboratory (Beer Division)	Field Breeding and Gene Analysis of Barley and Hop		Chemical Analysis

## FLOWER BREEDING OF KIRIN

In flower breeding, Agribio-related R&D institutes have been developing new technologies (Table 13.4) and varieties through cooperation with the affiliated companies, using biotechnology and conventional cross breeding. Target crops are various such as spray chrysanthemum, carnation, petunia, primrose, etc. In this section, breeding of petunia and spray chrysanthemum in Plant Laboratory is mentioned.

### PETUNIA

The petunia is one of the flowers most commonly planted in gardens throughout the world and a great number of varieties are available at present. However, fragility against rain and their rapid deterioration have been two major disadvantages. The plant Laboratory of Kirin, with the advice of Professor Ando from the Faculty of Horticulture of Chiba University, developed new F1 petunia varieties with improved characteristics by cross breeding.

“Purple Wave” (Fig. 13.1) is generating “waves” of magenta purple flowers due to its trailing habit, which is unique for a seed propagated petunia. This new class of petunia grows very vigorously, its foliage becomes more than one meter in diameter in one season, but creeps to the ground and makes

a great impact on the landscape, in hanging baskets and in window boxes. “Purple Wave” flowers continuously exhibit superior weather tolerance, particularly to drought and rain. “Purple Wave” is more tolerant against severe weather than any other seed petunia in the world. “Purple Wave” performed consistently at all AAS (All-America Selections) flower trial locations in North America and was the AAS winner for 1995.<sup>5</sup> “Pink Wave” is also available as “Wave series.”

“Bornfree” is a new concept of petunia for flower gardens, tubs and planters. This petunia, a bit more upright than the “Wave series,” has many flowers and a strong yet bushy habit without long shoots. Since their flowers are very resistant to rain, heat and cold, an abundance of flowers can be enjoyed for a long time. Flower colors available for “Bornfree series” are purple, rose, red, pink, white and light blue.

“Full Bloom” is for landscape gardening and ground covering. Radiant small flowers cover over the exterior of the 60 cm tall plant that expands to 1 m in diameter. It is very resistant to rain and high temperatures and has plenty of flowers for a very long time. The flower colors available are salmon rose and bright rose.

The sales of "Bornfree" and "Full Bloom" began in 1993, and "Purple Wave" in 1994 through Flower Gate affiliated companies, etc. The sales of young seedlings and seeds of these varieties increased up to about US \$20 million in 1995. Kirin continues to increase the number of colors available.

### **SPRAY CHRYSANTHEMUM**

As mentioned above the Kirin Agribio business group has the world top level of chrysanthemum breeding programs of Fides and SGP group. In addition, Kirin expects to expand its breeding variation by adapting Kirin's biotechnology; "ACE system" (Advanced Cell Engineering system) in the short-term and genetic engineering technique in the mid- to long-term (Fig. 13.2).

ACE is the abbreviation of Advanced Cell Engineering, consisting of somaclone, protoclone, protoplast fusion and selection system of disease resistance etc. By making use of the ACE somaclone system (Fig. 13.3), four new chrysanthemum varieties including "Royal Wedding" (Fig. 13.4), very popular in Japan, have been bred in Plant Laboratory.

### **"ACE SOMACLONE SYSTEM"**

In the ACE somaclone system, new types of varieties are developed by Kirin's original cell-culture method and somaclonal mutation technology for each targeted cell. Different cells or tissues have been selected and cultured by a suitable cell-culture method. Somaclone system can be used to uncover new varieties retaining the favorable qualities of an existing variety while improving some traits, such as flowering response time to short days, uniformity in growth, male sterility, shape of leaves and flowers, the formation and petal color of flowers, etc.

In the system, two selection steps serve as a sieve to permit recovery of population of regenerated plants suitable for a breeding program. One is that the culture medium and plant regeneration protocol provide a sieve for singling out cells from the original explant which possess genetic competence for plantlet regeneration. The other is that greenhouse selection permits identification of those regenerated plants that are capable of good growth. Many of the somaclones differ from the parent by a small number of genetic changes, and could be a valuable source of germplasm to isolate clones that acquired improved characteristics of interest (Fig. 13.3).

Somaclonal variation proved to involve such genetic variations as point mutations, amplification or deletion of DNA sequences, changes in chromosome number, etc.<sup>6</sup> In chrysanthemum, somaclonal variation of ACE system is widespread as mentioned above. Changes can occur at high

frequencies, i.e. about 20 to more than 50% of somaclones have changes in certain traits depending on the varieties tested. Variation occurs both in quantitative traits, i.e. response time, stem weight, etc. and in qualitative traits, i.e. shape of flowers, petal color, male sterility, etc (Fig. 13.5). As for the variation in ACE somaclone system, two sources are mentioned; one is the variation that arises from variegated cells by the regeneration of plants from these cells, the other is the variation that is induced during the culture phase.

### **"PROTOPLAST SYSTEM AND CELL FUSION"**

Chrysanthemum plants can be regenerated from differentiated tissue, cell aggregates, and from protoplasts. Protoclones, regenerated plants from protoplasts, are characterized by an outburst of variation, resulting in unique germplasm for breeding.<sup>1</sup> Protoclones differ from somaclones in that they are derived from cell wall-less, single cells. The attack of cell wall digesting enzymes can be a strong stress on plant cells. Genetic changes such as gene mutation, chromosome reconstruction and gross changes, etc. occur during the callus phase of protoplast culture. These stress and genetic changes induce more variations in the regenerated plants from protoplasts than those in somaclones.

The protoplast system offers another potential for the improvement of chrysanthemums, that is, the production of somatic hybrid plants mediated by protoplast fusion. Somatic fusions may provide a means by which traits from sexually incompatible species can be incorporated into chrysanthemum.

### **"COMBINATION WITH CELL SELECTION TECHNIQUE"**

Desirable clones from somaclones or protoclones can be identified in the test tube rather than in the greenhouse trial, somaclonal variation can be more efficient and a cost-effective system for breeding. This requires a correlation between the cellular level and whole plant response to specific selective substances such as chemicals, toxins, etc. used as a selective agent.

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## CHAPTER 14

# THE APPLICATION OF BIOTECHNOLOGY TO DATE PALM CULTURE

N. Bouchireb and M.S. Clark

### INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is an important commercial food crop in many Arab countries. Cultivation is largely confined to a belt across the top of Africa (including the Maghreb, Egypt and the Sudan) and most of the Near East. Commercial plantations have also been established in the New World, in particular in California. Accurate figures for current world date production are difficult to obtain, but can be estimated to be in the region of 3,200,000 MT, with the Near Eastern countries contributing 75%. While a large part of production goes for export to the USA and Europe, it must not be forgotten that the date palm is still (and will remain for the foreseeable future) a significant subsistence crop. The trees provide a shady micro-climate which makes it possible to cultivate other cash crops such as wheat, barley, alfalfa, oats, citrus and bananas. The rest of the tree is an important source of domestic requirements such as wood for furniture and firewood and also leaves for matting, roofing and baskets, etc. Alternative uses such as the potential for dates to be used as a source of oil and sugar are only just being explored.<sup>1</sup> In addition, date palms have been proven to be one of the most salt tolerant of fruit crops and, therefore, have the potential to help combat desertification processes.

However, the main consideration of this chapter will remain with the use of date palm as a major fruit cash crop. It is here that the main impetus for improvement lies. The development of modern date palm plantings for commercial exploitation of the crop needs to be based on the best available technology, an area ripe for biotechnological exploitation. This is not to say that such developments will not impact the small holder. It is without doubt that the greatest effect on farming at the subsistence level can be achieved using improved agricultural practices via government supported rural extension services.<sup>2</sup> The improvements obtained and field tested in commercial operations can be modified to meet local needs and technology transfer effected at the level of the small holder.

In order to appreciate the potential role of biotechnology, it is useful to review date palm culture and also the current situation with regard to date palm genetics and breeding.

## DATE PALM CULTURE

Date palm biology and production methods are well-documented.<sup>3-5</sup> The date palm is only found as a cultivated crop. It is a diploid, dioecious monocot, with separate male and female plants. These are produced in approximately 50:50 ratios from seed, but the sex cannot be determined until the plants mature, which is generally within the range of four to six years. The seeds are used for breeding studies only, as they show considerable variation and do not breed true to the parent. It is very rare for a seedling to produce as good a quality crop as the parent. Most palms are propagated by the removal and transplanting of offshoots that originate from the basal part of the stem. This is a difficult and laborious process, but also a very slow way of multiplying the crop, as each female tree only produces between six and 12 offshoots.<sup>6</sup>

For economic production, a 1:50 ratio of male: female is required.<sup>7</sup> The female palms start producing dates at an age of between four to six years with full production attained within 15-20 years. The average economic life of a date garden is estimated at between 40-50 years, although palms can survive for up to 150 years.

Date production is still very labor intensive. Equipment for mechanical harvesting and processing has so far only been designed for use with the semi-dry varieties such as Deglet Nour and Zahidi and is obviously only suitable for large-scale well-organized date plantations. The impetus behind this is the commercial date palm operations in California. The soft fruited varieties such as Medjool, Khadrawy, etc. still require manual harvesting and processing.

## DATE PALM GENETICS AND BREEDING

Virtually nothing is known about date genetics. While the date palm has been cultivated for thousands of years, it has not been subjected to systematic selection programs, as is the case with other cash crops such as wheat, barley, rice, etc. Selection has occurred on a local level, generally in the form of empirical selection of choice clones grown from chance seedlings and subsequent small-scale clonal propagation of offshoots. This has resulted in major problems concerning nomenclature. Different vernacular names may refer to the same cultivar and entirely different varieties may be given the same name. This is compounded by morphological modifications which occur when a cultivar is moved from one ecological zone to another.<sup>8</sup> Detailed evaluation of genetic resources has been in progress in Algeria for the past 10 years and so far over 800 varieties have been characterized.<sup>9-10</sup> As a

further complication, the pollen responsible for fertilization exercises a direct influence on the somatic tissue of the fruit outside the embryo and the endosperm. This effect is called "metaxenia."<sup>11-12</sup> Factors affected include the time of ripening and size of the fruits and seed. So pollen donors must be chosen with care to match local needs and environmental conditions.

Despite this long life cycle, some breeding trials have been conducted. At the turn of the century, the U.S. Department of Agriculture distributed seedling date palms to private farmers to evaluate potential new varieties. This was not a great success, although, several new local varieties originated from these seedlings. This was followed in 1910 by the first attempt to study inheritance in dates by the University of Arizona. Female Deglet Nour seedlings were pollinated from male seedlings of the same progeny.<sup>13</sup> After three generations, the study was discontinued with no production of novel varieties. In the 1940s two breeding programs (in the U.S.A. and Algeria) were initiated to systematically study the genetics of date palm with the aim of producing superior varieties.

In 1948, Nixon and Furr at the U.S. Date and Citrus Station, Indio, California began an ambitious breeding program with the following objectives: 1) develop backcross males that would approach the recurrent parental variety in genetic constitution; 2) use the males produced from the recurrent backcross program for "intervarietal crosses" to produce new and better fruiting varieties; 3) select superior seedlings and develop their potential as new varieties.

Thirty-five varieties, represented by 48 breeding lines, were used in these experiments and included the most popular varieties of Medjool, Abbada, Deglet Nour and Halawy. Results of this program have been varied. It took 20 years to develop the backcross males, before the intervarietal crosses could be produced. The program progressed as far as the backcross four generation in some lines by 1978, but no further results have been published. Many of the original lines in the backcross male program were discarded due to genetic weaknesses. None of the Deglet Nour intervarietal crosses produced fruit superior to that of the parent or resembled the female parent. A general problem was sterility and lack of vigor due to inbreeding depression and the appearance of albino leaves. The resulting backcrosses showed disappointing performances in the field and there was evidence of inbreeding depression.<sup>4-5,14-16</sup>

The research station Institut de Technologie et de Developpement de l'Agriculture Saharienne (ITDAS) of El-Arfane wilaya of El-Oued South

East Algeria started a breeding program in 1943.<sup>17</sup> The objectives were two-fold: 1) to produce a line of Deglet Nour that would breed sufficiently true to type so that propagation of the variety by seed would be feasible; and 2) produce new varieties, particularly males, that would flower early and/or yield a high quantity of pollen.<sup>4</sup> This has currently progressed as far as the backcross three generation and is still under evaluation.

These two breeding trials serve to exemplify the reasons why conventional breeding programs for date palm will ultimately fail to prove a commercial proposition. The out-breeding heterogeneous nature of the date palm genome is not predisposed to inbreeding without deleterious effects and research funding for, at least, a 50 year period would be required (untenable in this day and age). Breeders cannot possibly hope to produce by traditional methods new varieties within an acceptable time-scale and, in particular, in response to environmental challenges. So while the date palm is a plant breeders nightmare, it is possible to selectively use biotechnology to overcome some of the major problems associated with date palm culture, provide benefits for the farmers and create new opportunities particularly for those in marginal areas.

## AREAS IDENTIFIED FOR BIOTECHNOLOGY MANIPULATION

As with all the biotechnology programs, there is a wish list of potentially useful techniques that could be used if resources were unlimited and a much smaller practical list based on needs and available resources. The list below indicates major areas of study in date palm. The discussion, which follows, addresses each of these areas (which are often interrelated) both in terms of current progress and future potential based on current resources. Also related novel techniques, not yet in place, that may play a role in future developments are discussed.

### TISSUE CULTURE: MASS PRODUCTION OF SUPERIOR VARIETIES VIA CLONING

Improved farming practices could produce the most cost-effective increase in yields in the short-term. However, this could be greatly aided by the mass provision of *in vitro* plantlets of superior varieties. These could be supplied in bulk to commercial farmers so that they can be used to lay out date gardens with optimum growth conditions, spacing, male:female ratio, etc. and facilitate the use of mechanization. With regard to the subsistence farmer, they would have access to superior varieties, not normally available locally and present

a method of propagation more efficient than the current very slow ad hoc arrangements with removing and replanting offshoots. Most important is the rapid propagation of new varieties that are disease resistant (discussed in detail below).

Several groups have published successful protocols for the tissue culture of date palm.<sup>18-25</sup> Two systems are currently employed to regenerate date palms: 1) direct organogenesis with regeneration from auxiliary buds and apical meristems; and 2) indirect organogenesis/somatic embryogenesis utilizing 2,4-D as a growth inducer with all plants regenerating via a callus stage. Regeneration can occur from various meristematic areas including shoots, young leaves, stem, rachilla, etc.

The preferred technique is somatic embryogenesis and a considerable body of knowledge has been accumulated with regard to factors affecting success.<sup>26</sup> Regeneration via callus provides a far greater number of plantlets and, therefore, is amenable to mass production. The number of auxiliary buds that can be obtained from each palm is limited and also the regeneration procedure is not so efficient.<sup>21</sup> As with all tissue culture, each variety of date palm varies in its response and in the conditions required for optimization.<sup>24</sup> Alternative methods such as gynogenesis and androgenesis are also being evaluated, but are not yet at the stage of mass production.<sup>26</sup>

Morocco has a large-scale state-funded research program in tissue culture. This was initiated in response to the threat of Bayoud disease and is now at the stage where large numbers of tissue culture-derived plantlets are being made available to farmers in the first stages of field trials. Initial results show up to a 90% survival rate of *in vitro* palms when passed onto farmers.<sup>26</sup> Perversely, the only major commercial operation is based in the U.K.: Date Palm Developments (International Plant Laboratories) successfully exported some 70,000 plantlets, comprising 12 varieties, in 1995 to the Near East. This approach shows great promise and indeed palms produced by regional laboratories and the commercial operations of Date Palm Developments are already fruiting successfully and have been reported true to type.

The one potential problem with tissue culture: somaclonal variation<sup>27-28</sup> has, so far, only been lightly addressed. A couple of small-scale analyses using chromosome studies and isozymes<sup>20,29</sup> can be regarded as inconclusive, on the grounds that these techniques only detect gross genetic changes. Genome stability is of the utmost importance when cloning plants for commercial production. Field trials of regenerated date palms are proving successful with regard to genetic conformity and in other crop plants, numerous *in vitro* experi-

ments<sup>27-28</sup> indicate that diploidy exerts a strong selective constraint. Hence, expected variation should be minimal, but this issue still requires more in depth genetic analysis. Along these lines, Corniquel and Mercier<sup>30</sup> used RFLP and RAPD analysis on tissue culture derived plantlets, proving the utility of these techniques for date palm, however, once again, as with previous studies listed above, sample sizes were very limited. This aspect will be discussed in more detail below.

Tissue culture, as a technique, has many variants and uses. The primary aim is mass clonal propagation, but it is also useful for selection of novel clones and mutagenesis.<sup>28</sup> The aim of these with date palm would be the incorporation of novel characters, primarily disease resistance genes into popular commercial varieties such as Deglet Nour.

Potentially, the most useful of these techniques, which could be carried on alongside normal in vitro propagation, is in vitro selection. Letouze, in an EEC project report, cultivated regenerated plants through a tissue culture phase to which fungal extracts of Bayoud fungus (*Fusarium oxysporum* fsp. *albedenis*) were added to select for Bayoud resistant clones.<sup>31</sup> Plants were successfully regenerated, but no reports of post-in vitro performance have been published. Toxin selection is well-established and has been used with other species of *Fusarium oxysporum*.<sup>32,33</sup> This type of work is based on already established techniques and involves very little extra cost for the laboratory involved. As well as programs to select for disease resistance, it creates similar possibilities for selecting for salt tolerance.

Other tissue culture-type techniques such as a random mutagenesis program based on somaclonal variation, single gene transformation and somatic fusion are likely to be less important in the genetic improvement of date palm, certainly in the short term.

Somaclonal variation as a means of random genetic mutation, even as part of an active mutagenesis program, has the severe disadvantage that it is completely random; certain genes cannot be accurately targeted. Therefore, a large amount of effort has to be expended for uncertain results. With the extensive genetic heterogeneity of date palm, one could easily argue that research funds might be better used in a large-scale survey of the diversity of germplasm.

Single gene engineering and somatic fusion rely on the use of protoplasts. Regeneration from protoplasts has yet to be achieved with date palm and hence this technology is not immediately available. Of the two techniques, single gene engineering is potentially the most powerful, taking genes from

other crops (coding for example disease resistance) or using these as heterologous probes to clone the date palm homologs. These could then be used in transformation experiments (using electroporation, ballistic gun, etc.) in a focused attempt to improve varieties. Somatic fusion, although used in many other crop plants, is not sufficiently reliable, often producing aneuploid somaclonal variants and introgression of undesirable characteristics.<sup>34</sup> Since all cultivars of date palm appear to be sexually compatible, traditional crossing rather than 50:50 somatic fusion would produce more reliable (in terms of ploidy) offspring. The main use of somatic fusion could be envisaged as the use of asymmetric hybrids to reduce introgression of unwanted characters from the donor cultivar. It is here that the size and long life cycle of date palm becomes a problem, as the field trials to determine the quality of fusion products would take up much acreage and several years before fruiting and final product evaluation.

#### GENOME CHARACTERIZATION: DEVELOPMENT OF MOLECULAR MARKERS FOR CULTIVAR IDENTIFICATION

The date palm is rare among cultivated species in having been commercialized, but not subjected to genetic improvement by systematic breeding (for example, production of near isogenic lines, etc.), as is the case with other cash crops such as wheat, barley, rice, etc. Therefore, contained within the date palm genome is a tremendous amount of variation. Without inbred lines, it will prove virtually impossible to construct a genetic map and, consequently, to characterize the variation which is responsible for major traits such as fruit quality and yield, disease resistance, salt tolerance, etc. and to access the sequences controlling these processes.

This is not to say that molecular biology, in the form of molecular characterization of the genome (use of molecular markers), has no role to play in date palm biotechnology. There are alternative goals that are far more easily attainable. Efforts must be concentrated on the development of molecular markers for: 1) use in investigations to identify novel cultivars; 2) use in tissue culture experiments to verify the clonal nature of the products and to ensure that mutation in the form of somaclonal variation has not taken place, with potentially detrimental effects; 3) use in commerce to verify origin of cultivar; and 4) determine evolutionary relationships between cultivars.

Genome characterization has so far been limited in investigation. The classic study on date palm chromosomes was published by Beal in 1937.<sup>35</sup> Ten varieties were examined, all had a diploid content of 36 chromosomes. This is somewhat

disputed by recent studies reporting numbers ranging from  $2n=18$  to  $2n=64$ .<sup>36-38</sup> This matter obviously requires clarification. All sources agree that date palm is a diploid.

Previous studies on molecular markers have concentrated around isozymes. The largest survey carried out using isozyme markers took place in Algeria to examine the extent of genetic diversity.<sup>8</sup> Twenty enzyme systems were tested, of which seven showed sufficient reproducibility and polymorphism to act as molecular markers: alcohol dehydrogenase, diaphorase, aspartate aminotransaminase, acid phosphatase, endopeptidase, leucine aminopeptidase and phosphoglucomutase. These revealed seven polymorphic loci and 16 alleles. Genetic variability was greater in the west when compared to the east and 65% of all cultivars studied were identified from five enzyme systems. Cultivars were strongly heterozygous with a high percentage (70%) of polymorphic loci. Hmira and Tilemsu, cultivars known for their morphological similarity, were proved on isozyme analysis to be the same cultivar. Commercial crops, e.g. Deglet Nour, proved to be very homogeneous when compared with trees in traditional palm groves. In the latter case, much intra-cultivar diversity is present due to the coexistence of genetically different clones. In spite of these results, isozyme analysis was not sufficiently discriminatory as some cultivars displayed the same enzyme profile but were clearly morphologically distinct. This is because with this technique, using expressed proteins, only a small part of the genome can be sampled.

Similar surveys have revealed "identity cards" for cultivars.<sup>39,40</sup> The inheritance of single gene markers using isozymes on the American inbred lines has been demonstrated.<sup>41</sup> Alternative markers such as flavonoids and peroxidases have been used successfully, but merely emphasize that enzyme-based systems are limited in use.<sup>42,43</sup>

This naturally leads to the development of DNA probes for the date palm genome. The identification of "useful" molecular probes for the analysis of any genome is random. Normally the requirement is to find/use polymorphic markers that can be followed through inbred lines to identify genetic links with known characteristics. Since there are no widely available inbred lines with date palm, the normal criteria for molecular markers are reversed, due to the different aims of the study, in that non-polymorphic markers are required as landmarks. Preliminary studies indicated that molecular variation was normal rather than exceptional (Fig. 14.1).

Now that clonal propagation systems are available, the identification and propagation of novel cultivars from country-wide prospections is increasing. Potentially useful palms can be analyzed in the laboratory and in field trials, before being propagated for the mass market. However, there are several problems that arise in these investigations, namely, the identification of separate cultivars. As mentioned previously, nomenclature problems arise, particularly in rural areas where different vernacular names may refer to the same cultivar and entirely different varieties may be given the same name. These are based on vegetative and fruiting characteristics and morphological modifications also occur when a cultivar is moved from one ecological zone to another, further confusing the matter.<sup>8</sup> Confusion can arise even within popular marketable varieties. To determine where the selected clones under study are truly different, variety-specific genetic markers are required.

Also, it is necessary to study genomic stability through the tissue culture process. RFLPs and RAPDs have been applied to study the date palm genome.<sup>44</sup> A date palm cDNA RFLP probe revealed individual-specific profiles for a number of

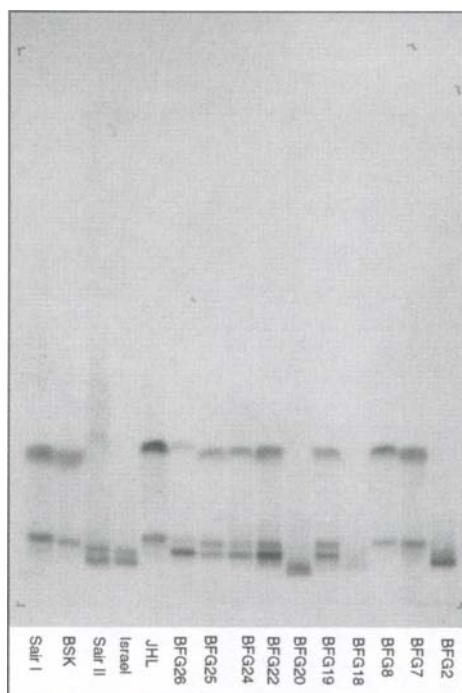


Fig. 14.1. EcoRI digest of various date palm DNAs probed with rDNA. BFG = Boufeggous; JHL = Jihel; BSK = Bouskari; Israel = Israeli date, probably Deglet Nour; Sair I and II = random clones, origin unknown. Note that the 10 Boufeggous DNA samples are as polymorphic as the entirely unrelated DNAs.

cultivars. This was substantiated by RAPD analysis, which showed additional bands not present in the RFLPs, indicating that perhaps RAPDs are more sensitive for this type of study. The aim of the study was to look at stability in tissue culture; however, one problem was the extraction and amplification of callus DNA. The first published study of its kind was carried out in France from plantation-derived material on a limited number of samples with close access to a laboratory, a situation very different from field conditions. To verify the use of this technology for date palm, it has to be applied in less rigorous conditions.

Studies by Bouchireb on plant material obtained from INRA (Institut National de Recherche en Agronomie), la wilaya d'Adrarn have revealed many technical problems.<sup>45</sup> Storage of leaves and the subsequent extraction of full length DNA is severely compromised by not having liquid nitrogen readily available. Extraction of DNA from date palm is difficult with a high percentage of polyphenols present and degradation often occurs, making such samples unsuitable for use in Southern blotting and RFLP analysis. Combined with the length of time required to obtain strong hybridization signals and the use of radiation, RFLPs are unsuitable for large-scale use in field analysis.

The use of RAPD markers is far more flexible; its efficiency is not compromised by the use of partially degraded DNA and it produces quick

results (within 12 hours) enabling a fast turnover. This is essential when analyzing a large number of samples. In the study, 50 RAPD primers were tested, of which eight proved informative.<sup>45</sup> Eight different cultivars were analyzed using the 31 polymorphic bands (66% of total) produced. With this, all eight cultivars were unequivocally distinguished by a mix of primers, some bands being specific to certain cultivars, while others were only shared by a certain number (Figs. 14.2, 3 and 4).

Statistical analysis using coefficients of similarity between individuals of the same cultivar and between cultivars revealed 100% homogeneity between individuals of Deglet Nour, Taquerbouche, Tinnaceur, Aghammu and Hartane, with intracultivar variation detected in individuals of Degla Beida, but most markedly with Gharss. This latter result is not surprising as the exact parentage of these plants is unknown as well as if the different individuals arose from seed. One can safely state that a date palm cultivar is not homogeneous like other crop plants, but is more a pool of clones where resemblances are not exact. Similarities between the cultivars studied varied from 24% between Degla Beida and Taquerbouche to 90% between Tilemsu and Aghammu. This data was used to draw up a dendrogram of evolutionary relatedness. Surprisingly, palms originating from the same region were no closer related than those from opposite parts of the country. Interestingly, Deglet Nour (Bayoud disease-sensitive) showed a close relatedness to the Bayoud resistant Taquerbouche (Fig. 14.5). This preliminary study shows promising results. To fully test the technology, a larger number of samples is required using DNA from trees from all over the country.

RAPDs were also used to study the backcross lines (previously mentioned in the section date palm genetics and breeding) from the research station Institut de Technologie et de Developpement de l'Agriculture Saharienne (ITDAS) of El-Arfiane wilaya of El-Oued South East Algeria (Fig. 14.6).

Unexpected anomalies appear in some of the RAPD profiles. For example, fragment 1 (arrowed) only appears in BCII. Because of the dominant nature of RAPD markers, this could not have "just appeared." Since known pollen was used from a palm that does not have this band, another female Deglet Nour, similar to the original, must have been used as one of the recurrent parents, introducing the variation. Fragment 2 appears in BCI since the male used in the production of BCII had this band, it should have appeared in BCII. Since it did not, this could potentially be the result of contaminated pollen. Fragment 3 still shows heterozygosity in BCII. This particular primer, along

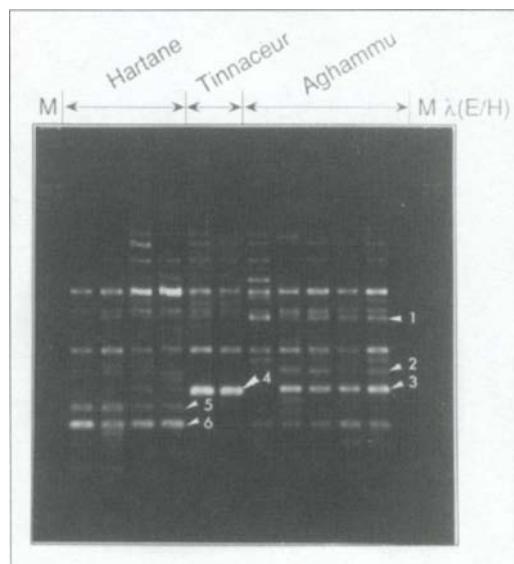
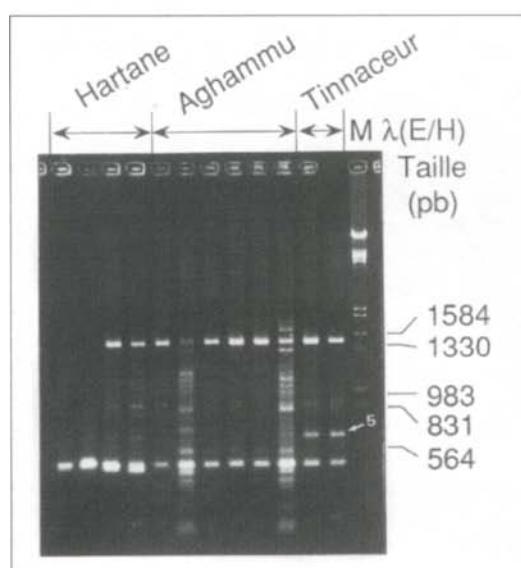
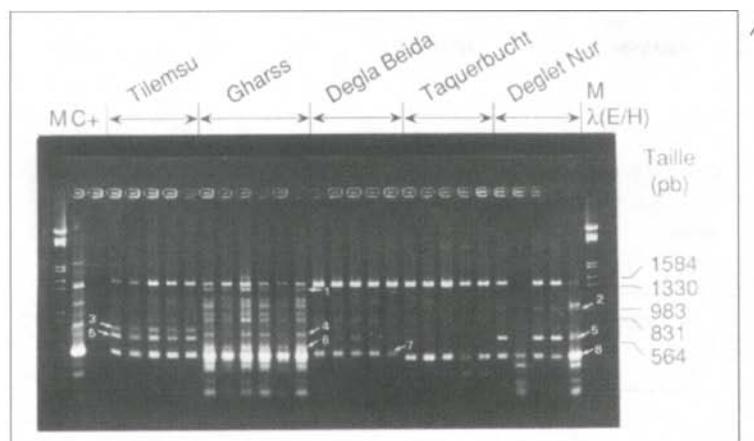
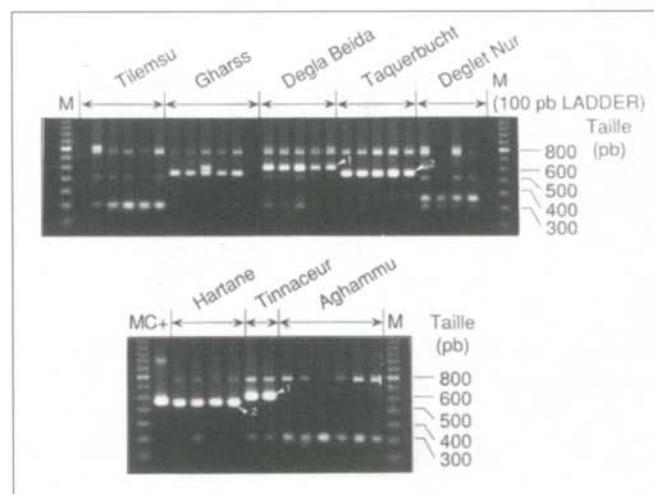


Fig. 14.2. Two percent agarose gel showing amplified products from three cultivars using primer SC10/48. Cultivar-specific bands in Aghammu (1,2,3); Tinnaceur (4) and Hartane (5,6). Marker = Lambda DNA cut with EcoRI and HindIII.



**Fig. 14.3 A and B.** Two percent agarose gel showing amplification products from primer SC10/92 that generated a total of eight polymorphic fragments between the cultivars tested. Seven of the bands were cultivar-specific: bands 1, 4 and 6 (Gharss), band 2 (Deglet Nour), band 3 (Tilemsu) and band 7 (Degla Beida). Band 5 was not specific to a single cultivar, but was only found in Deglet Nour, Tilemsu and Tinnaceur. No bands were found that were specific to either Hartane, Aghammu and Taquerbucht. Marker = Lambda DNA cut with EcoRI and HindIII.



**Fig. 14.4.** Two percent agarose gel showing amplification products from primer OPA/16. This illustrates the use of primers to exclude certain cultivars, with band 1 being specific only to Degla Beida and Tinnaceur and band 2, specific to Taquerbucht, Hartane and Gharss. Marker = 100 bp ladder.

with other results, indicates deficiencies in the production of the backcross lines. Statistical analysis calculates a homogeneity of 76.8% in BCI, but this is decreased in BCII and BCIII (to 58% and 68.4%, respectively) due to these errors. Therefore, one could hypothesize that the backcross program will not achieve the desired results. This study, although preliminary, emphasizes the utility and sensitivity of the RAPD technology. Hence, it has potential applications in many areas of genome characterization.

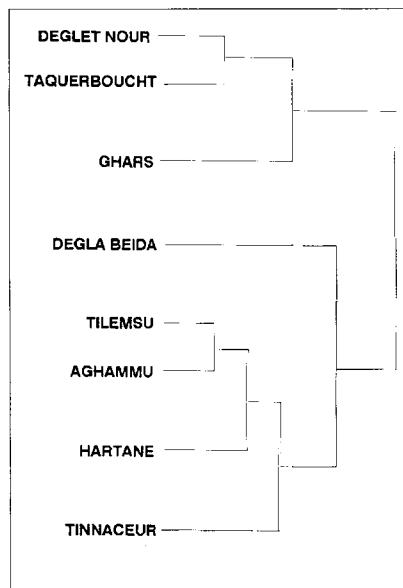


Fig. 14.5. Dendrogram showing the genetic relationship between the eight cultivars. This was generated using analysis of similarities on RAPD data.

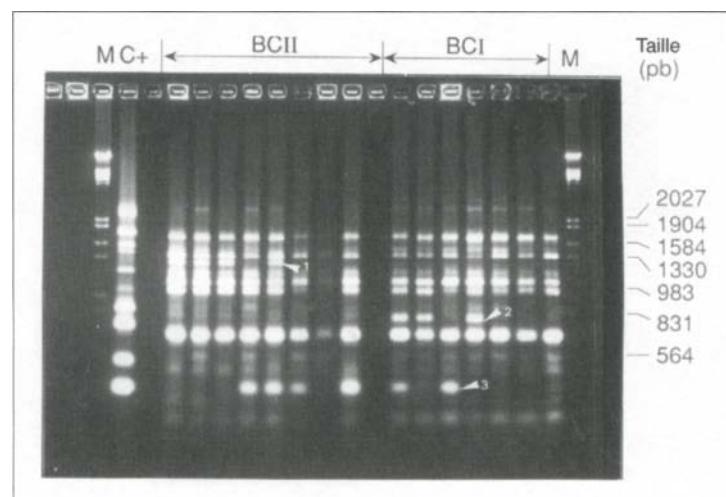
#### IMPROVED DISEASE AND PEST RESISTANCE

Date palm is affected by fungal and insect pests. However, by far, the most important is a fungal wilt disease (generally known as Bayoud disease) caused by *Fusarium oxysporum* fsp. *albehdinii*. It is thought that this disease originated in the Draa valley in the 1870s. Since then it has progressively spread across the whole of Morocco and the east of Algeria. So far, in excess of 13 million palms in Morocco and 3 million palms in Algeria have been affected. This has devastated the Moroccan export trade in dates. In addition to loss of foreign earnings, this disease has also caused an increase in desertification (as the date palm is one of the more marginal crops) and rural depopulation, as people have had to search for work other than in the traditional palm groves.<sup>46</sup> The fungus is still spreading and great fear is about the monocrops of Deglet Nour in Western Algeria, Tunisia and the Near East. Both Morocco and Algeria have programs concerned with the control and eradication of Bayoud.

While integrated control is a possibility, it is generally acknowledged that the most effective solution is the generation of new resistant cultivars that produce dates of equal or superior quality to those currently on the market.<sup>47</sup> This has lead to wide-scale investigations and evaluation of germplasm. As a result of the heterogeneity of the date palm, a wide range of resistance is found. In Morocco, six cultivars were identified that were presumed completely resistant, but all produced poor quality dates (Black Bou Sthammi, Iklane, Tadment, Sair Laylalet, Bou Feggous ou Moussa and White Bou Sthammi). In Algeria, the main resistant variety is Taquerboucht.

In 1972, a breeding program was initiated in Morocco with the aim of linking resistant charac-

Fig. 14.6. Two percent agarose gel showing amplification products of primer SC10/60 on backcross (BC) lines. Anomalous fragments are arrowed, explanations in text. Marker = Lambda DNA cut with EcoRI and HindIII. Positive control = potato DNA.



ters with the highest quality fruit and yield. High quality susceptible females were crossed with Bayoud resistant males and also the reverse was carried out with resistant females crossed with advanced backcrossed "varietal" males from Indio (U.S.A.). Over 400,000 seeds were produced from these controlled crosses and analysis is still continuing. Part of the testing program is problematic in that the only true test of resistance is to plant the palms in infested soil. However, it was found that over a 13-year period only 36% of the susceptible variety Jihel succumbed when planted in such soil at the field-testing station in Zagora.<sup>48,49</sup> Only 50.6% were infected after 25 years, i.e. the soil behavior varies in its receptivity to the disease. This has led to the development of more efficient ways of administering Bayoud fungal spores at Zagora such that within four years almost 90% of the variety Jihel succumbs.<sup>50</sup> Also a highly efficient testing system where in vitro plants acclimatized in the greenhouse are challenged with the fungal spores.<sup>51</sup> This has greatly improved the process of selection. It should be noted that although the method of producing the new palms was traditional crossing, the high throughput testing was only possible with the use of "biotechnology" and tissue culture.

This approach seems to be achieving success with several new presumed resistant varieties in the field trial stage.<sup>52</sup> If these are proved resistant, they can then be mass propagated by in vitro methods and provided to farmers in infested areas, hopefully regenerating the area. One problem that has arisen so far in these trials is persuading farmers to accept the in vitro plantlets instead of the traditional off-shoots. They also prefer the traditional commercial varieties rather than the unknown new ones. The approach in Morocco is to get the farmers to accept in vitro plants from known varieties first and, hopefully, they will be more amenable to the new Bayoud-resistant varieties in future years.

Some research has taken place on other aspects of Bayoud, in particular soil conditions and microfloral antagonism.<sup>50,53-57</sup> Certain chemicals have been associated with resistance, e.g. peroxidase and antifungal extracts, small circular plasmid-like DNAs, but the genetic nature of the disease and the mechanism of resistance remain largely undetermined.<sup>58-62</sup> Other crops are affected by wilt disease and with these more is known.<sup>63</sup> *Fusarium* resistance mechanisms are of two types: 1) monogenic, determined by a dominant gene (found in tomatoes, peas and cabbage); and 2) additive, determined by several genes. This is a very plastic type of resistance, influenced by the density of fungal spores and by plant-parasite nematodes.

In general, breeding for resistance selects for both major and additive resistance factors. *Fusarium* infects many crops and these resistance genes are often absent or difficult to isolate. In addition, some resistance genes are race-specific and, therefore, relatively easily overcome. Genes have been isolated in Europe that confer generalized resistance to fungal diseases: the fungal chitinases and  $\beta$ -1-3, glucanases. Experiments are continuing to transfer these genes into crop plants and assess the impact. Initial results are promising. The resistance has a general effect that is not broken down even when the fungus is mutated. The generalized nature of this resistance mechanism means that, potentially, it will be effective not only against *Fusarium* but also other major fungal diseases such as Inflorescence rot (*Mauginiella scaettiae*) and Graphiola leaf spot (*Graphiola phoenicis*). These genes are, therefore, potential candidates for genetic engineering of the date palm. One must be realistic that since certain technologies are not yet available in date palm, such as regeneration from protoplasts, this technology will be in the future. The more immediate and spectacular effects will be the result of controlled crosses and future investigations.

The main (and most widespread) date palm insect pests are Date Mite (*Oligonychus afriasiaticus*) Trombidioformes, Parlatoria Date Scale (*Parlatoria blanchardi*) Hemiptera and Dubas bug (*Ommatissus binotatus*) Homoptera. Biological control measures have been shown to be effective to a certain extent against these pests. However genetic engineering again may play a possible, more wide ranging role. Insect pests are affected by antimetabolites such as protease inhibitors. The cowpea trypsin inhibitor is active against a wide range of pests including lepidopterans, coleopterans and orthopterans (the families of which include date palm pests), and transformation experiments have shown initial success in other crop species. Other antimetabolites such as lectins and *Bt* toxins are also insecticidal. The full active range of these antimetabolites has yet to be determined.

Genetic engineering in date palm, at the moment, seems a long way off and imminent results will accrue more immediately from the investigations, controlled crossings, in vitro multiplication and improved storage, etc.

#### GERMPLASM CONSERVATION AND STORAGE

It is an internationally recognized necessity to preserve sources of genetic variation. Investigations to examine genetic variability in date palm and discover novel cultivars have demonstrated the

enormous genetic variability available. This has been used directly in breeding experiments for disease-resistant cultivars.<sup>52</sup> Vast tracts of the Near East are monocrops for Deglet Noor. This situation is likely to increase as tissue culture derived plants become more widely available, promoting the planting of new commercial palm groves. Because of market pressures for certain preferred varieties, i.e. Deglet Noor and Medjool, the commercial cropping of dates will consist of a handful of cultivars and all (in theory) genetically identical, if derived from tissue culture. Any pest or disease capable of destroying these chosen cultivars will cause mass devastation of the crop, as has been witnessed by the Bayoud disease problem in North Africa. This makes it increasingly important to preserve genetic reference stocks.

While the U.S.A. has a germplasm storage repository, other countries have botanical collections with only a small selection of the endemic variation available.<sup>64</sup> Algeria alone has over 800 different cultivars identified, many of which are single clones in private date gardens that will be lost in time as the trees become unproductive and are felled by the farmer.<sup>10</sup>

It would be advantageous to maintain a germplasm repository in North Africa where much of the genetic variation is actually reserved and accessible, with perhaps a further collection in the Near East. Moreover, a germplasm storage center in North Africa could circumvent the problems of importing potentially Bayoud disease infected material into a currently disease-free area.

Very little research has so far been carried out on the germplasm storage of dates. Date seed may be held in common storage at moderate temperatures for at least 5-6 years. Longer storage results in loss of viability. However, because date seeds are so heterogeneous, seed storage is obviously not the ideal method of preserving germplasm reference stocks. Tissue culture and cryopreservation techniques may prove the most effective.

There have been published reports on the use of cryopreservation for storing pollen, somatic embryos, callus and meristems.<sup>65-67</sup> However, the cryo-storage was performed for one hour only and then the samples used in fertilization or allowed to develop further on artificial media. In the latter case, after eight weeks of growth, a 40% loss was observed, a situation untenable for a major germplasm storage program.<sup>66</sup> It is clear that an urgent case can be made for further studies on the cryogenic preservation of date germplasm. Factors to investigate include examining the effectiveness of cryopreservation over a longer period of time (and subsequent ability to regenerate plants) and

also studying stability to determine possible mutagenic effects of the storage treatment.

#### OTHER FACTORS, INCREASED TOLERANCE TO SALINITY AND DROUGHT CONDITIONS, THAT MAY BE AMENABLE TO MANIPULATION

These other factors, in effect, constitute the "wish list." Whereby their requirement is not urgent, but production of such variants could potentially greatly increase the geographic range of date palm. In a survey of salt tolerance in fruit crops, date palm was the most tolerant followed by pomegranate, fig, olives, grape and cantaloupe. Palms can grow in soil with 3% salt content up to a limit of 6%.<sup>68,69</sup> The accumulation of salts is a major problem in desert cultivation. If varieties could be produced with increased salt and/or drought tolerance, farmers would be able to colonize marginal areas on a commercial scale and also provide a barrier to increased desertification.

Salt tolerance genes have been found in wheat, but introgress using additional lines and have not yet been cloned. There is a great international interest for example, at CIMMYT in the search for specific genes that are active during both high salt concentration and drought conditions. Collaborative efforts between international organizations may eventually lead to genetic engineering, when suitable genes have been identified in other crops. An alternative may be to select tissue culture-derived plantlets for increased tolerance after the introduction of stress conditions in the culture media. Neither of these options are short-term possibilities.

#### CONCLUSIONS

That date palm is a plant breeders' nightmare and, therefore, ripe for exploitation by biotechnology. The main restraint with regard to this so far has been geography; those countries most concerned with date palm improvement are those with the least molecular biology resources. Molecular biology is a very expensive application with no guarantee of success, certainly in the short term, and it is often felt that this money would be more productively spent on extension-type work.

However, biotechnology does have an important role to play in date palm culture, as has been proved by the work selecting and clonally propagating Bayoud resistant varieties. This was the result of a mix of traditional investigations and tissue culture. This points the way forward: a primary aim for all countries concerned in date production should be to establish a dedicated date palm tissue culture laboratory concentrating on *in vitro* culture of local superior varieties for both

commercial and small holder supplies. In tandem, the expansion of investigation studies and the establishment of germplasm resource centers should be occurring, in part, to help counteract the clonal crops resulting from tissue culture.

RAPD analysis has been proven as a technique for molecular characterization studies. The use of small amounts of tissue and rapid production of results make this the preferred technique for the large-scale analysis of the date palm genome. There are numerous immediate applications such as genome stability in tissue culture, varietal identification, selection of novel clones, analysis of genetic resources for germplasm collections etc.

It is these two techniques that are beginning to prove that biotechnology can work for date palm. However, it must not be forgotten that they cannot be used in isolation and that traditional investigations and field trials will still play a major role in future date palm improvement programs.

#### ACKNOWLEDGMENTS

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## CHAPTER 15

# BIOTECHNOLOGY AND GENETIC RESOURCES APPLIED IN OIL-SEED AND VEGETABLE *BRASSICA* IMPROVEMENT

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Plasticity in the genus *Brassica* has been utilized by humankind for breeding and selection, which has resulted in an array of different crops and species forms. The two major classes are oil-seed *Brassicas* and vegetable *Brassica* species. Most of the biotechnology development has been applied to oil-seed *Brassica*. Thus, in this chapter, we will provide a comprehensive review of the applications of cellular and molecular methods to oil-seed *Brassica* improvement, followed by a concise review of advances in genetic engineering of vegetable *Brassicas*.

### BIOTECHNOLOGY OF OIL-SEED BRASSICA CROPS

Several species comprise the oil-seed *Brassica* group. The winter and spring forms of oil-seed rape (or rapeseed), *Brassica napus* (var. *oleifera*), are the major oil crops in countries with oceanic and sub-Arctic climates of the temperate zone. Turnip rape, *Brassica campestris* (or *Brassica rapa* var. *oleifera*), is the major oil crop in the northern part of Europe and Canada as well as of substantial oil producers on the Indian subcontinent. Three *Brassica* mustard species, black mustard *Brassica nigra* (with genome constitution BB,  $2n=2x=16$  chromosomes), hybrid brown mustard *Brassica juncea* (AABB,  $2n=4x=36$ ) and abyssinian mustard *Brassica carinata* (BBCC,  $2n=4x=34$ ) are also known oil producers.

*B. campestris* (genome constitution AA,  $2n=2x=20$ ) and *B. nigra* (BB,  $2n=2x=16$ ) are ancestral forms of other *brassicas* characterized by high morphological diversity. The third ancestral form, *Brassica oleracea* (CC,  $2n=2x=18$ ), used in interspecific hybridizations, has an even larger morphological diversity, although oil cabbage, *B. oleracea* var. *oleifera*, has no remarkable importance in agriculture. Oil-seed rape, *B. napus* (AACC,  $2n=4x=38$ ), a tetraploid hybrid between *B. campestris* and *B. oleracea* has quite narrow genetic diversity, apparently, due to the same genome origin for all cultivated forms. On the other hand, from an engineering point of view, *B. napus*, fortunately, is a rather amenable plant for cell culture and *Agrobacterium tumefaciens* transformation.

Regeneration capacity of rapeseed is derived from the CC genome of *B. oleracea*, which has good regeneration potential; however, *B. oleracea* lines have weak

susceptibility to *A. tumefaciens* (disarmed C58, LBA4404 and ENA105) inoculation and are better transformed by several *A. rhizogenes* strains. The AA genome of *B. campestris* is characterized by quite good susceptibility to *A. tumefaciens* but has low regeneration potential (Kuvshinov et al, unpublished data).<sup>1,2</sup> Because oil-seed rape, *B. napus*, combines the desired tissue culture traits of the progenitors and is the most important oil-seed *Brassica* crop, it is also the most advanced of the *Brassica* species in biotechnology. Success in biotechnology of *B. napus* has been so substantial that oil-seed rape can be regarded as one of the model crops in biotechnology together with tobacco and potato.

The main lines of biotechnology research of oil-seed *Brassica* crops are as follows: 1) development of molecular biology and cell culture tools toward improvements in agronomic traits; 2) improvements of oil composition and productivity; 3) adjustment of storage protein composition for improved feed quality; 4) tolerance to biotic and abiotic stresses; 5) male sterility and self-incompatibility; and 6) environmental risk assessment.

#### DEVELOPMENT OF MOLECULAR BIOLOGY AND CELL CULTURE TOOLS FOR *BRASSICA* IMPROVEMENT

Progress in the development of recombinant oil-seed *Brassicas* is a result of intensive molecular and cell culture research over the past two decades. Almost all known transformation and cellular hybridization tools have been employed for oil-seed rape. Somatic hybrids of *B. napus* with *B. oleracea*, *B. campestris*, *B. nigra*, *B. carinata* and *B. juncea* have been reported.<sup>3</sup> Re-synthesized *B. napus* has been produced by many researchers through somatic hybridization of *B. campestris* and *B. oleracea*.<sup>4</sup> Rapeseed and other oil-seed *Brassicas* have also been hybridized within the tribe *Brassicaceae* with members of several genera, e.g. *Raphanus*, *Sinapis*, *Diplotaxis*, *Eruca* and *Arabidopsis*.<sup>5</sup> Improvement of storage oil and protein composition, disease and osmotic stress tolerance, male sterility and self-incompatibility traits and herbicide resistance have been transferred to oil seed *Brassicas* through symmetric and asymmetric hybridization of protoplasts.

Embryo culture is useful for rescuing hybrid embryos. Anther and pollen culture, which have enabled the production of (di)haploid lines, are very important in hybridization programs. High frequency of haploid embryo production has been reported for many *Brassica* species and commercial cultivars. This has enabled the development of homozygous lines for breeding programs. Culture

systems have also been developed for protoplast isolation and regeneration from haploid tissues of *Brassica*, which have been utilized in somatic fusion programs.

The use of *Agrobacterium tumefaciens* is routine for *B. napus*,<sup>6</sup> *B. juncea*<sup>7</sup> and *B. nigra*<sup>8</sup> transformation. There are also two reports on the successful transformation of *B. campestris* hypocotyles by *A. tumefaciens*.<sup>9,10</sup> Explants from mature plants are not so amenable to transformation; however, our recent data show that it is possible to obtain transformed shoots from peduncel segments of *B. campestris* (Kuvshinov et al, unpublished data).

Direct DNA delivery is also widely used in recombinant technologies. Electroporation and PEG osmo-transformation of protoplasts have resulted in several successful introductions of different transgenes into the genomes of *Brassicas*.<sup>11,12</sup> Particle bombardment of different explants has also proven effective in *B. napus* transformation.<sup>13</sup> It is, however, more difficult to use these procedures for *B. campestris* transformation due to low shoot regeneration potential.

The basis of successful solutions for *Brassica* improvement by biotechnological methods lies in the molecular investigation of different physiological mechanisms. Research directed toward that recognition and isolation of tissue- and ontogenesis-specific signal and promoter sequences in *Brassica* genomes can be used for the construction of recombinant genes. Promoter sequences specifically expressed in anther tapetum, seeds, shoot and root apical meristems and induced by different abiotic stresses have been cloned from *Brassica* plants and are available for genetic transformation.

Oil-seed *Brassicas* are also used as a gene pool for the improvement of other crops. For example, cloned storage protein genes have been expressed in different plants. Several nuclear and cytoplasmic genes of *Brassicas* have been transferred to other species through asymmetric and symmetric hybridization.

#### IMPROVEMENTS IN OIL COMPOSITION AND PRODUCTIVITY

The value and suitability of seed oil for nutritional and industrial purposes is largely determined by its fatty acid composition (for detailed reviews see refs. 14-15). Originally, seeds of *Brassicas* contained two anti-nutritional components: erucic (cis-13-docosenoic acid, C<sub>22:1</sub>) acid present formerly in oils of cruciferous species in proportions as high as 25-50%; and glucosinolates—a group of chemically related thioglucosides. The seeds of *Brassica* species contain 40-44% of oil, which is primarily used for human consumption.

The meal cake, residue from crushed seeds, after removal of the oil contains 38–41% protein and is used as animal feed.<sup>16</sup> Erucic acid is poorly digested<sup>17</sup> and causes myocardial lesions in animals.<sup>18</sup> During the crushing of the seeds, myrosinase released from plant tissue hydrolyzes glucosinolates into anti-nutritional compounds.<sup>19</sup> Because of the anti-nutritional factors, *Brassica* oil-seeds did not find wide uses in Europe and North America. Only after the release of canola quality type of rapeseed and turnip rape by methods of conventional genetics and selection, was the possibility for its use as a vegetable oil opened. Double-low seed type canola implies that it contains less than 2% of erucic acid in the oil and less than 30 mol of the aliphatic glucosinolates per gram of oil-free meal.<sup>20,21</sup> Recently, *B. juncea* has also been developed to Canola quality, which could have a significant impact on the Indian subcontinent where brown mustard is a major oil crop. Largely due to the major improvements in seed quality described above, *Brassica* oil-seed crops have become the third most important world source of vegetable oil after soybean and oil palm providing over 13% of the world's edible oil supply.

Rapeseed oil contains primarily five fatty acids: palmitic, stearic, oleic, linoleic and linolenic acid. Compared to other edible fats, such as milk and pork fat, rapeseed oil contains a clearly lower level of saturated fatty acids. Especially, the content of linoleic acid, the only n-3 fatty acid of vegetable origin, is high. The desired fatty acid composition depends on the end use (margarine, cooking oil) but in general, improvement aims at an increase in palmitic, oleic and linoleic acid and a decrease in linolenic acid.<sup>22</sup> Recent interest in rape oil is based on an observation that the combined action of monounsaturated fatty acids and plant sterols lower serum cholesterol by inhibiting cholesterol absorption in the intestine.<sup>23</sup>

Attempts to improve fatty acid composition have included conventional breeding, cellular manipulations and transgenic approaches. Interspecific crosses have been successfully used for improving fatty acid composition. Embryo rescue and somatic hybridization—interspecific, intergeneric and inter-tribal—have further expanded the germplasm utility for *Brassica* oil improvement (for review see ref. 5). An interesting utilization of microspore culture has been its application to study lipid biosynthesis and accumulation in *B. napus* and *B. rapa*. Microspore-derived embryos of *B. napus* have also been used to screen for oil quality in vitro. Iqbal et al<sup>24</sup> showed that the biosynthesis level of glucosinolates in vitro plantlets derived from embryos correlated with the level

in seeds. In breeding programs in which the double haploid technique is applied, this could be useful for selecting genotypes with low glucosinolate content at an early stage during in vitro culture.

A powerful tool to speed up breeding for nutritionally improved fatty acid composition is molecular marker-assisted breeding. Linkage maps of *B. napus*, *B. oleracea*<sup>25</sup> and *B. rapa*<sup>26</sup> are available (for review see ref. 27). Markers associated with linolenic acid content in *B. napus*,<sup>28</sup> erucic acid content in *B. napus*<sup>29</sup> and *B. rapa*<sup>26</sup> palmitic and oleic acid content in *B. rapa*<sup>27</sup> have been reported and are utilized in breeding.

During the last three decades interest in industrial (non-nutritional) uses of vegetable oils has increased. Industrial needs in terms of oil composition are sometimes quite opposite of those required nutritionally. High erucic acid-containing oils are used as lubricants for two-cycle engines. Microspore-derived embryos have been used for in vitro selection of high erucic acid content and doubled-haploid lines used in combination with conventional breeding have yielded new high erucic acid breeding lines.<sup>30</sup> Rapeseed oil-derived methyl esters on a small scale have proven useful as a diesel fuel substitute. However, this application is not economic at the moment due to the low price of diesel fuel. Different oil quality modifications by means of molecular biology tools are now in progress. Another long chain fatty acid (in addition to erucic acid) of industrial interest is nervonic acid. Protoplast isolation and fusion to combine production properties of *B. napus* and high nervonic acid content of *Lunaria annua* are under intensive efforts.<sup>31</sup>

Potential for genetic engineering of fatty acid composition has sparked an interest in investigations of fatty acid biosynthesis pathways. The formation of storage oil from sucrose involves many enzymatic reactions, each of which could be a potential object for engineering. Expression of *E. coli* analogous enzymes from the lipid biosynthesis pathway in oil-seed rape have shown that, apparently, fatty acid biosynthesis is not controlled by one rate-limiting enzyme, such as acetyl-CoA carboxylase<sup>32</sup> or malonyl CoA-acyl carrier protein transacylase.<sup>33</sup> Recently, several genes for coding lipid biosynthesis enzymes and proteins have been cloned and sequenced including stearoyl-acyl carrier protein desaturase.<sup>34,35</sup> Expression of this gene in antisense orientation in rapeseed has led to reduction of the enzyme activity in embryos, blockage of conversion of stearoyl-ACP to oleoil-ACP and consequently to the rise of stearate level from 2% to 40%.<sup>34</sup>

Many lipid metabolism enzymes are insoluble trans-bilayer membrane proteins that are much more difficult to purify than soluble proteins. A good approach being used for cloning of such enzyme genes is T-DNA tagging mutagenesis and chromosome walking. Using this approach 3 fatty acid desaturase has been cloned from *A. thaliana* by *A. tumefaciens* T-DNA tagging mutagenesis and used as a hybridization probe for isolating the gene from *B. napus*.<sup>36</sup>

Several other modifications in oil composition could be performed in oil-seed *Brassicas*. Stearic acid (C<sub>18</sub> saturated fatty acid) serves as a major ingredient in margarines and cocoa butter substitutes. Transgenic oil-seed rape plants with high content of stearic acid have been obtained by Knutzon et al.<sup>34</sup> The transgenic plants are now in field experiments in Scotland and the U.S.A. and show normal establishment, vigor, yield and pest/disease-resistance characteristics.<sup>37</sup> Lauric acid (C<sub>12</sub> saturated fatty acid) is an important industrial product for the manufacture of detergents, soaps and surfactants. The gene for lauroyl-acyl-carrier protein thioesterase enzyme synthesizing lauric acid has been cloned from the California Bay plant *Umbelluria californica* and inserted into rapeseed (which does not contain lauric acid), resulting in a novel cultivar with a seed oil containing almost 25% lauric acid.<sup>38</sup> There are also some other attempts to produce high levels of euric, petrocelinic, linolenic, ricinolic fatty acids and Jojoba wax in rapeseed.<sup>14</sup>

#### ADJUSTMENT OF STORAGE PROTEIN COMPOSITION FOR IMPROVED FEED QUALITY

Rapeseed ranks third in the production of oil-seed meal after soybean and cotton. Its meal contains 40% protein after oil extraction. The protein pool for *B. napus* consists of 20–30% heterologous 2S albumin seed storage protein, napin, and 60% 11S globulin seed storage protein, cruciferin.<sup>39</sup> Another remarkable protein in rapeseed, oleosin, plays an important role in oil storage. Napsins have an average molecular weight of 13 kDa and are composed of a large (9 kDa) and a small (4 kDa) subunit linked by disulfide bonds. They are coded by a multi-gene family and several cDNA and genomic clones have been isolated as early as the 1980s.<sup>40</sup> Napin genes exhibit more than 90% sequence identity among themselves. Rapeseed storage proteins, like most other cultivars, have unbalanced amino acid composition and levels of certain essential amino acids; particularly methionine and lysine are too low for optimum feed quality. Increases in the levels of both methionine and lysine would make rapeseed meal more competitive with soybean meal.

Expression of napin gene in antisense orientation led to a significant reduction in napin content in rapeseds. Reduction of the napin level was compensated for by elevated cruciferin synthesis. Because albumins have more sulfur-containing amino acids and lysine than globulins, shift of protein synthesis from albumin (napin) to (globulin)—cruceferin has led to further reduction in cysteine and lysine.<sup>41</sup> Insertion of Brazil nut methionine-rich 2S albumin in rapeseed genome resulted in the accumulation of heterologous methionine-rich protein at levels that range from 1.7 to 4.0% of the total seed protein and contain up to 33% more methionine.<sup>42</sup> Use of chimeric 2S albumin (napin) with an increased methionine and lysine content for insertion into the rapeseed genome led to the accumulation of methionine-enriched protein, up to 2% of the total high salt-extractable seed protein.<sup>43</sup> In contrast to these data, in field experiments, expression of the chimeric 2S albumin gene in *B. napus* was at an undetectable level.<sup>44</sup>

Oil-seeds contain abundant hydrophobic proteins named oleosins, which are also under intensive research. Oleosins are small oil body proteins (16–24 kDa) tightly associated with the oil body membrane. Each oil body of 1 m in diameter contains a matrix of triacylglycerols surrounded by a layer of phospholipids embedded with oleosins. The possible functions of oleosins include maintenance of the structural integrity of oil bodies and serving as a recognition signal for lipase binding during oil mobilization in seedlings. Genes coding for oleosins have been cloned and investigated quite thoroughly by deletion analysis using GUS fusions.<sup>45</sup> It was shown that oleosin and napin gene promoters are under the same regulation and are interchangeable.<sup>46</sup> Also, analogous genes like maize oleosin can be expressed in *B. napus* and correctly targeted to seed oil bodies.<sup>47</sup>

#### TOLERANCE OF NIOTIC AND ABIOTIC STRESSES

One of the most developed lines of biotechnology research of *Brassicas* is the development and management of resistance to weeds, fungi, insects, diseases and abiotic stresses. Development of weed control strategies started as early as the 1980s when transformation technology and genes coding for herbicide resistance became available. Oil-seed rape plants resistant to Bialaphos and Glufosinate (phosphinothricin) were obtained using *A. tumefaciens* mediated transformation of the *bar* (phosphinothricin acetyl transferase) gene isolated from *Streptomyces hygroscopicus*<sup>48</sup> and the synthesized gene.<sup>49</sup> Cloning of a mutant aceto-

hydroxyacid or acetolactate synthase (with blocked allosteric control by isoleucine and leucine) from *Arabidopsis thaliana* into oil-seed rape resulted in resistance of rapeseed to sulphonylurea herbicides chlorsulphuron, imidazolinone, flumetsulam, imazamethabenz, metsulfuron and imazethapyr.<sup>50</sup> Similarly, transfer of a gene from *aroA* locus from *Salmonella typhimurium* into *B. campestris* resulted in glyphosate (Roundup) resistance.<sup>51</sup>

Resistance to fungi could be developed by transforming plants with genes coding for enzymes able to degrade mycelium cell walls of phytopathogenic fungi (chitin and glucan). Transgenic canola plants expressing bean chitinase gene under CaMV 35S promoter have shown reduced or delayed seedling mortality in comparison to non-transformed controls after inoculation with *Rhizoctonia solani*.<sup>52,53</sup> Another approach of fungal resistance is to degrade oxalic acid that is produced by several plant pathogenic fungi including *Sclerotinia sclerotiorum* and is thought to have a primary role in pathogenesis. A gene coding for the enzyme oxalate oxidase was isolated from barley roots and introduced into oil-seed rape as a means of degrading oxalic acid in vivo. Transgenic plants were shown to contain an active oxalate oxidase enzyme and were tolerant to exogenously supplied oxalic acid.<sup>54</sup>

There are also some successful reports on engineered disease resistance in oil-seed rape. Transgenic *B. napus* plants obtained by direct transfer of two separate plasmids containing the cauliflower mosaic virus coat protein gene IV and a selectable marker gene have shown expression of the transgenes. Assessment of CaMV virus resistance is on-going.<sup>55</sup> As a result of *A. rhizogenes* transformation of *B. napus* with the 3' untranslated region of the turnip yellow mosaic virus (TYMV) genome the transgenic rapeseed plants showed partial resistance to TYMV RNA or virion infection.<sup>56</sup> Resistance against rapeseed pathogens *Phoma lingam* (black leg disease) and *Plasmoidiophora brassicae* (club root disease) harbored by the *Brassica nigra* (BB) genome has been transferred to *Brassica napus* (AACC) through asymmetric somatic hybridization. Regenerants have revealed resistance and susceptibility to both diseases and also only to one pathogen.<sup>57</sup>

Engineered insect resistance in *Brassicas* has dealt mainly with the transformation of plants by *cry* (crystallogenic) protein (*Bt* toxins or delta-endotoxins) genes of *Bacillus thuringiensis*. The mechanism of action of *Bt* toxins implies high specific binding to receptor molecules (some kind of proteins) in the insect midgut and, subsequently, ion pore formation in the mid-gut epithelium cell membrane that results in ion paralysis of the cell

and eventually of the epithelium. This action leads to the death of the insect larvae. *Cry* genes are divided into five classes according to their specificity of action. The most harmful *Brassica* pest of the *Lepidoptera* family is the diamond back moth *Plutella xylostella*. *cryIAc* gene from *B. thuringiensis* ssp *Kurstaki* HD73 effective against *P. xylostella* has been fused with the *uidA* (GUS) gene and cloned in *Brassica juncea* plants.<sup>58</sup> Although the presence and expression of the gene by histochemical GUS assay and Northern blot was clearly shown, it was established that native *cry* genes are not expressed in plants sufficiently to kill insects and to be detectable by Western blot. The *cry* genes, especially from *cryI* group, should be truncated and resynthesized with changed codon preference. Unfortunately, there are no data on rapeseed transformation by any of the *cryIII* genes, which would be effective against blossom beetle *Meligethes aeneus*—a dangerous Coleopteran pest of oil-seed *Brassicas*. Under discussion now are other methods of blossom beetle control such as transformation of crops by different proteinase inhibitors.<sup>59</sup>

Abiotic stress tolerance implies mainly tolerance to temperature and osmotic stress. Both properties depend on complicated molecular mechanisms. In many species, osmotic regulation in response to drought and high salinity is related to the overproduction of proline and betaines. Mechanisms of interactions of betaines, proline and polyamides in *Brassica napus* are being studied.<sup>60-62</sup> Altered gene expression in response to drought in *B. napus* has also been detected and some of the corresponding genes cloned.<sup>63,64</sup> An additional application of transgenesis in rapeseed is heavy metal tolerance conferred by the gene of human metallothionein-II. Transgenic plants can be cultivated on heavy metal contaminated soils.<sup>65</sup>

## ENGINEERING MALE STERILITY AND SELF-INCOMPATIBILITY

Cultivated forms of oil-seed *Brassicas* are almost all self-compatible and do not possess male sterility (MS) traits. Male sterility prevents self-fertilization and can be used for the production of hybrid seed. A major concern in oil-seed *Brassicas* breeding for MS has been to find a functional male sterility system that would be genetically stable and not influenced by environmental conditions. Two forms, cytoplasmic (CMS) and genetic (GMS) male sterility, are under development. There are several successful approaches to obtain CMS forms through somatic hybridization with related species that harbor the CMS trait.

The donor-recipient protoplast fusion method was used in somatic hybridization of *Brassica*

*tournefortii* and *Brassica napus*. Some of the hybrid plants carried rapeseed nuclear genome and *Brassica tournefortii* mitochondrial (and plastid) genome with the CMS traits encoded by the *atp6* region.<sup>66</sup> CMS line of brown mustard *B. juncea* has been developed by combining the cytoplasm originating from the somatic hybrid *Trachystoma ballii* + *B. Juncea* by repeated backcrossing with brown mustard.<sup>67</sup>

Plant Genetic Systems N.V.<sup>68</sup> has developed an elegant GMS system. The PGS hybridization system for oil-seed rape is based on the recombinant DNA GMS system that was developed for *A. tumefaciens* transformation of *barstar-barnase* genes. The *barnase* gene encodes a specific RNase that disrupts normal cell functions. Under the control of PTA29 promoter, *barnase* expression is confined to the tapetum cell layer of the anthers. Due to the exact timing of expression, early anther development is arrested leading to GMS. The *barstar* gene, controlled by the same promoter, has no effect on plant phenotype. The protein encoded by *barstar* is a specific inhibitor of the *barnase*-derived RNase. When both are expressed in the same plant, *barstar* interferes with *barnase* and prevents the induction of male sterility. Thus, during hybridization of *barstar* and *barnase* expressing lines, pollen of *barstar* line restores pollination capacity of *barnase* line embryos.<sup>69</sup> The PGS male sterility system has already been employed for commercial production of hybrid canola. A report is also available on double (CMS + GMS) male sterile *B. napus* developed by means of conventional genetics.<sup>70</sup>

Another original approach, which could be used in hybridization, is to arrest embryo development mediated by expression of modified *Pseudomonas aeruginosa* exotoxin A in transgenic *Brassica napus* under napin regulatory sequences.<sup>71</sup>

Self-compatibility of oil-seed *Brassicaceae* is also a barrier to field hybridization, which could be removed by biotechnological tools. *Brassica oleracea* genome harbors glycoprotein (SLG) and S receptor kinase (SRK) genes localized in the S locus responsible for self-incompatibility that are under research in transgenic rapeseed. However, engineered self-incompatibility of *B. napus* has not been obtained yet because of the polymorphism of S locus proteins.<sup>72,73</sup>

## RECENT ADVANCES IN ENGINEERING VEGETABLE *BRASSICAS*

Vegetable *Brassicaceae* include four major species:

*Brassica oleracea*

- var. *botrytis* / cauliflower

- var. *capitata* / cabbage
- var. *gemmifera* / Brussels sprout
- var. *gongylodes* / kohlrabi
- var. *italica* / broccoli
- var. *medullosa* / narrow stem kale
- var. *sabauda* / Savoy cabbage
- var. *sabellica* (var. *acephala*) / common kale
- var. *viridis* / fodder kale

*Brassica chinensis* / chinese mustard (pak-choi)

*Brassica pekinensis* / chinese cabbage

*Brassica perviridis* / spinach mustard

*Brassica oleracea* includes the major vegetable *Brassica* forms, and most of the biotechnology applications have been reported on this species. *Brassica oleracea* carrying the CC-genotype is recalcitrant to genetic transformation but relatively amenable to regeneration in cell culture conditions. However, several important vegetable *Brassicas* have already been transformed with various methods including *Agrobacterium*-mediated transformation. Table 15.1. lists the reports of *Agrobacterium*-mediated genetic transformations of *B. oleracea*, which compose the most important group of vegetable *Brassicas*. Also few stable transformations of other vegetable *Brassicas* have been published.<sup>74</sup>

In gene transfer work, different explant sources such as hypocotyls, cotyledons, stem segments, leaf discs, flower stems and seed have been included. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* strains, both disarmed and oncogenic ones, have been used. Also different selective agents have been applied: kanamycin, phosphinotrycin<sup>6</sup> and hygromycin.<sup>75</sup> Variability in regeneration capacity is generally higher at the cultivar level than at the *Brassica* species level<sup>76</sup> and thus the potential for transformation of different cultivars also differs.<sup>77</sup> Regeneration capacity does not necessarily correlate with susceptibility to *Agrobacterium*; therefore, in transformation work, different cultivars should be screened both for regeneration capacity and susceptibility to *Agrobacterium*.

De Block et al<sup>6</sup> discovered that the use of silver nitrate was a prerequisite for efficient shoot regeneration of *Brassica oleracea* var. *botrytis* under selective conditions and that it was beneficial to increase aeration in the culture vessels by using porous tape to close them. *B. oleracea* plant material frequently became necrotic at the cut or wounded surface in the transformation with *Agrobacterium*. The necrosis can be reduced by keeping transformed plant material in the dark for two weeks.<sup>78</sup> However, wounding the plant material is not necessary, and *B. oleracea* var. *botrytis* seeds have successfully been used for transformation. Surface sterilized seeds were infected with *A. tumefaciens* by using an insect

**Table 15.1. Agrobacterium-mediated genetic transformations of *Brassica oleraceae***

Variety	Explant type	Agrobacterium strain	Reference
cauliflower	hypocotyl	<i>A. rhizogenes</i> strain 8196, oncogenic	David C & Tempe J. 1988 <sup>81</sup>
cauliflower	leaf	<i>A. tumefaciens</i> strain C58C1, oncogenic	Srivastava V et al. 1988 <sup>87</sup>
cauliflower	hypocotyl	<i>A. tumefaciens</i> strain C58C1, disarmed	De Block M et al. 1989 <sup>6</sup>
cauliflower	flower stem	<i>A. tumefaciens</i> strain pCIB542	Toriyama K et al. 1991 <sup>77</sup>
cauliflower	seeds	<i>A. tumefaciens</i> strain LBA4404, disarmed	Eimert K et al. 1992 <sup>78</sup>
cauliflower	leaf	<i>A. tumefaciens</i> strain LBA4404, disarmed	Eimert K et al. 1992 <sup>78</sup>
cabbage	petiole and leaf	<i>A. rhizogenes</i> strain A4, oncogenic	Berthomieu P & Jouanin L. 1992 <sup>75</sup>
acephala	cotyledon petiole and leaf	<i>A. rhizogenes</i> strain A4, oncogenic	Christey M & Sinclair BK. 1992 <sup>82</sup>
cabbage	cotyledon	<i>A. tumefaciens</i> strain ASE-1, disarmed	Baily et al. 1993 <sup>84</sup>
cauliflower	seedling, in vitro plantlet and mature plant	<i>A. tumefaciens</i>	Ovesna J et al. 1993 <sup>88</sup>
cabbage, cauliflower	hypocotyl and cotyledon	<i>A. tumefaciens</i> strain LBA4404, disarmed	He Y et al. 1994 <sup>89</sup>
cabbage	in vitro plant	co-cultivation of oncogenic <i>A. tumefaciens</i> strain 82.139, and disarmed strains <i>A. tumefaciens</i> strains C58pMP90 or LBA4404	Berthomieu P et al. 1994 <sup>86</sup>
Brussels sprout	leaves	<i>A. rhizogenes</i> strain IFO13257	Hosoki T & Kigo T. 1994 <sup>90</sup>
cabbage, broccoli	hypocotyl cotyledon petiole and peduncel	<i>A. tumefaciens</i> strain ABI, disarmed	Metz T et al. 1995 <sup>85</sup>

pin after dipping it into a suspension of *Agrobacterium*.<sup>79</sup> Also in vitro plants have been used for transformation with *A. tumefaciens* and *A. rhizogenes* in the co-cultivation method, where oncogenic and disarmed *Agrobacterium* were used simultaneously. When co-cultivation method is used, transformed shoots can be regenerated from shooty tumors on kanamycin containing basal medium without hormones. The co-cultivation method is based on the fact that some wild-type *A. tumefaciens* strains can induce shooty tumors in the *Brassicaceae*. For example, wild-type *A. tumefaciens* 82.139 has been used to transform rapid cycling cabbage<sup>75</sup> and cauliflower, and *A. tumefaciens* C58 has been used to transform cabbage.<sup>80</sup> Oncogenic *A. rhizogenes* can also be used in gene transfer work as it induces transformed roots that can be regenerated into shoots by using appropriate hormones

in the growth medium.<sup>75,81,82</sup> *A. rhizogenes* can cause reduced apical dominance, reduced fertility, shortened internodes, wrinkled leaves, late flowering and plagiopropic roots.<sup>83</sup>

There are three examples of a transferred gene being used for an agronomic application: two of them are transgenic plants expressing a *Bacillus thuringiensis* insecticidal *Bt*-toxin gene in cabbage (*B. oleracea* var. *capitata*)<sup>84</sup> and broccoli (*B. oleracea* var. *italica*)<sup>85</sup> and the third is transformation of cauliflower (*B. oleracea* var. *botrytis*) by cauliflower mosaic virus coat protein.<sup>86</sup> Cabbage and other vegetable *Brassicaceae* have many severe insect pests that endanger the harvest if chemical insecticides are not used. Transformation of *Brassica* with *Bt*-toxin genes could be an effective biological method for controlling insect pests as shown by Metz et al.<sup>85</sup> Many *Bt*-toxin genes against lepidopteran pests are

known. In addition to insect pests, *Brassicas* suffer from a number of diseases like clubroot caused by *Plasmidiophora brassicae* and storage decay causing fungi; *Botrytis cinerea* and *Sclerotinia sclerotiorum*. It might be possible in the future to transfer naturally occurring resistance genes from resistant cultivars to susceptible ones or to find genes against fungi like those coding for chitinases that degrade chitin in fungal cell walls. Transgenic *B. napus* expressing a bean chitinase gene has shown reduced or delayed seedling mortality after inoculation with *Rhizoctonia solani*.<sup>53</sup>

For industrial use, cabbage could be a suitable source to produce proteins as a bioreactor grown in the field. Protein content in cabbage is 2-3% and harvest per hectare is 50-60 tons of fresh material, which amounts to 1500-1800 kg protein per hectare. In addition to producing large biomass, cabbage does not contain toxic compounds like potato or tobacco. Therefore, for the food and feed industry, there would be possibilities to express enzymes of interest in transgenic cabbage. Difficulties in transformation are serious limiting factors for developing biotechnological applications in vegetable *Brassicas*. Routine transformation still requires optimization and information on culturing parameters that affect the transformation. Probably it would be useful to search for new, more effective *Agrobacterium* strains or to adapt the known direct DNA transfer systems, like particle bombardment, to vegetable *Brassicas*.

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CHAPTER 16

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# TRADITIONAL MEDICINAL PLANT GENETIC RESOURCES AND BIOTECHNOLOGY APPLICATIONS

K. Shimomura, K. Yoshimatsu, M. Jaziri and K. Ishimaru

## INTRODUCTION

From carbon dioxide, water and inorganic ions, plant enzymes manipulate organic syntheses that result in a complex array of natural product molecules. This metabolic activity leads to the elaboration of two classes of metabolites: primary metabolites and secondary metabolites (Fig. 16.1).<sup>1</sup>

Primary metabolites, such as sugars and amino acids, are substances widely distributed in nature. They occur in one form or another in virtually all organisms, and are directly implicated in the basic life process of the plant: plant growth and development. Secondary metabolites are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families). Secondary metabolites, which show extreme structural diversity, are supposed to be implicated in plant-environment interactions, tending to be synthesized in specialized cell types and at distinct developmental stages in contrast to primary metabolites. Contrary to the previous belief that secondary metabolites have little explicit function in plant organism, there is an increasing awareness that numerous secondary products play a physiological role. Secondary metabolites may provide plants with adjustment to changing circumstances and are, thus, a measure of the fitness of the plant to survive. Secondary metabolites often have an ecological role; they are pollination attractants, represent chemical adaptations to environment stresses, or serve as chemical defenses against microorganisms, insects and higher predators, and even other plants (allelochemicals).

From the earliest times, humans have had to distinguish between those plants which are poisonous and those which are not, and a knowledge of naturally occurring drugs has gradually developed. Various kinds of plants have been used in the traditional systems of medicines that have been uniquely developed in various areas of the world,<sup>2</sup> and they have greatly contributed to the fundamental establishment of modern medicine. Nowadays, plants are still the most important source of modern allopathic medicines (Table 16.1). Some are extracted and isolated from plant materials as a pure substance of therapeutic value (e.g. morphine as an analgesic, codeine as an antitussive and vinblastine and vincristine as antitumor drugs), some others are semi-synthetic products that depend on plant sources for starting materials (e.g. steroid hormones).<sup>3</sup> In this

chapter, plants for traditional medicines, plants as a source of medicines and biotechnological application for conservation of medicinal plant resources and alternative source of pharmaceuticals are described.

## PLANTS FOR TRADITIONAL MEDICINE

Traditional medicine is a rather vague term loosely used to distinguish ancient and culture-

bound health care practice that existed before the application of science to health matters in official modern scientific medicine or allopathy. Until the beginning of the 19th century, all medical practice was what we now call traditional medicine and it has made a great contribution to the welfare of all nations.<sup>2</sup> Traditional medicines, of which a major portion involves the use of plant extracts, naturally varied according to the plants available in a particular climatic area.<sup>4</sup> It has been estimated that from the

Fig. 16.1. Primary and secondary metabolism in plant.

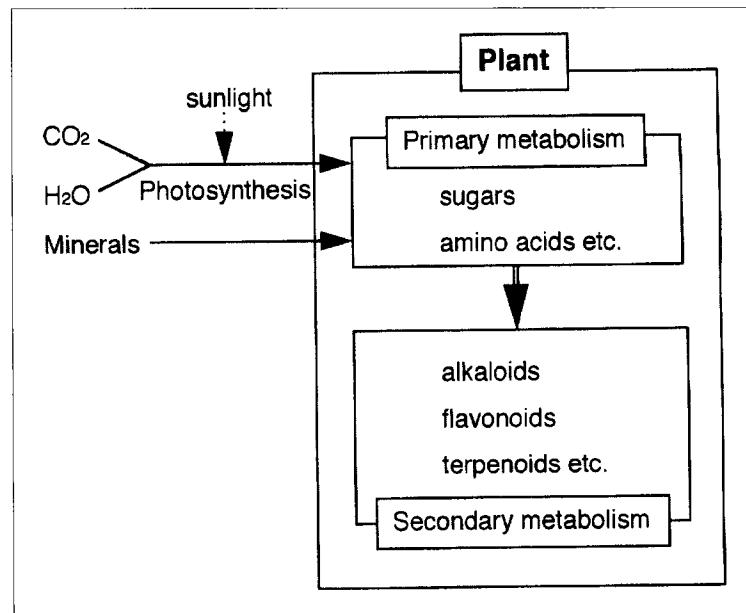


Table 16.1. Some plant-derived drugs obtained commercially from plant sources

Plant name	Compound	Product	Major therapeutic category
<i>Dioscorea</i> spp. (Mexican yams)	diosgenin	steroidal hormones	oral contraceptives, corticosteroids
<i>Digitalis lanata</i> (Grecian foxglove)	digoxin	methyl digoxin	cardiac glycosides
<i>Atropa beladonna</i> (Belladonna)	hyoscyamine	atropine sulfate	parasympatholytic agents
<i>Duboisia</i> spp.	scopolamine	N-butylscopolamine bromide	parasympatholytic agents
<i>Papaver somniferum</i> (Opium poppy)	morphine	morphine hydrochloride	narcotic analgesics
	codeine	codeine phosphate	antitussives
<i>Catharanthus roseus</i> (Periwinkle)	vincristine	vincristine sulfate	antineoplastics
	vinblastine	vinblastine sulfate	antineoplastics hydrate
<i>Colchicum autumnale</i> (Autumn crocus)	colchicine	colchicine	antigout agents
<i>Taxus brevifolia</i> (Pacific yew)	paclitaxel	paclitaxel	antineoplastics
<i>Ephedra sinica</i>	ephedrine	ephedrine hydrochloride	bronchodilator

250,000 to 750,000 species of higher (flowering) plants existing on earth, some have not yet been botanically described. Although there is no way to determine the accurate number of these species that have been used in traditional medicine, a reasonable estimate would be about 10% of all the species (from 25,000 to 75,000).<sup>5</sup> These collections of herbal medicines, compiled over centuries by trial and error and presumably using the patient as the experimental animal throughout, must surely contain some material worthy of further investigation and should not be too readily discarded.<sup>4</sup> Phytotherapy, including the traditional systems in the industrialized countries, had declined during the first half of the 20th century;<sup>6</sup> however, approximately 80% of the people in developing countries rely chiefly on traditional medicines for their primary health care needs.<sup>7</sup> Even in the industrialized countries, there has been an immense revival interest in the phytotherapy in recent years, because the prevalence of chemotherapy by synthetic drugs in these countries has led to a true drug-related chemical pollution, superimposed on the environmental pollution.<sup>6</sup>

For many areas of the world, the plants used in traditional medicine have been adequately recorded. However, for other regions, e.g. in South America with its vast flora of potentially useful plants, the art of their systems of medicines in aboriginal societies is in rapid decline owing to a changing mode of life of the people.<sup>4</sup> There is a great need to register local knowledge before it is completely lost. Ethnobotanists are currently fighting a battle against time to record such information before it is lost in a generation or so.<sup>4</sup>

Among the numerous traditional medicines including codified and non-codified systems, there are two most developed systems, namely, traditional Chinese medicine and Indian ayurvedic medicine, which are now receiving scientific attention.<sup>3</sup> These two systems are formalized and have been recognized by their respective national governments.<sup>8</sup> Both of them have a unique theoretical system that can neither be replaced nor explained by modern science. Both systems explain that illness occurs due to imbalance in the equilibrium of the basic elements. In the case of traditional Chinese medicine, yin (represents the negative and/or passive force), and yang (represents the positive and/or active force) are two primordial cosmic forces in the human body (microcosm).<sup>9</sup> In ayurveda vata (motion), pitta (energy) and kapha (inertia) are the basic constituents of the physiological systems.<sup>10</sup>

## PLANTS AS A SOURCE OF MEDICINES

Plant-derived compounds have been used in medicine throughout history. There are about 121 clinically useful prescription drugs worldwide that are derived from higher plants of 95 species.<sup>7</sup> About 74% of them have been discovered through follow-up investigation of their traditional medicinal uses.<sup>11</sup> One recent successful discovery is the isolation of the anticancer alkaloids, vincristine and vinblastine, from *Catharanthus roseus*, which has been used for the treatment of diabetes by the indigenous people in Madagascar. Although modern investigators have been unable to confirm this property, Canadian workers, during 1955-1960, discovered that extracts of the leaves produced leukopenic actions in rats. These observations led researchers at Eli Lilly and Co. to undertake an intensive phytochemical investigation of the plant with the view to isolate constituents of value in cancer chemotherapy. The investigation resulted in the discovery of vincristine and vinblastine.<sup>12,13</sup> Thus indigenous knowledge is helpful to select plant materials from immense genetic pools of plant species for drug discovery because a long history of use by humans indicates the presence of a biologically active constituent in a plant,<sup>11</sup> but it is not entirely essential. Since only 15 to 17% of all flowering plant species have been studied for their medical potential,<sup>11</sup> undoubtedly the plant kingdom still holds many species of plants containing substances of medicinal value that have yet to be discovered. Widely adopted, the plant drug discovery approach at present is the screening of a large set of diverse plant samples for one or more biological activities.<sup>11</sup> Compared to traditional medicinal use based drug discovery approach, the discovery reservoir is much deeper in biodiversity-based search; at least 80% of the flowering plants on earth (200,000 species) remain to be investigated, the greater proportion of which are tropical forest plants.<sup>11</sup> Needless to say, plants must be taxonomically classified and/or identified for any drug development program. Large numbers of plants are constantly being screened for their possible pharmacological value.<sup>3,11</sup>

The discovery of a new drug and its development into a new commercial drug are an extremely expensive and complex, multifaceted process that may take many years and a large expenditure of money.<sup>14</sup> Key steps in the discovery process include: 1) selection or characterization of plants for study; 2) design or application of novel screens; 3) isolation, purification and structure elucidation of a new

active lead (compound with many of the characteristics of a desired new drug that will be used as a model for chemical modification); 4) discovery and characterization of novel, unobvious and useful biological properties of the new lead; and 5) synthesis of useful new analogs or derivatives.<sup>14</sup>

### DISCOVERY OF PACLITAXEL AS A NEW ANTIPLASTIC

Over a period of 20 years (1960-1981), the National Cancer Institute (NCI) screened more than 32,000 higher plant species for their ability to inhibit various tumors. About 7% of these plant species exhibited activity and several hundred cytotoxic and/or antitumoral compounds were discovered during the NCI program. This hard and time-consuming work resulted in the discovery of paclitaxel, a new antitumor compound developed into a drug to treat ovarian and breast cancer, from the bark of Pacific yew (*Taxus brevifolia*).<sup>15-17</sup>

The history of paclitaxel discovery is presented in Figure 16.2. Paclitaxel was not patented by its discoverers; probably because its concentration in the bark of *Taxus* was found to be very low (approximately 0.001% dry weight basis). In addition, because of the difficulty in obtaining the

substance and its insolubility in water, which makes any pharmaceutical formulation difficult, the pharmacological importance of paclitaxel was not recognized until the late seventies, when it was first realized how it functioned. Nowadays, paclitaxel is considered to be the most promising anticancer drug of the past 15 years. Indeed, NCI considered paclitaxel of 'prime importance' and decided to treat 12,000 patients a year with it. At this point, the problem of availability has arisen. Yew bark is thin (about 3 mm), and a century-old plant yields an average of 3 kg of bark, corresponding to 300 mg of paclitaxel (approximately a single dose in the course of a cancer treatment). Thus, three century-old *Taxus* plants are needed in order to obtain 1 g of paclitaxel.<sup>26</sup> A course of treatment with paclitaxel requires about 2 g of product, which means six century-old trees are needed. The decortication of the stem kills the tree. Therefore, environmentalist groups strongly oppose the demands of the medical community and pharmaceutical industry, because such large-scale harvesting of *Taxus* stem barks would mean the disappearance of the Pacific yew within a few years. Today's priority of a research program is to solve the problem of product availability.

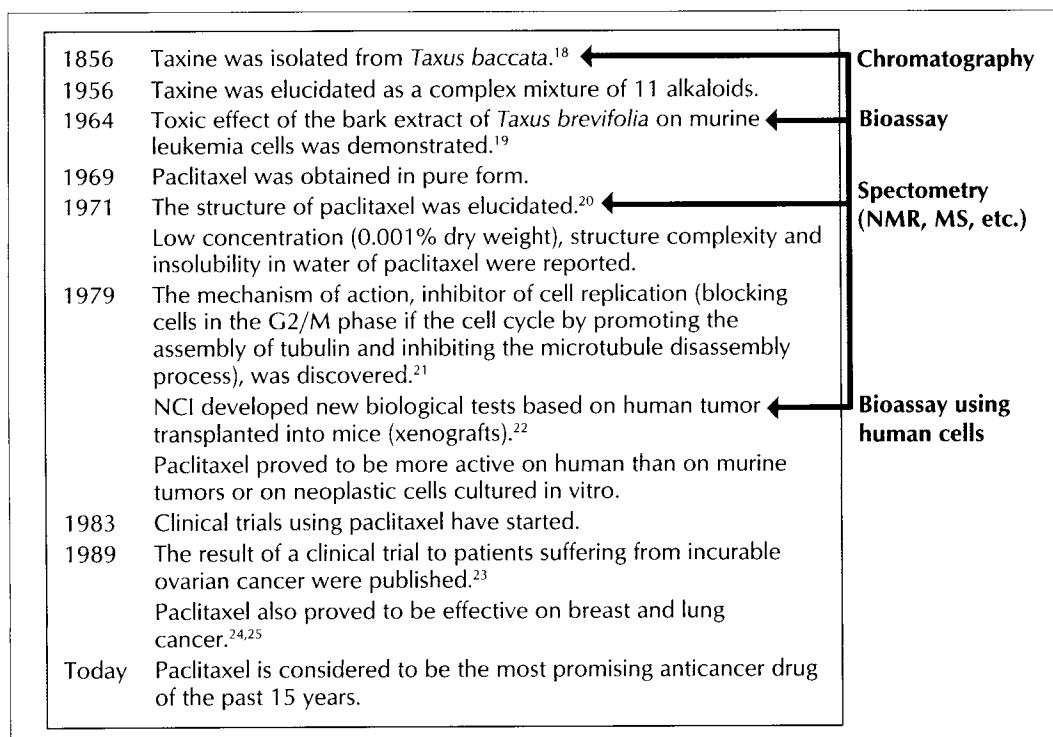


Fig. 16.2. History of Paclitaxel discovery as an antineoplastic agent.

### NEW APPROACH TO IMPROVE PRODUCT AVAILABILITY OF PACLITAXEL

The discovery of paclitaxel is strongly linked to the development of appropriate chromatographic and biological methods for separation and localization of the active fractions (Fig. 16.3). However, this method may eliminate compounds whose structures are closely related to the active ones but not biologically effective. The compounds that do not exert biological activity alone but have closely related structures to the active principle could be a valuable material such as precursor to produce the active compound by hemisynthesis. Which method will allow the detection of such structurally related compounds in crude plant extracts? Although chemical reagents are indeed used for such purposes, they can only be used for the detection of one class of compounds. For example, the Dragendorff's reagent is widely used for the detection of alkaloids by TLC analysis, but this reagent cannot distinguish between the several subclasses of alkaloids showing different molecular structures. In general, reagents are specific for a functional group rather than for the molecular structure itself. Thus, a specific chemical reagent for the taxane skeleton is not yet available. The approach was based on the intrinsic properties of two biological tools: antibodies and receptors, both specific proteins, for the detection of the taxane molecular structures can overcome the matters mentioned above (Fig. 16.4). In both cases, biochemical reactions are involved in which the target compound is recognized from its particular structure by its corresponding antibody or its corresponding naturally occurring receptor. To utilize this specific recognition, two different biochemical assays have been developed and used for the research of new biologically active taxanes in *Taxus* plant extracts: 1) the production of anti-paclitaxel antibodies and the establishment of an immunoassay; and 2) the use of *in vitro* assembly and disassembly of microtubules.

### IMMUNOASSAY FOR THE DETECTION OF TAXOIDS

Compounds with low molecular weight, such as paclitaxel, do not induce the formation of antibodies when injected into animals. They must be conjugated to an immunogenic carrier, such as protein, and thus form the antigen. One of the procedures frequently used consists of the synthesis of derivatives containing a free carboxylic function, followed by a reaction of this carboxylic function with a free amino group of the protein carrier to form a stable and non-hydrolyzable peptide bond, and the antigen is thus constructed (Fig. 16.5).<sup>27</sup> The antibodies obtained after immunization of rabbits, for example, are then used for the establishment of an immunoassay. In the case of paclitaxel, an enzyme-linked immunosorbent assay (ELISA) has been developed. Using serial dilutions of pure paclitaxel, a typical standard curve can be drawn that allows a quantitative determination of paclitaxel-related compounds in crude plant extracts (Fig. 16.6). Cross-reactivity with other pure taxanes showed that the anti-paclitaxel antibodies recognize equally well paclitaxel and cephalomanine, which have different

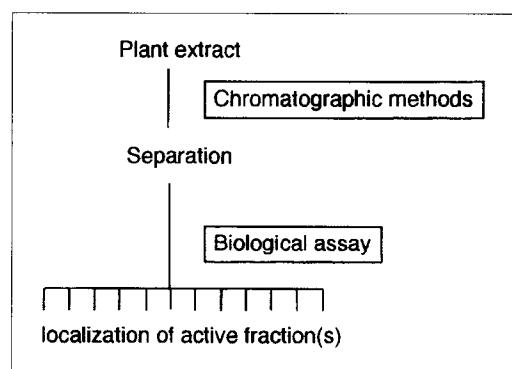


Fig. 16.3. Separation and localization of the active fractions.

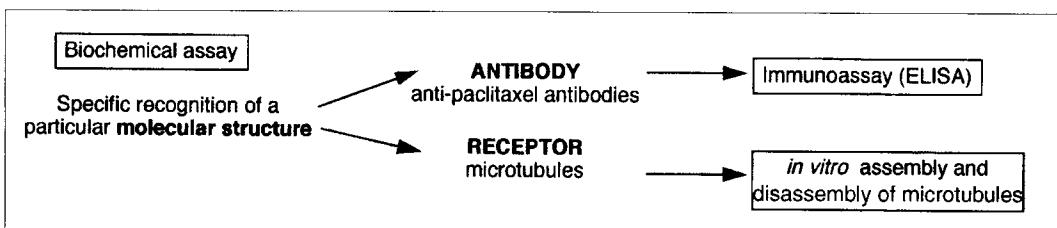


Fig. 16.4. Strategy for the detection of structurally related compound.

side-chain substituents at C-13 position. Baccatin III, which differs only by the C-13 substituent, exhibits an affinity much lower than that of the two other compounds. Finally 10-deacetylbaccatin III, which differs from baccatin III only by the acetate at C-10 position, is not recognized at all (Fig. 16.6).

Figure 16.7 shows the experimental procedure used for the separation and isolation of taxane compounds from stem bark extract of *T. baccata*. ELISA monitoring was used in each chromatographic step. The dichloromethane fraction was subjected to silica gel column chromatography and 13 fractions were obtained and assayed by ELISA. Fraction 11 showed the highest immunosignal. The examination of this fraction by TLC and HPLC indicated that paclitaxel and cephalomanine were the major constituents. Fraction 10 was also subjected to HPLC fractionation and ELISA moni-

toring. And this led to the isolation of two new taxane molecules; one of them, paclitaxel C, exhibits a cytotoxic activity twice as high as paclitaxel.<sup>28</sup>

This result shows that the strategy is successful and biologically active taxoids can be detected efficiently. This strategy is now routinely used for screening programs. In addition, a second ELISA using anti-10-deacetylbaccatin III antibodies, which open a wide field of investigation for the search for new taxane molecules, have developed. Several new taxoids have been isolated and identified.<sup>28-30</sup>

#### IN VITRO ASSEMBLY AND DISASSEMBLY OF MICROTUBULES FOR THE DETECTION OF TAXOIDS

The second approach developed to search for new biologically active taxoids in chromatographic fractions is the use of receptors that also recognize molecular structures. The naturally occurring receptor for paclitaxel is localized on the microtubules. Microtubules are filamentous structures found in virtually all eukaryotic cells. Microtubules are mainly composed of tubulin, a heterodimeric protein having similar  $\alpha$ - and  $\beta$ -subunits; each has a molecular mass about 55 KDa. In the presence of guanosine-triphosphate (GTP), tubulin polymerizes in a hollow cylindrical structure to form protofilaments, 13 of which form the basic microtubule wall that is made up of a helical array of alternating  $\alpha$ - and  $\beta$ -tubulin subunits. Tubulin *in vivo* is remarkable because of its ability to rapidly assemble and disassemble in response to a wide variety of chemical agents and physical conditions (Fig. 16.8). The dynamic instability of microtubules is exploited in cell development, for example, the formation of the mitotic spindle during cell division.

Recently, it has been demonstrated that the manipulation of chemical or physical conditions, such as temperature, allows the assembly and disassembly of the supernatant obtained from centrifuged brain homogenates. This observation leads to the establishment of an *in vitro* bioassay system. *In vitro* microtubule formation is induced by heating the tubulin from 4°C to 37°C (the addition of GTP is necessary) and is monitored by the measurement of turbidity, which is a reliable determinant of the mass of tubulin assembled into higher molecular weight structure, the microtubule. This polymerization is reversed by cooling to 4°C (Fig. 16.8).

A number of natural products and synthetic compounds have been shown to act as antimitotic agents because of their ability to disrupt the dynamic cycling of tubulins. Many of these compounds, such as colchicine, vincristine, vinblastine,

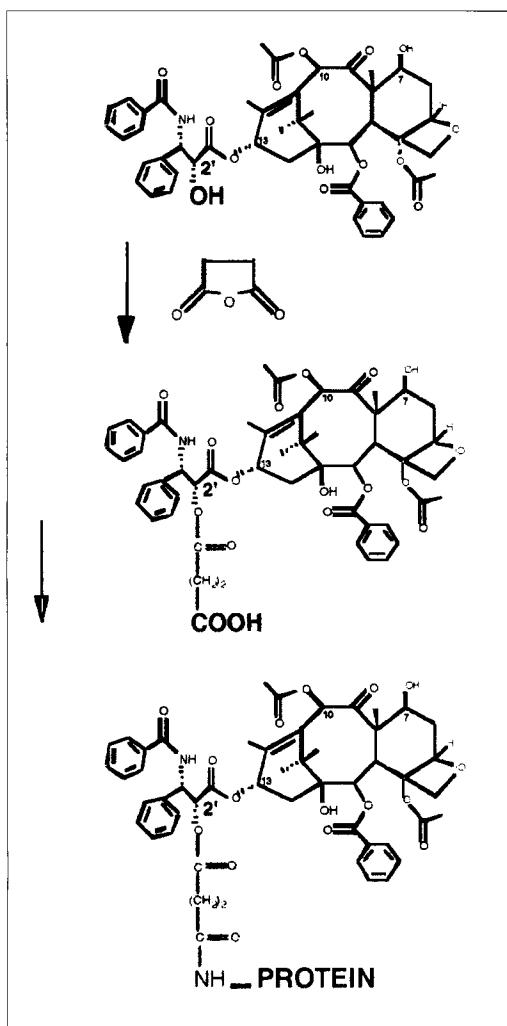


Fig. 16.5. Synthesis of paclitaxel-protein conjugate.

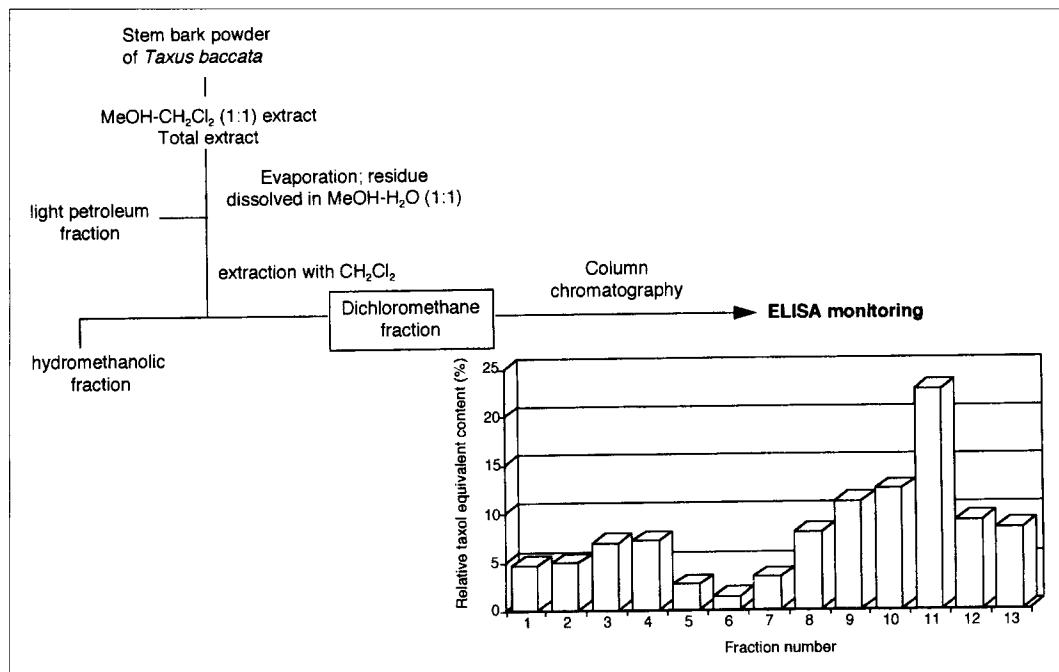


Fig. 16.6. Typical standard curves of the ELISA for paclitaxel and cross-reactions with its closely related compounds.

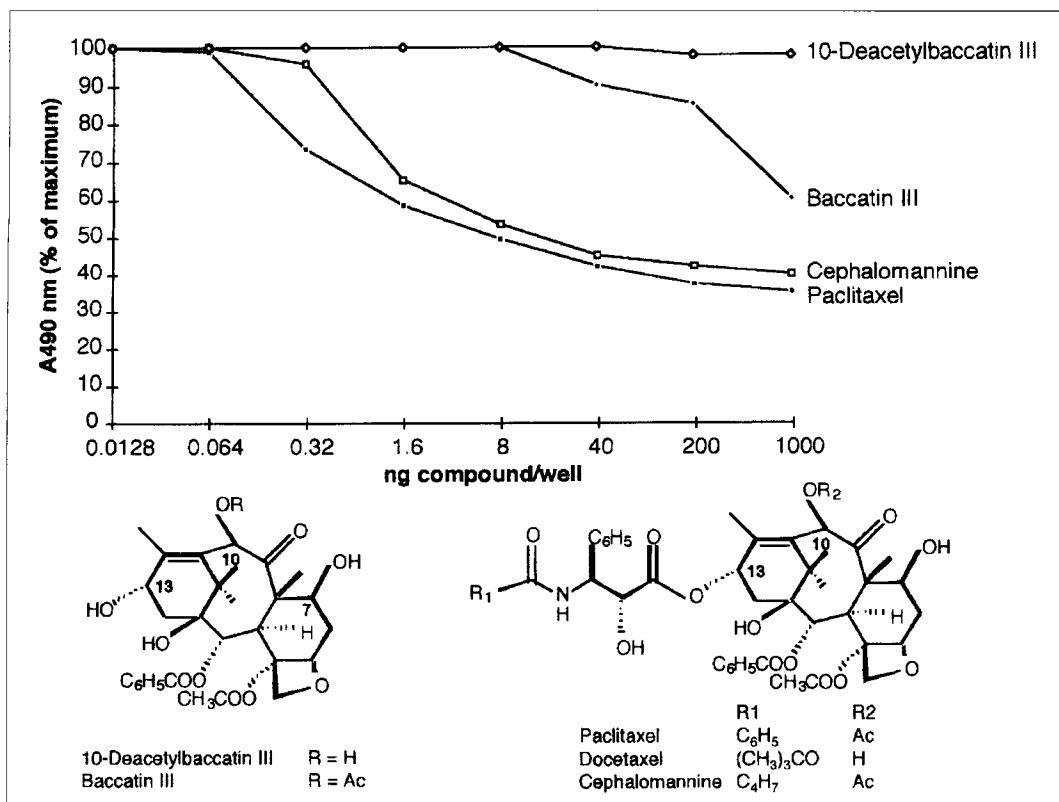


Fig. 16.7. Combination of chromatographic and immunoenzymatic methods: application for the search of new taxoids.

podopyllotoxin, etc., bind to soluble tubulin and prevent its polymerization into microtubules. This ultimately leads to the elimination of microtubules in both the mitotic spindle and the cytosol and, consequently, to the prevention of cell division. In contrast, paclitaxel and related taxoids have unusual effects on the *in vitro* assembly of tubulin. They enhance tubulin assembly by reducing tubulin loss at the depolymerization end of the microtubules. In addition, paclitaxel-treated microtubules are not able to depolymerize at low temperature. This evidence suggests that there is a paclitaxel binding site on the microtubules.

An alternative approach to the development of microtubule-stabilizing drugs is to identify new substances that act similarly to paclitaxel. The *in vitro* assembly and disassembly of tubulin assay is easy to establish. Mammalian brain is a rich source of tubulin, which can be extracted by repeated cycles of assembly and disassembly. The kinetics of *in vitro* polymerization and depolymerization of tubulin is altered by the addition of paclitaxel and particularly during the depolymerization process. As shown in Figure 16.8, the speed of depolymerization is inversely proportionate to the concentration of paclitaxel.

These biological properties have been used for monitoring chromatographic fractions. Using this

system, Potier's group from Gif-sur Yvette, France, has isolated several new taxane molecules. One of them showing a very weak effect in the tubulin assay was isolated from *Taxus* leaf extract and its structure was elucidated as 10-deacetylbaccatin III.<sup>31</sup> This compound is present in the needles of *Taxus* at relatively high concentrations (0.1% dry weight), almost 10 times higher than paclitaxel in the stem bark. Several processes for converting 10-deacetylbaccatin III into paclitaxel have been developed.<sup>31-33</sup> Generally, they concern the esterification of the hydroxyl group at the 13th position of 10-deacetylbaccatin III with various amino acids of the phenylisoserin type. This strategy has led to the production of 'Docetaxel,' a semisynthetic analog of paclitaxel, which is being developed by Rhone-Poulenc Rorer. Docetaxel appears to be somewhat more active than paclitaxel, at least in some pharmacological assays.<sup>34</sup> In addition, the hemisynthesis approach has many advantages since it originates from a renewable source, the needles of the *Taxus* plant.

As described above, two different biochemical assays combined with chromatographic methods are useful for the screening of natural product collections for additional sources of paclitaxel or new compounds with the same biological activity (microtubule-stabilizing) as paclitaxel.

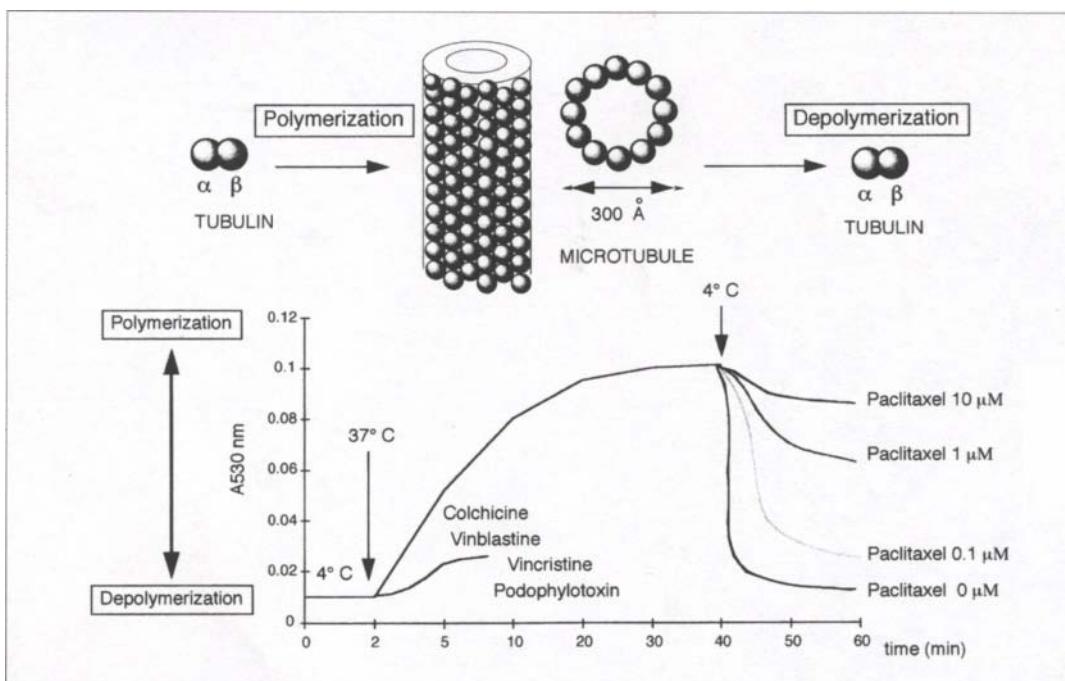


Fig. 16.8. Kinetics of *in vitro* polymerization and depolymerization of microtubules and effect of plant-derived drugs on a dynamic system.

Another potential long-term source of paclitaxel and its derivatives concerns a biotechnological approach based on the extraction of paclitaxel from *Taxus* cell or tissue cultured in vitro.<sup>35</sup>

#### SCREENING OF NATURAL PRODUCTS FOR CHEMOTHERAPEUTIC ACTIVITY

Until quite recently, most bioassays were carried out using living animals. In occidental countries, there is political pressure to discourage the use of animals for research, especially in the early stages of drug development. The other disadvantages of use of animal models are the limited number of parameters that can be measured in a single experiment, and that the pharmacological study of antidiabetic, anticancer, anti-inflammatory, antihypertensive and immunomodulatory agents is time consuming and very expensive. Furthermore, a poor correlation between the results obtained from animals and those from humans is observed in some biological activities.

Once a crude plant extract with an interesting biological activity is discovered, the bioassay has to be used to monitor the chromatographic fractionation until the active principles are discovered. This becomes even more expensive and requires large amounts of plant extracts, which may not be available in the early stages of the drug discovery process. In order to overcome these problems, in vitro bioassays have been developed. These assays are based on the inhibition or the stimulation of receptor binding, biochemical reactions or enzyme systems. These assays, which can be mechanically driven, need very little plant material compared with the animal models and are usually easy to perform; the procedures are also amenable to automation, thereby reducing costs. Some industrial laboratories have the capacity to perform in vitro bioassays on 1000 to 5000 different samples per day.

During 1985-1990, the NCI replaced the use of *in vivo* mouse leukemia primary screen with a new in vitro screening assay employing over 60 human cancer cell lines.<sup>36</sup> In 1986, with the advent new screening techniques, the NCI stepped up exploration of natural products as new therapeutic agents for cancer and AIDS and began worldwide collections of plants in tropical and subtropical regions.<sup>36</sup> The NCI program of drug discovery using the in vitro anti-human immunodeficiency virus (anti-HIV) screen is as follows:<sup>37</sup> plants are air or heat dried in the field and packaged in 0.5-1.0 kg samples, which are sent to the Frederick Cancer Research Facility for extraction and study. Duplicate specimens are deposited in herbaria in the country where the plant was collected and the bulk samples are sent to the U.S.A. for study by botanists.

Dried plant samples are stored at -20°C for a minimum of 48 hours immediately after arrival at the NCI. This period of freezing is a US Department of Agriculture requirement as a precaution to reduce risk of release of alien pests. Each sample is labeled (bar-coded label), logged into a raw material database and sent to the Natural Products Extraction Laboratory for grinding and extraction. A small portion of each sample is removed and kept as a voucher. The rest of the sample is then ground and extracted by slow percolation at room temperature with a dichloromethane: methanol (1:1 mixture), followed by a methanol washing. The combined extracts are concentrated in vacuo and finally dried under high vacuum to give an organic extract. After methanol washing, the residual plant material is extracted by percolation at room temperature with distilled water; lyophilization of the percolate gives the water extract. All extracts are returned to the natural products repository for storage at -20°C until required for testing. For the in vitro anti-HIV screening, human T lymphoblastic cells infected with the HIV virus are incubated for six days with varying concentrations of the extracts. Untreated infected cells do not proliferate and die rapidly. Infected cells treated with extracts containing effective antiviral agents will proliferate and survive at moderate extract concentrations, whereas high concentrations of extracts generally will kill the cells. The degree of activity is measured by the level of protection provided by extracts at less toxic concentrations. The same approach has been used by the NCI for the search of antitumoral drugs.

#### BIOTECHNOLOGICAL APPLICATION OF MEDICINAL PLANTS

##### CONSERVATION OF MEDICINAL PLANT RESOURCES BY IN VITRO CULTURE AND CRYOPRESERVATION

Since more than half of all plant species are found in the tropical forests, and at least one-half (about 120,000 species) are in the tropical rain forests that comprise only 7% of the world's land surface, the question becomes obvious as to which part of the world should be given priority in the quest for new medicinal drugs from plants, a quest now known as biodiversity prospecting.<sup>11</sup> Extermination of plant species in these forests through commercial logging, fuelwood consumption, cattle ranching, forest farming and so forth is progressing at an alarming rate (estimated at about 1% of the forest annually and at least two rain forest angiosperm daily),<sup>7</sup> even before the plants have been recorded,

much less studied chemically, the need arises for increased efforts directed toward the conservation of gene pools of locally used medicinal plants. The conservation of natural resources including plants is now an international concern together with the rights of countries to their plant and animal genetic resources (biodiversity).<sup>38</sup>

For long-term ex situ storage of plant germplasm to maintain the genetic diversity of species and to preserve the ones threatened with extinction, seed banks have long been used taking advantage of a natural plant preservation mechanism, dry seed.<sup>39</sup> The seeds of most agricultural species have desiccation-tolerance, can often remain viable for many years and longevity can be increased further by storing the seeds at low temperatures.<sup>40</sup> Their seeds are generally adaptable to cryopreservation (storage in liquid nitrogen).<sup>39</sup>

Tropical rain forest species have a very narrow genetic tolerance so that they may be difficult to propagate outside of their own habitat. This concerns ex situ medicinal genetic resource conservation such as seed banks being done with crop plant species.<sup>7</sup> However, a number of tropical species as well as species from several threatened habitats that are desiccation-sensitive, or recalcitrant (short-lived seeds) cannot be stored intact using the conventional methods of drying.<sup>7,40</sup> Combination of tissue culture and cryopreservation techniques may enhance germplasm conservation for

these species once suitable methodologies are established.<sup>40</sup>

One of the Amazonian medicinal plants named *Cephaelis ipecacuanha*, known in commerce as Rio or Brazilian ipecac and used for medicinal purpose worldwide (expectorant, emetic or amoebaside), is now becoming difficult to obtain from the wild habitat because of tropical deforestation.<sup>41</sup> Its seeds are kind of recalcitrant, which makes conventional propagation or conservation of this species difficult despite of its economical value.<sup>42</sup> Recently several in vitro micropropagation methods of ipecac<sup>43,44</sup> and evaluation of regenerated plants through tissue culture<sup>45</sup> have been reported. These works prove the efficacy of in vitro culture for conservation of plant resources as well as the commercial mass propagation.

Plant tissue culture technique enables us to preserve not only various plants in the world but also plant strains which exhibit desirable properties.<sup>46,47</sup> In addition, plants can be mass-propagated with the following advantages: limited space for their preservation, disease-free condition and possibly easy control of their growth. However, in vitro cultures are always exposed to danger of microbial contamination or unexpected trouble at the culture facilities. Cryopreservation of plant tissue cultures is a reliable method for long-term preservation, since almost all the metabolic functions of living cells are at a standstill in liquid nitrogen (-196°C).<sup>48</sup> During the last two decades,

significant progress has been made and literature has accumulated on the cryopreservation of plant cell, tissue and organ culture<sup>40,49-51</sup> and many medicinal plant species have been successfully cryopreserved.<sup>51</sup> Recently, two novel cryogenic protocols—the complete vitrification method and the encapsulation (alginate-coated)—dehydration techniques have been developed (Fig. 16.9).<sup>48-50</sup> The principle of these methods is based on physical phenomenon, namely vitrification.<sup>49</sup> Vitrification refers to solidification (glassification) of the system during cooling without ice formation. Sufficiently high concentrations of solutes in the cells (caused by a highly concentrated cryoprotective solution in the former and by extremely enhanced sucrose concentration

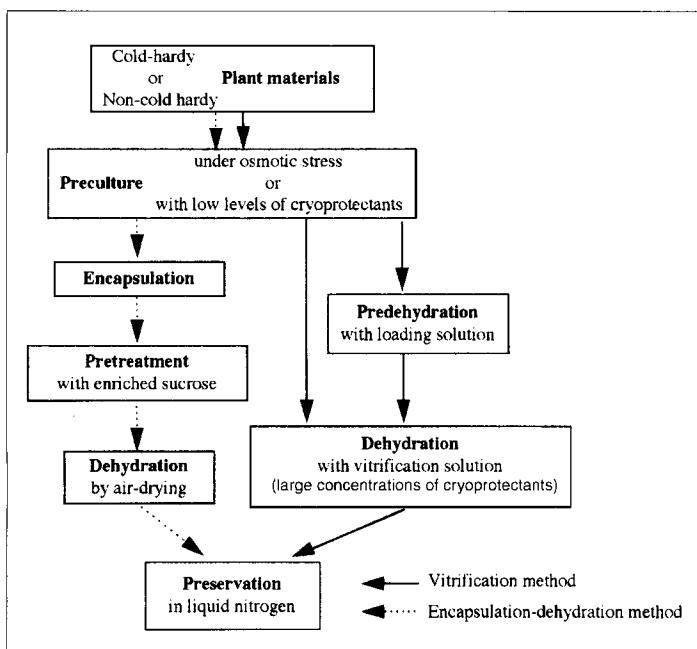


Fig. 16.9. Two novel cryogenic protocols for plant cell tissue cultures.

during the drying process in the latter) become so viscous that it solidifies into an amorphous, in other words, metastable glass (vitrifies) by ultra-rapid cooling.<sup>40,49,50</sup> Vitrification has long been proposed for cryopreservation of biological materials because the potentially detrimental effects of extra- and intracellular freezing (ice crystallization) can be avoided.<sup>49,50</sup> As glass is exceedingly viscous where all metabolic functions of biological materials might cease, its formation may lead to perpetual dormancy and stability.<sup>49</sup> These simple and inexpensive methods are popular because both of them do not require sophisticated and expensive cryostats for controlled freezing rates that are prerequisite for the conventional slow freezing method. By the former technique, genetically transformed roots (hairy roots) of *Panax ginseng* have also been successfully cryopreserved as well as shoot meristems and cell suspensions of medicinal plant species, and it has been proved that the cryopreservation of root tips did not influence the biochemical capabilities of hairy roots for ginsenoside.<sup>52</sup>

## BIOTECHNOLOGICAL APPLICATION OF MEDICINAL PLANTS AS AN ALTERNATIVE SOURCE FOR PHARMACEUTICALS

Medicinal plants are still an important source for the production of pharmaceuticals, as mentioned above. Not only are the active principles found in plants, the origins for exploiting new medicines, but also raw materials isolated from the plant materials are sometimes used therapeutically. Since most of them have a rather complex structure, it is not easy to synthesize these bioactive compounds chemically at a low price. Therefore, plant biotechnology is one of the desirable systems to produce the target bioactive compounds efficiently. Many researchers have investigated the production of target secondary metabolites from different plant tissue cultures such as callus, cell suspension, adventitious shoot, adventitious root, hairy root (transformed with soil bacterium *Agrobacterium rhizogenes*) cultures, etc. Some examples are described (Table 16.2).

**Table 16.2. Secondary products in transformed root cultures (hairy root) of medicinal plants**

Compound groups	Products	Material plant
<b>Phenolics</b>	geraniin, coriagin sanguinin, hydroxyl tannin condensed tannin  Anthraquinone xanthone	Geranium thunbergii Sanguisorba officinalis Phyllanthus niruri Lotus corniculatus  Rubia tinctorum Swertia japonica
<b>Terpenes</b>	amarogentin, amaroswerin hernandulcin shikonin digitoxin β-ecdysone ginesnoside	Swertia japonica Lippia dulcis Lithospermum erythrorhizon Digitalis purpurea Ajuga reptans Panax ginseng
<b>Alkaloids</b>	tropane alkaloid  indole alkaloid morphinan alkaloid lobeline hyalbidone cinchona alkaloid vinca alkaloid	Hyoscyamus albus, H. niger Scopolia tangutica, Datura innoxia, D. candida Amsonia elliptica Papaver somniferum Lobelia inflata Hyoscamus albus Chinchona ledgeriana Catharanthus roseus
<b>Others</b>	polyacetylene	Tagetes patura Lobelia assilifolia, L. inflata Platycodon grandiflorum

## SECONDARY METABOLITE PRODUCTION BY ROOT CULTURES

Induction of adventitious (non-transformed) roots to establish adventitious root leaf-segments (ca. 5 x 5 mm) of axenic shoot culture or disinfected leaf of intact plants were used. Leaf segments were placed on solid nutrient medium such as Murashige-Skoog, containing 1 mg/l IAA or 0.1 mg/l NAA in the dark at 25 °C. The roots were sub-cultured every four to eight weeks in liquid medium (50 ml/100 ml flask) containing the same phytohormone as used for root induction. Root cultures of *Hyoscyamus* species were established from the roots of axenic plants in vitro and subcultured in phytohormone-free medium.

Induction of hairy roots: pathogenic soil bacteria, *Agrobacterium* sp., induce plant tumors, so-called crown gall or hairy roots at the infected sites of plants and these morphologically characteristic tumors produce a unique amino acid called opine.<sup>53,54</sup> The detailed mechanism of these phenomena is elucidated by a number of studies on the infection of plant cells with *Agrobacteria*; T-DNA (transferred DNA) region of Ti (tumor-inducible) or Ri (root inducible) plasmid in *Agrobacterium* is integrated into plant genomic DNA and the genes coded on the T-DNA are expressed in the infected plant cells.<sup>53,54</sup> *Agrobacterium tumefaciens* harboring Ti plasmid induces crown gall while *Agrobacterium rhizogenes* harboring Ri plasmid induces hairy root at the infected site.

In plant tissue culture, phytohormones, such as auxin and cytokinin, are generally indispensable to induce and/or maintain callus or adventitious root cultures. However, plant tumors mentioned above grow vigorously on phytohormone-free nutrient medium after the complete elimination of bacteria by antibiotics. In the case of hairy root, each root developed at the infected site can be esteemed as a clone and much research indicates that hairy roots have the capability to produce plant metabolites that are biosynthesized in the root part of the plants.

## PHENOLS

In the hairy root cultures (transformed by *A. rhizogenes* A4) of *Geranium thunbergii* (Geraniaceae), the traditional medicinal plant mainly used for diarrhea, the metabolism of the tannin constituent geranin, which is the major pharmaceutical in the aerial part of the plant, was regulated by NH<sub>4</sub><sup>+</sup> content in the culture medium.<sup>55,56</sup> Biosynthesis of geranin in *G. thunbergii* hairy roots was activated in Gamborg B5 medium (containing 2 mM NH<sub>4</sub><sup>+</sup>) and not in NH<sub>4</sub><sup>+</sup> rich medium (1/2 MS, containing 10.3 mM NH<sub>4</sub><sup>+</sup>).

Rosaceous medicinal plant *Sanguisorba officinalis* also contains high molecular polyphenol compounds (particularly hydrolyzable-type tannins), such as sanguin H-6 and sanguin H-11, which are presumed to be active constituents when treated for hemostatic, anti-phlogistic and astringent properties. Adventitious root cultures of *S. officinalis* showed the best growth and tannin production (major phenolic was sanguin H-6, 7.3 mg/flask in 50 ml medium) in MS liquid medium in four weeks of culture whose yield level was improved by the addition of 1 mg/l naphthalene acetic acid (NAA).<sup>57</sup> Among six clones with hairy roots, induced by the infection with *A. rhizogenes* A4 and cultured in hormone-free 1/2 MS liquid medium, five clones produced mainly sanguin H-6 [0.217–0.569%, as fresh weight (fw)] whereas the other one clone produced an especially high level of 1,2,3,6-tetra-O-galloyl-β-D-glucose (0.322% fw) and sanguin H-11 (0.221% fw) whose levels were over double compared to those in the parent plant.<sup>58,59</sup> These root cultures of *S. officinalis*, by careful selection of the clone and the determination of the effects of some additives (medium constituents, auxin, etc), showed a stable supply of these high molecular tannins, coincidentally affording a useful system for biosynthetic studies of hydrolyzable tannins.

The hairy roots (induced by *A. rhizogenes* 15834) of *Swertia japonica* (Gentianaceae) produced plenty of xanthones, i.e. bellidifolin, methylbellidifolin, swertianolin and a new glycoside 8-O-primeverosylbellidifolin, which originated as bright yellow coloration in the tissues.<sup>60</sup> It was biosynthetically noteworthy that the hairy roots produced only 1,3,5,8-oxygenated xanthones, although in vivo plants produce both 1,3,5,8- and 1,3,7,8-oxygenated derivatives. *S. japonica* hairy roots also yielded two new phenyl glucosides, 5-(3'-glucosyl)-benzoyloxygentisic acid and 2,6-dimethoxy-4-hydroxyphenol 1-glucoside together with 1-sinapoylglucoside.<sup>61</sup> In transformed cell cultures (hairy roots), the activation of biosynthetic capability of glycosylation had occurred, which made it possible to produce some new phenylglycosides.

Growth and (+)-catechin production in root and cell suspension cultures of *Rheum palmatum* (Polygonaceae) was regulated by auxin content in the culture medium.<sup>62</sup> In MS liquid medium containing 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), the content of (+)-catechin in *R. palmatum* cells reached 0.38% [dry weight (dw)] at week 7 of the culture. In contrast, the root cultures in MS liquid medium with 2 mg/l NAA showed the highest level of (+)-catechin (0.24% dw) at week 1 of the culture.

Eight clones of *Phyllanthus niruri* (used as an euphorbiaceous folk medicine) hairy roots, induced by *A. rhizogenes* A4 or *A. tumefaciens* R-1000 + 121 (having two plasmids, a root-inducing plasmid pRi A4b and a minor Ti plasmid pBI121 containing genes encoding for NPT-II and GUS on the T-DNA), produced seven phenolics: gallic acid, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin 3-O-gallate and (-)-epigallocatechin 3-O-gallate.<sup>63</sup> Although phenolic constituents in the aerial parts of *P. niruri* (both *in vitro* and *in vivo* plants) were mainly hydrolyzable tannins such as geraniin, corilagin and galloylglucose, the hairy root cultures contained flavan-3-ols, whose constitutional pattern was similar to that observed in leaves of *Thea sinensis* (green tea). These cultures were expected to be new medicinal materials such as antiviral (hepatitis, flu, anti-HIV etc) and antitumor drugs.

#### ALKALOIDS

Production and biosynthetic regulation of alkaloidal metabolites in medicinal plants using biological approaches have been achieved worldwide by numerous researchers. Successful modifications (in sufficient levels of amount and/or content) of various alkaloid metabolisms (tropane-, piperidine-, indole-, quinoline-, isoquinoline-type, etc) in plant tissue cultures were performed, and offered both commercial and scientific merits to conventional cultivars of medicinal plants.

Transformed (*A. rhizogenes* A4) root cultures of *Amsonia elliptica* (Apocynaceae) produced a new yohimbane derivative 17 $\alpha$ -O-methylyoimbine together with previously known indole alkaloids, vallesiachotamine and pleiocarpamine, in B5 liquid medium.<sup>64</sup> The addition of 0.5 mg/l NAA to the culture medium remarkably enhanced the growth and alkaloid production (to ca. 10 times of the parent tissues *in vivo*) of the hairy roots. In the light condition, the hairy roots accumulated chlorophylls to be 'green hairy roots' and the production of vallesiachotamine and pleiocarpamine was promoted.<sup>65</sup>

Callus and adventitious root cultures of *Cephaelis ipecacuanha*, Amazonian medicinal plant 'ippecac,' were established and skillfully used in the production and biosynthetic studies (regulation by auxins and basal medium) of emetic alkaloids, emetine, cephaelin, etc.<sup>66,67</sup>

Solanaceous medicinal plants such as *Datura*, *Hyoscyamus*, *Scopolia*, *Duboisia*, etc. are major sources of tropane alkaloids supply. Among numerous research projects on secondary metabolism in plant tissue cultures, those concerning tropane

alkaloid biosynthesis using various solanaceous plant cells (including transformants) have been most actively and largely performed, resulting in several useful products.<sup>68</sup>

Adventitious and hairy root cultures of *H. albus*, *H. niger*, *S. tangutica*, *Datura innoxia*, *D. candida*, *Duboisia* hybrid (M-II-8-6: *D. myoporoides* x *D. leichhardtii*), etc. mainly produced four tropane alkaloids hyoscyamine, 6 $\beta$ -hydroxyhyoscyamine, 7 $\beta$ -hydroxyhyoscyamine and scopolamine, supplying important medicines.<sup>69-72</sup> Particularly, successful isolation and chemical structural characterization of 7 $\beta$ -hydroxyhyoscyamine, a biosynthetically interesting intermediate in the conversion of hyoscyamine to scopolamine occurring a little in natural plant tissues, has become practicable through the utilization of *H. albus* and *Duboisia* hybrid hairy root systems. In a recent work, genetic engineering of *Atropa belladonna* (root cultures and the regenerated plantlets) by *Agrobacterium*-mediated transformation (hyoscyamine 6 $\beta$ -hydroxylase gene transfer) succeeded where high (enhanced) amounts of scopolamine were produced.<sup>73</sup> In the future, development of several transgenic medicinal plants in which the secondary metabolism being modified for high productivity of their useful pharmaceuticals is expected.

Even in one of the most historical medicinal plants, opium poppy (*Papaver somniferum*), a gene transfer experiment was done by *A. rhizogenes* MAFF 03-01724 mediate method.<sup>74</sup> Numerous transformed shoots obtained in the system yielded morphinan alkaloids at comparable level to that in non-transformed shoots.

Other alkaloidal constituents such as piperidine-type lobeline (in *Lobelia inflata*),<sup>75-77</sup> piperidone-type hyalbidone<sup>78</sup> (a new alkaloid in *H. albus*), etc. also profitably synthesized in each tissue culture system.

#### TERPENOIDS

Genetic transformation of *Digitalis purpurea* (fox glove), one of the most common medicinal plants in the world, was done by *Agrobacterium*-mediated system.<sup>79</sup> The 'green' hairy roots obtained in the experiment highly produced cardioactive glycosides.

Other principles, sometimes used as natural additives for drugs and/or food chemicals, such as sweet sesterterpenoid hernandulcin (in *Lippia dulcis*),<sup>80</sup> bitter glycosides amarogentin and amaroswerin (in *Swertia japonica*),<sup>60</sup> shikonin (in *Lithospermum erythrorhizon*),<sup>81</sup> etc., were also yielded in *Agrobacterium*-transformed tissues (hairy roots) of each species.

## OTHER PHARMACEUTICS

Polyacetylene compounds popularly occur in several varieties in Asteraceae, Umbelliferae, Araliaceae, etc. Particularly, the important biological activity (cytotoxicity for tumor cells) of *Panax* species (Araliaceae), whose alcoholic extract (of roots) has been widely used as an anticancer drug in home treatment, is presumed to have originated in their polyacetylene constituents. Recently, in hairy root cultures of some campanulaceous plants such as *Platycodon grandiflorum*, *L. inflata*, *L. chinensis*, *L. sessilifolia*, etc, new polyacetylene derivatives, lobetylol, lobetylolin and lobetylolinin, which have mild cytotoxic activity, were produced in rich amount.<sup>82-87</sup>

## BIOTRANSFORMATION AND PRODUCTION OF NEW COMPOUNDS

In addition to micropropagation of important medicinal plant varieties, tissue culture techniques have been available for both production and biosynthetic experiments of the secondary metabolites. Biotransformation attempts of some chemicals using plant cell cultures (with active enzymes) offered good systems for yielding new useful metabolites found in poor amounts in nature.<sup>88</sup> Particularly, for the synthesis that needs positional selectivity in the reactions, plant cells cultured in suitable condition are very profitable when used as the bioreactors. Hairy root cultures sometimes show high productivity of glycosylated secondary metabolites. Recently, *L. sessilifolia* hairy roots were used for biotransformation of some phenolics (flavan-3-ols and C6-C1 phenols), which succeeded in the production of new glucosylated compounds.<sup>89</sup> In the present study, the metabolism in biotransformation of five phenolics (as substrates), protocatechuic acid, gallic acid, trans-cinnamic acid, p-coumaric acid and caffeic acid in hairy root cultures of herbal plants, i.e. *L. sessilifolia*, *L. cardinalis*, *Campanula medium* (Campanulaceae), *Ocimum basilicum* (Lamiaceae) and *Fragaria x ananassa* (Rosaceae) was determined and succeeded on the site-specific glycosylation of phenolics, whose achievement is difficult when using the technique of organic chemical synthesis.

In vitro experiments of some important medicinal plants, the biosynthetic pathways of the secondary metabolism were arranged (newly and/or selectively activated) to lead to the high amount production of new metabolites in the plant cells. The discovery of new chemicals, expected to have some new biological activities through biotechnological approaches, is very important for medicinal plants because these approaches present other methods of exploitation of the traditional plants.

In several medicinal plants, both traditional and unknown (expected as future drugs), biotechnological approaches will continuously help the versatile supplement of their useful secondary metabolites, i.e. natural pharmaceutics.

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## CHAPTER 17

# BIOTECHNOLOGY OF INDUSTRIALLY IMPORTANT TREE SPECIES IN DEVELOPING COUNTRIES

S.M. Jain

### INTRODUCTION

Large-scale industrialization and ever-increasing population growth are placing tremendous pressures on existing water resources, arable land and sound forestry practices. This, in turn, is causing environmental hazards—depletion of atmospheric ozone, acidic rain, erratic weather conditions, insect and pest problems, diseases, global warming and an increase in ultraviolet-B (UV-B) radiation levels on earth. In the long run, forest plantations and other woody species will be seriously affected by these developments, which may lead to the destruction of these resources.

Deforestation is a serious problem in developing countries due to a large number of people who use forest trees as firewood and in the construction of houses. Forest plantations are often destroyed by the mismanagement of officials that can lead to the loss of invaluable germplasm. In India, deforestation is a serious problem and partly carried out illegally.<sup>1</sup> All this destruction is causing a serious concern to the state governments and the industry. Industrially important tree species are being destroyed at a much faster pace than the pace of reforestation, resulting in heavy economic losses. In some cases, for example, the paper industry is forced to import wood. Thus, it has become increasingly more important to reforest and preserve invaluable germplasm for continuous supply of elite genetic material for breeding and raw material for the industries. Reforestation is a slow process and it is difficult to restore the vegetation of the primary forest.<sup>2</sup> Extensive afforestation programs have been undertaken in India for the mass propagation of forest trees such as *Shorea robusta*, *Tectona grandis*, *Betula spp*, *Butea sp*, *Pinus spp*, *Accacia sp* and many other species.<sup>1</sup> The demand will increase requiring the supply of superior planting material of many tree species. The conventional methods of tree improvement and selection offer only limited possibilities of meeting the rapidly growing demands. Therefore, new innovative techniques are needed for the creation of new hybrids, early selection and testing of desirable genotypes, rapid vegetative propagation of selected genotypes and improvement through biotechnology.<sup>3</sup> Quality of the product and cost effectiveness of propagation are two fundamental measures of success in any tree improvement program. Furthermore, there is a need to produce genetically uniform stocks of the selected genotypes of planting material, and for testing their resistance to fungal and bacterial diseases and

environmental stress factors. For this purpose, the support of both the public and private sectors as well as international funding organizations is needed.

## PROBLEMS IN DEVELOPING COUNTRIES

In spite of heavy demand on wood products, the progress in biotechnology of forest and other industrially important tree species has been slow in developing countries as compared with developed countries. Developing countries are still continuing to struggle to develop national biotechnology programs whereas developed countries have a strong economic base and exhibit a more dynamic and aggressive approach toward developing and utilizing biotechnology in forestry and industrial woody plants to support industry and basic research.

The following factors hinder progress of biotechnology in the developing countries: 1) Shortage of trained manpower. There are few trained hands to carry out sophisticated research, especially in the biotechnology of woody plants. This constraint is amplified by the fact that highly trained people either move abroad or try to operate on reduced efficiency. 2) Lack of funds to support basic and applied research leaves no incentives for research and development. The available manpower tends to immigrate to developed countries and create "brain drain." 3) Poor facilities and maintenance of laboratory equipment, frequent power failures, poor library and computer facilities and infrequent water supply contribute to the lack of progress. 4) Due to lack of coordination between industry and academia most of the research funds come from the government, which is not sufficient enough to carry out efficient research programs. In many cases, over-employment in the research establishments slows down the progress of research and development and consumes most of the money allotted by the government to the research organizations. There is no coherent government policy either to transfer the technology from the research laboratories to the industry or to encourage interaction between industry and academia. 5) Lack of patent law protection. A majority of foreign investors are reluctant to transfer patented technologies because of a fear of losing money. 6) Crunch for foreign exchange prevents the purchase of books, journals, computers, chemicals, enzymes and equipment from abroad. Moreover, researchers are not able to keep up with the latest progress in the field as a result of poor library facilities. 7) Rigid bureaucracy and mismanagement. The freedom of work and efficiency of researchers are very much hampered by the rigid

bureaucracy and mismanagement of resources. This demoralizes the working environment and reduces momentum in the research programs. The above listed concerns call for strong policy action by the national governments supported by the international community.

## BIOTECHNOLOGY: DEFINITION AND PROGRESS

There are several definitions of biotechnology; however, it can be simply defined as the manipulation of natural biosystems with multiple disciplines for human benefits—agriculture, environment, forestry, medicine and industries. Biotechnology has also been defined by the Office of Technology Assessment of the United States Congress as "any technique that uses living organisms, or substances from those organisms, to make or modify a product, to improve plants or animals, or to develop micro-organisms for specific uses."<sup>4</sup> Plant cell and tissue culture, molecular biology, biochemistry and plant breeding are basically the main pillars of biotechnology, and further interaction with other disciplines can be achieved depending on the nature of the research project. This chapter will describe the main pillars of biotechnology for improving industrially important tree species in the developing countries.

The commercial importance of woody plants is usually dependent on several factors such as climate, population and social structure for each individual developing country. For example, some of the important regional woody plants in developing countries are: 1) Asia-Pacific region—*Acacia spp*, *Albizia spp*, *Azadirachta indica*, *Camellia sinensis*, *Cocos nucifera*, *Elaeis guineensis*, *Eucalyptus spp*, *Hevea brasiliensis*, *Mangifera indica*, *Santalum album*, *Tamarindus indica*, etc;<sup>2</sup> and 2) African region—*Acacia spp*, *Citrus spp*, *Eucalyptus spp*, *Olea europaea*, *Phoenix dactylifera*, *Cocos nucifera*, *Camellia spp*, *Vitis spp*.<sup>5</sup> Woody legumes can be the predominant species in certain ecosystems, especially in the tropics,<sup>6</sup> and their impact is immense in tropical rainforests including the impact of substantial amount of nitrogen fixed through the symbiotic association between legumes and *Rhizobium*. Woody legumes also play a key role in the development of agroforestry systems.<sup>7</sup> Agroforestry is the integration of a woody tree or shrub species into traditional crop or livestock production system<sup>8</sup> and offers potential relief from the substantial deforestation in the tropics. Trigiano et al<sup>6</sup> have listed the potential ecological (soil improvement - nitrogen fixation, erosion control, wind breaks, reclamation of mining sites, green manure) and economical values and uses of woody legume genera.

These include use as fodder or forage, as forest products (timber-lumber, carpentry, pulp for paper production, fuel wood or charcoal), as agroforestry nurse trees (support for vine crops, shade for coffee, tea, cacao), and as ornamental, pharmaceutical, soap, gum, tannin, fish stupefier and insecticide uses.

### PLANT CELL AND TISSUE CULTURE

Tissue culture offers great potential for the rapid multiplication of elite lines in large-scale production.<sup>9,2</sup> This technology is important for woody plants that have long maturation periods or are difficult to multiply through conventional means. Several important woody plant species (legumes, conifers, etc.) cannot be improved through selection of elite trees for seed production owing to self-incompatibility or low seed viability.<sup>6</sup> Furthermore, tissue culture may eventually provide the primary means for clonal propagation of superior genotypes or can serve to enhance conventional breeding efforts by large-scale multiplication of intra- or interspecific hybrids.<sup>6</sup>

The most commonly used tissue culture methods in forest biotechnology are: 1) micropropagation, 2) somatic embryogenesis, 3) haploid production, 4) somaclonal variation, 5) cryopreservation, and 6) somatic cell hybridization.

### Micropropagation

Rapid large-scale uniform plant multiplication of forest trees is done by micropropagation either by direct in vitro shoot formation (organogenesis) or by somatic embryogenesis (embryos from somatic cells). More than 1000 plant species have been micropropagated, including more than 100 forest species.<sup>10,11</sup> The basic micropropagation protocol has been reviewed by several authors.<sup>6,12,13,14</sup>

Plant multiplication by tissue culture requires: 1) culture initiation; 2) sequential production of multiple adventitious shoots regenerated from adventitious buds, axillary buds, embryos and cotyledons; 3) rooting of shoots; and 4) acclimatization of regenerated plants. The most common media that have been used are MS,<sup>15</sup> B5,<sup>16</sup> Nitsch's medium,<sup>17</sup> LS medium,<sup>18</sup> WPM,<sup>19</sup> DCR<sup>20</sup> and SH.<sup>21</sup> The development of multiple shoots requires a medium supplemented with either a cytokinin or a combination of cytokinin and auxin. The regenerated shoots can be further recultured for re-multiplication on a fresh shoot regeneration medium or for rooting either in vitro on a rooting medium containing low concentration of auxin or in vivo. The most common growth regulators that have been used are: 1) Auxins—indoleacetic acid (IAA), naphthalene acetic acid (NAA), 2,4-

dichlorophenoxyacetic acid (2,4-D), indole butyric acid (IBA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); and 2) Cytokinins—kinetin, zeatin, bezylaminopurine (BA) and 2-isopentyl adenine (2iP). Recently, thidiazuron (TDZ) has been used for woody plant tissue culture.<sup>14</sup> Care must be taken while using TDZ for clonal propagation because it stimulates auxillary shoot proliferation as well as callus formation and shoot organogenesis.<sup>14</sup> The combination of TDZ with either auxin or cytokinin in the medium can significantly enhance shoot proliferation. Additions of TDZ to a BA-containing medium enhanced axillary shoot formation of *Acer x fremanii*,<sup>22</sup> *Fraxinus americana*,<sup>23</sup> *Pyrus communis*,<sup>24</sup> *Vitis rotundifolia*<sup>25,26</sup> and *Vitis vinifera*.<sup>27</sup> Sometimes gibberellic acid (GA<sub>3</sub>) and abscisic acid (ABA) are also added in the culture medium for shoot multiplication. Jain et al<sup>28</sup> added 2 mg/l GA<sub>3</sub> in MS medium containing 6 mg/l BA, 0.5 mg/l NAA for shoot multiplication of *Camellia sinensis*. Sen et al<sup>29</sup> improved shoot organogenesis in loblolly pine (*Pinus taeda*) by adding ABA in the culture medium. The reduction of the medium strength (lower salt concentration) is helpful in adventitious bud formation<sup>2,30</sup> and shoot multiplication.<sup>31,32</sup> Selection of suitable explants from juvenile and mature plants is important since they show varied growth response in vitro.

In vitro micropropagation is ideal for large-scale multiplication of forest and woody plants. In developing countries, micropropagation is highly suitable for mass propagation of woody plants because it is labor intensive, chemicals used are inexpensive and readily available in the country, micropropagated material is easy to transport and does not require sophisticated infrastructure and manpower. Kozai<sup>33</sup> suggested to use a photo-autotrophic micropropagation system, using carbon dioxide as a carbon source instead of adding sugar in the culture medium. This system needs modification for making it cost-effective in the developing countries. It has several advantages: 1) prevention of contamination of plantlet; 2) enhancement of plantlet growth; 3) reduced labor cost; 4) no requirement acclimatization; and 5) reduced variation in plantlets. Labor costs for multiplication, rooting and acclimatization of plantlets account for approximately 60% of the total production costs in conventional micropropagation. Other reasons for high cost of production in conventional micropropagation include: 1) the long period (often several weeks or more) required for each culture stage; 2) low multiplication rate; 3) loss of plantlets due to biological contamination and physiological and morphological disorders arising during the multiplication stage; 4) relatively high percentage of dead

plantlets due to serious environmental stresses during the acclimatization stage; 5) large variation in size, quality and morphology of plantlets; 6) significant costs for lighting, air conditioning, sterilization, washing, etc.; and 7) significant costs for gelling agents, culture vessels, and chemicals. Many developing countries including China, India, Bangladesh, Pakistan, Sri Lanka, Indonesia and some African countries are working on micropropagation of woody plants and producing millions of plants per year. Some of the important forest and fruit trees micropropagated include cardamom, date palm, oil palm, neem, tea, apples, pines, spruces, birch, eucalyptus, grapes, olive, roses and ornamental trees. Developed countries, private enterprises and international funding agencies should support developing countries by providing funding for establishing micropropagation facilities.

### Somatic Embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells, which is achieved through a series of developmental stages most of which are similar to those of zygotic embryogenesis. Steward et al.<sup>34</sup> reported this process for the first time in *Daucus carota*. Somatic embryogenesis is now routinely exploited in several plant species and woody perennials, which include both angiosperms and gymnosperms.<sup>35</sup> Embryogenesis can be induced either directly from an explant without a callus phase or indirectly after a proliferation of callus tissue,<sup>36</sup> and offers several advantages for plantlet production: 1) cost effective plant production in large numbers; 2) both root and shoot meristem development occurs in the same step of the process; and 3) quick and easy scale-up can be achieved via liquid culture. Somatic embryos are suitable for long-term germplasm storage via cryopreservation, and manufactured seeds (or artificial seeds) and a direct delivery system of manufactured seeds can be used to establish emblings (plantlets regenerated from somatic embryos). Other advantages of somatic embryogenesis are rapid genetic gains of forest trees and continuous supply of manufactured seeds throughout the year to seed orchards. Gupta and Grob<sup>36</sup> have reviewed somatic embryogenesis in conifer species including *Abies alba*, *Larix spp*, *Picea spp*, *Pinus spp*, and *Pseudotsuga menziesii*. All reports indicated that somatic embryogenic cultures of *Pseudotsuga menziesii*<sup>37</sup> and *Pinus* species<sup>37-41</sup> resulted from immature embryos at precotyledonary stages. Both immature and mature embryos of *Picea spp*<sup>42-45</sup> and *Abies spp*<sup>46,47</sup> resulted in the induction of somatic embryogenesis. However, in conifers, the efficiency of somatic embryogenesis from mature embryos has not been

as high as from immature embryos. The female gametophyte may play an important role in the production of embryonic suspensor masses (ESM). Durzan and Gupta<sup>48</sup> reported that immature embryos of *Pseudotsuga menziesii* excised with female gametophyte still attached via the suspensor system produced higher percentages of embryogenic cultures than those lacking the female gametophyte. Several culture media have been used in conifer somatic embryogenesis such as DCR,<sup>20</sup> LP,<sup>49</sup> NS III,<sup>42</sup> MS<sup>15</sup> and WPM.<sup>19</sup>

Somatic embryogenesis has also been achieved in woody angiosperm plants including *Actinidia spp*, *Albizia spp*, *Azadirachta indica*, *Bambusa spp*, *Citrus spp*, *Cocos nucifera*, *Coffea spp*, *Elaeis guineensis*, *Eucalyptus spp*, *Hevea brasiliensis*, *Juglans spp*, *Litchi sinensis*, *Malus spp*, *Mangifera indica*, *Olea europaea*, *Phoenix dactylifera*, *Populus spp*, *Prunus spp*, *Pyrus spp*, *Santalum album*, *Tilia cordata* and *Theobroma cacao*.<sup>35</sup> This technology can be used for clonal mass propagation by making artificial seeds (encapsulated somatic embryos) and in bioreactors for growing somatic embryos. For most of the forest trees and woody plants, this technology is still at the developmental stage from a commercial point of view. Some paper companies in the U.S.A. are testing somatic embryo plants of conifers as planting material and field trials are ongoing for their evaluation. Developing countries can use somatic embryogenesis in forest and woody plants to their advantage. The cost of production of plant material may decrease by continuous production of secondary embryos and increase by conversion rate of somatic embryos into plantlets.

### Haploid Production

Haploids are autonomous, sporophytic plants that have gametophytic chromosome number because they originate from a gametic cell in the embryo sac or in the pollen grain.<sup>50</sup> The haploid embryo can either be gynogenic (arise from an egg cell) or androgenic (arise from male gametes), or can originate from a gametophytic cell other than the egg cell (apogamy). The first natural haploid was observed in *Datura stramonium* by Blakeslee et al.<sup>51</sup> Since then, haploids have been reported in many plant species including cereals, vegetables, ornamentals<sup>52</sup> and woody plants.<sup>53</sup> There are different methods for haploid production—anther, ovary and ovule cultures, microspore cultures, chromosome elimination and use of irradiated pollen. These methods are well-described in a recently published book by Jain et al.<sup>53</sup>

The importance of haploids in genetic analysis and plant breeding has been known for a long time.

Several reviews have appeared on the application of haploids in woody plants and crop improvement.<sup>54-56</sup> One of the main applications of haploids is to produce diploid homozygous pure lines in a single generation, thus saving many generations of back-crossing to reach homozygosity by traditional means or in crops where self-pollination is not possible.<sup>53</sup> Every gene is hemizygous at the haploid level and after chromosome doubling, which in theory makes an identical copy of each haploid chromosome, every gene is homozygous. Therefore, doubled haploid plants are completely homozygous.<sup>57</sup> Haploids are also applicable in mutation breeding, genetic engineering, somatic cell fusion, genome mapping and chromosome engineering.<sup>53</sup>

Androgenic haploids have been produced in several woody species such as *Albizia lebak*, *Populus* spp., *Coffea arabica*, *Vitis vinifera*, *Camellia sinensis*, *Betula pendula*, *Citrus aurantifolia*.<sup>2</sup> For other woody plants (including gymnosperms and angiosperms) see the review by Baldursson and Ahuja.<sup>54</sup> In a recent review by Ochatt and Zhang,<sup>58</sup> haploidy in fruit trees has been described in detail covering *Citrus* species, *Litchi chinensis*, *Coffea arabica*, *Cocos nucifera*, *Pyrus* species, *Malus* genotypes, *Carica papaya*, etc. Androgenesis in woody legumes has been accomplished mainly in tropical species such as *Tamarind indica*, *Cassia* spp., *Cajanus cajan*, *Albizia lebbeck*.<sup>6</sup> The current progress on haploidy in woody plants clearly indicates that there is a vast potential to utilize haploid and doubled haploid breeding of woody plants in the developing countries. The combination of haploidy, mutagenesis, breeding and micropropagation could be very well-applied in improvement of woody plants for the isolation of useful variants. This technology does not require sophisticated equipment; however, it is highly labor intensive. So far, China is the only country in the world that has successfully exploited this technology in woody plants and other crop plants for haploid production.<sup>59</sup>

### Somaclonal Variation

Somaclonal variation is a major obstacle in the production of genetically uniform and stable plants. Usually genetic variability in tissue culture-derived plants is heritable (transmitted through meiosis), occurs randomly and is usually irreversible. The changes may be heritable but reversible resulting from altered gene expression.<sup>60</sup> Only alterations in the genetic information would give rise to true stable changes. Another class of variation, epigenetic variation (non-genetic) frequently appears in regenerated plants as a result of physiological responses, but these changes are not heritable,<sup>61,62</sup> and both are predictable and reversible. Methyla-

tion can cause changes in gene activity that can be transmitted to the sexual progeny, but may revert under certain conditions.<sup>63</sup> This raises serious doubts about the stability of somaclonal changes after self- and cross-pollination in breeding programs.<sup>64</sup> Somaclonal variation does not seem to be a simple phenomenon, and may reflect pre-existing genetic variation in somatic cells or tissue culture-induced variability. The variation may be generated through several types of nuclear chromosomal rearrangements and losses, gene amplification or de-amplification, non-reciprocal mitotic recombination events, transposable element activation, apparent point mutations, or reactivation of silent genes in multigene families, as well as alterations in maternally inherited characteristics.<sup>65,66</sup> The genotype of the explant is an important factor influencing the frequency of genetic variation,<sup>67</sup> hormonal influence<sup>68-71</sup> and DNA methylation.<sup>72-74</sup> Genetic stability is also affected by stresses experienced through an in vitro culture regime, including subculture interval, level of growth hormones and state of growth medium.<sup>75</sup> Genetic variation has also been shown to increase with prolonged in vitro culture<sup>61</sup> as a result of loss in totipotency, which may be explained by either loss or mutation of genes responsible for regeneration or by changes in ploidy level.

Somaclonal variation can be detected by biochemical and molecular markers. Isozymes have proven to be useful markers for somaclonal variation among regenerants from apple root stocks.<sup>76</sup> Isozyme polymorphism was observed among regenerants based on banding patterns, and root stocks and regenerants could be distinguished. Sabir et al<sup>77</sup> calculated the frequency of genetic changes giving rise to somaclonal variation on the basis of isozyme variation in sugarbeet. However, they failed to detect all genetic variants by isozyme patterns. Bouman and De Klerk<sup>78</sup> suggested to examine developmentally and physiologically stable enzymes for isozyme analytical studies—alcohol dehydrogenase, malate dehydrogenase, phosphoglucomutase and phosphoisomerase, and not to use those that are very variable such as peroxidases and esterases. Since somaclonal variation may occur as a result of either genetic or epigenetic changes, it is essential to identify them at an early stage of plant development in woody plants, e.g. in oil palm, coffee, tea, mango, conifers, cocoa, etc. and prevent economic disasters at a later stage due to the long life cycle of woody plants.

The most common molecular marker techniques such as restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) have been used for

somaclonal variation detection in oil palm,<sup>79,80</sup> *Picea abies*,<sup>81</sup> and *Populus deltoides*.<sup>82</sup> Heinze and Schmidt<sup>81</sup> found no gross somaclonal variation in somatic embryos and somatic embryo plants of *Picea abies* by RAPD analysis. Another sensitive molecular marker method such as amplified fragment length polymorphism (AFLP),<sup>83</sup> microsatellites or short sequence repeats (SSR),<sup>84</sup> and DNA amplification fingerprinting (DAF)<sup>85</sup> could be applied to uncover somaclonal variation. However, these methods have not yet been tried for the identification of somaclonal variation in woody plants. In most developing countries, molecular biology techniques may be difficult to use due to lack of facilities and manpower. However, countries like China, India, Korea, Brazil and Malaysia have sufficient expertise in molecular biology and can easily use these techniques for the identification of somaclonal variation in woody plants. In Malaysia, somaclonal variation in oil palm is a serious problem, that is causing economic losses. Early detection of somaclonal variation with molecular biology techniques will help Malaysian researchers develop a molecular marker-based diagnostic kit for the identification of unwanted variation.

### Cryopreservation

This technique is used for long-term storage of cells, tissues and somatic embryos by freezing under controlled conditions and storage in liquid nitrogen. It is a reliable method for long-term storage of germplasm, requires minimum space and maintenance and causes few genetic alterations. The most important aspect of cryopreservation is reliability of plant regeneration from cryopreserved material without genetic variability and quality deterioration. By cryopreservation, plant regeneration has been successful in *Citrus sinensis*, *Coffea arabica*, *Larix sp*, *Phoenix dactylifera*, *Picea spp*, *Pinus taeda*, *Pyrus spp*, and *Vitis vinifera*.<sup>86</sup> There are several other methods of short-term storage (few months to a year) of tissue cultures: 1) low temperature; 2) desiccation; 3) mineral oil overlay; 4) use of growth retardants; and 5) low atmospheric pressure.<sup>87</sup> Here the main task is to reduce the growth of the plant tissue that has proven to be the most practical for many plant species including *Daucus carota*, *Solanum spp*, *Chrysanthemum morifolium*, *Vitis vinifera*, *Fragaria sp*, *Malus domestica* and *Pinus radiata*. The periodic transfer or sub-culturing is minimal—once a year appears to be sufficient to maintain plant tissues in a slow growing stage.<sup>87</sup>

Both long-term and short-term storage of plant materials are very essential in the developing countries for the preservation of biodiversity and elite genetic material for the future uses. A lot of valua-

ble genetic material is being lost due to deforestation and economic development. International funding agencies should support developing countries in establishing National Germplasm Centers. The government of India has established a National Germplasm Center in New Delhi, India with the assistance of international funding agencies. In this center, germplasm is stored on both a long-term and short-term basis from all over the country and the center also assists researchers in the exchange of germplasm.

### Somatic Cell Hybridization

Protoplasts can be isolated and regenerated from different tissues including cell suspensions. There are several applications for protoplast cultures such as somatic cell hybridization (asymmetric and symmetric), direct DNA transfer, organelle uptake, protoclonal variation, mutation and microinjection of DNA. Usually, woody plant protoplasts are recalcitrant in terms of sustainable division and plant regeneration and, thereby, little work has been done on woody plant protoplasts as compared with crop plants. Tibok et al<sup>88</sup> have extensively reviewed protoplasts of forest and woody trees. Plants can be regenerated from protoplasts of *Eucalyptus sp*, *Populus spp*, *Santalum album*, *Sesbania spp*, *Ulmus campestris* and other fruit trees such as apple, Kiwi, *Citrus spp*, coffee, passion fruit, cocoa, rose, cherry, pear, papaya etc. Similarly, Bekkaoui et al<sup>89</sup> have listed protoplast culture and regeneration of forest trees including *Abies alba*, *Biota orientalis*, *Larix spp*, *Picea spp*, *Pinus spp* and *Pseudotsuga menziesii*. Somatic embryos can be obtained from protoplasts of *Pinus taeda*, *Picea abies*, *Picea glauca* and *Pseudotsuga menziesii*.<sup>89</sup> By protoplast fusion, fertile fruit trees have been obtained from protoplast fusion products of *Citrus sinensis* and *Citrus sinensis* × *Pocirus trifoliata*,<sup>90</sup> *C. sinensis* and *Murraya paniculata*,<sup>91</sup> *C. unshiu* and *C. jambhiri* or *C. junos*<sup>92</sup> and *C. aurantiifolia* and *Feroniella lucida* or *Swinglea glutinosa*—an intergeneric somatic hybrid.<sup>93</sup>

Protoplast technology is also useful in partial genome transfers in making fertile asymmetric somatic hybrids, both intergeneric and interspecific. These hybrids will create new germplasm. This type of research can easily be conducted in the developing countries with some national biotechnology capacity, e.g. China, India, Brazil, Indonesia, Malaysia, Korea and even poorer countries like Bangladesh, Sri Lanka.

### GENETIC ENGINEERING

Recently, rapid progress has been achieved in genetic engineering for plant improvement. Successful genetic engineering of plants requires:

1) gene isolation and identification; 2) insertion of isolated genes with selective marker genes into a cloning vector; 3) transformation of recipient plant cells; 4) regeneration of transgenic plants; and 5) testing the inheritance and expression of transgenes in transgenic plants.<sup>94</sup> Progress in gene transfer technology has facilitated genetic transformation of cereals, woody plants and vegetable and oil crop plants. Christou et al<sup>95</sup> suggested certain criteria for the development of a practical gene transformation system for any crop: 1) a cultivar or genotype-independent transformation system; 2) the recovery of large number of transgenic plants for the evaluation of transgene expression; and 3) minimizing tissue culture manipulations in order to avoid somaclonal variation. Most gene transfers have been accomplished with *Agrobacterium tumefaciens* and *A. rhizogenes*, soil-borne bacterial pathogens with a wide range of host dicot plants. However, difficulties have been encountered with *Agrobacterium*-mediated transformation of monocot plants.<sup>96</sup> Now alternative gene transfer methods such as electroporation, sonication, UV laser and biolistics are available. A biolistic bombardment method is being used routinely for the transformation of most plants including cereals and woody plants. Gene transfer into crop plants requires selection (e.g. neomycin phosphotransferase type II or NPT II) and reporter (e.g.  $\beta$ -glucuronidase or GUS) genes for testing the gene expression and allowing favorable growth of transformed cells.<sup>96</sup>

The progress in transgenic research in forest trees and other woody plants has recently been reviewed in Somatic Embryogenesis in Woody Plants, book vol. 1.<sup>35</sup> It can be concluded that progress in woody species has been slower than in crop plants due to their long life cycle and lack of identified genes. In developing countries, transgenic research of woody plants cannot be successful unless developed countries collaborate and provide national programs either with isolated genes or transgenic plants for large-scale multiplication and field trials. For example, useful genes for protecting trees against abiotic and biotic stresses such as insects and pests, herbicide, drought, salinity and air pollutants need attention in transgenic research of woody plants.

## SOLUTIONS FOR IMPROVING BIOTECHNOLOGY IN THE DEVELOPING COUNTRIES

Developing countries can improve industrially important woody plants by recent advances in biotechnology. There are, however, certain conditions that need to be addressed before setting up

effective national biotechnology programs. In this context, the following recommendations should be considered by the policy makers.

First, it is very important that the policy makers of each developing country assess and evaluate their own requirements before formulating an agenda for biotechnology, e.g. identification of research targets and their research priorities. One of the basic requirements before taking up this program is to assess the availability of manpower for basic and applied research, and chalk out the education and training requirements to meet the needs.<sup>97</sup> Education and training of researchers should include inter-related sciences and technologies so that a strong scientific foundation can be laid for the development of biotechnology programs. Second, linkage of developed technologies to product development is important. The policy makers should make sure that the researchers have a right to patent their discoveries and use them in product development. This can be possible when industry and academia work together. Improving the patent laws will protect licensed technologies, encourage investments from national and international private enterprises, encourage researchers to be more innovative and productive and improve the quality of research. Third, funds should be raised to develop infrastructure, initially, at selected universities and institutes. International funding agencies may assist in providing foreign currency for the purchase of chemicals and equipments. Finally, should be provided to allow researchers to participate in international conferences, which will enable them to interact with international scientists and learn the latest in the field.

## CONCLUSIONS

The success of biotechnology in developing countries is very much dependent on the creation of basic infrastructure, training of manpower, co-ordination of academia and industry and careful selection of appropriate biotechnology. Micropropagation and somatic embryogenesis techniques are very effective for large-scale mass production of woody plants to secure continuous supply of plant material to the industry as well as for reforestation. These technologies are ideally suited for developing countries because they are labor intensive, simple and effective and do not require excessive economic input. For example, selection of useful mutants can be done by in vitro selection, mutagenesis or somaclonal variation. Genetically stable variants and mutants can be directly incorporated in tree improvement programs. Long-term storage facilities would be needed for storing elite

germplasm and protecting biodiversity for future uses both for research and exchange of germplasm. Cryopreservation and low temperature storage facilities should be provided.

The role of sophisticated technologies such as genetic engineering for the improvement of woody plants in most of the developing countries is yet to bear fruits. However, countries like China, India, Korea, South Africa, Brazil and Argentina have started working on genetic transformation in woody plants because these countries have a strong base in molecular biology. Currently, more emphasis should be given to develop tissue culture technology in countries like Bangladesh, Nepal, Pakistan, Bhutan, most of the African and South and Central American countries, Sri Lanka and Vietnam, and gradually as they develop basic infrastructure and human power base molecular biology and other sophisticated technologies can be introduced. Developing countries cannot wait longer for the genetic engineering to bear fruits in agriculture and forestry because of the increases in human population growth that is causing tremendous pressure on cultivated lands and forests. Developing countries in consultation with developed countries and international funding agencies should identify the immediate needs and set up a time frame (short- or long-term) of progress in developing a national biotechnology program in forestry and woody plants. The frame work should be pursued in setting up programs (basically labor-oriented) including development of infrastructure and human resources. Private sector and academia interaction should be encouraged to make sure that the developed technologies are properly implemented. Finally, laws for the protection of intellectual property rights must be strictly enforced in developing countries so that more international investment is encouraged.

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Fig. 13.4. "Royal Wedding" chrysanthemum, pink and white bi-coloured flowers, produced by "ACE somaclone system."

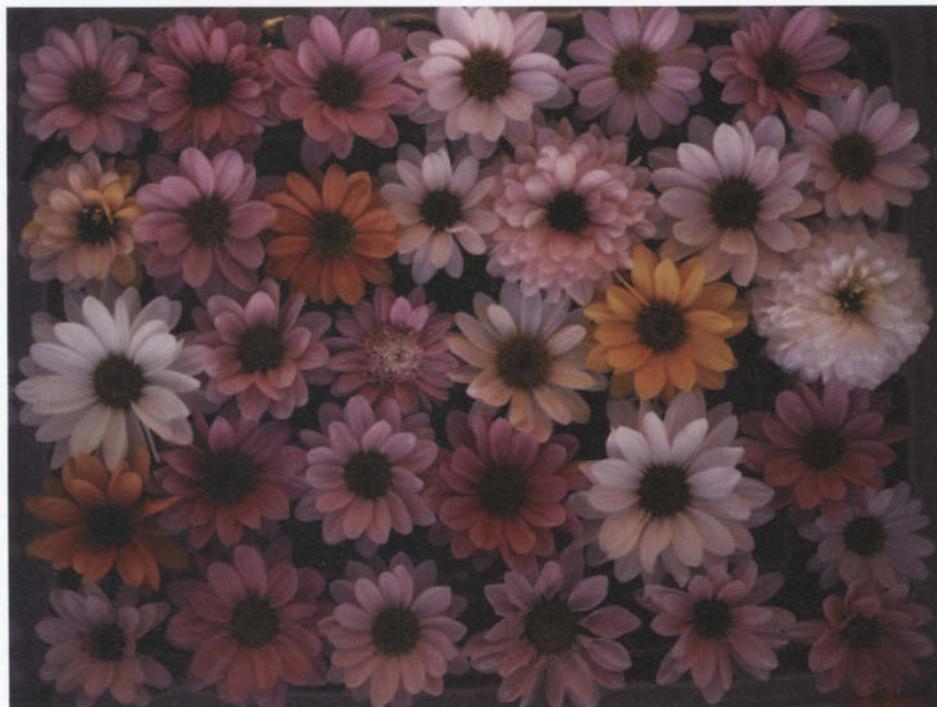


Fig. 13.5. Somaclonal variation in flowers produced by the application of "ACE somaclone system" to cv. Lineker (arrow) chrysanthemum. Variation is found in shape, color and number of petals, male sterility, flower type, etc.