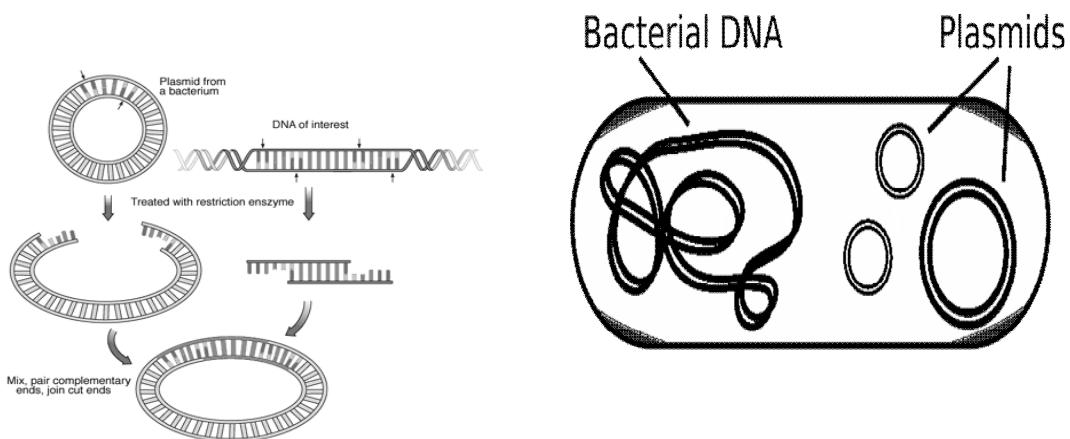
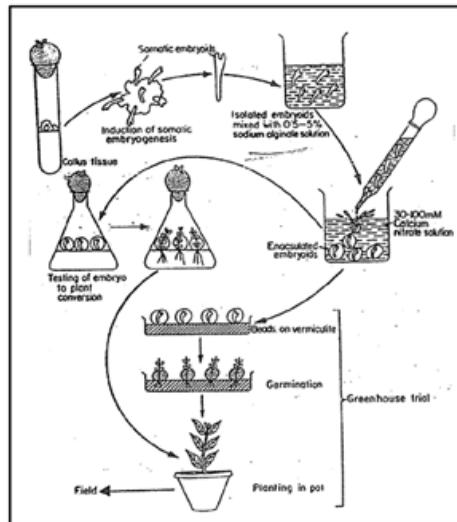




## Vardhman Mahaveer Open University, Kota



## Biotechnology, Molecular Biology and Genetic Engineering of Plants



**Vardhman Mahaveer Open University, Kota**

**Biotechnology, Molecular Biology and  
Genetic Engineering of Plants**

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**Production: 2017**

**ISBN No:**

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Printed and Published on behalf of the Registrar, V.M. Open University, Kota.

Printed By: Rajasthan State Cooperative Press Ltd. , Jaipur



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## **Vardhman Mahaveer Open University, Kota**

### **Preface**

The present book entitled “**Biotechnology, Molecular Biology and Genetic Engineering of Plants**” has been designed so as to cover the unit-wise syllabus of MBO-08 course for M.Sc. Botany (Final) students of Vardhman Mahaveer Open University, Kota. The basic principles and theory have been explained in simple, concise and lucid manner. Adequate examples, diagrammes, photographs and self-learning exercises have also been included to enable the students to grasp the subject easily. The unit writers have consulted various standard books on the subject and they are thankful to the authors of these reference books.

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## Unit-1

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# Biotechnology and Plant Tissue Culture

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### Structure of the Unit:

- 1.0 Objectives
  - 1.1 Introduction
  - 1.2 Concepts of Biotechnology
  - 1.3 History of Biotechnology
  - 1.4 Scope of Biotechnology: The Indian Advantage
  - 1.5 History of Plant Tissue Culture
  - 1.6 Cellular Differentiation and Totipotency
  - 1.7 Culture Media
    - 1.7.1 Inorganic nutrients
    - 1.7.2 Micro nutrients
    - 1.7.3 Organic supplements
    - 1.7.4 Other organic supplements
  - 1.8 Aseptic Culture Technique
  - 1.9 Summary
  - 1.10 Glossary
  - 1.11 Self-Learning Exercise
  - 1.12 References
- 

### 1.0 Objectives

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After studied this unit students found the knowledge about

- biotechnology and plant tissue culture

## 1.1 Introduction

---

Biotechnology is the use of living systems and organisms to develop or make useful products. Modern biotechnology is mainly based on recombinant DNA (rDNA) and hybridoma technology is addition to bioprocess technology. rDNA technology (recombinant DNA) is the main tool used to not only produce genetically modified organisms, including plants, animal and microbes, but also to address the fundamental questions in life sciences. Infact, modern biotechnology began when recombinant human insulin was produced and marketed in the United States in 1982. Recent year's biotechnology has assumed enormous significance due to its major impact on human welfare. In this unit brief history scope and concept of biotechnology and also the concepts, history scope, cellular differentiation, totipotency, culture medium and various sterilization techniques of the plant tissue culture.

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## 1.2 Concepts of Biotechnology

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Biotechnology is the use of living systems and organisms to develop or make useful products, or "any technological" application that was biological systems, living organisms or derivatives these of make or modify products or processes for specific use.

For thousands of years, humankind has used biotechnology in agriculture, food production and medicine.

The term itself is largely believed to have been coined in 1919 by Hungarian engineer Karoly Ereky.

In the late 20<sup>th</sup> and early 21th century, biotechnology has expanded to include new and diverse sciences such as genomics, recombinant gene technologies, applied immunology and development of pharmaceutical therapies and diagnostic tests.

### Definitions

**Biotechnology is 'the controlled use of biological agents, such as microorganisms or cellular components for beneficial use (US National Science Foundation).**

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### 1.3 History of Biotechnology

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- **500 B.C.:** In China, the first antibiotic, moldy soybean curds are put to use to treat boils.
- **1675:** Leeuwenhoek discovers bacteria.
- **1761:** English surgeon Edward Jenner Pioneers vaccination, inoculating a child with a viral smallpox vaccine.
- **1964:** Pasteurization
- **1965:** Medal and modern genetics
- **1970:** Breeders crossbreed cotton, developing hundreds of varieties with superior qualities.
- **1870:** The first experimental core hybrid is produced in a laboratory.
- **1900:** Drosophila (Fruit flies) used in early studies of genus.
- **1911:** American pathologist Peyton Rous discovers the first cancer – causing virus.
- **1926:** Hybridization
- **1928:** Scottish Scientist Alexander Fleming discovers Penicillin.
- **1933:** Hybrid corn is commercialized
- **1942:** Penicillin is mass produced in microbes for the first time.
- **1950's:** The first synthetic antibiotics created.
- **1951:** Artificial insemination of livestock is accomplished using frozen semen.
- **1953:** Discovery of DNA structure.
- **1978:** Recombinant human insulin is produced for the first time.
- **1979:** Human growth hormone is synthesized for the first time.

- **1980:** Small pox is globally eradicated following 20 year mass vaccination effort.
- **1980:** The U.S. Supreme Court approves the principle of patenting organisms, which allows the Exxon oil company to patent an oil-eating microorganism.
- **1981:** Scientists at Ohio University produce the first transgenic animals by transferring genes from other animals in to mice.
- **1982:** The first recombinant DNA vaccine for livestock is developed.
- **1982:** The first biotech drug, human insulin produced in genetically modified bacteria, is approved by FDA. Genetech and Eli Lilly developed the product.
- **1985:** Genetic markers are found for kidney disease and cystic fibrosis.
- **1986:** The first recombinant vaccine for humans a vaccine for hepatitis-B, is approved.
- **1986:** Interferon becomes the first quicancer drug produced through biotech.
- **1988:** The first pest-resistant corn, *Bt*-corn is produced.
- **1990:** The first successful gene therapy is performed on a 4 year old girl suffering from an immune disordered.
- **1992:** FDA approves bovine somatotropin (BST) for increased milk production in dairy cows.
- **1993:** FDA approves Betaseron the first of several biotech products that have had a major impact on multiple sclerosis treatment.
- **1994:** The first breast cancer gene is discovered.
- **1994:** The Americans are certified polio free by the international commission for the certification of polio Eradication.
- **1995:** Gene therapy, immune system modulation and recombinant produced antibodies enter the clinic in the war against cancer.
- **1996:** A gene associated with Parkinson's diseases is discovered.

- **1996:** First genetically engineered crop is commercialized.
- **1997:** A sheep named Dolly in Scotland becomes the first animal cloned from an adult cell.
- **1998:** FDA approves Herceptin a pharmacogenomic breast cancer drug for patients whose cancer over expresses the HERZ receptor.
- **1999:** A diagnostic test allows quick identification of Bovine Spongiform Encephalopathy (BSE also known as "mad cow" disease) and Creutzfeldt-Jacob disease (CJD).
- **2000:** Kenya field – tests its first biotech crop, virus resistant sweet potato.
- **2001:** A gene targeted drug for patients with chronic myeloid leukemia. Gleevec is the first gene targeted drug to receive FDA approval.
- **2002:** EPA approves the first transgenic rootworm resistant.
- **2002:** The beuteng an endangered species is cloned for the first time.
- **2003:** China grants the world's first regulatory approval of a gene therapy product, Gendicine, which delivers the P<sup>53</sup> gene as a therapy for squamous cell head and neck cancer.
- **2003:** The human genome project completes sequencing of the human genome.
- **2004:** A food and agriculture organization endorses biotech crops, stating biotechnology is a complementary tool to traditional forming methods that can help poor farmers and consumers in developing nations.
- **2004:** FDA approves the first antiangiogenic drug for cancer, avastin.
- **2005:** The Energy Policy Act is passed and signed into law, authorizing numerous incentives for biotechnology development.
- **2006:** FDA approves the recombinant vaccine Gradsil, the first vaccine developed against human papilloma virus (HPV), an infection implicated in cervical and throat cancers, and the first preventive cancer vaccine.

- **2006:** USDA genetics Dow Agrosciences the first regulatory approval for a plant made vaccine.
- **2007:** FDA approves the H5N1 vaccine, first vaccine approved for avian flu.
- **2009:** Global biotech crop acreage reaches 330 million acres.
- **2009:** FDA approves the first genetically engineered animal for production of a recombinant form of human antithrombin.
- **2009:** Cedars – Sinai Heart Institute uses modified SAN heart genes to create the first viral pacemaker in guinea pigs, now known as iSAN's.
- **2010:** Researcher at the J. Craig Venter Inst. Create the first synthetic cell.
- **2012:** 31 years old Zac Vawter successfully uses a nervous system controlled bionic leg to climb the Chicago Willis Tower.

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## **1.4 Scope of Biotechnology: The Indian Advantage**

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Biotechnology may be as old as human civilization but modern biotechnology is less than three decades old. Traditional biotechnology that led to the development of processes for producing products like yeast, vinegar, alcohol and cheese was entirely empirical and bereft of any understanding of the mechanisms that led to the product. There were no possibility of deliberate design to produce a desired new product.

In modern biotechnology, we use the in depth understanding we have gained in the last five decades. The variety of functions is performed by living organisms to produce a desired new or old product. In the case of an established product, the biotechnological process is cheaper and better in many respects than the earlier processes. Modern biotechnology has been, infect, an historical imperative. Its emergence on the world scene was predicated at least four decades ago.

The term genetic engineering was coined independently in 1973 by the author of an article in the Guardian in the UK, and in a syndicated article by the present author in India.

## Importance of Biotechnology

In the past, biotechnology concentrated on the production of food and medicine. It also tried to solve environmental problems. In the 19<sup>th</sup> century, industries linked to the fermentation technology had grown tremendously because of the high demand for various chemicals such as ethanol, butanol, glycerine, acetone etc. The advancement in fermentation process by its interaction with chemical engineering has given rise to a new area for bioprocess technology. Large scale production of proteins and enzymes can be carried out by applying bioprocess technology in fermentation. Applying the principles of biology, chemistry and engineering sciences processes are developed to create large quantities of chemicals, antibiotics, proteins, and enzymes in an economical manner.

Bioprocess technology includes media and buffer preparation, upstream processing and downstream processing. Upstream processing provides the micro-organisms the media, substrate and the correct chemical environmental to carry out the required biochemical reaction to produce the product. Downstream processing is the separation method to harvest the pure product from the fermentation medium. Thus fermentation technology changed in to biotechnology, now known as classical biotechnology.

Now we look at biotechnology, we find its application in various fields such as food, agriculture, medicine, and in solving environmental problems. This has led to the division of biotechnology into different areas such as agricultural biotechnology, medical or pharmaceutical biotechnology, industrial biotechnology and environmental biotechnology.

Modern biotechnology is mainly based on recombinant DNA (rDNA) and hybridoma technology is addition to bioprocess technology. rDNA technology is the main tool used to not only produce genetically modified organisms, including plants, animal and microbes, but also to address the fundamental questions in life sciences. Infect, modern biotechnology began when recombinant human insulin was produced and marketed in the United States in 1982. The effort leading up to this landmark event began in the early 1970's what research scientists developed protocols to construct vectors by cutting out and pasting pieces of DNA together to create a new piece of DNA that could be inserted into the bacterium, *E. coli* (transformation). If one of the pieces of the new DNA includes a gene for insulin

or any other therapeutic protein or enzyme, the bacterium would be able to produce that protein or enzyme in large quantities by applying bioprocess technology.

Gene Therapy, Immune-Technologies, Stem Cell Techniques, Enzyme Engineering and Technology, Photosynthetic Efficiency, New DNA Technologies, Plant Based Drugs, Peptide Synthesis, Rational Drug Design, Pharmaceuticals, Assisted Reproductive Technologies. Organ Transplantation, New Drug Delivery Systems, DNA Vaccines, Biosensor, Use of Microbes, Bioremediation, Bioinformatics including Genomics and Proteomics, Nano Biotechnology; these all are branch and work of Biotechnology.

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## **1.5 History of Plant Tissue Culture**

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### **Concept of Cell Culture**

German botanist Gottlieb Haberlandt (1902) developed the concept of *in-vitro* cell culture he was the first to culture isolated cells in a nutrient medium containing glucose, peptones and knops salt solution Haberlandt realized that asepsis was necessary when culture media are enriched with organic substances metabolized in his culture free from contamination. Cells were able to synthesized starch as well as survived for several weeks. However Haberlandt failed in its goals to induce these cells to divide. He is constituted to be Father of Tissue Culture.

### **Development of Tissue Culture**

Hanning in (1904) initiated a new line of investigation involving the culture of embryo genetic tissue which later becomes an important applied area of investigation using *in-vitro* tech. He excised nearly mature embryo of crucifers and successfully grew them to maturity on mineral salts and sugar solution.

### **Root Tip Culture**

Kotte (1922) Germany and Robbins (1922) USA both was successful in an establishment of excised root tip *in-vitro*. They postulated that a true *in-vitro* culture could be made easier by using meristematic cells such as those that are present in root tips or buds.

However in 1934 pioneering work of growing excised root of tomato *in-vitro* was demonstrated by white initially white used a medium containing yeast extract

inorganic salts and sucrose. But later east extract was replace by 3 vitamins namely pyridoxine, thiamine and nicotinic acid.

White synthetic media is used today as one of the basic media for the culture of various cells.

Gautheret (France), White (U.S.A.) and Nobecourt frame published independently studies on successful cultivation of cambial tissue of carrot root (Gautheret, 1939), Tomato (White, 1939) and Carrot (Nobecourt, 1939).

During early 1950 a number of inquiries were initiated. It was realized that plant growth hormone enhanced the multiplication of totipotents cell.

Miller and Skoog 1953 worked on bud formation from cultured pith explants of tobacco let to the discovery of kinetin. Now many synthetic as well as natural compounds which show shoot bud proliferation. Muir reported that if fragments of *Tagetes erecta* and *Nicotiana tabacum* are transferred to liquid medium and agitated on rotator shaker and then the callus fragment break up to give a suspension of a single cell and the further develop proper raft nurse technique.

An important technique of cloning large number of single cells of higher plants was developed Bergman (1960). Toshio Murashige in Wiskonsin University. Later professor in the University of California guard the most universally used high salt media along with Skoog i.e. MS medium in 1962 in addition to mineral salts. The media contains on energy source vitamins and growth regulators.

In 1958 regeneration of somatic embryo *in-vitro* from nucleus of *Citrus* ovule was cloned by Maheshwari and Rangaswami.

Maheshwari and Rangaswami in 1959 was regenerated somatic embryo from callus clumps and cell suspension of *Daucus carota*. Kocking in 1960 discovered enzyme cellulose and pectinase with solubilize the cell wall in buffer solution in optimum pH caused protoplast isolation and culture.

Guha and Maheshwari reported direct development of embryo from microspores of *Datura innoxia* by culture of excised anther.

Fasil and Helderbrandt (1975) observed colonies arising from cloning of isolated cells of the hybrids *Nicotiana glutinosa*. The phenomenon of somatic embryogenesis leading to plantlet formation in cultures was later reported in many

species. All these discoveries contributed to establishment of totipotency of somatic cells under experimental condition. These by accomplishing the goals set by Haberlandt.

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## 1.6 Cellular Differentiation and Totipotency

---

Totipotency is the basis of plant cell and tissue culture techniques. Term was coined by Morgan in the year 1901.

### Definition

**Potential of a cell to grow and develop a multi-cellular or multi-organized higher organism is termed as totipotency.**

### Cellular Totipotency

Plant body and cellularity is maintained by the zygote and this zygote contains all the information referred to the plant. This information remains localized in the DNA, due to the mitosis, zygote divides into the cells formed which carry genetic information. Many of the genes that remain inactive in differentiated tissues or organs are able to express under adequate culture conditions. To express totipotency differentiated cell first undergoes de-differentiated then re-differentiation.

S.C. Steward exemplified totipotency by using in a model system. In tissue culture, cells obtained from stem, root or other plant part and are allowed to grow in cultural medium containing mineral nutrients, vitamins, hormones etc. to encourage cell division and growth. As a result, the cells in culture will produce an unorganized mass of proliferative cells of a Callus Tissue. The cells that comprise the callus are totipotent thus a callus tissue may be in a broader sense totipotent. Theoretically, totipotency of all the cells are expressed at a time, it is expected that equal number of shoots or embryoid will be represented from such cells but in experiment such results are not obtained.

### Various reasons behind the limited expression of totipotency may be:

1. Variation in chromosome number of cells of callus tissue.
2. An association of cells may be sometimes necessary to provide the appropriate environment for certain individuals to express their totipotency.

3. The endogenous hormone level of a cell and exogenously supplied hormone make a threshold level which actually induces the totipotent cell to express culture but the cells that comprise callus tissue absorb hormones and nutrients at a gradient, availability of hormones is not equal to all cells and thus all the cells do not express totipotency.

### **Importance of Totipotency in Plants**

1. Vegetative propagation can be done to produce plants of economic, medicinal and agricultural importance.
2. Genetic modification of plants.
3. Production of homozygous diploid plants.
4. Desired characters can be obtained in the plants by plant breeders and commercial plant growers.
5. Germplasm conservation.

### **Ways of Totipotency expression**

1. Axillary bud proliferation.
2. Direct somatic embryogenesis.
3. Adventitious shoot bud formation.
4. Organogenesis in callus and suspension cultures.
5. Embryogenesis in callus and suspension cultures.
6. By androgenesis, gynogenesis.
7. Apical bud formation.

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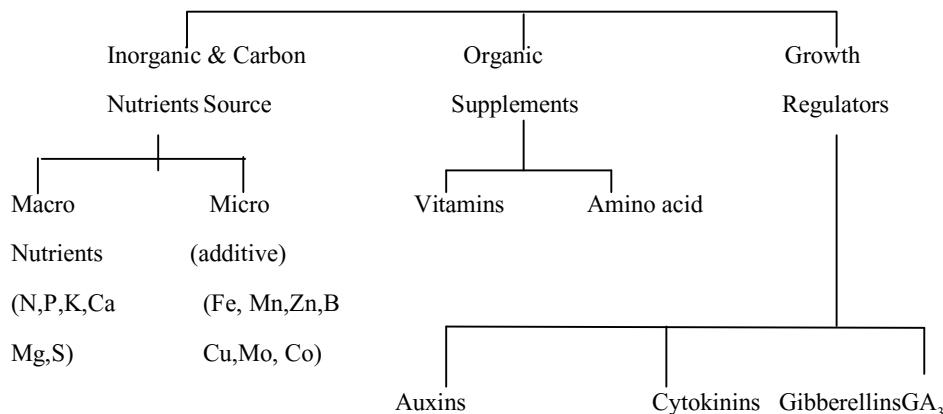
## **1.7 Culture Media**

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Growth and morphogenesis of plant tissue *in-vitro* are largely governed by composition of the culture media. Although the basic requirement of cultured plant tissue are similar to those of whole plants in practice. Nutritional requirements for optimal growth of tissues *in-vitro* may vary with the species. Even tissue from different part of the plant may have different requirement for satisfactory growth. As such no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organ. A medium containing only chemically defined

compounds is refer to as synthetic medium. In Plant tissue culture media milli moles per lit is used for expressing the concentration of nutrients and organic nutrients require in large amount and micro mole or lit. for nutrients require in small amount.

### Main components of Plant tissue Culture medium



Some tissues grow on simple media containing only inorganic salts and utilizable carbon source but for most others it is essential to supplement the medium with vitamins, amino acid and growth substances.

Examples of media used are:

- (i) **White's medium:** Used for root culture and to induced organogenesis and regeneration.
- (ii) **M.S. medium:** Basal media
- (iii) **L.S.: (Linsmaier and Skoog Medium).**
- (iv) **B5 medium:** For cell suspension or callus culture.
- (v) **N<sub>6</sub> medium:** For anther culture.

The minerals present in the Plant Tissue Culture media can be used by plant cells as building blocks for the synthesis of organic molecules.

The concentrations of the dissolved salts play an important role in the osmotic regulation and in maintaining the electrochemical potential.

Nitrogen, sulfur and phosphorus are components of proteins and nucleic acid.

Mg and micronutrient are essential part for catalysis of various metabolic reactions.

### **1.7.1 Inorganic nutrients**

- Ca – Important for strength of the Cell-wall and resistance against fungal infection and regulation of cell potential.
- P – Used as  $\text{H}_2\text{PO}_4$  form.
- K – In highest concentration in medium used as ionic barrier, osmotic regulation.
- Mg – Plays important role in photosynthesis.
- N – Used as a source of protein and amino acid. It helps in osmoregulation, maintaining cation-anion balance.
- S – Absorbs by plants in the form of sulphates use in amino acids and Proteins.

### **1.7.2 Micro nutrients**

- B – Use in lignifications of cell wall and differentiation of xylem.
- Cl – Absorb in  $\text{Cl}^-$  form.
- Fe – Use in chelated form with EDTA.
- Cu – As co-factor in enzymes.
- Co – Important in  $\text{N}_2$  fixation.

Mn and Mo – They bind to several metalo proteins.

### **Carbon and Energy Source**

The most important Carbon source is sucrose. Glucose and fructose are also used as a source of carbon. During autoclaving of medium sucrose get converted of medium sucrose get converted into glucose and fructose.

The other carbohydrates used are lactose and galactose.

### **1.7.3 Organic Supplements**

#### **Vitamins and Amino Acids**

These vitamins are inositol, Nicotinic acid Pyridoxine HCl pentothonate and myoinositol use in (0.1 to 10 mg or lit.) Amino acids and additives are important for stimulating cell growth. Tyrosine should be used only when agar is added to the media. Amino acid added singly proves to be inhibitory to cell growth while used in combinations proves to be beneficial.

#### **1.7.4 Other Organic Supplements**

Protein hydrolysate coconut milk yeast and extract ground banana, orange juice and tomato juice.

**Activated charcoal** –Stimulate growth and differentiation, it also enhances secondary metabolites as well toxins production by the plant.

**Antibiotics**–Generally used streptomycin and kanamycin. To control the contamination the use of antibiotics is essential as it retards the growth of cell.

**Growth regulators-** commonly used auxins are e.g. IAA, NAA, IBA, 2,4-D, 2,4,5-D (2,4-dichlorophenoxy acetic acid) (Tri chloro).

Auxins are added by dissolving in ethanol or dil. NaOH; they induce cell division, cell elongation of stem and internode tropism, apical dominance and excision and rooting.

**Cytokinin-** Dissolve in dil. HCl or NaOH, responsible for cell division modification of apical dominance shoot differentiation. eg. BAP (6-benzyl amino purine), BAA (6-benzyl adenine), 2IP (2-N<sup>6</sup>-β-2-isopentynyl adenine)

**Kinetin-** N-(2-furfuryl amino-1-H-purine, 6-amine)

**Zeatin-** 6-(4-hydroxy-3-methyl trans-2-butyl amino) purine

High auxin and low cytokinin, it induces root initiation.

Low auxin and high cytokinin initiates axillaries and shoot formation.

**Gibberellins-** GA<sub>3</sub> is most common gibberellins that promotes the growth and enhances callus growth and induces dwarf plantlets to elongate.

**Solidifying agent-** Mainly used is agar. It is digested by the explant. It does not react with media constituents normally used in 0.5 to 1%. Other solidifying agents are phyto-gel alginate.

**pH-** Plant cells require optimal pH for growth and development in culture as the pH affects uptake of ions for the mostly used Plant Tissue Culture media pH 5.0 to 6.0.

## 1.8 Aseptic Culture Technique

All tissue cultures are likely to end up contaminated if the inoculums or explants used are not obtained from properly disinfected plant material. To obtain sterile plant material is difficult because in the process of sterilization (aseptic) living materials should not lose their biological activity; only bacterial or fungal contaminants should be eliminated. Plant organs or tissues are, therefore, only surface-sterilized by treatment with a disinfectant solution at suitable concentrations for a specified period. The disinfectants most widely used and their concentrations in the solution are given in Table 1.1

Fairly hard explants are treated directly with disinfectants. For example, in the culture of mature seeds or mature endosperm (euphorbiaceous plant), whole seeds or de-coated seeds are surface-sterilized. An explant that carries a heavy load of micro-organisms needs to be washed in running tap-water for 1-2 hr prior to its treatment with disinfectant solution. Ethyl alcohol or isopropyl alcohol is used to surface sterilize delicate tissues such as shoot apices.

**Table 1.1: Disinfectants used for Sterilizing Plant Material**

<b>Disinfectant</b>	<b>Concentration</b>	<b>Duration of treatment (min)</b>
Benzyl chloride	0.01-0.1%	5-20
Bromine water	1-2%	2-10
Calcium hypochlorite	9-10%	5-30
Ethyl alcohol	75-95%	-
Hydrogen peroxide	3-12%	5-15

Mercuric chloride	0.1-1.0%	2-10
Silver nitrate	1%	5-30
Sodium hypochlorite	0.5-5%	5-30

Pollen grains, and shoot or flower buds. Such explants are given a rinse in 70% ethanol for a few seconds and then left exposed in the sterile hood until the alcohol evaporates. Usually shoot apices or pollen grains are free from micro contaminants and may be used for inoculation without surface sterilization. Addition of a few drops of a surfactant (Triton-X or Tween-20) to the solution or treating the plant material in a solution of Cetavlon for 2 min before exposing to sterilant may enhance sterilization efficiency.

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## 1.9 Summary

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Recent year's biotechnology has assumed enormous significance due to its major impact on human welfare. In this unit brief history scope and concept of biotechnology and also the concepts, history scope, cellular differentiation, totipotency, culture medium and various sterilization techniques of the plant tissue culture.

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## 1.10 Glossary

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- **Biotechnology:** Biotechnology is a technology using biological phenomena for copying and manufacturing various kinds of useful substance.
  - **Totipotency:** Potential to regeneration whole plant from a single cell.
  - **Sterilization:** To prevent the contamination of plant tissue cultures.
- 

## 1.11 Self -Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Define biotechnology.
2. What is tissue culture?
3. Define totipotency.
4. Define surface sterilization.

5. What is the basic technique in plant tissue culture?

**Section B : (Short Answer Type Questions)**

1. Write any two scope of biotechnology.
2. What is the composition of culture medium?

**Section C : (Long Answer Type Questions)**

1. Write short notes of history and concepts of biotechnology.
  2. Write a note on plant tissue culture technique.
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## **1.12 References**

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- S.S. Bhojwani, Plant tissue culture applications and limitations.
- S.S. Bhojwani and S.P. Bhatnagar. The embryology of Angiosperms.
- M.K. Razdan, Plant tissue culture.
- Rastogi Publication, Elements of Biotechnology.

## Unit - 2

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# Organogenesis, Micropropagation and Somatic Embryogenesis

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### Structure of the Unit

- 2.0    Objective
- 2.1    Introduction
- 2.2    Fundamental Aspects, Techniques and Utility of Organogenesis
  - 2.2.1 Callus Cultures
  - 2.2.2 Callus Formation
  - 2.2.3 Characteristics of Organogenesis
  - 2.2.4 Factors Affecting Organogenesis
  - 2.2.5 Morphology of Callus
  - 2.2.6 Internal structure of Callus
  - 2.2.7 Chromosomal Variation in Callus Tissues
  - 2.2.8 Significance of Callus
- 2.3    Micropagation
  - 2.3.1 Sterlization of Explant
  - 2.3.2 Inoculation of Explant
  - 2.3.3 Multiplication of Shoots or Somatic Embryo formation
  - 2.3.4 Germination of Somatic Embryo and Rooting of regenerated shoot
  - 2.3.5 Proliferation of Shoots in the Multiplication Medium
  - 2.3.6 Acclimatization of Plant transferred to Soil
  - 2.3.7 Browning of the Medium
  - 2.3.8 Advantages of Micropropagation product development
- 2.4    Somatic Embryogenesis

- 
- 2.5 Synthetic Seeds and their Applications
  - 2.6 Summary
  - 2.7 Glossary
  - 2.8 Self-Learning Exercise
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- 

## **2.0 Objective**

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The objective of this unit is to explain

- techniques & utility of organogenesis,
  - detail procedure of micropropagation and
  - somatic embryogenesis in a very simple way.
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## **2.1 Introduction**

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In this unit we will study about the fundamental aspects, techniques and utility of organogenesis, micropropagation, somatic embryogenesis and synthesis of artificial seeds and their application

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## **2.2 Fundamental Aspects, Techniques and Utility of Organogenesis**

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### **2.2.1 Callus Cultures**

If organized tissue diverted into an unorganized proliferation mass of cells they form callus tissue. Some times deep, large wounds in branches and crumps of the intact plants results in formation of soft mass of unorganized parenchymatous tissue which are rapidly form on or below the injured surface of the organ concern. Such callus tissue is known as wound callus and is formed by division of cambial tissues. Secondly sometimes unorganized, compact, white, light outgrowth or callus like masses on stem, leaf, root and formed by stimulus of cell division in fully differentiated cell due to some diseases.

**Definition:** Callus tissue means an unorganized, proliferative mass of cells produced from isolated plant cell, tissues organs. When grown aseptically or artificial nutrient media under cultured conditions.

**Principle-** For successful initiation of callus culture 3 important criteria should be accomplished.

1. Aseptic preparation of plant material: First washed with liquid detergent with generally 5% made by Teepol) than surface sterilize by 0.1% made by volume  $MgCl_2$ , 0.8% to 1.6% sodium hypochloride.
2. Selection of suitable media supplement with appropriate ratio of auxins and cytokinin.
3. Incubation of culture under controlled physical condition of light, temperature and humidity.
  - Temperature  $25\pm2^{\circ}C$
  - Light duration – Totally dark for 16 hrs.
  - Light intensity – Cool white light 2000 to 3000 lux approximately.
  - Humidity – 60%

Once the growth of the callus tissue is well established, portions of callus tissue can be removed and transferred directly on to fresh media.

### **2.2.2 Callus Formation**

Formation of callus is outcome of cell division of cells of explants. During formation of callus tissue explants losses its original characteristics. For initiation of callus culture, tissue from young seedlings and juvenile part of a mature plant are generally taken. As the explant absorbs exogenously supplied hormones along with other nutrients, it makes a continuous nutrient gradient among the different cell of the explants on the basis of their location. As a result, cell divide asynchronously depended upon the availability of nutrients and hormones.

Both auxins and cytokinins required for indefinite growth and cell division in callus culture. Sometimes only 2,4-D is sufficient as auxins promote growth and cytokinins promote cell division.

After formation of visible unorganized mass of cells at cut end, gradually the old tissue is involved to form callus.

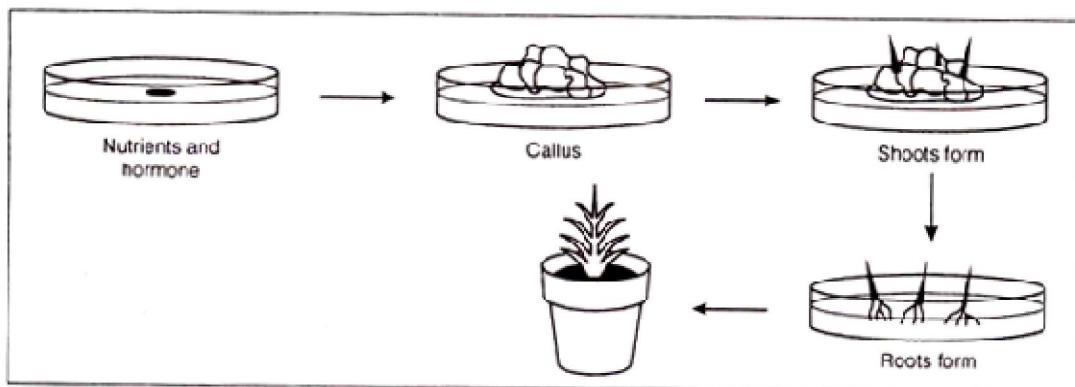
**Caullogenesis**— formation of shoots induction or proliferation

**Rhizogenesis** – Formation of roots

**Organogenesis** – The development of adventitious organ or primodia from undifferentiated cell mass in tissue culture by the process of differentiation.

When periphery cell start dividing it forms abnormal growth and it will degenerates after some time.

Some anomalous abnormal structure which are structurally similar to the organ between the dermal vascular and ground tissue present in plant tissue. But they differ from the true organ because they are obtained directly from peripheral cells of the callus and not from the organized meristemoid (patches of actively dividing cells of the callus).



**Fig. 2.1 : Callus Culture**

### 2.2.3 Characteristics of Organogenesis

Unlimited growth of callus should be formed for proper differentiation.

Initiation of shoot buds prior to rooting between rooting potential persist for a longer time and the shooting potential is for smaller time therefore rooting is done prior than shooting it is very difficult to initiate shooting.

Once Rhizogenesis starts callus obtained is yellow in colour and it turns green when caulogenesis starts.

Organogenetic capacity is a capacity of callus to differentiate into different organ.

The use of the growth regulators – when we use it the cell may undergo some mutation (aneuploidy, polyploidy) if we subculture the callus, the callus formed

may undergo some change in genetic makeup and hence they may lose their organogenetic potential.

**Habituated callus** – It shows the undifferentiated growth this habituated callus gives organogenetic potential.

#### 2.2.4 Factors Affecting Organogenesis

Following factors affects process of organogenesis -

1. Size of explants.
2. Age of the explants.

A younger leaf produces roots and older leaves produce shoots after organogenesis.

3. Seasonal explants

Explants excised during summer and winter do not produce shoot at all.

4. Oxygen gradient

Low – caulogenesis

High – Rhizogenesis

5. Colour spectrum or quality or intensity of light.

Blue colour of spectrum

Red colour promotes the rooting, light duration is 16 hrs and light intensity is 2000-3000 lux and temperature is 25°C.

6. Ploidy level.

Large number of plant species including economically important medicinal plants, horticultural important plant etc. have been successfully regenerated from the callus.

- Regeneration of whole plants from explant is of special interest in mutagenic studies.
- Regeneration of whole plants from somatically mutant cell types new strains of mutant plant are obtained through organogenesis.
- New source of genetic variability is also available in plant regenerated for cell culture. This somaclonal variation is a useful source of variability.
- A chromosomal variation is associated with phenotypic variation including agriculture by use of characters such as disease resistance.

### **2.2.5 Morphology of Callus**

Callus tissue proliferates as an amorphous mass of cell having no regular shape. All calluses derived from different plants look alike externally but can be distinguish on basis of internal structure.

### **2.2.6 Internal Structure of Callus**

Cellular composition of callus tissue is extremely heterogenous ranging from cell with dense cytoplasm, plant cells with vacuolated cytoplasm, shape of cells very strong spherical to elongated plant elongated cells are usually non dividing having large central vacuole while small cells are actually dividing cells which have reduced vacuole size and dense cytoplasm, formation of xylem and phloem with in callus which is known as cytodifferentiation.

Soft callus is friable in nature and is made of heterogeneous mass of cells having minimal content. Hard callus consist of giant cell which closely packed that is compact in nature.

### **Colouration**

Generally creamish yellow in colour. Sometimes callus tissue may be pigmented; pigmentation may be uniform or patchy – some time Callus Tissue grows in dark and turn green after transferring in light condition due to development of chloroplast.

Yellow	-	Carotinoids
Purple	-	Xanthocyanin
Brown	-	Phenolic

### **Habituation**

Sometimes, after repeated subculturing the callus tissue gains ability to grow on a std. basal medium which is devoid of growth hormones. This property is called as habituation and callus tissue is known as or habituated callus tissue.

Cells in habituated callus tissue appear to have developed a capacity to synthesize adequate amount of auxins and or cytokinins. These can't be distinguish from normal callus accept in their hormonal requirement.

### **2.2.7 Chromosomal Variation in Callus Tissues**

1. **Genetic basis of variation in callus tissue**

Endoreduplication is of frequent occurrence in differentiated tissue of higher plants and such cells remain in mitotic state. Therefore callus tissue may get such genomic heterogeneity possibly due to non selective induce of asynchronous division of both diploid and endoreduplicated cells. Variation of chromosomes no ranges from aneuploidy to different level of polyploidy.

2. **Epigenetic basis**

Highly meristematic cells are expected to be diploid but callus derived from meristem also shows variation in chromosome no. it is also found that prolong sub culture may lead to establishment of one karyotype and other are gradually eliminated. Sometimes structure changes of chromosome like deletion, translocation etc. may occur in culture. Ideal callus culture is characterized by the possession of numerical or structural stability in long term culture. But it is very rare that cells of callus tissue may be haploid if it is derived from microspore culture.

#### **2.2.8 Significance of Callus**

1. Whole plant can be regenerated in large number from callus tissue which manipulation of the nutrient and hormonal consequence in culture medium. This phenomenon is known as plant regeneration, organogenesis or morphogenesis.
2. Callus tissue is good source of genetic or karyotyping variability, so regenerate the plant from genetically variable cells of callus tissue
3. Cell suspension culture is moving liquid medium and can be initiated from callus culture.

We use only tissue culture technique and it is part of the clonal propagation and in clonal we use conventional and tissue culture also.

Clonal propagation through tissue culture popularly known as micropropagation and can be achieved in a short time and space. Thus, it is possible to produce plants in large number starting from a single individual – use of tissue culture for micropropagation was inflated by G. Morel (1960). Products of this rapid vegetative propagation can be regarded as done only when it is established that the cell, they comprise are genetically identical.

## 2.3 Micropropagation

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*In-vitro* clonal propagation is a complicated process requiring many steps or stages. Murashige (1978) proposed distinct stages that can be adopted from overall production technology of clones under *in-vitro* culture conditions. The sole objective of the technique is the demonstration of totipotency of differentiated plant cells.

### 2.3.1 Sterilization of Explant

This process includes 3 steps:

1. Washing under running water using liquid detergent and sodium hypochloride.
2. Two rinsing and washing with distilled water.
3. Explant sterilization with aqueous mercuric chloride in the laminar air flow bench.

### 2.3.2 Inoculation of Explant

**In the nutrient medium** – the process of inoculation is carried out in the laminar air flow technique. Keeping all the sterile culture conditions the explant is inoculated on the medium, using a pair of sterile forceps.

**Stage 1** last for 3 months to 2 years and requires at least 4 passages of the subculture usually explant carrying a performed vegetative bud are suitable for enhanced axillary branching. If stock plants are tested virus free than the most suitable explants are nodal cuttings. The disadvantages of using small size explant are that they have a low survival rate and show slow initial growth.

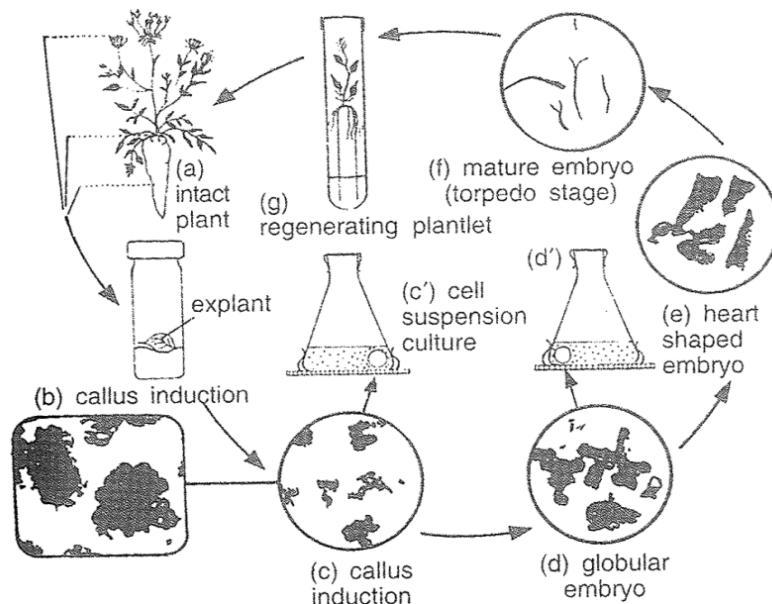
### 2.3.3 Multiplication of Shoots or Somatic Embryo formation

Plant tissue culture technique also leads to the development of shoots and somatic embryos in vitro. The process includes various stages which are described below:

**Stage 2** – This stage involves the maximum proliferation of regenerated shoot using a defined culture medium. Various approaches for micropropagation include–

1. Multiplication growth and proliferation of meristems excised from apical and axillary shoot of the parent plant.

2. Induction and multiplication of adventitious meristems through process of organogenesis or somatic embryogenesis on direct explants.
3. Multiplication of calli derived from organ tissue, cell etc. and the subsequent expression of either organogenesis or somatic embryogenesis in serial subcultures.



**Fig. 2.2 : Schematic representation of Somatic Embryogenesis in carrot**

### 2.3.4 Germination of Somatic Embryo and Rooting of Regenerated Shoot

**Stage 3-** Shoots proliferated from stage 2 is transferred to a rooting (storage) medium. Sometimes shoots are directly established in soil as microculturing to developed roots. The shoots are generally rooted *in vitro*. When the shoots or plantlet are prepared for soil it may be necessary to evaluate several factors such as:

- (i) Dividing the shoots and rooting them individually.
- (ii) Hardening the shoots to increase their resistance to moisture stress and disease.
- (iii) Rendering plants capable of autotrophic development in contrast to heterotrophic state induced by culture.
- (iv) Fulfilling requirements of breaking dormancy.

**Stage 3-** Requires 1-6 weeks Transfer of pellets to sterilized soil or green house.

**Stage 4-** Steps taken to ensure successful transfer of the plantlets of stage 3 from the aseptic environment of the laboratory to the environment of the green house comprises stage 4. This is known as acclimatization. It takes 4-16 weeks for the finished product (plant size in the range of 3-6 inches) to be ready for sale.

### **2.3.5 Proliferation of Shoots in the multiplication medium**

*in-vitro* multiplication of shoot involves 3 main approaches.

#### **(a) Multiplication of axillary and apical shoots**

Axillary and apical shoots contain quiescent or active meristem depending upon the physiological state on the plant. The axillary buds are treated with hormones to break dormancy and produce shoot branches. The shoot are then reported and rooted to produce plants division. Generally the technique of proliferation by axillary shoots is applicable to any plant that produces regular axillary shoots are respond to cytokinins such as BAP and Zeatin.

Apical shoots (1-5 mm) are normally cultured on media containing mixture of auxin and cytokinin. The presence of cytokinin in the media inhibits root development, cultured material is transferred in stage 3 to a rooting medium which contain either no or reduce levels of cytokinin.

#### **(b) Multiplication by adventitious shoots:**

Adventitious shoots are stem and leaf structures that arise naturally on plant tissues located inside other than at normal leaf axil region. These structures include stems, bulbs, corns, tubers and rhizomes. Almost every one of these organs can be used as culturing in conventional propagation e.g. (leaves of Begonia) in culture similar type of shoot formation can be induced by using appropriate condition of growth regulators in media I 25°C some cultures may require initial low temperature for morphogenic resistance.

Genotype screening and selection of genotypes among segregating populations could be fruitful approaches in the improvement of micropropagation capabilities of plant which are recalcitrant in tissue culture.

### **2.3.6 Acclimatization of Plant transferred to Soil**

Micropropagation on a large scale can be successful only when plants after transfer from culture to soil show high survival rates and the cost involved in the process is low. Plants are transferred to the soil usually after the *in-vitro* rooting stage it is essential that the dark parts of tissue culture plants or shoots be washed thoroughly before their transfer to the potting mixture.

Transplanted plantlets or shoots are immediately irrigated with an inorganic nutrient solution and maintained under high humidity for the initial 10-15 days. This is required between plantlets, dividing culture all adopted to almost 90-100% humidity attempts have also been made to harden the shoot system by inducing anatropism and development of surface wax on *in-vitro* leaves. During large scale micropropagation of some plants certain (bacterial) contamination persists even after critical surface sterilization of explants. Addition of antibiotics or fungicides to the culture medium may control the contamination.

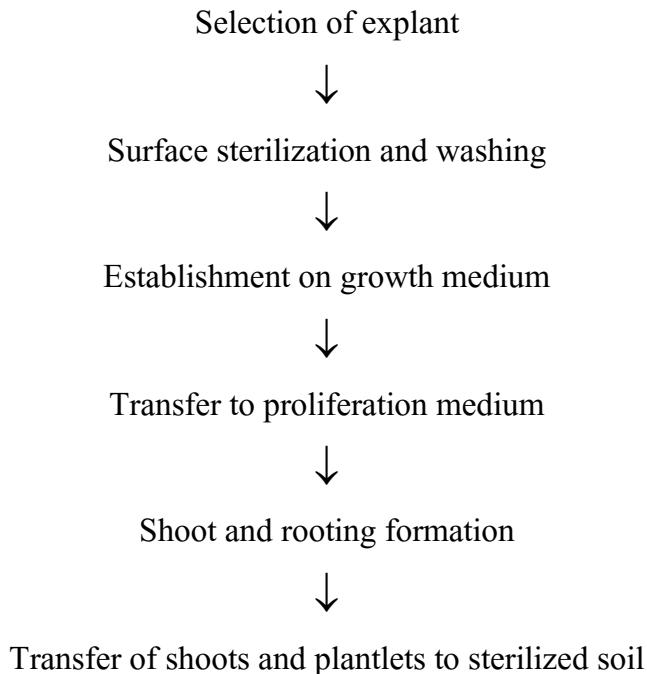
### **2.3.7 Browning of the Medium**

Explants from the adult tissue of some woody species often produce excessive amount of phenolic substances which turn the medium dark brown. Such a medium is toxic to tissues and inhibits their growth. Browning may be prevented by incorporating ascorbic acid or citric acid in culture media or by repeated subculturing.

### **2.3.8 Advantages of Micropropagation product development**

- 1. Rapid multiplication:** Micropropagation provides a method for rapid increase in the both asexually propagated and sexually propagated materials many flowers and vegetable used.
- 2. Product uniformity:** The resulting product can have a high degree of phenotypic uniformity hence the crops can be artificially manipulated in the laboratory to yield a large plant population at the same growth.
- 3. High volume:** Large population can be produced in relative by smaller growing space and in a reduced time frame.
- 4. Elite selection:** It is possible to effectively capitalize on the selections of one desirable plant and then micropropagate it into large number and release as superior selection.

5. **Germplasm stage:** Storage methods for effective preservation of valuable selection can be accomplished by combining micropropagation with cold storage and even cryopreservation in liquid N<sub>2</sub>.
6. **Diseased induced plants:** Technique to index or eliminate specific diseases particularly viruses can readily be incorporated into micropropagation procedure.
7. **Non-Specific production:** Micropropagation gives propagation uses such as minitubers or minicorms for plants multiplication throughout the year irrespective of the season.
8. **Cloning of dioecious species:** Multiplication by cloning of dioecious species is extremely important when the seed progeny yield 50% males and 50% females and plants of one of series are desired commercialized
9. 30°C is optimum temperature for cellulose formation.
10. **Shoot tip culture and virus free plant**



### Major stage of micropropagation

Morel and Martin, 1952, developed the technique of meristem culture for *in vivo* virus eradicate of Dahlia.

We use the axillary bud or meristem tissue:

- Because the high concentration of auxin, virus is not able to survive.
- The cell division is too fast so virus is not able to replicate in this region.

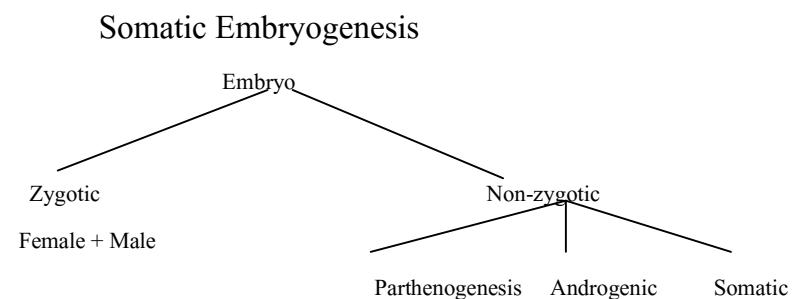
### **Shoot tip or meristem culture**

Cultivation of axillary or apical shoot meristem via meristem culture

**Explant:** Shoot apical meristem lies in the shoot tip beyond the youngest leaf and first leaf primordium.

- It measures up to about 100  $\mu\text{m}$  in diameter and 250  $\mu\text{m}$  in length.
- Thus a shoot tip of 100-500  $\mu\text{m}$  in  $\mu\text{m}$  contains 1-3 leaf primordia in addition to the apical meristem.
- 1 mm – shoot tip used for virus elimination 1 cm for clonal propagation.
- Shoot tip may be cut into five pieces to obtain more than one plantlet from each shoot tip.
- Meristem of shoot tip is cut or isolated from stem by applying a U shaped cut with a sterilized knife.

## **2.4 Somatic Embryogenesis**



**Definition:** The developmental pathway of numerous well organized small embryos resembling zygotic embryos from the embryogenic potential of a somatic plant cell of the callus tissue or cells of suspension culture is known as somatic embryogenesis. J. Reinnert (1958-1959) first reported somatic

embryogenesis in *Daucus carota*. Capability of somatic plant cells of a culture to produce embryoids is known as embryogenic potential.

**Principles:** It can be done by 2 ways:

1. **Direct or PEDC (preembryogenic determined cells):**

Predetermined fate to form embryo and await some kind of stimulation from embryoid.

2. **Indirect or IEDC Induced embryogenic determined cells**

**Callus:** Cells on callus then form embryoid. No predestined fate, it is induced embryoid form.

(i) Somatic embryogenesis is initiated in 2 different phases:

**(a) Direct:** In some cultures the somatic embryogenesis is initiated by preembryogenic determined cells (PEDC). In PEDC's the embryogenic pathway is predetermined and the cells appear to only wait for synthesis of an inducer or removal of an inhibitor to resume independent mitotic divisions, in order to exhibit their embryogenic potential.

**(b) Indirect:** In some cases, the somatic embryo development needs some kind of callus formation and embryoids emerge from induced embryogenic determined cells (IEDC's). IEDC's require redetermination to the embryogenic state by exposure to specific growth regulators such as 2,4-D. In most of the cases, indirect embryogenesis occurs. In indirect somatic embryogenesis, where it has been induced under *in-vitro* conditions, 2 different media are required.

1. For initiation of embryogenic cells we require induction medium:

The induction medium must contain auxins to initiate somatic embryogenesis; it is removed or reduced to minimum levels in the second medium which supports the subsequent development of these cells into embryoids.

2. Embryoids are generally initiated in callus tissue from the superficial clumps of cells associated with large vacuolated cells that do not take part in embryogenesis.

3. Embryonic cells are characterized by dense cytoplasmic contents, large starch grains and a relatively large nucleus with a darkly stained nucleolus.

Each developing embryoid passes through 3 sequential stages of embryo development:

Globular, Heart-shaped stage and Torpedo stage. Torpedo stage is a dipolar structure which ultimately gives rise to a complete plantlet.

### **Factors affecting Somatic Embryogenesis**

1. **Auxins:** Presence of auxins in the growth medium is generally essential for embryo initiation tissues or calli maintained continuously in an auxin free medium, generally do not form embryos, therefore somatic embryogenesis is achieved in 2 steps:
  - (i) Callus is initiated and multiplied on a medium rich in auxin. For eg. 2,4-D in a concentration of 0.5 mg or lit which induces the differentiation of localised groups of meristematic cells known embryogenic plants. This medium is known proliferation medium.
  - (ii) Embryogenic clumps developed into mature embryos when transferred to a medium with a low level of auxin (0.01 to 0.1 mg or lit.) or no auxins at all. This medium is c or a embryo development medium.
2. **Cytokinins:** Gaiatin stimulate somatic embryogenesis when cells are cultured in an auxin free medium but the same process is inhibited by addition of either kinectic or benzyl amino purine. The inhibitor effect may be due to the selective stimulation of cell division of non-embryogenic cells of culture.
3. **Reduced nitrogen:** The substantial amount of nitrogen usually in reduced form is required for both embryo initiation and maturation. It is convenient to use ammonium in combination with nitrate ( $\text{NH}_4^+ - \text{NO}_3^-$ ). eg. In carrot, addition of  $\text{NH}_4\text{Cl}$  to the embryogenic medium already contains  $\text{KNO}_3$  produces near optimal number of embryos. Other sources of nitrogen can also be used. eg. Coconut milk, casein hydrolysate,  $\alpha$ -glutamine,  $\lambda$ -alanine or mixture of these can be used as an alternative.
4. **Other constituents:** An essential feature for embryogenesis in carrot is presence of high concentration of K that is 20 millimole in medium. Amount of dissolved  $\text{O}_2$  in the medium is critical and should be as low as

1.5 ml gm or lit. A low amount of dissolved O<sub>2</sub> results in synthesis of higher levels of ATP while higher amount of dissolved O<sub>2</sub> favours direct rooting.

Activated charcoal also improves embryogenesis as it absorbs a wide variety of inhibitory substances as well as hormones.

**Importance of Somatic Embryogenesis:** Potential application of somatic embryogenesis and organogenesis are more or less similar.

1. The mass production of adventitious embryos in cell culture is still regarded as the ideal propagation system.
2. The indirect pathway involving IEDC's generates a high frequency of somaclonal variation mutations during embryogenesis may give rise to mutant embryo which will form a new strain of the plant species.
3. Synthesis of Artificial Seeds: Somatic embryo has no food sources but suitable nutrients could be packed and embryo enclosed in protective coating to form artificial seeds which can form plantlets when shown directly into fields. These artificial seeds have been proposed as low cost high volume system.
4. Synthesis of metabolites: Oils and pharmaceuticals etc.
5. Conservation of genetic resources
6. Genetic transformation

## 2.5 Synthetic Seeds and their Applications

Artificial seeds are the living seed like structure which are made experimentally by a technique where somatic embryoids derived from plant tissue culture are encapsulated by a hydrogel and such encapsulated embryoids behave like true seeds if grown in soil and can be used as a substitute of natural seeds.

### Method for making Artificial Seeds

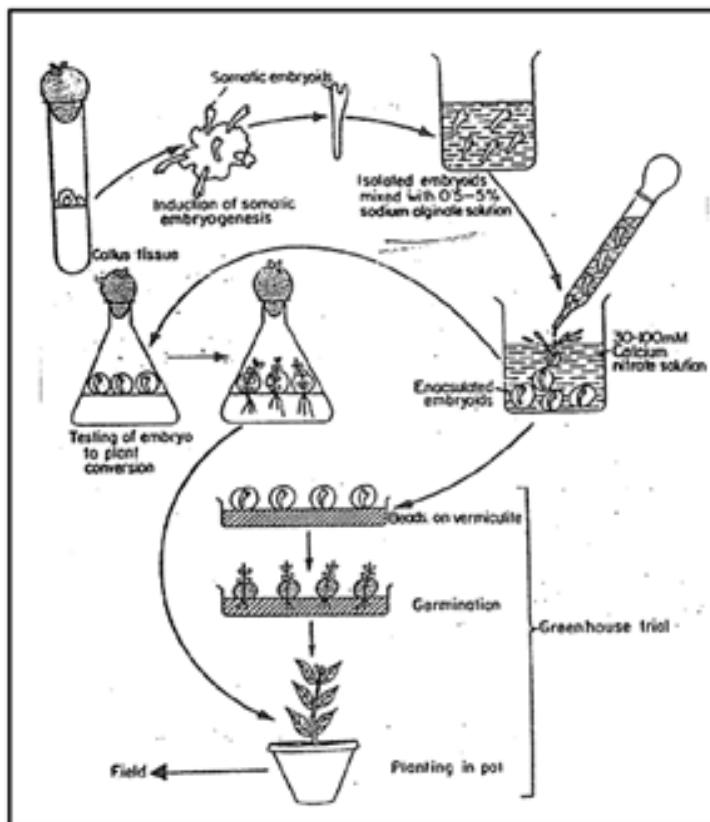
Several steps are followed for making artificial seeds:

- (1) Establishment of callus culture
- (2) Induction of somatic embryogenesis in callus culture
- (3) Maturation of somatic embryos
- (4) Encapsulation of somatic embryos

After encapsulation, the artificial seeds are tested by two steps –

- (1) Test for embryoid to plant conversion
- (2) Green house and field planting.

Establishment of callus culture and the induction of somatic embryogenesis in callus culture have already been discussed in details previously.



**Fig. 2.3 : Flow diagram showing the method for making Artificial Seeds**

### Importance of Artificial Seeds

The potential importance of artificial seeds are more or less similar to that of somatic embryogenesis, still it has some practical applications –

- (1) The seeds are produced in plant at the end of reproductive phase by the process of complex sexual reproduction. A plant may take a long or short time to attain the reproductive phase. So we have to wait upto the end of reproductive phase of a plant for getting seeds. But artificial seeds are available within at least one month. Nobody has to wait for a long time.

- (2) Plants bear the flower and produce the seeds at particular season of a year. But the production of artificial seeds is not time or season dependent. At any time or season, one may get the artificial seeds of a plant.
  - (3) Artificial seeds help to study the role of endosperm and seed coat formation.
  - (4) Somatic embryogenesis has been observed in a great many species to date, which indicates that it may be possible to produce artificial seeds in almost any desired crops.
  - (5) Artificial seeds also protect the meiotically unstable, elite genotypes.
  - (6) Artificial seeds will be applicable for large scale monocultures as well as mixed genotype plantations.
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## **2.6 Summary**

Callus tissue means an unorganized, proliferative mass of cells produced from isolated plant cell, tissues organs. When grown aseptically or artificial nutrient media under cultured conditions. Callus Culture means an unorganised proliferative mass of cells produced from isolated plant cells, tissues or organs when grown on artificial nutrient.

Several tissues are organized together to form an organ, such as leaves, roots, flowers and the vascular system. The process of initiation and development of an organ is called organogenesis. In plant tissue culture, inducing organogenesis is an important way to regenerate plants from the culture.

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply noble plants such as those that have been genetically modified or bred through conventional plant breeding methods.

Somatic embryogenesis is a process where a plant embryo is derived from a single somatic cell or group of somatic cells. Somatic embryos are formed from plant cells that are not normally involved in the development of embryos, i.e. ordinary plant tissue. No endosperm or seed coat is formed around a somatic embryo.

Synthetic seed are the artificial encapsulation of somatic embryo, shoot buds or aggregates of cell or any tissues which has the ability to form a plant in *in-vitro* or

*in-vivo* condition. Synthetic seeds can be stored for a long time in appropriate condition.

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## 2.7 Glossary

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- **Organogenesis:** Type of morphogenesis which result in the formation of different organs like shoot, root.
  - **Micropropagation:** *in-vitro* propagation of plants.
  - **Somatic embryogenesis:** Formation of a bipolar structure containing both shoot and root meristems.
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## 2.8 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Define organogenesis.
2. Define micropropagation.
3. Define explants.
4. Define somatic embryogenesis.

### Section B : (Short Answer Type Questions)

1. What is the difference between differentiation and redifferentiation?
2. Write any two significance of somatic embryogenesis.

### Section C : (Long Answer Type Questions)

1. Write a note on artificial seed.
  2. Write a note on synthetic seeds applications.
- 

## 2.9 References

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- S.S. Bhojwani, Plant tissue culture applications and limitations
- S.S. Bhojwani and S.P. Bhatnagar. The embryology of Angiosperms
- M.K. Razdan, Plant tissue culture
- S. Chand, Rastogi Publication, Elements of Biotechnology.

## Unit-3

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# Protoplast Culture

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### Structure of the Unit

- 3.0 Objectives
- 3.1 Introduction
- 3.2 Protoplast
- 3.3 Isolation of Protoplast
  - 3.3.1 Source of Plant Material
  - 3.3.2 Techniques of Isolation of Protoplast
  - 3.3.3 Osmoticum
- 3.4 Purification of Protoplast
  - 3.4.1 Sedimentation and Washing
  - 3.4.2 Floatation
- 3.5 Protoplast Viability Testing
- 3.6 Culture of Protoplasts
  - 3.6.1 Nutrient media
  - 3.6.2 Methods of Culture
- 3.7 Cell Wall Regeneration
- 3.8 Regeneration of Plant
- 3.9 Applications of Protoplast Culture
- 3.10 Summary
- 3.11 Glossary
- 3.12 Self-Learning Exercise
- 3.13 References

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## 3.0 Objectives

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After going this unit will able to understand about:

- Protoplast
  - Isolation and Purification of Protoplast
  - Protoplast Viability Testing and Culture of Protoplasts
  - Regeneration of Cell Wall and plant
  - Applications of Protoplast Culture
- 

### 3.1 Introduction

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One of the most significant developments in the field of plant tissue culture is the isolation culture and fusion of protoplast. Protoplast are plant cells devoid of cell wall i.e. cytoplasm surrounded by the cell membrane. Hanstein (1880) introduced the term “**Protoplast**” to designate the living matter enclosed by plant cell membrane.

Protoplasts (cell minus cell wall) are the biologically active and most significant material of the cell. When cell wall of a cell is removed mechanically or enzymatically, the isolated protoplast is known as “**naked plant cell**” on which recent researches are based. The potential of protoplast was also observed as a tool to study cellular phenomenon. Cocking (1960) first used enzymes to release protoplasts. Since then, this developed technique of enzymatic digestion of cell walls has been extended as a model system to others.

Successful enzymatic degradation of cell wall and isolation of protoplast led to significant development in the field of somatic cell genetics in higher plants. This was appreciated by cell biologists and E.C. Cocking at Nottingham UK (1960). According to them, cell wall of constituent cells of tomato fruit could be removed effectively by cellulase and protoplast becomes an ideal material for experimental studies.

Rapid progress has taken place in the development and application of plant protoplast technology. Since then protoplasts have become important tool for genetic manipulation through methods like protoplast fusion, electroporation and micro injection. The essential ingredients of this technique include isolation of

protoplasts, culture of protoplasts, introduction of foreign DNA in to protoplasts and somatic hybridization.

Plant genetic transformation, production of somatic hybrid or cybrid and experiments in plant virus infection could also be achieved with isolated protoplast. Cultured Protoplasts can be used not only for somatic cell fusions, but also for taking up foreign DNA, cell organelles, bacteria and virus particles for genetic modification or transformation.

The genetic modification through uptake of foreign DNA involves uptake, integration, replication, expression and transmission. In view of this, isolation and culture of protoplast has become a very important area of research within the realm of plant biotechnology.

Isolated Protoplast can also be induced to divide in culture to form callus. From callus, whole plants can be regenerated either through embryogenesis or organogenesis.

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### 3.2 Protoplast

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Protoplast is the living part of a plant cell, consisting of the cytoplasm and nucleus with the cell wall removed. Protoplast (cell wall minus cell) is the biologically active and most significant material of cells. Cell wall is consists of a complex mixture of cellulose, hemi cellulose, pectin, lignin, lipids and protein. If the cell wall is mechanically or enzymatically removed the isolated protoplast is called "**naked plant cell**". Mostly recent researches are based on isolated protoplast. The role of cell wall is to protect the cytoplasm from microbial invasion and environmental stress. In other words plant cell wall acts as a physical barrier..

For dissolutions of different components of cell wall, cell wall degrading enzymes are used. Cocking 1960 first time isolated protoplasts of plant tissues by using cell wall degrading enzymes viz., cellulase hemicellulase, pectinase and protease. Usually a mixture of pectinase or macerozyme (0.1-1.9%) and cellulase (1-2%) is appropriate for most plant materials. Hemi cellulase is necessary for some tissues.

Protoplasts can be isolated from whole plant organs or tissue cultures. If they are then placed in a suitable nutrient medium, they can be induced to reform a cell wall and divide. A small cluster of cells eventually arises from each cell.

The potential of protoplast as tools to study cellular phenomena was appreciated by cell biologists and E.C. Cocking at Nottingham, UK (1960). They showed that the walls of the constituent cells of the tomato fruit could be removed effectively by cellulose that sufficient material could be generated for experimental purposes. Since then, Protoplasts have become ideal material for genetic manipulations

Although protoplast culture is one way of producing plants in culture, it is not routinely used for micropropagation. Protoplasts are currently used in experiments in plant virus infection, Plant genetic transformation, Production of somatic hybrids or cybrids.

### **3.3 Isolation of Protoplast**

#### **3.3.1 Sources of Plant Material**

The degree of success depends upon nature of starting plant material. Protoplasts have been isolated from various plant parts. Cells suspension and leaf mesophyll cells are most suitable material to obtain viable protoplast. In leaves uniformity of cell component is maintained. The mesophyll cells in leaves are loosely arranged so chemicals used for isolation have an easy access to cells. Protoplast isolation has been seen properly from the plant growing in green houses or in growth controlled room.

In-vitro raised shoots are also used as source material by many workers' (Butenko and Kuch Ko, 1980). Some species of cereal crops show difficulty in isolating culturable protoplast from leaf cells for them alternative source is the suspension culture. Vasil and vasil (1979) used protoplasts isolated from suspension cultures.

The yield of protoplast from cultured cells depends on the growth rate and growth phase of the cells.

#### **3.3.2 Techniques of Isolation of Protoplast**

There are several different method by which protoplast may be isolated:-

- (i) **Mechanically breaking of cell wall:** Conventionally in the past protoplast were isolated by mechanical methods from pieces of sectioned plant material. This method yielded small number of intact protoplast.
- (ii) **Enzymatic digestion method of the cell wall:** Discovery of protoplast isolation through enzymatic digestion method given by E.C. Cocking

abandoned the mechanical method. Protoplasts are obtained from mesophyll cells, callus and cells suspension on treating with enzyme mixture containing cellulase, hemi-cellulase and pectinase. The cells tissues are soaked in a high concentration of enzymes for several hours. The isolation protoplasts is carried out under sterile conditions whether from plants organs or in-vitro culture by means of different protocols suitable for specific materials. The principal care, which is taken, is to ensure removal of cell wall without injury to the released protoplasts.

### **Enzyme used for Protoplast Isolation**

The cellulase and pectinase enzymes are regarded essential to isolate protoplasts from plant cell. The first successfully used commercially available enzyme preparation came from microbes particularly of fungal origin. Macrozyme and Onozuka cellulase have been widely used. Some commonly used commercially available enzymes for protoplast isolation are:

Cellulase R-10	(from <i>Trichoderma virida</i> )
Hemicellulase H-2-125	(from <i>Thizopus</i> )
Macrozyme R – 10	(from <i>Rhizopus</i> )
Pectinase	(from <i>Aspergillus</i> )
Zymolase	(from <i>Arthrobacter luteus</i> )
Driselase	(from <i>Irpex lactes</i> )

Commercially available preparations which are often mixtures of enzymes from a fungal or bacterial source generally contain pectinase, cellulase and hemi-cellulase activity. Some of the commercially available enzymes include:

- Onozuka cellulase (P-1500, R-10) (Kinki)
- Cellulase (Cellulysin) (Calbiochem)
- Driselase (Kyowa)
- Macerozyme R-10 (Pectinase) (Kinki)
- Pectinase (Sigma)
- Rhozyme (HP-150) (Hemi-cellulase)

### Hemicellulase (Sigma)

The choice of enzyme is also important in protoplast isolation as their selection depends on the structural composition of the cell wall.

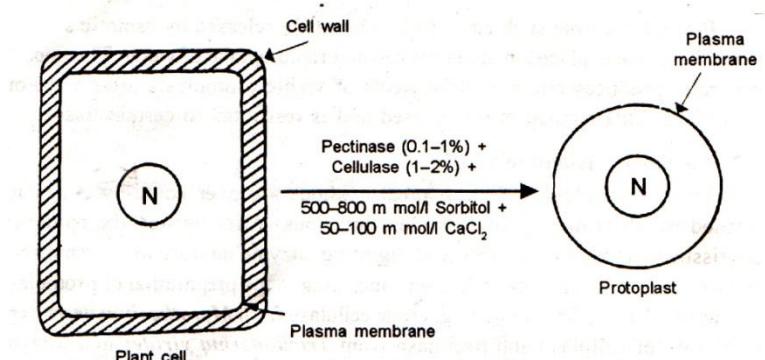
### Enzymatic Treatment

Before using enzymatic mixture for protoplast isolation the chemical nature of cell wall is to be understood. To degrade the complex cell wall a mixture of enzyme should be selected according.

While treating with enzyme solution, the enzyme should penetrate the intercellular spaces of leaf cell. While keeping plant tissues (peeled one) with enzyme solution the tissue should directly be in contact with enzyme mixture. If peels of plant material is not being used then pieces or section of leaf and tissue are placed in the solutions. This is the most common method used for isolation of protoplast.

There is a Vacuum infiltration or seitz filters (.45um) are also used to facilitate the penetration of enzymes in to the tissue.

The use of enzymes on the plant tissue depends on some factors. These factors are



**Fig. 3.1: Protoplast Isolation by Enzyme Treatment with Osmoticum**

Isolation of Protoplasts by enzyme treatment (enzymes have been shown above the arrow) osmoticum (below the arrow) is added to stabilize the protoplast and prevent them from bursting pH of enzyme should be 4 to 5 and 5 to 6

- The concentration of enzyme
- Duration of enzyme treatment

- Optimum temperature for the activity of enzymes is 40-50 °C and for protoplast isolation 25-30 °C temperature is adequate.

### **3.3.3 Osmoticum (Osmotic Stabilizer)**

During isolation, possibility of bursting of isolated Protoplast is a great problem.

To prevent this bursting, addition of osmotic stabilizer in the enzyme solution is advisable. So before attempting to isolate protoplast from a tissue it is advisable to determine the correct level of osmoticum. The freshly isolated protoplast in a proper osmolarity appears spherical. The protoplasts are more stable in a slightly hypertonic rather than isotonic solution. (Fig. 3.1)

The concentration of osmotic stabilizer varies depending upon the plant tissue. Use of mannitol solution (0.45 – 0.8m) as an osmotic stabilizer was appropriate for cultured cells and green house plant. Sorbitol, an isomer of mannitol is also used.

In enzymatic isolation method solution of various sugars or sugar alcohol are used. In case of mechanical isolation method salt solutions ( $\text{CaCl}_2$  – 50 – 10 m  $\text{mol}^{-1}$  or l) were used predominantly as osmotic stabilizer.

## **3.4 Purification of Protoplast**

During the process of isolation of protoplast, the protoplast are not only component present in the incubated enzyme solution but subcellular debris e.g. chloroplast, vascular elements, undigested cells and broken protoplast may be present.

The removal plant debris and segregation of healthy protoplast require purification procedure in centrifuge tube.

### **3.4.1 Sedimentation and Washing**

Crude protoplast suspension is centrifuged at low speed (50-100g or 5min). Protoplasts accumulate as pellet in the tip of the tube. Supernatant is carefully pipette out which contains cells debris. Pellets are then resuspended in fresh culture medium with osmoticum.

### **3.4.2 Floatation**

A concentrated solution of osmotic with enzyme-protoplast mixture in specialized bottles, Babcock bottle is combined and centrifuged at low speed. Protoplasts can be pipette off from the top of the tube.

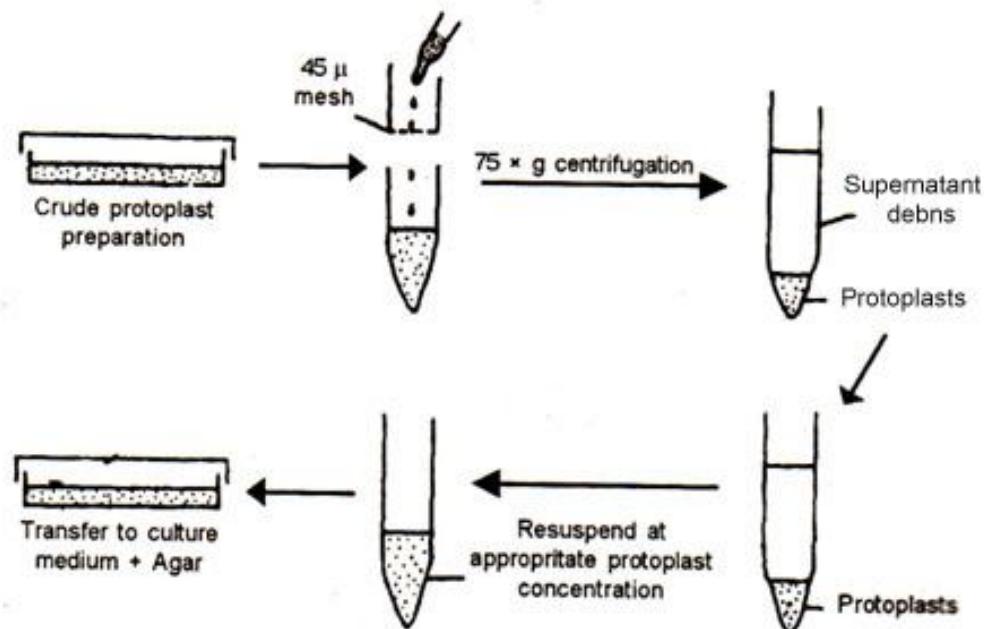


Fig.3.2: Purification of Isolated Protoplasts by Sedimentation and Washing

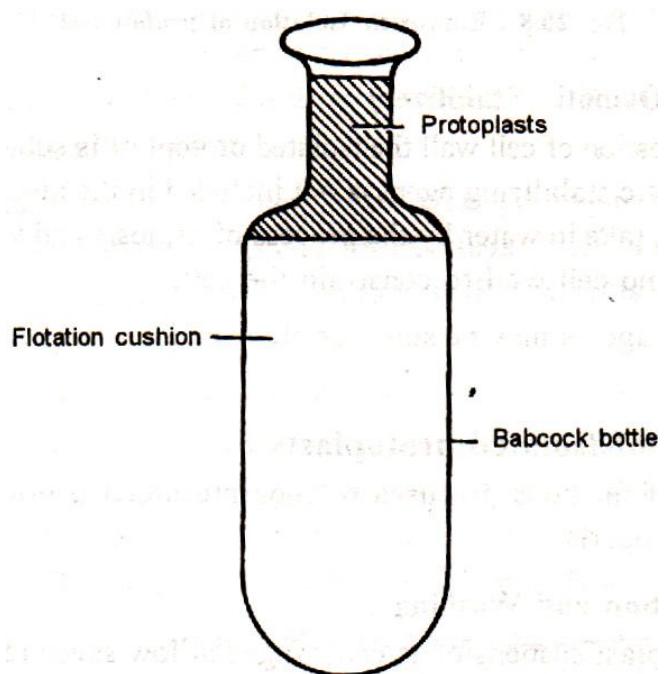
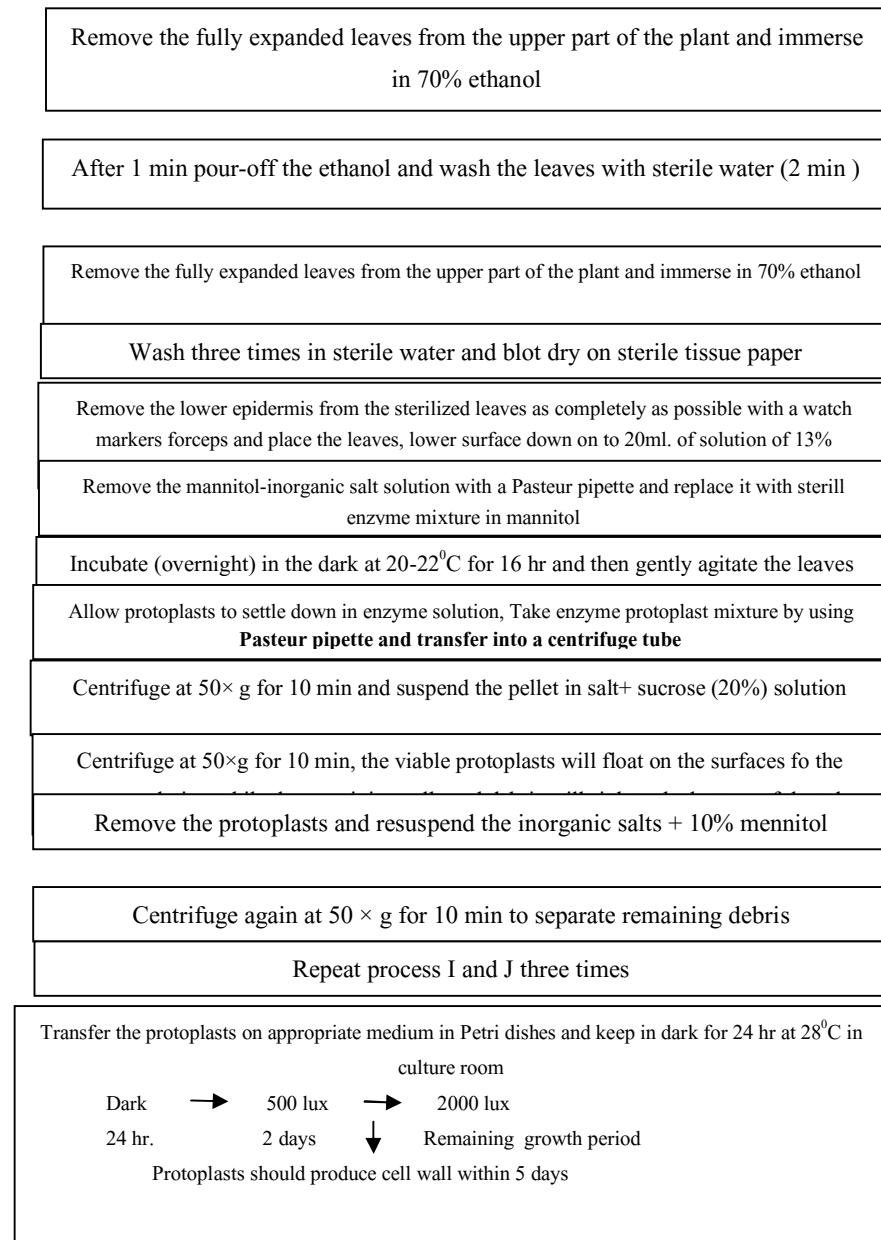


Fig.3.3: Flotation of the protoplasts in a Babcock bottle facilitates the removal of purified protoplast from the sucrose cushion

## Isolation, purification, culture sand regeneration of protoplasts



They yield of protoplasts and their viability depends upon the age, the physiological state of the plant, the purity of enzymes used, the P<sup>H</sup> of the medium and the period of incubation.

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### 3.5 Protoplast Viability Testing

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The most frequently used method for estimation of viability is the use of fluorescein diacetate (FDA) stain. As the FDA accumulates within the plasmamembrane, viable protoplasts appear fluorescent green or white under fluorescence microscope. Another stain commonly used is phenosafranine.

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### 3.6 Culture of Protoplasts

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The protoplasts which are obtained after cleaning have to be suspended in a suitable medium in order to allow them to reform a cell wall and initiate divisions.

#### 3.6.1 Nutrient media

The nutrient requirements of isolated protoplasts are very similar to those of cultured cells and tissues. Since protoplasts lack cell wall, they tend to be very efficient in the uptake of nutrients from the medium. Hence, the nutrient media used for the culture of protoplasts are generally modified to contain reduced levels of inorganic substances. Due to the absence of cell wall, leakage of some metabolites may also take place from protoplasts. This also necessitates modification of the nutrient medium. The Murashige and Skoog's medium is modified by reducing the levels of inorganic nutrients like  $\text{NH}_4^+$  salts which is detrimental to protoplast survival.  $\text{Ca}^{++}$  concentrations are increased 2-4 times as it is important for protoplast membrane stability. Concentrations of Zn and Fe are also lowered. Osmotic stabilizer and plant growth substances are two important ingredients in protoplast culture medium.

Proper osmolarity is achieved through the inclusion of mannitol, sorbitol, sucrose or xylose either alone or in combination. The most commonly used growth substances are synthetic auxins such as 2, 4-D, NAA, IAA and Cytokinins such as BA and kinetin.

#### 3.6.2 Methods of Culture

The methods employed in protoplast culture are the modifications of the methods used for culture of plant cells. The protoplast may be cultured on a suitable medium in a variety of ways:

- (a) **Suspension or Droplet Cultures:** Protoplasts are suspended in a liquid medium at a density of about  $10^5$  or ml either in conical flasks or plastic petridishes. This technique consists in placing approximately 50ml or droplets containing protoplasts previously adjusted to  $1 \times 10^4$  or  $1 \times 10^5$  or ml in plastic petridishes. The petridishes are sealed with parafilm and incubated at  $25^{\circ}\text{C}$  to  $30^{\circ}\text{C}$  at low light intensities or in the dark.
- (b) **Bergman's plating technique:** Protoplasts are suspended in a liquid medium in a petridish at double the concentration that is planned for the experiment and mixed gently but quickly with an equal volume of medium containing double the agar concentration that is used for culture of cells and tissues. The petridishes are with parafilm and incubated upside down in continuous light (1000-2000 lux) at  $23^{\circ}\text{C} - 25^{\circ}\text{C}$ .
- (c) **Microculture Chambers:** This method requires the culturing of 30-50  $\mu\text{l}$  of medium containing one or more protoplasts on a microscope slide which is enclosed by a cover glass resting on two other cover glasses placed on either side or the drop. The cultures are sealed with sterile paraffin oil and incubated in light at  $25^{\circ}\text{C}$ .
- (d) **Feeder layers and Nurse Cultures:** Non-dividing but metabolically active, X-irradiated protoplasts embedded in nutrient agar support the growth of protoplasts pated at very low densities (5-50 protoplasts or ml) above them. Nurse cultures are also used where the fast growing protoplasts aid the recalcitrant (slow growing) species.
- (e) **Micro-drop Array Technique:** This method consists of hanging droplets of 40 ml representing one combination of regeneration factors to be tested. The droplets are arranged in a regular array of  $7 \times 7$  drops on the lid of 9 or 10 cm petridish.

Generally it has been observed that MS (Muraschige and skoog 1962) and B5 media and their modification are used for Protoplast culture. The media are supplemented with a suitable osmoticum and an auxin and cytokinin. Types of auxin and cytokinin depend on plant species. After 7-10 days of culture protoplast regenerate cell wall. The macroscopic colonies are transferred on to normal tissue

culture media. Protoplasts are very sensitive to light therefore they are cultured in diffuse light or dark for the first 4-7 days.

### 3.7 Cell Wall Regeneration

Protoplasts which are cultured in an appropriate medium show rapid cytoplasmic streaming respiration, synthesis of RNA, protein and polysaccharides, increase in size, formation of numerous cytoplasmic; and most of the cell organelles particularly the chloroplasts aggregate conspicuously around the nucleus.

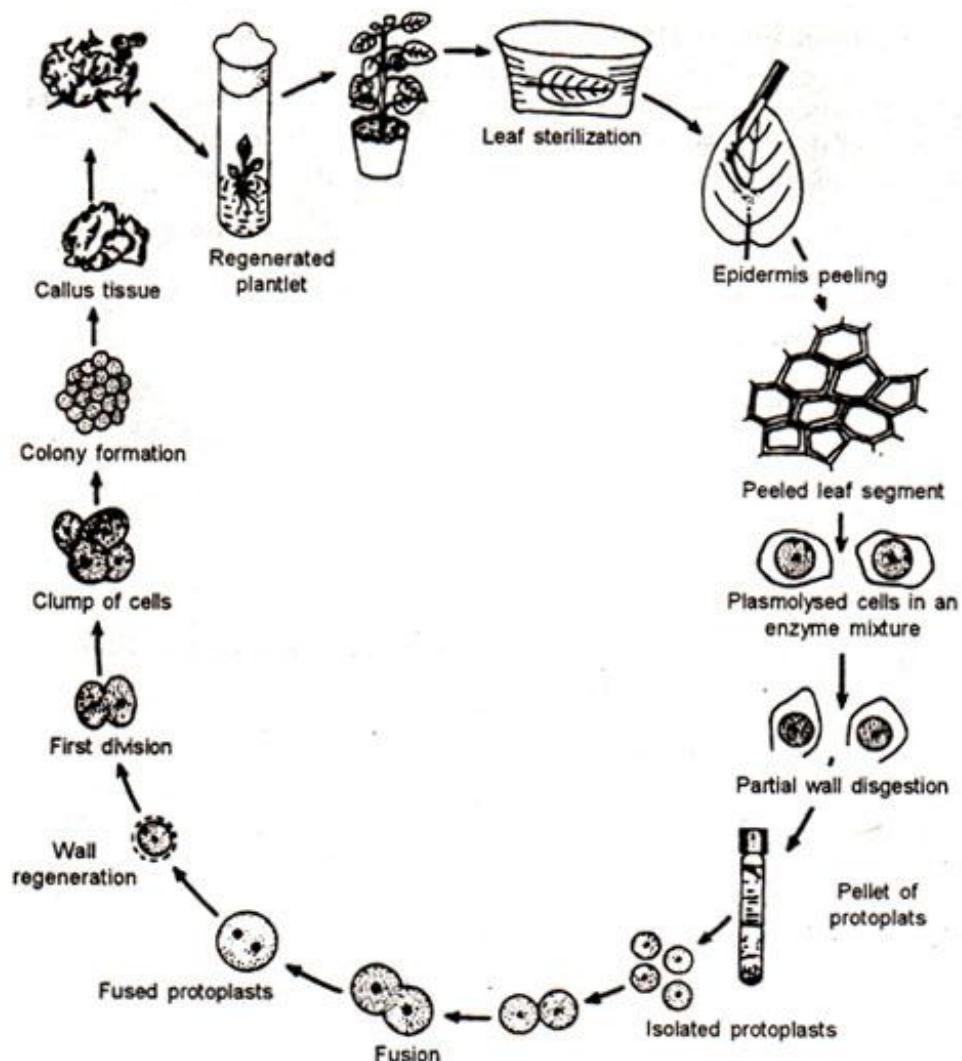


Fig.3.4: Isolation Culture and Fusion of Leaf cell Protoplasts

Cell wall formation starts within 24 to 48 hours of culturing protoplasts. Cell wall regeneration takes place by the deposition of cellulose microfibrils on the surface of the plasma membrane. The newly deposited cell wall is composed of loosely organized cellulose microfibrils, which later become more organized to form a typical plant cell wall.

The first cell division after the formation of new cell wall usually occurs between 2-7 days after the culture. Successive sustained cell division of the daughter cells leads to the formation of multicellular colonies after one to three weeks of culture, which can be transferred to an appropriate nutrient media for further growth and multiplication of the protoplast-derived calli.

### **3.8 Regenerations of Plant**

The regeneration of protoplast-derived calli into whole plants is either through organogenesis or through embryogenesis. In general, organogenesis consists of the transferring protoplast-derived callus to cytokinin free medium for the induction of shoots and then to root inducing medium.

The conditions for isolation and culture of protoplasts and regeneration of complete plants have been standardized for a large number of plant species. Successful plant regeneration has been achieved in protoplast for example: *Cucumis sativus*, *capsicum annuum*, *Beta vulgaris*, *chrysanthemum spp.*, *Rosa spp.*, *Beta vulgaris* are few plant species.

The naked protoplasts isolated from different plants cells have been used by plant breeders for various genetic manipulations. But protoplast regeneration in cereals is still difficult.

The isolated protoplasts are used for various purposes like:

- (i) Bio-chemical and metabolic studies.
- (ii) Fusion of two different somatic cells to get somatic hybrids.
- (iii) Fusion of nucleated (containing nucleus) and enucleated (without nucleus) cells to produce cybrid (cytoplasmic hybrid)
- (iv) Genetic manipulation.
- (v) Drug sensitivity

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### **3.9 Applications of Protoplast Culture**

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The fundamental properties of plant cell have become established on the basis of isolated protoplast studies. The physioloical activity with totipotent nature made this study wide open in many field of biological sciences. Its application in somatic hybridization and genetic engineering is the greatest contribution so far known today. The importance and application of protoplast culture are:

#### **1. Cell wall formation**

During cell wall formation, the nature of deposition of cellulosic microfibril and their orientation on the protoplast surface has provided detail information in cell biology research.

#### **2. Isolation technique**

Like isolated protoplast technique, the isolation of different cell organelle like chloroplast, mitochondria even bacteriods from root nodule became convenient. This method ensures freedom of the worker to isolate and study the biological importance of the organelle. This technique provides clues for the isolation of chromosomes and its introduction for some other major studies of chromosomes research.

#### **3. Viral study**

From virus infected plant tissue virus can be isolated and its method of infection and maturation inside the host tissue can also be traced clearly at molecular and cellular level by the study of cultured isolated protoplast. It is interesting to note that the intensity of viral infection can be checked by the study.

#### **4. Photosynthetic study**

The biochemical aspects of photosynthesis study has opened a new vista in plant; science by the introduction of protoplast culture study. This study is used in various ways to unlock the mystery of photosynthesis mechanism.

#### **5. Gene transfer study**

The introduction of exogenous DNA into experimenting protoplast brings some genetic modification and has the chance to produce new type plant. The genetic diversity study can be achieved by this study using the DNA virus as vector. It may

be possible to insert foreign DNA into the viral genome. So DNA virus is suitable vectors for gene transfer.

The genetic manipulation of plant protoplast can be studied in detail in pathogenic bacteria (*Agrobacterium tumefaciens*) that cause crown gall. The part responsible for tumour information is Ti plasmid. The autonomous growth of the tumour cell is caused by the integration of a piece of DNA from a Ti plasmid. This naturally transformed system of Ti plasmid DNA can be used in experiment to transform plant protoplasts into hormone independent cells. This is genetic engineering plasmid technology. And with the similar approach the 'nif' gene can also be transferred from leguminous plant to non leguminous plant thus giving the plant a self-fertilizing properties.

## **6. Transplantation study**

Incorporation of bacteria, isolated nucleus, yeast etc. into protoplast is the basic step of studying endosymbiotic association with higher plants, nuclear cytoplasmic interaction with foreign and genetic information respectively. The introduced microorganism may beneficial effect. Their association may also exert some effect on the protoplast which may be used in other types of study. The nuclear transplantation establishes different hybrid variety of cultivated crops.

## **7. Hormone study**

The protoplast culture is influenced by different types of growth hormone. Cell division, callus formation and increase of cell permeability are the effect of hormone which helps to understand the biological activity of the protoplast. The site of auxin action can be ascertained by this study.

## **8. Study of morphogenesis**

The protoplast culture involves cell wall synthesis, regeneration and formation of a new plant that helps in study of morphogenesis species.

## **9. Somatic hybridization**

The possibility of protoplast fusion between different species has led the formation of hybrid plant. This plant has genome of any one partner and cytoplasm of both. Thus without sexual union there is potentialities in formation of new types of plant which are used in crop improvement programme.

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### 3.10 Summary

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Plant protoplasts are cells without cell wall. Protoplasts are naked cells which are potentially capable of cell wall regeneration, growth and division.

The absence of cell wall makes the protoplast suitable for a variety of experimental manipulations which are not possible with intact cells. Plant protoplasts have a great potential in securing genetic recombinations through somatic hybridization in sexually incompatible crosses. Protoplasts are most useful for plant cell manipulations.

Plant modification studies involve the uptake of DNA and organelles and uptake of single cells of bacteria and algae into protoplasts or selective transfer of beneficial genes or gene in to protoplast. Protoplast culture helps in crop improvement by somatic hybridization and cell modification. The protoplast in culture can be regenerated into an entire plant.

The technique in future will be one of the most frequently used research tools for tissue culturists, physiologists, pathologists, molecular biologists, cytogeneticists and biotechnologists.

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### 3.11 Glossary

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- **Protoplast** : Plant cell whose cell wall has been removed and cell take a shape of spherical shape or cell devoid of cell wall is called “**protoplast**”.
- **Osmoticum** : Reagents that increase the osmotic pressure of a liquid .
- **Fusogenic agent**: An agent that triggers protoplast fusion is called “**fusogenic**” agent or fusion inducing agent.
- **Visual Marker**. A strategy used for selection of hybrid protoplast. eg. pigmentation .
- **Cybrid**: Acytoplasmically hybrid cell with organelle from both parent cell sources obtained by fusion of cytoplasm of both parent cell and a nucleus of only one cell. Nucleus of other cell denatured.
- **Protoplast fusion** : When two protoplast gets fused with each other process is called “**protoplast fusion**”.

- **Electrofusion Technique :** This technique is a low voltage ( $65-80 \text{ V cm}^{-1}$ ) electric current pulses to align the protoplast in a row and they get fused by a very brief pulse of high voltage.
- **Somatic hybridisation :** Production of hybrid plants through fusion of protoplasts of two different plant species or Varieties is called “**Somatic hybridisation**”.
- **Homokaryon.** Fusion product of two or more protoplasts of the same species., or A cell with two or more identical nuclei as a result of fusion.
- **Heterokaryon** Protoplasts from both the parental species.
- **Fusogen** : A fusion inducing agent used for agglutination of protoplast in somatic hybridization.

### 3.12 Self-Learning Exercise

#### Section A : (Very Short Answer Type Questions)

1. Who did isolation of Protoplast for the first time and in which year?
2. What do you mean by “hybrid” or “synkaryocyte”?
3. What is “heterokaryon”?
4. Name three cell wall degrading enzymes which are used in protoplast isolation.
5. Name the osmoticum used during protoplast culture.
6. Mention two uses of isolated protoplasts.
7. Define “Cybrid”.
8. Write full form of P.E.G.

#### Section B : (Short Answer Type Questions)

1. Explain fusogenic chemicals give example also.
2. Describe the whole method of isolation of protoplast from mesophyll cells.
3. Discuss the selection of “**Somatic hybrid cells**”.
4. Write a note on applications of Protoplast culture.
5. Explain “Intra Specific protoplast fusion”.

#### Section C : (Long Answer Type Questions)

1. Describe the method of protoplast fusion and the development of somatic hybrid.

2. Discuss the brief history and detailed account of role of enzymes in protoplast isolation.
3. Write Notes on
  - (i) Somatic hybrid and their use in crop improvement
  - (ii) limitation of Protoplasts culture and in hybridization somatic
4. Give a detailed note on the use of protoplasts in hybridization and Crop improvement giving suitable examples.

**Answer Key of Section -A**

- 1 Cocking 1960
- 2 When as hybrid or synkaryocyte (constable 1978)
- 3 When nuclei of two protoplast do not fuse even after fusion of cytoplasm, the binucleate cells are known as “ hetero karyon or heterocyte”
- 4 Cellulase, Hemi cellulase, Macerozyme
- 5 Sorbitol and manitol.
- 6 (i) To obtain somatic hybrid (ii) Genetic manipulation
- 7 Cybrid a cytoplasmically hybrid cell with organelle from both parental sources (obtained through fusion of cytoplasm) and a nucleus of any one cell, nucleus of the other cell denatured.
- 8 Poly ethylene glycol

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### 3.13 References

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## Unit- 4

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# Somatic Hybridization

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### Structure of the Unit:

- 4.0 Objectives
  - 4.1 Introduction
  - 4.2 Somatic Hybridization (Protoplast Fusion)
  - 4.3 Poly Ethelene Glycol (PEG) Treatment
  - 4.4 Hybrid Selection
  - 4.5 Regeneration of Hybrid Plants
  - 4.6 Applications of Somatic Hybridization
  - 4.7 Problems and Limitations of Somatic Hybrdization
  - 4.8 Summary
  - 4.9 Glossary
  - 4.10 Self -Learning Exercise
  - 4.11 References
- 

## 4.0 Objectives

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After studying this unit you will be able to understand about:

- somatic hybrdization.
  - PEG (Poly Ethelene Glycol) Treatment
  - Applications, Problems and Limitations of Somatic Hybrdization
- 

## 4.1 Introduction

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Somatic hybridization or protoplast fusion is the most important aspect of biotechnology. **Production of hybrid plants through the fusion of protoplasts of two different plant species or varieties is called somatic hybridization such hybrid is called somatic hybrid.** One of the major advantages of tissue culture technique is protoplast fusion and somatic hybridization. The technique is

particularly used for hybridization between two different species and different genera where crosses are not possible by conventional method i.e. sexual reproduction.

Protoplast fusion has been suggested as a means of developing unique hybrid plants which cannot be produced by conventional sexual reproduction.

The somatic hybridization or Para sexual hybridization in plants has now become more popular and useful because hybrid cells of plants can be easily regenerated to whole plants due to totipotency. Somatic hybridization involves following four steps:

- (i) Isolation of protoplasts
- (ii) Fusion of protoplasts of desired varieties or species
- (iii) Selection of somatic hybrid cells
- (iv) Culture of hybrid cells and regeneration of hybrid plant from them.

A number of strategies have been used to induce fusion between protoplasts of different strain or species. Three fusion techniques have been proved relatively more successful. They are as follows:

- (i) High PH high  $\text{Ca}^{++}$  treatment
- (ii) Poly Ethylene Glycol (PEG) induced protoplast fusion
- (iii) Electro-fusion technique.

Best example of the use of protoplasts to improve crop production is that of Nicotiana where the somatic hybrid products of a chemical fusion of protoplasts have been used to modify the alkaloid and disease resistant traits of commercial tobacco cultivars.

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## **4.2 Somatic Hybridization (Protoplast Fusion)**

Protoplast Fusion or Somatic hybridization technique is the most important aspect of biotechnology. This technique is used for hybridization between two species or genera, where crosses are not possible by conventional method. i.e. sexual reproduction. Somatic hybridization (Parasexual- hybridization) in plants has become more popular and useful because hybrid cells of plants can be easily

regenerated to whole plants (due to totipotency). Protoplast fusion may occur by two ways

**(i) Spontaneous fusion (ii) Induced fusion**

**(i) Spontaneous fusion:** Spontaneous fusion generally occurs during isolation of protoplast by enzymatic method. At the time of enzymatic treatment, the dissolution of cell wall and liberation of protoplasts provide opportunity for spontaneous protoplast fusion. This fusion generally occurs when protoplasts are isolated from actively dividing cells or young leaves.

This fusion results in results in to “Homokaryons” or Homo-karyocytes. . Spontaneous fusion is strictly intraspecies.

**(ii) Induced fusion:** Induced fusion may be induced by physical or chemical method. Such fusion occurs when fusing protoplasts belongs to different species or genera or from other sources. This fusion requires ‘fusogen’ (suitable chemical or physical agent).

Fusion of protoplasts of genetically different lines or species which show physical or chemical compatibility in normal sexual crosses is called **Somatic hybridization**.

Somatic-hybridization of crop plants represents a new challenge to plant breeding and crop improvement. Somatic-hybridization represents that, it is possible to recover fertile and stable amphidiploid somatic hybrids after protoplast fusion.

In induced fusion, many chemicals are required e.g. sodium nitrates, polyvinyl alcohol, and dextrin or polyethylene glycol.

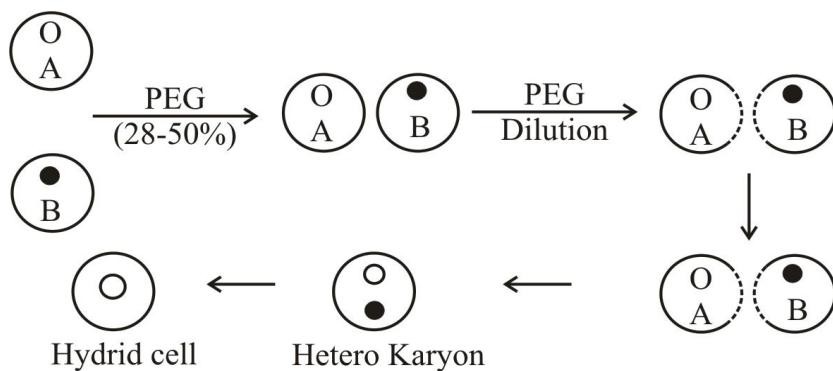
### **4.3 (Poly Ethylene Glycol (PEG) Treatment**

This PEG treatment is most commonly used because as it induces reproducible high frequency fusion with low toxicity to most cell types. This PEG treatment is given by following methods

**(i) Direct Method:** When isolated protoplasts are available in sufficient amount. One ml. of culture medium with suspended protoplast is mixed to 1 ml of 56% PEG solution in a test tube for 15-30 minutes. Tube is shaken for few seconds (4-5 second). It is followed by gradual washing of the protoplasts to remove PEG,

protoplast fusion occurs during washing and then Protoplast are allowed to settle for 10 minutes. The washing medium is alkaline (PH 9-10) and contains a high  $\text{Ca}^{+2}$  ion concentration ( $50\text{m mol l}^{-1}$ ).

The sediment is washed with growth medium and examined for fusion of protoplast. This approach is a combination of PEG and high PH-high  $\text{Ca}^{++}$  treatments and is considered more effective. PEG is negatively charged and may bind to cations like  $\text{Ca}^{++}$  which in turn may bind to the negatively charged molecules present in plasmalemma. During the washing process, PEG molecules pull out the plasmalemma; components bound to them which would disturb plasmalemma organization and may lead to the fusion of protoplasts close to each other.



**Fig.4.1: PEG induced Protoplast Fusion**

- (ii) **Drop culture technique:** Drop culture technique is used when the protoplasts are available in micro quantities. In this method, the protoplasts belonging to two distant species are mixed in equal amounts. Micro drops (4-6) of this mixture are placed in small petri-dishes and allowed for some time to settle at room temperature. 2-3 micro drops of PEG solution are added from periphery. This results in to fusion of protoplast which is finally washed and replaced to culture medium for regeneration.
- (iii) **Treatment with calcium ions ( $\text{Ca}^{++}$ ) at high PH:** In this method, isolated protoplasts are mixed in a solution containing  $0.05\text{ M CaCl}_2 \cdot 2\text{H}_2\text{O}$  in  $0.4\text{ M}$  mannitol at PH 10.5 and centrifuged for about 30 minutes. After centrifugation the tubes are kept in a water bath at  $37^\circ\text{ C}$  for 40-50 % minutes. This results into fusion of 20- 50 % protoplasts.

- (iv) **Sodium nitrate treatment:** In this method, isolated protoplasts are suspended in an inducing mixture containing 5.5% Sodium nitrate on 10% sucrose solution and placed on water bath maintained at 35°C. Then mixture is centrifuged and the pellet is re-suspended in the inducing mixture. After this, mixture is replaced by a liquid medium and the protoplasts are incubated again. This process is repeated twice or thrice, and then the protoplast is plated on solid medium.
- (v) **Electro fusion technique:** It is a selective and less drastic approach. In this technique low, voltage electric current pulses are flown to align the protoplasts in a single row like a pearl chain. These aligned protoplasts are moved with a micro manipulator and pairs of protoplasts may be isolated in individual micro electro fusion chambers. The pair of protoplasts are fused by a very brief (few micro seconds) pulse of higher voltage ( $500\text{--}1000\text{ V cm}^{-1}$ ). High voltage creates transient disturbances in the organization of plasmalemma which leads to the fusion of neighbouring protoplasts. The entire operation is carried out manually in electroporator.

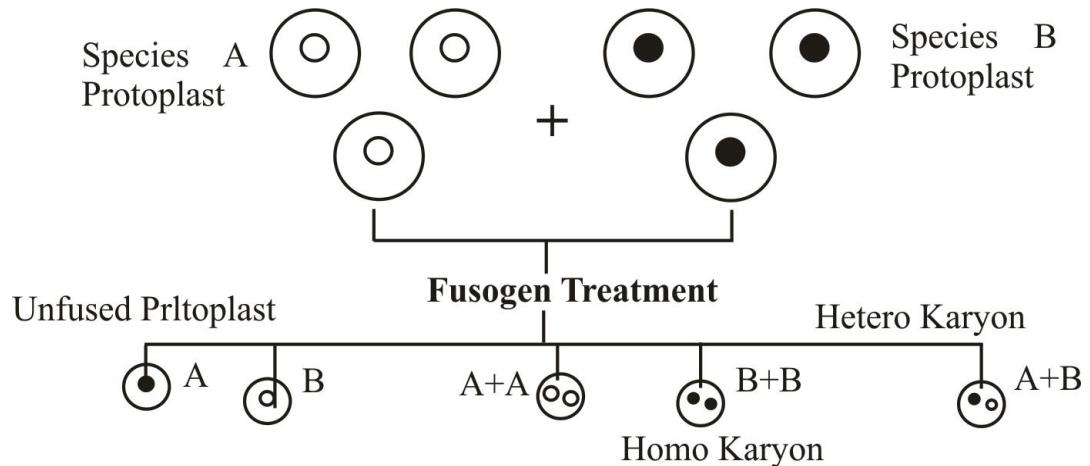
#### 4.4 Hybrid Selection

After treatment with fusogen (fusion inducing agent), Protoplast suspension contains following type of cells:

- (i) Unfused Protoplast of two species
- (ii) **Homokaryons:** Products of fusion between two or more protoplasts of two same species.
- (iii) **Hybrid:** Protoplasts produced by fusion between one or more protoplasts of each of the two different species

In somatic hybridization experiments, the heterokaryotic or hybrid protoplasts resulting from fusion between one protoplast of each of the two spp. are of interest but they form a very small amount of the population (5-10%)

So the effective strategy has to be employed for the identification and isolation. This step is called **selection of hybrid cells**. Selection of hybrid cell is most critical step in the whole phenomenon and it is still an active area of investigation.



**Fig. 4.2: Protoplast Fusion**

There are various selection methods for the selection of fusion products. Selection methods are dependent on:

- Physical properties of fused cells
- Biological properties of fused cells
- Biological properties of colonies formed from fused cells

Somatic hybrids cannot be identified so biochemical markers are required. A number of strategies have been used for the selection of used hybrid protoplasts.

- Visual markers:** Pigmentation of parental protoplasts is used for the identification of hybrid cells under microscope. They are mechanically isolated and cultured.
- Deficiencies or other property of parental species:** Deficiencies are not expressed in the hybrid cells due to complementation between their genetic systems. The properties may be sensitivity to culture medium constituents temperature, anti-metabolites and inability to produce essential biochemicals (auxo-trophic mutants) etc.

These properties may be naturally present in the parental species e.g. one parent is chlorophyll-deficient (Non-green) chloroplasts with the protoplasts of other parents. This helps in visual selection of heterokaryon under microscope.

The selection strategy exploits those natural properties of the two parental species which show complementation in the hybrid cell. Genetic engineering has been used to transfer resistance to an antibiotic or herbicide in one fusion parent and that to another one in to the other parent. The hybrid cells are selected using a medium containing both the concerned antibiotics and herbicide.

- (iii) This is most widely accepted strategy. In this strategy, the entire protoplast population is cultured without applying any selection for the hybrid cells. In this case identification can be done on the basis of callus morphology, chromosome constitution, protein and enzyme banding patterns.

Successful somatic hybridization has been done in many crop plants. First somatic hybrids were obtained by somatic hybridization between *Nicotiana glauca* and *N. langsdorffii* (Carlson et al 1972).

Somatic Hybrids have been found between the species of Brassica, Petunia, and Solanum. **Pomato** is an example of intergeneric somatic hybridization between **Potato and Tomato**.

However somatic hybridization is more useful in agriculture if the protoplast (cytoplasm and nucleus of one parent) is allowed to fuse with the cytoplasm of another parent.

## 4.5 Regeneration of Hybrid Plants

Plant regeneration is a pre-requisite for crop improvement. Hybrid plants should be partially fertile and essentially have a useful property to be of any use in breeding schemes and they should be fertile.

## 4.6 Applications of Somatic Hybridization

Through Somatic hybridization many characters can be introduced in another plant. Kuchko (1985) obtained somatic hybrid of wild and cultivated potatoes (*S. tuberosum* and *S. Chacoense*) and succeeded in the induction of organogenesis. The somatic hybrid plant inherited many characters viz, stomata, intermediate leaf morphology prolonged flowering, large fertile pollen grains, high yield etc.

Through Somatic hybridization, production of novel interspecific and intergeneric crosses between plants is possible which are difficult to hybridize conventionally. Somatic hybrids over comes sexual incompatibility barriers. Melcher's and co-workers (1978) created pomato by creating fusion between protoplasts of *Lycopersicon esculentum* (Tomato) and *Solanum tuberosum* (Potato). A symmetric hybrid is also achieved when there is a partial hybridization. These asymmetric hybrids have abnormal or wide variation in chromosome number than the total of two species.

Attempts to overcome conventional breeding barriers by interspecific fusion of rice with 4 wild species: *Oryza brachyantha*, *Oryza Cichingeri*, *Oryza officinalis* and *Oryza Perrieri* were more successful. Mature plants with viable pollen could be obtained in some species.

In Graminaceous crops also fusion technology has been successful in production of fertile inter specific diploid Rice hybrid plant.

**Gene transfer through Somatic Hybridization:-**

**Disease Resistance:-** Many disease resistant genes viz Potato leaf roll virus, leaf blight, *Verticillium phytophthora*, have been transferred to *Solanum tuberosum* from other species where normal crossing would not be possible due to taxonomic and other barrier. Efforts have been made to introduce tolerance in *Brassica napus* against alternaria (Primard *et al.* 1988).

Resistance has been introduced in tomato against various diseases like TMV, spotted wilt virus, insect pest and cold tolerance.

**Abiotic Stress Resistance:** In Fabaceae, Brassicaceae, Poaceae, Solnaceae, work related to abiotic stress resistance has been done.

Somatic hybridization have been developed by fusing cultivated Potatoes (*S. tuberosum*) and wild relative (*S. acaule*) possessing several disease and early frost resistance character.

**Production of autotetraploids** Somatic hybridization has been used to obtain tetraploids. Somatic hybridization can be used to produce fertile diploids and polyploids. Somatic hybridization is possible only in plants that are still in the juvenile phase.

Somatic cell fusion is useful in the study of cytoplasmic genes and their activities. This information can be applied in plant breeding experiments.

Through, somatic hybridization there is a production of unique nuclear cytoplasmic combinations. Unique combinations using protoplasts will add the development of a novel germ plasm not obtainable by conventional methods.

Protoplast fusion in fungi is the improvement of strains to be used for commercial purpose. By this method, compatibility barrier can be overcome between several species.

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## 4.7 Problems and Limitations of Somatic Hybridization

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- (i) Efficient plant regeneration from somatic hybrid is essential but it is limited up to few species.
  - (ii) Efficient selection method for fused product is also a great problem.
  - (iii) The development of chimeric calluses in place of hybrid is a great problem during somatic hybridization. Formation of amphidiploids from somatic hybridization of two diploids is not favourable except tetraploids. So fusion between two haploid protoplasts is recommended. Genetic stability during protoplast culture is poor.
  - (iv) Regeneration products after somatic hybridization are often variable due to somaclonal variation, chromosome elimination, translocation and organelle segregation.
  - (v) Expression of particular characteristic is not certain after somatic hybridization.
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## 4.8 Summary

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Somatic hybridization is the most important aspect of plant biotechnology. This technique is particularly used for hybridization between two different species or two different genera which cannot be made to cross by conventional method of sexual hybridization. Through somatic hybridization, it has been possible to transfer useful genes e.g. disease resistant genes, nif gene and rapid growth genes from one species to another to widen the genetic base for plant breeding. By somatic hybridization, production of cybrid help in transfer of cyto-plasmic genetic

information from one plant to another as few characters are carried in cytoplasmic inheritance instead of nuclear genes e.g. cyto-plasmic male sterility, susceptibility, resistance to some of the pathotoxins and drugs. Information of cybrids can be helpful in plant breeding experiment.

Somatic hybridization is more useful in agriculture if the protoplast of one parent is allowed to fuse with the cytoplasm of another parent.

## 4.9 Glossary

- **Somatic hybridization:** Technique of hybrid production through the fusion of somatic cells from two genetically different plants.
- **Hetrokaryon:** A cell in which two or more nuclei of unlike genetic make up are present.
- **Homokaryon:** A Cell in which two or more nuclei of same genetic make up is present.
- **Protoplast:** A plasmalemma bound vesicle consisting of a naked cell formed as a consequence of the removal of cell wall by mechanical or enzymatic means.
- **PEG:** Poly Ethylene Glycol.
- **Fusogenic agent:** An agent that triggers protoplast fusion is called fusogenic agent or fusion inducing agent.

## 4.10 Self -Learning Exercise

### Section A : (Very Short Answer Type Questions)

1. “Protoplast fusion” what does it mean?
2. Mention three steps involved in Somatic Hybridization.
3. Mention three strategies which have been considered successful for Protoplast fusion.
4. What is “Fusogen”?
5. Name 2 methods of protoplast isolation.
6. Induced fusion by  $\text{NaNO}_3$  was reported by whom for first time and in which year?

7. Name three important “fusogen”?
8. Who obtained “Pomato”? Mention the year and the name of parent Protoplast?

**Section B : (Short Answer Type Questions)**

1. Name six application of Protoplast culture
2. Out line the different steps of Protoplast isolation.
3. Explain “symmetric hybrids”
4. Explain Protoplast fusion Product “hybrid” and “cybrid”
5. Discuss the method of selection of somatic hybrid cells in brief.

**Section C : (Long Answer Type Questions)**

1. Discuss following
  - (i) Intraspecific Protoplast fusion.
  - (ii) Inter specific Protoplast fusion.
2. What is the role of somatic hybridization and crop improvement?
10. Discuss in detail, illustrate potential of somatic hybridization.
4. Describe the full method of somatic hybridization.
5. Discuss the problems and limitations of somatic hybridization phenomenon.

**Answer key of section-A**

1. Mixing of Protoplasts of different genome.
2. (i) Fusion of protoplast (ii) selection of hybrid cells (iii) identification of hybrid plant.
3. (i) PEG induced fusion (ii) Electro fusion (iii) High  $\text{Ca}^{++}$ , High PH treatment.
4. “Fusogen” is a fusing agent.
5. (i) Mechanical method (by injuring cells of tissue or leaves)  
(ii) Cell wall degrading Enzyme mixture.
6. Power et al 1970.

7. Con Cavalin A, Polyvinyl alcohol, PEG, NaNo<sub>3</sub>, Lysozyme, PEG, antibodies etc. (any three).
  8. Melchers et al (1978) *Lycopersicon esculentum* and *Solanum tuberosum*.
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## 4.11 References

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## Unit-5

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### Haploid Culture:

### Anther, Pollen and Ovule Culture

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#### Structure of the Unit:

- 5.0 Objectives
- 5.1 Introduction
- 5.2 Haploids
- 5.3 Androgenic Haploid Production
- 5.4 Induction and Procedure of Anther Culture
- 5.5 Microspore Culture (Pollen Culture)
- 5.6 Induction of Androgenesis
- 5.7 Process of Androgenesis
- 5.8 Identification of Haploids
- 5.9 Diploidization
- 5.10 Factor Affecting Androgenesis
- 5.11 Application of Anther and Microspors Culture
- 5.12 Merits & Demerits
- 5.13 Limitations
- 5.14 Gynogenic Haploids
- 5.15 Factors Affecting Gynogenesis
- 5.16 Applications of Ovule culture
- 5.17 Summary
- 5.18 Glossary
- 5.19 Self-Learning Exercise
- 5.20 References

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## 5.0 Objectives

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After studying this unit we understand about -

- haploid Culture, pollen and ovule culture.
  - applications, merits and limitation of haploids.
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### 5.1 Introduction

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The value of haploids in genetics and plant breeding has been known for a long time. Haploids are defined as sporophytes with gametophytic chromosome number. Haploid plants have a single set of chromosome that in turn may be useful for the improvement of many crop plants (Sunder, 1979).

Guha & Maheswari (1964) made a remarkable discovery by culturing pollen grains of an angiospermic plant *Datura inoxia* on the nutrient agar medium and also developed torpedo shaped embryoids that metamorphosed into plantlets through the process. Haploid materials remain unaffected from the problems of dominance or segregation. It is feasible to uncover desirable recessive traits in haploid and can develop homozygous diploid pure lines in a single step. So produced homozygous lines can be used in creating desirable recombinants. Haploids have considerable potential in plant breeding and also for production of homozygous plants and in their studies on the detection of mutation.

Haploid flowering plants simplify the product identification and selection of mutants which can be of great help in applied and fundamental genetics.

Anther and pollen culture has become standard tool for plant breeders e.g. pollen derived plants have been incorporated in the breeding programs for many crops e.g. Tobacco, Potato, Oil, Rape Seed, Rice and Wheat.

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### 5.2 Haploids

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The term “**haploid**” refers to those plants which possess a gametophytic number of chromosomes (single set) in their sporophytes. The interest in haploids is due to their considerable potential in plant breeding, mainly for the production of homozygous plants and the studies of the detection of mutations.

The significance of haploids in the fields of genetics and plant breeding has been realized for a long time. However, their exploitation remained restricted due to low frequency in nature. Spontaneous production of haploids usually occurs through the process of apomixes or parthenogenesis (embryo development from an unfertilized egg). Artificial production of haploids was attempted through distant hybridization, delayed pollination, application of irradiated pollen, hormone treatment and temperature shock. However, none of these methods were dependable and repeatable.

It was in 1964 that Guha and Maheshwari reported the direct development of embryos and plantlets from microspores of *Datura innoxia* by the culture of excised anthers. Bourgin and Nitsch (1967) obtained complete haploid plants of *Nicotiana tabacum*. Since then, anthers containing immature pollen have been successfully cultured for important species.

Haploids may be grouped into two broad categories: (a) monoploids (monohaploids), which possess half the number of chromosomes from a diploid speceis, e.g. maize, barley; and (b) polyhaploids, which possess half the number of chromosomes (gametophytic set) from a polyploidy speceis, e.g. potato, wheat.

Here, the general term haploid is applied to any plant originating from a sporophyte ( $2n$ ) and containing ( $n$ ) number of chromosomes.

Three in-vitro methods have been used to generate haploids. They are:

- (i) Culture of excised ovaries and ovules (Gynogenesis)
- (ii) Chromosome elimination following interspecific hybrdisation bulbosum technique of embryo culture.
- (iii) Culture of excised anthers and pollen.

Haploid production through anther or microspore culture has been referred to as **androgenesis** while **gynogenesis** is the production of haploid plants from ovary or ovule culture.

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### **5.3 Androgenic Haploid Production**

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The androgenic method of haploid production is from the male gametophyte of an angiosperm plant, i.e. microspore (immature pollen). The underlying principle is to

stop the development of pollen cell whose fate is normally to become a gamete, i.e. a sexual cell, and to force its development directly into plantlets.

Haploids can be obtained by the culture of excised anthers and culture of isolated pollen (microspore) (Fig. 5.1).

An angiosperm stamen consists of a **filament**, **connectivetissue** and **anther**. A typical anther shows two anther lobes, an each lobe possess two microsporangia or pollen sacs. During microsporogenesis in a young anther, there are four patches of primary sporogenous tissue, which either directly function as pollen mother cells (PMCs) or undergo several divisions. The pollen mother cells form pollen tetrads by meiosis and when the callose wall of tetrad dissolves, the four pollen grains or microspores are liberated. The newly released microspore is uninucleate, densely cytoplasmic with the centrally located nucleus. As vacuolation occurs, the nucleus is pushed towards the periphery. At first pollen mitosis, the microspore nucleus produces a large vegetative and a small generative nucleus. The second pollen mitosis is restricted to generative nucleus and forms two sperms and takes place in either the Pollen or Pollen Tube.

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#### **5.4 Induction and Procedure of Anther Culture**

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The technique of anther culture is rather simple quick and efficient. Young flower buds (with immature anthers in which the microspores are confined within the anther sac at the appropriate stage of pollen development) are surface sterilized and rinsed with sterile water. The calyx from the flower buds is removed by flamed forceps. The corolla is slit open and stamens are removed and placed in a sterile petridish. One of the anthers is crushed in acetocarmine to ascertain the stage of pollen development. If it is found of correct stage, each anther is gently separated from the filament and the intact uninjured anthers are inoculated horizontally on nutrient media. Injured anthers may be discarded because wounding often stimulates callusing of the anther wall tissue. In case of minute flowers such as *Brassica* and *Trifolium*, use of stereo microscope for dissecting the anthers is necessary. In case of cereals, spikes are harvested at the uninuclear stage of microspore development and surface sterilized. The inflorescence of most cereals at this stage is covered by the flag leaf.

Anthers can be plated on solid agar media in Petri dishes. Normally 10-20 anthers are plated in a 6 cm Petri dish. Anthers can be cultured on a liquid media. In responsive anthers, the wall tissue gradually turn brown and within 3-8 weeks they burst open due to the pressure exerted by the growing pollen callus or pollen plants. After they have attained a height of about 3-5 cm, the individual plantlets or shoots emerging from the callus are separated and transferred to a medium that would support further development. The rooted plants are transferred to sterile soil in the pots.

Anther culture has an advantage over Pollen culture in being very quick for practical purposes, and also sometimes the anther wall has an influence on the development of microspores in it, acting as a conditioning factor.

## **5.5 Microspore Culture (Pollen Culture)**

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Haploid plants can be produced through *in-vitro* culture of male gametophytic cells, i.e. microspores of immature pollen. In the procedure of microspore culture, anthers are collected from sterilized flower buds in a small beaker containing basal media .The microspores are squeezed out of the anthers by pressing them against the side of beaker with a glass rod (Fig. 5.1) Anther tissue debris is removed by filtering the suspension through a nylon sieve. It has been observed that smaller microspores do not regenerate and larger, good and viable regenerate, thus larger, good and viable microspores can be concentrated by filtering microspore suspension through nylon sieves.

This pollen suspension is then centrifuged at low speed for 5 min. The supernatant containing fine debris is discarded in fresh media and it was washed at least twice. The microspores obtained are then mixed with an appropriate culture medium at a suitable density. The final suspension is then pipetted into small petri dishes.

To ensure good aeration, the layer of liquid in the dish should be as thin as possible. Each dish is sealed with parafilm to avoid dehydration and is incubated. The responsive microspores form embryos or calli and its subsequent development to plant formation can be achieved by transferring to suitable media.

Culture of anthers has been proved to be an efficient technique for haploid induction. But it has one main disadvantage that is, plants not only originate from pollen but also from various other parts of the anther also (especially in dicots) so

resulting plant population is of various ploidy levels. This difficulty can be overcome by culture of isolated microspores.

**Advantages of pollen culture are as follows:**

1. Uncontrolled effects of the anther wall and other associated tissues are eliminated and various factors governing androgenesis can be better regulated. But it may be disadvantageous where anther wall has a stimulatory effect.
  2. The sequence of androgenesis can be observed starting from a single cell.
  3. Microspores are ideal for uptake transformation and mutagenic studies as microspores may be evenly exposed to chemicals or physical mutagens.
  4. Higher yields of plants per anther could be obtained.
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## **5.6 Induction of Androgenesis**

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The anthers normally start undergoing pollen embryogenesis within 2 weeks and it takes about 3-5 weeks before the embryos are visible bursting out of the anthers. In rice this may take up to 8 weeks. Haploid plantlets are formed in two ways (i) **direct androgenesis**: embryos originating directly from the microspores of anthers without callusing or (ii) **indirect androgenesis i.e. organogenic pathway**: microspores undergo proliferation to form callus which can be induced to differentiate into plants.

Pollen embryogenesis can be induced on a simple mineral-sucrose medium in plants like tobacco. For androgenesis, addition of certain growth regulators is required. For example, cereal anthers require both auxins and cytokinins and optimal growth response depends on the endogenous level of these growth regulators. However, direct embryogenesis can be promoted with simple media with low levels of auxins. When the response of cereal cells and tissues to different growth regulator concentrations and combinations are compared with dicots, generally growth hormones are needed in the monocots whereas high amount of phytohormone autotrophy is present in the dicots.

Activated charcoal in the medium enhances the percentage of androgenic anthers in some species presumably by removing the inhibitors from the medium.

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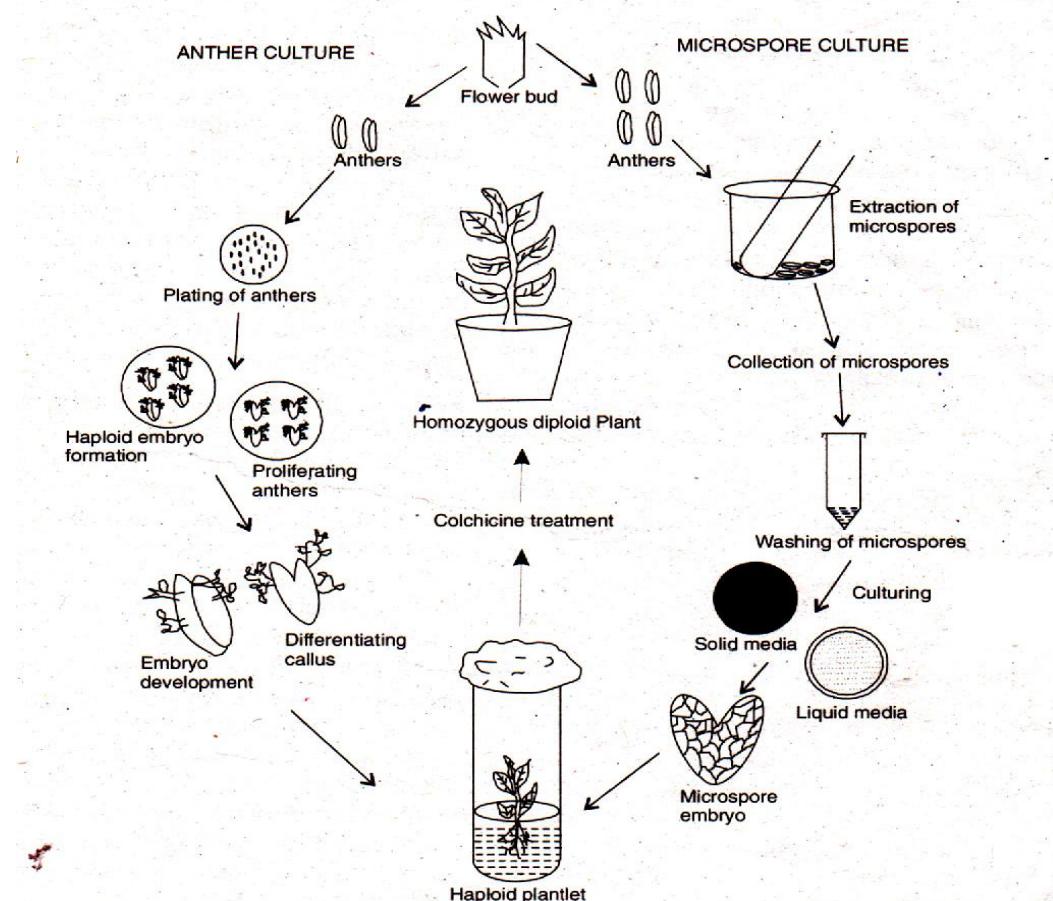
## 5.7 Process of Androgenesis

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In the process of androgenesis, microspores undergo divisions and a 40-50 celled **proembryo** is formed. The embryos, mostly at globular stage, burst out of the exine and are released. The embryos undergo various stages of development, simulating those of normal zygotic embryo formation. However, when the microspore takes organogenic pathway, it looks to be larger than embryonal type of microspore after 2-3 weeks and contain only a few cells. These cells increase in size, exerting pressure on the exine which bursts open and the contents are released in the form of a callus. These calli then differentiate into plantlets.

The plants with well developed shoots and roots are then transferred to pots. The plantlets originating from the callus generally exhibit various levels of ploidy.

The physical environmental conditions in which the cultures are to be placed can enhance the differentiation. The cultures generally are incubated at 24-28<sup>0</sup>C. In the initial stages of induction of morphogenesis, darkness is normally more effective or cultures should be kept in low light intensity (500 lux). After induction, macroscopic structures are transferred to a regeneration medium (in cereals with reduced sucrose and auxin concentration) and kept at 14 h day-light regime at 2000-4000 lux.



**Fig.: 5 .1 : Diagrammatic illustration of Anther and Microspore culture for production of Haploid Plants and Diploidization**

## 5.8 Identification of Haploids

### The ploidy level and Chromosome doubling

The ploidy of plants derived from anther or microspore culture is highly variable. The wide range of ploidy levels seen in androgenetic plants has been attributed to endomitosis or fusion of various nuclei. Haploid tissues are quite susceptible to changes in ploidy level during cell proliferation and growth in vitro. For obtaining homozygous lines, the plants derived through anther culture must be analysed for their ploidy status. Approaches to determine ploidy level are as follows:

- Counting of plastids in the stomata:** The ploidy level of a plant may be determined by counting the number of plastids in the stomata of a leaf.

2. **Chromosome number:** It can be counted from pollen mother cells of buds which can be collected from the regenerated plants and fixed in Carnoy's solution. Acetocarmine can stain cells. Root tips are also utilized for chromosome counting. Fixed root tips are normally hydrolyzed in 1 N HCl at 60 °C for 10 min followed by staining with acetocarmine.
3. **Number of nucleoli:** Haploid plants contain one nucleolus while diploids contain two nucleoli. Number of nucleoli is directly related to the ploidy status of a plant. Leaves can be incubated overnight with orcein and number of nucleoli can be counted.
4. **Flow cytometric analysis:** Leaves of potential haploid plants are finely chopped and intact interphase nuclei are freed from the cells. At this stage nuclear DNA content reflects the ploidy state of the donor which is determined by flow cytometry. This method is very quick.

## 5.9 Diploidization

Haploids can be diploidized to produce homozygous plants by following methods:

1. **Colchicine treatment:** Colchicine has been extensively used as a spindle inhibitor to induce chromosome duplication. It can be applied in the following ways:
  - (i) The plantlets when still attached to the anther are treated for 24-48 h with 0.5% colchicine solution, then washed thoroughly and replanted.
  - (ii) Anthers can be plated directly on a colchicines supplemented medium for a week after first division; they are transferred to colchicine free medium for androgenesis process to take place. This method can be followed in maize where male and female flowers are borne separately and diploidization is a problem.
  - (iii) Colchicine- When the plants are mature; lanolin paste (0.4%) may be applied to the axils of leaves. The main axis is decapitated to stimulate the axillary buds to grow into diploid and fertile branches.
  - (iv) Repeated colchicine treatment to axillary buds with cotton wool plugs over a period of time (e.g. 14 days in potato).

(v) In cereals, vigorous plants at 3-4 tiller stage are collected; soil is washed from the roots and is cut back to 3 cm below the crown. The plants are placed in glass jars or vials containing colchicine solution (2.5 g colchicines dissolved in 20 ml dimethyl sulfoxide and made up to a liter with water). The crowns are covered with colchicines solution. The plants are kept at room temperature in light for 5 h, the roots are washed thoroughly with water and potted into light soil.

Plants should be handled with extra care after colchicine treatment for few days and should be maintained under high humidity.

2. **Endomitosis:** Haploid cells are unstable in culture generally and have a tendency to undergo endomitosis (chromosome duplication without nuclear division) to form diploid cells. This property of cell culture has been exploited in some species for obtaining homozygous plants or for diploidization. A small segment of stem is grown on an auxin-cytokinin medium to induce Callus formation.

During callus growth and differentiation, there is a doubling of chromosomes by endomitosis to form diploid homozygous cells and ultimately plants.

## 5.10 Factors Affecting Androgenesis

- (i) **Genotype of Donor plant:** Success in anther culture is predominantly dependent on the genotype of the anther donor plant material. Various species and cultivars exhibit different growth responses in culture. Generally, anther culture ability is genetically controlled. Thus a general survey for tissue culture response of various cultivars must be undertaken with simple media as complex media rich in growth regulators tend to favour proliferation of somatic anther tissue giving rise to callus of various ploidy levels.
- (ii) **Physiological status of the donor plants:** The physiological status of the plants at the time of anther excision greatly affects the sporophytic efficiency of microspores. It is very important to start the pollen culture with healthy pollen cells. Therefore, it is good to culture anthers from plants

grown under the best environmental conditions. The donor plant should be taken care of from the time of flower induction to the sampling of pollen.

The use of any kind of pesticide should be avoided for 3-4 weeks preceding sampling. The response in culture is predominantly influenced by the different growth conditions during various seasons. The reason of this variation may be due to the difference in the endogenous level of growth regulators. Critical environmental factors are light intensity, photoperiod, temperature, nutrition and concentration of carbon dioxide. It has been generally observed that plants grown outdoors during natural growing seasons are more responsive than greenhouse grown material. Flowers from relatively young plants taken in the beginning of the flowering season are more responsive. Therefore it is of prime importance that plants be grown under optimal growth conditions, watered with minimal salt solutions periodically and relatively young plants should be used.

(iii) **Stage of pollen:** Selection of anthers at an appropriate stage of pollen developments is most critical in culture. Anthers with microspores ranging from tetrad to the binucleate stage are responsive. But as soon as starch deposition has began in the microspore, no sporophytic development and subsequently no macroscopic structure formation occurs. It has been established that uninucleate microspores are more prone to experimental treatment for culture just before or during first mitosis. There is an optimum stage for each speceis. For example, pollen at or during first mitosis in *Datura innoxia*, *Nicotiana tabacum*.In cereals early bicellular stage of microspore development has been found to give the best results.

(iv) **Pretreatment of anthers:** The underlying principle of androgenesis is to stop the development of the pollen cell (whose fate is to become a gamete) and to force its development directly into a plant. This abnormal pathway is possible if the pollen cell is taken away from its normal environment and placed in other specific conditions. This induction of androgenesis is enhanced by giving certain treatments to the whole anthers, or flower bud or to the spike.

(i) **Cold pretreatment:** In general, cold treatment between 3 and 6 °C for 3 to 15 days gives good response. Maize responds better to a

temperature of 14 °C. The degree of cold that should be given is dependent on the species. As a result of cold treatment, weak or non-viable anthers and microspores are killed. It is possible that cold pretreatment retards aging of the anther wall, allows a higher proportion of microspores to change their developmental pattern from gametophytic to sporophytic.

- (ii) **Hot treatment:** In some species when floral buds or entire plants are subjected to 30°C (for 24 h) or 40°C (for 1 h) stimulates embryogenesis (e.g. Brassica)
- (iii) **Chemical treatment:** Various chemicals are known to induce parthenogenesis. 2-chloroethylphosphonic acid (Ethrel) has a pronounced effect in increasing the haploid production in various species. Plants are sprayed with an ethrel solution (e.g. 4000 ppm in wheat) just before meiosis in pollen mother cells which results in multinucleated (4-6) pollen with fewer starch grains. It is possible that multinucleated pollen might be induced to form embryos when cultured.
- (iv) **Culture media:** The composition of medium is one of the most important factors which not only determine the success of anther culture but also decide the mode of development. Normally only two mitotic divisions occur in a microspore, but androgenesis involves repeated division. It is difficult to decide to which medium is most suitable as species or even genotypes may demand different nutritional conditions. Basal medium of MS, White (1963), Nitsch and Nitsch (1969) and N<sub>6</sub> for solanaceous crops; and B<sub>5</sub>, N<sub>6</sub>, LS (Linsmaier and Skoog, 1965) and Potato2 medium (Chuang et al. 1978) for cereals have been used
- (v) **Sucrose:** is essential for androgenesis. Sugars are indispensable in the basal medium as they are not only the source of carbon but are also involved in osmo-regulation. The usual level of sucrose is 2-4% however; higher concentration (6-12%) favours androgenesis in cereals. Chelated iron has been shown to play an important role in

the differentiation of globular embryos into heart-shaped embryos and further into complete plants.

Nitrogen metabolism is quite an important feature. The presence of nitrate, ammonium salts as well as amino acids appears to play a very special role at different stages of the developmental process. However, glutamine is probably beneficial for most plant species as an aid to achieving the in-vitro differentiation of a cell to a complete plant.

## **5.11 Applications of Anther & Microspore Culture**

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- 1. Development of pure homozygous lines:** In the breeding context, haploids are most useful as source of homozygous lines. The main advantage is the reduction in time to develop new varieties. A conventional plant breeding programme takes about 6-8 years to develop a pure homozygous line, whereas by the use of anther or microspore culture, the period can be reduced to few months or a year. Thus, homozygosity is achieved in the quickest possible way making genetic and breeding research much easier. Homozygosity is still more important for those plants which have a very long juvenile phase (period from seed to flowering) such as fruit trees, bulbous plants and forestry trees. Even if repeated self-pollination is possible, achievement of homozygosity in this group of plants is an extremely long process.
- 2. Developing asexual lines of trees or perennial species:** Rubber tree taller by six meters which could then be multiplied by asexual propagation to raise several clones has been reported by researchers. Pollen derived haploid plantlets have been obtained in other woody species also such as *Aesculus hippocastanum*, *Citrus microcarpa*, *Vitis vinifera*, *Malus prunifolia*, *Litchi chinensis*.
- 3. Hybrid development:** As a result of complete homozygosity obtained from diploidization of haploids, one can rapidly fix traits in the homozygous condition. Pure homozygous lines can be used for the production of pure F<sub>1</sub> hybrids.
- 4. Induction of mutations:** Haploid cell cultures are useful for studying somatic cell genetics, mutation and cell modification. Majority of induced mutations are recessive, so not expressed in the diploid cells because of the presence of

dominant allele. Mutants which are resistant to antibiotics, herbicides and toxins etc. have been isolated in a number of plant species by subjecting haploid *Nicotiana tabacum* cells to methionine sulfoximine.

Carlson (1973) regenerated mutant plants which showed a considerably lower level of infection to *Pseudomonas tabaci*. Wenzel and Uhrig (1981) developed mutants in potato through anther culture which were resistant to potato cyst nematode. Mutants have also been isolated for various temperatures, rediosensitivity to ultraviolet light, gamma radiation, amino acid, e.g. valine, and various antibiotics and drugs.

- 5. Induction of genetic variability:** By anther culture not only haploids plants of various ploidy levels and mutants are also obtained and can be used into the breeding programmes.
- 6. Generation of exclusively male plants:** By haploid induction followed by chromosome doubling it is possible to obtain exclusively male plants. For example, in *Asparagus officinalis* male plants have a higher productivity and yield earlier in the season than female plants.
- 7. Cytogenetic research:** Haploids have been used in the production of aneuploid. Monosomics in wheat, trisomics with  $2n=25$  in potato and nullisomics in tobacco were derived from haploids obtained from monosomics which could not be produced nullisomics on selfing. Haploids also give evidence for the origin of basic chromosome number in a speceis or a genus.
- 8. Significance in the early release of varieties:** Based on anther culture many varieties have been released. In Japan, a tobacco variety F 211 resistant to bacterial wilt has been obtained through anther culture. In *Brassica napus*, anther- derived doubled haploid lines had low erucic acid and glucosinolate content. Similarly, in sugarcane, selection among anther culture derived haploids led to the development of superior lines with tall stem and higher sugar content. In bell peppers, haploid lines exhibited all shades of color ranging from dark green to light green. These reports have encouraged many plant breeders to incorporate anther culture in breeding methods.
- 9. Hybrid sorting in haploid breeding:** One of the essential steps in haploid breeding involves selection of superior plants among haploids derived from  $F_1$

hybrids through anther culture. It is properly described as hybrid sorting and virtually means selections of recombinant superior gametes. The haploid method of breeding involving hybrid sorting is considered superior over pedigree and bulk methods. Firstly because of the frequency of superior gametes is higher than the frequency of corresponding superior plants in  $F_2$  generation. Secondly haploid breeding reduces significantly the time required for the development of a new variety.

**10. Disease resistance:** Haploid production has been used for the introduction of disease resistance genes into cultivars. An established cultivar is crossed with a donor for disease resistance. Either  $F_1$  or  $F_2$  generation anthers are plated and haploids are developed from them. These haploids are screened for resistance and then diploidized. Resistance to barley yellow mosaic virus has been introduced into susceptible breeding lines by haploid breeding (Foroughi and Friedt, 1984) A barley accession Q 21681 was found to be resistant to various diseases. This line was crossed to susceptible breeding lines and anthers of  $F_1$  plants were cultured to develop double haploid lines which were resistant to stem rust, leaf rust and powdery mildew (Steffenson et al. 1995). Rice varieties Zhonghua No 8 and No 9 have been developed with blast resistance genes, high yield and good quality using haploids integrated in conventional breeding approaches.

“**Hwasambye**”, a rice variety bred through anther culture showed resistance to leaf blast, bacterial leaf blight and rice stripe tenui virus (byeong-geun *et al.* 1997 ) In tobacco , a variant that showed resistance to a highly necrotic strain of potato virus Y (PVY) from a population of doubled haploids was reported by Witherspoon *et al.* . (1991).

**11. Insect resistance:** A medium-late maturing rice variety ‘Hwacheongbyeo’ derived from anther culture showed resistance to brown plant hopper. This variety was also resistant to blast, bacterial blight and rice tenui virus and showed cold tolerance (Lee *et al.* , 1989) .In rice , promising anther culture lines have been developed which show resistance to rice water weevil (N’Guessan *et al*, 1994), pests (Zapata *et al.* , 1991)

**12. Salt tolerance:** Salt tolerant breeding lines have been developed in different crop species which have been integrated in conventional breeding. Miah *et al.*

(1996) developed doubled haploid salt tolerant breeding line of rice that showed tolerance.

**13. Doubled haploids in genome mapping:** A recent application of double haploid lines is their use in genome mapping. For molecular screening studies a much smaller sample of double haploids is required for desirable recombinants. In a population of Double Haploid lines, the identification of markers is much more secure, as most intermediate phenotypic expressions are excluded due to heterozygosity. A gene will segregate in a 1:1 ratio both molecular marker and the phenotype at the plant level. Diploid haploid is used for genome mapping for major genes and quantitative traits in barley, rice.

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## 5.12 Merits and Demerits

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The significance of haploid in genetics and plant breeding has been known for a long time.

- (i) Haploids remain unaffected from the problem of dominance and segregation. One can easily uncover the desirable recessive traits in haploids and one can develop homozygous diploid pure lines in a single step. These homozygous lines can be used in creating desirable recombinants.
- (ii) Haploid plants are very useful in (i) indirect screening of recessive mutation because in diploid or polyploid, screening of recessive mutation is not possible. (ii) development of homozygous diploid plants can be obtained by doubling of chromosomes of haploid plants cell by mutagenic agents like colchicine in one generation.
- (iii) Besides, haploid flowering plant can simplify the production, identification and selection of mutants. This can be of great help in applied as well as fundamental genetics. Haploid Production through anther culture or microspore culture has been referred to as androgenesis.

### Demerits

1. High level of management and expertise is required to operate the tissue culture production of haploids.

2. Diploids and tetraploids often regenerate at the same rate as the haploids.
3. Selective cell division must take place in the haploid microspores and not in other unwanted diploid tissues. This selective cell division is often impossible.
4. Callus formation whether it has arisen spontaneously or has been induced by growth regulators is usually detrimental.
5. The relatively high incidence of albinism in some types of anther and pollen culture.
6. The lack of selection of traits during the derivation of haploid material.
7. There is little chance of isolating a haploid from a mixture of haploids and higher ploidy levels since latter ones are easily outgrown.

The doubling of a haploid does not always result in the production of a homozygote.

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### **5.13 Limitations**

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Haploid breeding has not given the desired and expected dividends despite substantial investments made on haploid research during last decades. Following are some limitations in haploid production:

- (i) Haploids can not be obtained in high frequency.
- (ii) The cost benefit ratio in haploid breeding is often not favourable thus discouraging the use of haploid breeding inspite of advantages.
- (iii) Haploid will express recessive deleterious traits and deleterious mutations may show up during anther culture.
- (iv) The cost benefit ratio in haploid breeding is often not favourable thus discouraging the use of haploid breeding despite its advantages.
- (v) Haploid will express recessive deleterious traits and deleterious mutations may show up during anther culture.
- (vi) Because of different ploidy levels, haploid status may need to be confirmed cytologically.

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## 5.14 Gynogenic Haploids

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Recent advances in plant tissue culture have resulted in the successful induction of haploid plants from ovary and ovule culture. This means that megasporangia or female gametophytes of angiosperms can be triggered in-vitro to sporophytic development. These plants have been described as **gynogenic** as compared to androgenic plants derived from microspores. In-vitro culture of unpollinated ovaries and ovules represents an alternative for the production of haploid plants in species for which anther culture has given unsatisfactory results (e.g. albino plants) or has proven insufficient, e.g. Gerbera.

The first successful report on the induction of gynogenic haploids was in barley by San Noeum (1976). Subsequently haploid plants were obtained from ovary and ovule cultures of rice, wheat, maize, sunflower, sugar beet, tobacco, poplar, mulberry, etc.

Ovaries can be cultured as pollinated and unpollinated. For haploid production, flower buds are excised 24-48 h prior to anthesis for unpollinated ovaries. The calyx, corolla, and stamens are removed and ovaries are then surface sterilized. Before culturing the tip of the distal part of the pedicel is cut off and the ovary is implanted with the cut end inserted in the nutrient medium. The normal Nitsch's (1951), or White's (1954) or MS or N<sub>6</sub> inorganic salt media supplemented with growth substances are used. When liquid medium is to be employed, the ovaries can be placed on a filter paper raft or float with the pedicel inserted through the filter paper and dipping into the medium. Sucrose as a carbon source is essential, although maltose and lactose have been shown to be equally favourable. The various species studied so far seem to have few requirements in common for growth in vitro. Some species require only the basal medium and sucrose for growth, although addition of an auxin brought about greater stimulation of growth.

The origin of gynogenic halpoids differs in species as reported to be from synergids in rice, whereas egg or antipodal cell develops into embryos in barley.

In-vitro culture of unfertilized ovules has been the most efficient and reliable technique for the production of haploid and doubled haploid plants of sugar beet. In tree species, gynogenic plants were reported in mulberry (*Morus indica*), an important tree for nourishing silkworms for use in the silk industry. Ovaries were

cultured on MS medium supplemented with 1 mg or 1 BA and 1 mg or 1 kinetin which resulted in direct regeneration of plants without a callus phase.

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## 5.15 Factors Affecting Gynogenesis

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1. **Genotype:** Genotype of the donor plant is one of the most important factors since each genotype shows a different response. For each genotype, a specific protocol must be followed for maximal efficiency.
2. **Growth condition of the donor plant:** It has been found that embryo induction from ovules harvested from lateral branches gives high response than ovules harvested from stem apex. Ovules harvested from first formed lateral branches (at the base of plant) give higher response than from the sixth formed lateral branches in sugar beet.
3. **Stage of harvest of ovule:** Excision of ovules at an early developmental stage where no self pollination can occur or use of highly self incompatible donor plants helps to minimize the problem of fertilization in sugar beet and other crops so that proper stage of unfertilized ovule could be harvested.
4. **Embryo-sac stage:** It has been reported that complete maturation of ovule is not necessary for induction of gynogenesis but an appropriate stage is more important.
5. **Culture conditions:** Although solid media have been used more frequently for gynogenic culture, few investigators have used liquid medium for the induction of gynogenic culture. Few investigators have used liquid medium for the induction of gynogenic calli followed by dissection and transfer of embryogenic structures to solid medium for differentiation. MS, B5, Miler's basal medium have been most commonly used. The amount of sucrose has been reported to be important for embryogenesis. In rice 3-6% sucrose was effective, whereas in onion, 10% sucrose give maximum response.
6. **Seasonal effects:** Seasonal variation is an important factor. In sugar beet highest embryo yield was obtained from the summer grown plants. Callus induction from ovules of Gerbera occurred at a higher frequency during autumn as compared to spring.

7. **Physical factors:** Certain physical factors (treatments) given to explants or plant parts from which explants are taken prior to culture may have a strong influence on embryo induction. For *Beta vulgaris*, cold pretreatment of flower buds at 4°C for 4-5 days increases embryo yield from cultured ovules. Similarly cold pretreatment of inflorescence prior to ovule isolation increased haploid callus frequency in *Salvia selarea*. Other physical factors influencing ovary and ovule culture are light and temperature of incubation.

## 5.16 Applications of Ovule Culture

In raising hybrids which normally fails to grow due to abortion of the embryo at a rather early stage when its excision and culture is not possible. Ovule culture holds a good potential.

The development of fibre in *Gossypium hirsutum* is dependent on the day of anthesis in culture of ovule. Bassley and Tiny (1973) cultured the ovule of *Gossypium* on the day of anthesis and 2 days after anthesis, the development of fibres showed promising result. Thus ovule culture projects the developmental pattern of fibre development which may be used by cultivators.

Ovule culture has been successfully employed in interspecific crosses to obtain hybrid seedlings. In the cross between *Brassica chinensis* and *Brassica pekinensis* a true hybrid is obtained by culturing the fertilized ovule.

In angiospermic parasites, the host parasite relation is maintained throughout. The ovule culture of a root parasite Stiga or Orobanchae, the formation of shoots has been demonstrated. This study opened the door to understand the physiology of host and parasite relation.

The seeds of orchid plants take more time to germinate which live in association of fungus. To overcome this time factor and presence of fungus, Poddubhanay Arnolddi (1960) successfully grew ovules of orchids in culture.

## 5.17 Summary

Haploid plant production from anther or microspore culture is a relatively new technique. Haploid plant production has been reported in more than 200 species. The production of haploid plants from anther culture technique offers a rapid achievement of homozygous lines for early release of new crop varieties. The

culture of anthers or microspores gives rise to haploid plants whose chromosomes can be doubled by suitable treatment (e.g., colchicine) to produce homozygous diploid plants. Chromosome doubling of haploids allows the production of homozygote which can be used directly in hybrid production.

Besides, it allows the use of haploid cells and protoplasts for the induction and selection of recessive mutants. Haploids facilitate recovery of recessive mutants and unique genetic recombination.

Parthenogenesis of egg culture has also been reported. Since the beginning of modern plant breeding practices, intensive efforts have been made to speed up the production of homozygous lines which normally requires at least six inbreeding generations. The starting material for the production of homozygous lines in just one generation is the haploid gametes.

## 5.18 Glossary

- **Androgenic embryos** : embryos derived from pollen is known as androgenetic embryos
- **Anther or pollen culture** : Placing of pollen and anthers on a suitable culture medium to have haploid plants is called anther or pollen culture.
- **Pollen Culture** : Isolated pollen grain when cultured in-vitro give rise to haploid embryos.
- **Totipotency** : A property of a normal cells that they have the genetic potential to give rise to a complete individual.
- **Totipotent** : The ability at a cell to respond according to environmental stimuli and divide to form differentiated cell type
- **Organ culture** : Culture of an organ **invitro**
- **Organogenesis** : Formation of any organ. Root or shoot in-vitro
- **Auxins** : A class of plant growth regulators that stimulate cell division, cell elongation ,apical dominance, root initiation e.g. 2,4-D, IAA, IBA ,
- **Micro Propagation**: Use of small piece of tissue such as meristem grown in culture to produce large number of plants.
- **Morphogenesis** : Development of organized structure .

- **Regeneration** : It refers to development of organized structures like roots, shoots, flower buds, somatic embryos etc from cultured cells or tissues.
- **Aseptic** : Free from all organism.
- ***in-vitro*** : Any process carried out in sterile cultures in laboratory condition
- ***in-vivo*** : In natural condition in which organism live
- **Cell Culture** : Cell Culture is growing cells in-vitro in liquid medium.
- **Tissue Culture** : The rearing of cells or tissues in an artificial medium under controlled condition.
- **Explant** : A Piece of tissue or any excise part of plant which is merismatic is used to initiate, tissue cultures is called explants.
- **Excise** : Cut out with knife or scalpel etc. and prepare a tissue organ etc for culture.
- **Embryoid**: Embryo like structures produced as a result of differentiation process like embryogenesis and. Andro genesis.
- **Somatic embryogenesis** : Process of development of embryo from a somatic cell is called Somatic embryogenesis.
- **Callus** : Unorganised mass of cells, capable of cell division and growth in-vitro
- **Caulogenesis** : Shoot induction from callus.

## 5.19 Self-Learning Exercise

### Section A : (Very Short Answer Type Questions)

1. Who obtained “androgenic haploids” for the first time and in which plant, mention the year also. ?
2. Define “pollen culture”
3. Define androgenesis.
4. What do you understand with doubled haploids?
5. Why haploids are suitable for studies of genetics and plant breeding?
6. What is the role of “Ether” ( 2 Chloro ethyl phosphonic acid)?
7. Mention the factor which influences androgenesis and haploid production?

8. Why mutations can easily be detected in haploids?

**Section B : (Short Answer Type Questions)**

1. What are the benefits of pollen and anther culture?
2. How do you differentiate direct androgenesis and indirect androgenesis?
3. Mention four uses of haploid plants?
4. Mention two advantage of “Mentor pollen technology”.
5. What is the use of homozygous diploids plants in crop improvements?

**Section C : (Long Answer Type Questions)**

1. Describe history of discovery of haploid production in culture.
2. Explain Method of haploid production.
3. Discuss the role of haploid plants in crop improvement
4. Discuss role of haploids in “Cytogenetic research”.
5. Discuss in brief the method of “Diplodization in haploids”

**Answer key of section-A**

1. Guha & Maheshwari (1964) in *Datura innoxia* by culturing pollen grain.
2. Isolated pollen grains when cultured *in-vitro* gives rise to haploid embryos or callus. This approach is called **pollenculture**.
3. Development of haploid plants from the male gametophyte following a development pattern resembling embryogenesis resulting from the culture of anthers or micro spore.
4. When the chromosomes of haploid plants are doubled to make homozygous line which is suitable for crop improvement programme.
5. Because from haploids pure homozygous lines can easily be obtained in short time.
6. It is used to induce parthenogenesis.
7. (i) Donor plant (ii) culture medium (iii) anther preculture treatment
8. Because there is not interference of dominant all etc in haploid plant.

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## 5.20 References

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## Unit- 6

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# Applications of Plant Tissue Culture

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### Structure of the Unit:

- 6.0 Objectives
  - 6.1 Introduction
  - 6.2 Clonal Propagation
  - 6.3 Production of Disease Resistant Plant
  - 6.4 Production of Disease Free Plants
  - 6.5 Development of Androgenic Haploids
  - 6.6 Embryo Culture
  - 6.7 Production of Secondary Metabolites
  - 6.8 Germplasm Storage
    - 6.8.1 *ex-situ* conservation
    - 6.8.2 *in-situ* conservation
  - 6.9 Summary
  - 6.10 Glossary
  - 6.11 Self-Learning Exercise
  - 6.12 References
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## 6.0 Objectives

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The objective of this unit is to explain

- various applications of plant tissue culture in a very simple way.
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## 6.1 Introduction

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Plant cell and tissue culture is a technique which is used to propagate plants from cell or tissue under aseptic conditions. In this unit author emphasize the applications of plant tissue culture in various aspects such as clonal propagation

production of disease resistant plants, production of disease free plants and organic haploids embryo rescue, somaclonal variations, distant hybridization, production of artificial seed, production of secondary metabolites germ plasm storage.

## 6.2 Clonal Propagation

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**Definition:** Multiplication of genetically identical copies of a cultivar by asexual reproduction is known as clonal propagation. A population derived from a single individual by asexual reproduction is known as a clone. It is the only method for crop such as grapes, banana, fig, chrysanthemum etc. which produce little number viable seeds.

**Technique:** *in-vitro* clonal propagation is a complicated process requires many steps. Murashige in 1978 proposed distinct stages that can be adopted for overall production of clone's stage 1-3 are followed under *in-vitro* condition while stage 4 is accomplished in greenhouse environment. Debergh and Maine (1981) suggested an additional stage 'O' for various micro propagation systems. The adoption of all these stages not only simplifies the daily operation but allows for better yielding communication with other labs.

**Stage 1:** This is an initial step in which stock plant used for culture initiation are grown for at least 8 months under carefully monitored conditions stock plants are grown at a relatively low humidity and H<sub>2</sub>O either with irrigation tube or by capillary sand bags. This stock plant preconditioned stage also includes, majors to be adopted for reduction of surface and microbial contamination.

**Stage 2:** Murashige in 1974 define this stage as initiation and establishment of aseptic cultures. The main steps involved as:

- 1) Preparation of explant followed by its establishment on suitable culture medium. Most commonly used explant for commercial micropropagation is shoot tip and axillary bud.
  - (a) Explant isolation: Explant used to describe the initial piece of the plants used to start *in-vitro* cultures practically any part of plant can be successfully cultured *in-vitro* and can regenerate plantlet. This explant physiological stages for development in potent.
  - (b) Sterilization of explant: This includes 3 steps:

- (i) Washing under running water using liquid dependent and NaOCl.
- (ii) Through rinsing and washing with double distilled water.
- (iii) Explant sterilization with aq. HgCl<sub>2</sub> in the LAF.

**Stage 3:** It is for first 4 or 3 months to 2yrs and regeneration for at least 4 passages of the subculture. Usually explant carrying a preform vegetative bud are suitable for enhanced axillary branching of stock plant are treated virus free then the most suitable explant are nodal cuttings. The disadvantages of using small size explant are that they have a low survival rate and slow initial growth. So subtermined or slightly older segments are desirable.

**Stage 4:** This stage involves maximum proliferation of regeneration shoots using a defined culture medium.

### 6.3 Production of Disease Resistant Plant

A plant that has the ability to resist certain types of diseases is known as disease resistant plants (while other varieties of the same plant are typically susceptible).

The resistance may be naturally occurring, or it may be the result of breeding programs designed to enhance the plant's ability to resist the specific disease (s).

An example is the tomato, which may be selected for its resistance to Verticillium, Fusarium, root nematodes and or or tobacco mosaic diseases indicated by the letters. VFNT on the plant's or seed's packaging.

Among flowering plant's varieties resistant to powdery mildew are often preferable to those which are more susceptible, especially in areas where summer humidity is a problem.

- Relative to a susceptible plant, disease resistance is the reduction of pathogen growth on or in the plant, while the term disease tolerance describes plants that exhibit little disease damage despite substantial pathogen levels.
- Plant disease resistance is crucial to the reliable production of food, and it provides significant reduction in agricultural use of land, water, fuel and other inputs.

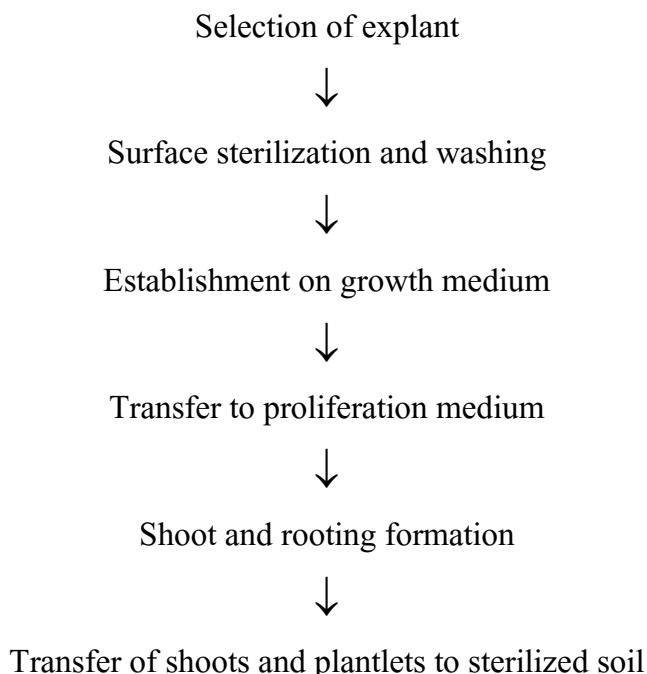
- Plants pathogens can spread rapidly over great distances vectored by water, wind, insects and humans.
- Across large regions and many crop species, it is estimated that diseases typically reduce plant yield by 10% every year in more developed nations or agricultural systems, but yield loss to diseases often exceeds 20% in less developed setting, an estimated 15% of global crop production.

## 6.4 Production of Disease Free Plants

Another utility of the technique of Plant Tissue Culture is the production of disease free plant and this can be achieved by culturing shoot tips as explants under in-vitro culture conditions because shoot apical meristem and some young tissues surrounding them often usually are free from viruses.

The technique for the production of virus free plants is described below:

### **Shoot tip culture and virus free plant**



### **Major stage of micropagation**

Morel and Martin, 1952, developed the technique of meristem culture for *in vivo* virus eradicate of Dahlia.

We use the axillary bud or meristem tissue:

- Because the high concentration of auxin, virus is not able to survive.
- The cell division is too fast so virus is not able to replicate in this region.

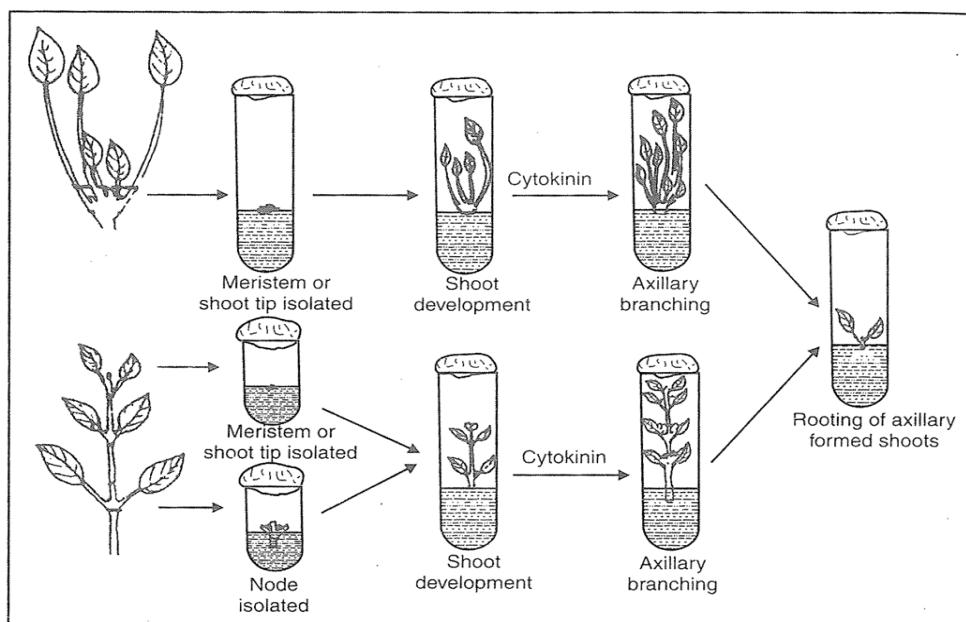
### **Shoot tip or meristem culture**

Cultivation of axillary or apical shoot meristem known as meristem culture

**Explant:** Shoot apical meristem lies in the shoot tip beyond the youngest leaf and first leaf primordium.

- It measures up to about 100  $\mu\text{m}$  in diameter and 250  $\mu\text{m}$  in length.
- Thus a shoot tip of 100-500  $\mu\text{m}$  in  $\mu\text{m}$  contains 1-3 leaf primodia in addition to the apical meristem.
- 1 mm – shoot tip used for virus elimination 1 cm for clonal propagation.
- Shoot tip may be cut into five pieces to obtain more than one plantlet from each shoot tip.

Meristem of shoot tip is cut or isolated from stem by applying a U shaped cut with a sterilized knife.



**Fig. 6.1 : Scheme showing the *in-vitro* Clonal Propagation of plants by the Axillary bud Method** (Upper row as applied to rosette plants; bottom row: applied to plants which elongate, (adopted from Pierik 1989))

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## 6.5 Development of Androgenic Haploids

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### A. Pathway of microspore division

Four pathways based on use few initial divisions in the microspores have been identified as leading to *in-vitro* androgenesis.

**Pathway-I** The microspores divide by an equal division and two identical daughter cells contribute to the sporophyte development. eg. *Datura innoxia*.

**Pathway-II** The division of uninoculated microspores is unequal, resulting in the formation of a vegetative and a generative cell. The sporophyte arises through further divisions in the vegetative cell either does not divide or does so once or twice before degenerating eg. *Triticum aestivum*.

**Pathway-III** The microspore undergoes or normal unequal division but the pollen embryos are predominantly formed from the generative cell done eg. *Hyoscyamus niger*.

**Pathway-IV** The division is unequal. Both vegetative and generative cell divide vegetative and generative cells divide further and contribute to the development of the sporophyte eg. *Datura metel*, *Atropa belladonna*.

### (B) Later development

Irrespective of the early pattern of divisions, the embryogenic pollen grains ultimately become multicellular and burst open, gradually assuming the form of a globular embryo. This is followed by the normal stages of post globular embryogeny until the development of the plant. Alternatively, bursting pollen grains may proliferate to form a callus which may later differentiate into whole plant. Gynogenesis can be obtained *in-vitro* either by organogenesis or embryogenesis as long as ploidy of resulting plant is haploid there are families (Compositae Liliaceae) where androgenic response is very poor or nil, but gynogenesis has given positive results. Further the only approach to produce haploids of male sterile or dioecious species is gynogenesis.

### Haploid Production and their applications

A.D. Bergner discovered haploid plant is *Datura stramonium* in 1921 and since the many workers have intensively worked to obtain haploids through *in vivo* or *in-vitro* techniques.

In haploids induced mutations are readily detected, and doubled haploids provide immediate homozygosity and the secondary of alleles not detected in heterozygous state. Hence, the production of haploids and dihaploids in large numbers is of great value to plant breeders.

In nature, haploids arise as a result of the parthenogenesis and these plants rarely produce characters of the male parent.

### **Techniques for haploid production (androgenesis)**

The two techniques and their modifications routinely used for *in-vitro* androgenesis are anther culture and pollen culture.

#### **Anther culture**

For raising haploids from microscope or pollen grains generally the entire anther is cultured. Young plants grown under controlled conditions of temperature, light and material for anther and pollen culture the stage of pollen is a critical factor for *in-vitro* androgenesis. Take most productive anthers and choose which contain uninucleate microspores midway between release from the tetrad and the first pollen grain mitosis. Floral buds with pollen at the first haploid mitotic. One collected from vigorously growing young greenhouse plants. They are surface sterilized with calcium hypochlorite (5% w or v) for 7 mins, mashed thrice with distilled water and anthers isolated. The anthers are placed horizontally and are slightly embedded in the culture medium. This prevents premature desiccation of the anthers and also facilitates fast migration of nutrients to the pollen grains.

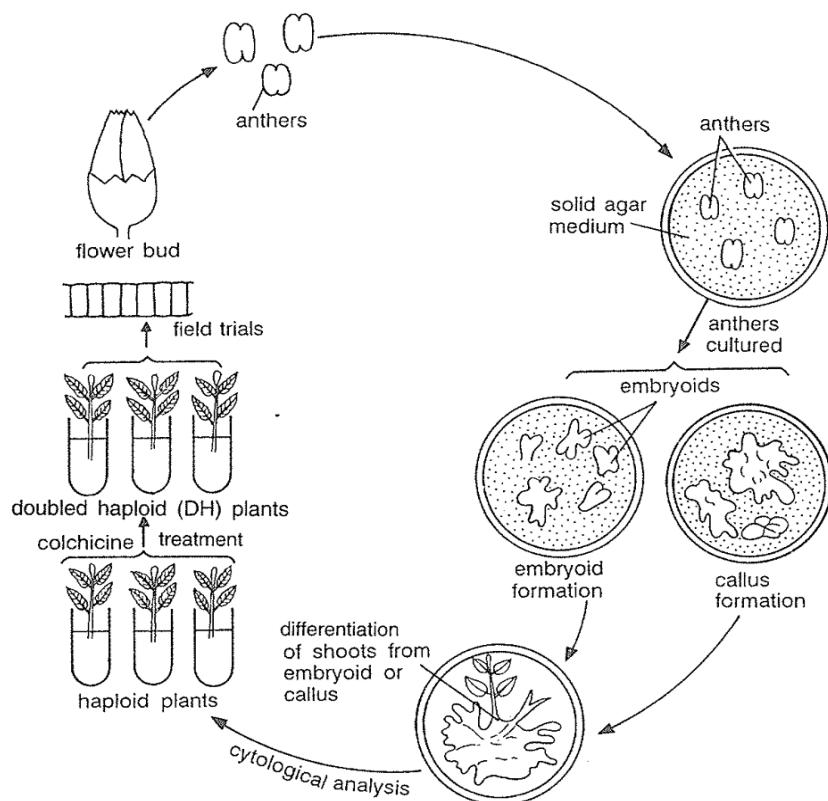
In anther cultures, depending on the species and the composition of nutrient medium, the pollen may either directly develop into embryos or first form a callus which would regenerate plants.

#### **Isolated Pollen culture**

Isolated pollen grains can also undergo embryogenesis. Advantages of isolated pollen culture are:

1. Homogenous population of pollen grains at the developmental stage most suitable for androgenesis can be obtained by gradient centrifugation.
2. Isolated incorporates can be genetically modified by mutagenic treatment or genetic engineering before culture and genotypes selected at an early stage.

3. Direct pollen embryogenesis is of considerable value in studies on plant cell differentiation, embryogenesis and morphogenesis.
4. The frequency of pollen embryogenesis could be increased by preculture of anthesis for 4 to 7 days before isolation of pollen grains.



**Fig. 6.2 : Different steps involved in the Production of Haploid Plants using Anther Culture**

#### Factors influencing haploid production in anther and pollen culture

The factors that significantly affect haploid production in anther and pollen culture are-

**(i) Physiology of the donor plant-** The environmental conditions and the age of the donor plants which influence their phytology significantly effect the androgenic response in anther and microspore cultures.

Generally the first flush of flower provides more responsive anthers than those borne later. However exceptions are there.

Treatment of donor plants with feminizing agents such as ethrel or exposing them to water or nutrient stress have also been shown to produce androgenesis in many plants for eg. Plants starved of nitrogen may give more responsive anthers compared to those that are well fed with nitrogenous fertilizers.

Anthers taken from plants grown under short day (8 hrs) conditions and highlight intensity regime show relatively better response than long day (16 hrs) donor plants at the same intensity. Therefore, it is recommended that only materials grown under controlled environmental conditions be used for microscope androgenesis.

**(ii) Stage of pollen development –** For the induction of androgenesis the stage of pollen development at which the anthers are cultured is very crucial. There is staging optima for each species. Generally, the most responsive stage of anthers is when pollen is first before, at or just after the pollen mitosis.

The commonly used external marker to pick up anthers at the right stage without testing each one of them before culturing is the length of the flower bud, which, under fixed environmental conditions, shows a reasonably good correlation with the developmental stage of the anthers.

**(iii) Genotype of the donor plant –** The genotype of the donor plant plays significant role in determining the frequency of pollen plant production. Striking variations occur between and within species. For eg. Among the crop *Brassicas*, *B. napus* is most responsive and *B. juncea* the least. In *Melandrium album*, which shows chromosomal basis of sex determination, only pollen with X chromosome are competent to form pollen plants and therefore, the androgenic plants are phenotypically and cytologically females. In tetraploid Melandrium even a single Y chromosome is able to suppress the effect of three X-chromosome.

**Culture medium-** Culture medium and culture density there is as single anther culture medium applicable to all systems since the requirements vary with the genotype and probably the age of the anther as well as conditions under which donor plants are grown.

Most Solanaceous species exhibit androgenesis on a complete nutrient medium of Nitsch or MS of the various minerals iron seems crucial for pollen embryo development in cultures that are 3 or 4 weeks old.

In the majority of non-solanaceous plant species known to exhibit androgenesis, pollen embryo formation occurs only when the medium is fortified with growth adjuvants.

Several culture media developed by Chinese scientists one now widely used for another culture of cereals eg. N<sub>6</sub> and Yu-Pis media.

Sucrose is another essentially constituents of the medium as long as the tissue in culture is unable to photosynthesize for itself. It is generally used at a concentration of 2-4%.

Culture activity could be a critical factor particularly with regard to isolated pollen culture. The minimum density regained for embryogenesis is 3000 pollen or ml of the culture media.

**(iv)Physical factors** – Several physical treatments such as temperature shock centrifugation and  $\gamma$ -irradiation, to the flower buds or cultured anthers or microspores. Promoted androgenesis in different systems of these promotions by temperature shock is most widely observed.

The buds treated with cold temperature at 3°C or 5°C for 72 hr induced approximately 58% anthers to yield pollen embryos. The induction of androgenesis has been found very effective if other cereals are stored at low temperatures prior to another culture. The pretreatment of anthers at elevated temperatures (35°) stimulate androgenesis in some *Brassica* and *Capsicum* species.

The frequency of haploids formed and the growth of plant although pollen plants from same genotypes grew both in the light and in the dark.

**Anther wall factors** –Pollen from one cultivar of tobacco would successfully develop into an embryo even if transferred into anthers of another cultivar. It is suggested that a gradient of critical substances of tapetal origin is instrumental in the induction of embryonic division in the pollen grains.

### **Applications of Haploids in Plant Breeding**

1. Mutations are difficult to detect in higher organisms because they are usually recessive and do not express themselves in the presence of their dominant alleles on the homologous chromosomes on the other hand mutations induced in haploids can be easily detected because they have only a single set of genes and consequently there is no interference by their dominant alleles haploids with desirable mutation can be picked up and their chromosomes duplicated to get fertile diploids with all desirable mutation in a single generation.
2. Haploid plants provide a source of haploid tissue which can be maintained *in-vitro* in undifferentiated state and can then dissociated into free haploid cells. In this may it unit be possible to obtain rear suspensions of haploid cells and to carry out new genetics researches on the higher plants by applying the techniques used for microorganisms, such as mutation studies at the physiological biochemical analysis, etc.
3. For using hybrid vigour as a method of crop improvement, homozygous, true breeding cultivars are extremely important. In this respect haploids can be easily treated with colchicine to produce homozygous diploids in a single generation.
4. The commercially desirable features of *Asparagus officinalis* are uniform, male plants with inflorescences having low fibre content. In this dioecious crop plant an inbred population is produced through subcrosses between pistillate (XX) and staminate (XY) plants are male and 50% female. Theminin (1974) and Tsay *et al.* (1982) cultured anthers from the male plants and raised homozygous super males (YY) when such males are crossed with female (XX) plants the entire progeny consists of males.

5. Anther culture is also a good source of genetic variation and enables analysis of gametic variation at plant level. Pollen grains within an anther form a highly heterogeneous population because they are formed as a result of meiosis which involves recombination and segregation. Therefore, each pollen plant is genetically different from the other variation in pollen plant is especially high when the parent plant is a recent hybrid.

## 6.6 Embryo Culture

E. Hanning (1904) was the first to perform removal of mature embryos from the seeds of *Raphanus sativus*.

**Definition:** The embryo of different developmental stages formed within the female gametophyte to sexual processes can be isolated aseptically from the bulk of maternal tissue of ovule, seed or capsule and cultured *in-vitro* under aseptically controlled conditions.

**Different categories of Embryo Culture and their objectives are following:**

1. **Culture of mature intact seed embryo:** to analyze the metabolic and biochemical aspect of dormancy.
2. **Culture of surgically dissected embryo:** Mature seed embryos can be dissected surgically into a number of segments to analyze relationship of different parts of embryos to its final form in the culture.
3. **Culture of immature embryos or pro-embryos:** Globular or heart staged stages of embryos are appropriately called pro-embryos. The objective of such culture is to understand the control of differentiation and nutritional requirements of such progressively developing embryos.
4. **Culture of intact seeds containing undifferentiated embryo:** For eg. Orchid, each fruit of an orchid plant develops several thousand tiny seeds which contain morphologically undifferentiated embryos. There is no storage tissue like endosperm in the seeds and seed coat is reduced to a membranous structure for this reason, the entire seed of orchid containing undifferentiated embryos is cultured and treated as embryo culture. In nature these seeds germinate only in association with a proper fungus as else they perish. As a result numerous seeds

are lost *in-vitro* culture of orchid seeds is routinely employed for orchid propagation.

- 5. Culture of adventitious embryos from polyembryonic seeds:** Besides zygotic embryos, produced from egg cells, some additional embryos are produced from nuclear tissue in polyembryonic seeds like lemon and oranges. Such additional (viable) aborated embryos can be exploited in culture for clonal propagation.
- 6. Culture of non-viable or abortive embryos:** In many interspecific or intergeneric breeding experiments, sometimes non-viable or abortive embryos may develop due to unsuccessful crosses, it is possible to raise a hybrid plant by growing these embryos *in-vitro*.

### **Principles of Embryo Culture**

1. The underlying principle of method is aseptic excision of embryo and its transfer to a suitable nutrient medium.
2. In general, it is relatively easy to obtain pathogen free embryos, since the embryo is lodged in sterile environment of ovule or seed or capsule or fruit, so surface sterilization of embryos as such is not necessary, only the seed or the fruit containing the ovule needs to be surface sterilized.
3. Seeds with hard seed coat are generally surface sterilized and then soaked in sterile water for a few days, aseptically so that a seed can be cut easily to free the embryo.
4. In the isolation of comparatively smaller embryos, it is important that they are removed carefully from ovule without any injury.
5. In culture, embryos are not induced to form callus tissue but they are allowed to form a plantlet. After the embryos have grown into plantlet *in-vitro*, they are generally transferred to sterile soil or vermiculture and grown to maturity in the greenhouse.

### **Nutritional Requirements of Embryos**

The most important aspect of embryo culture is the selective of the medium necessary to sustain continued growth of the embryos. The younger the embryo, the most complex is its nutritional requirement. Upto a certain stage as globular stage in capsella, the embryo is heterotrophic as it derives some part of its nutrition

from the endosperm. Beyond this stage, the embryo becomes autotrophic and is able to synthesis its biochemical requirements from simple nutrients like salt and sugar. In general, older an embryo, the simpler are its nutritional means.

Embryos are quite sensitive to the salt solution of culture medium

Sucrose is the best carbon source, 8-12% sucrose is generally used which approximates the osmotic potential of the young embryo sac. Mannitol at 120 gm or 1 is suitable for heart shaped embryos; generally growth regulators are not used since embryos are usually autotrophic in this sharply with embryo growth so that for best result embryos need to be transferred to new media regularly as they continue to grow during culture.

### **Applications of Embryo Culture**

1. It helps in determining of factors that regulate the growth of the primordial organ of seedling for Biological studies
2. Culture of surgically dissected embryos has facilitated the understanding of relationship of different plants of the embryos to their final form in the plant.
3. An embryo undergoes a gradual transition from the dependence of the zygote to the relative autonomy of the mature embryo changes in the nutritional requirements of the embryos at different stages can be demonstrated by embryo culture.
4. Role of accessory tissue can be studied by culturing isolated embryos.
5. Seeds and embryos of several obligatory parasites have been grown under aseptic conditions to study their dependence on the host.

### **Applied aspect**

It is used to raise the healthy plant from non-viable or abortive embryo eg. apple, peach, pea etc. interspecific cross or intergeneric cross.

### **Hybrid Embryo Rescue**

Mostly of the wide crosses are difficult to obtain by conventional methods non realization of hybrids may be due to pre or post fertilization barriers. In most of the crosses where fertilization does take place, the hybrid embryo aborts before maturation. Even if the seeds are formed, they either fail to germinate due to lack of endosperm or produce weak seedlings which do not survive. It is mainly due to

disharmony between parental genomes resulting in embryo mortality, endosperm breakdown, seed in viability and hybrid sterility. In *Brassica* embryos, embryo rescue was first used for obtained interspecific hybrids of *Brassica oleracea* cross *Brassica campestris* following of this many interspecific and intergeneric hybrids have been raised by embryo culture methods.

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## 6.7 Production of Secondary Metabolites

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- Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism.
- Primary metabolites, absence of secondary metabolites does not result in immediate death.
- Primary metabolism consists of chemical reaction that allows the plant to live.
- Secondary metabolites play an important role in plant defense against herbivore and other interspecies defenses.
- Human use secondary metabolites as medicines, flavoring and recreational drugs.
- In order for the plants to stay healthy, secondary metabolism plays a principle role in keeping all the of plant's systems working properly.

### History

Plant secondary metabolites were "by products" of the primary metabolism and were not crucial to the plant's survival. The study of plant metabolites is thought to have started in early 1800s when Friedrich Willhelm Serturner isolated morphine from opium poppy and after that new discoveries were made rapidly. In the early half of the 1900s, the main research around secondary plant metabolism was dedicated to the formation of secondary metabolites in plants, and this research was compounded by the use of tracer techniques which made deducing metabolic pathways much easier. In the 1970s, however, new research showed that secondary plant metabolites play an indispensable role in the survival of the plant in its environment.

- Secondary metabolites contain organic and inorganic moiety concept given by A. Kossel in 1891.

- Alkaloid was given by W. Meibner, which mean alkali like.
- The first alkaloid to be synthesized was coniine in 1886 by Ladenburg which had already been isolated in 1827.
- Recently, the research around secondary plant metabolites is focused around the gene level and the genetic diversity of plant metabolites.

### **Primary vs Secondary Plant Metabolism**

- Primary metabolism in a plant comprises all metabolic pathways that are essential to the plants survival.
- Primary metabolites are compounds that are directly involved in the growth and development of a plant whereas secondary metabolites are compounds produced in other metabolic pathways that, although important, are not essential to the functioning of the plant. However, secondary plant metabolites are useful in the long term, often for defense purposes, and give plants characteristics such as colour.
- Secondary plant metabolites are also used in signaling and regulation of primary metabolic pathways.
- Plant hormones, which are secondary metabolites, are often used to regulate the metabolic activity within cells and oversee the overall development of the plant.
- Secondary plant metabolites help the plant maintain an intricate balance with the environment, often adapting to match the environmental needs.
- Plant metabolites that colour the plant are a good example of this, as the colouring of a plant can attract pollinator and also defend against attack by animals.

### **Classification**

Secondary metabolites derived from the primary metabolites so they have more complexity to primary metabolites. They classified in many groups.

1. Alkaloids
2. Plant amines
3. Phenolic compounds – flavanoids

4. Sapogenines C-27 steroids
5. Iso prenoids and terpenoids
6. Polyisoprenes and rubber like substances
7. Rare amino acids ethers.

**Table-6.1 Source and Uses of Secondary Metabolites**

<b>Class</b>	<b>Example compounds</b>	<b>Example sources</b>	<b>Some effects and uses</b>
<b>A. Nitrogen containing</b>			
(1) Alkaloids	Nicotine cocaine theobromine	Tobacco coca plant chocolate (cacao)	Interfere with neurotransmission block enzyme action
<b>B. Nitrogen and Sulphur containing</b>			
(i) Glucosinolates	Sinigrin	Cabbage	
(ii) Terpenoids			
(iii) Monoterpens	Methanol linalol	Intand relative many plants	Interfere with neurotransmission block ion transport anesthetic
(iv) Sequiterpenes	Partenolid	Parthenium and relative (Asteraceae)	Contact dermatitis
(v) Diterpenes	Gossypol	Cotton	Block phosphorylation toxic
(v) Triterpenes, cardiac glycosides	Digitogenin	Digitalis (Foxglove)	Stimulate heart muscle, alter ion transport

Tetra terpenoids	Carotene	Many plants	Antioxidant : orange colouring
Terpene polymers	Rubber	Hevea (rubber) trees, deudation	Gum up insects, airplane tires
Sterols	Spinasterol	Spinach	Interfere with animal hormone action
Phenolics			
Phenolic acids	Coffeic, chlorogenic	All plants	Cause oxidative damage, browning in fruit and wine
Coumarins	Umbelliferone	Carrots, parsnip	Cross link DNA, block cell division
Lignans	Podophyllin urushiol	Mayapple positioning	Cathartic, vomiting allergic dermatitis
Flavanoids	Anthocyanin catechin	Almost all plants	Flower, leaf colour, inhibit enzymes, anti and prooxidants, estrogenic
Tannins	Gallotannin, condensed tannin	Oak, hemlock trees, birds foot trefoil, legumes	Bind to proteins, enzymes, block digestion, antioxidants
Lignin	Lignin	All land plants	Structure, toughness, fibre

Nitrogen and sulfur-containing chemicals, glucosinolates protect these plants from many enemies. The astringency of wine and chocolate derives from tannins. The use of spices and other seasonings developed from their combined uses as preservatives (since they are antibiotic) and flavourings.

### 1. Alkaloids

Alkaloids are secondary metabolites. They are primarily composed of nitrogen and are widely used in medicine. They can also be highly toxic.

**A. Morphine** was the first alkaloids to be found. Morphine comes from the plant *Papaver somniferum* or the Opium poppy.

It is used as a pain reliever in patients with severe pain leaves and cough suppressant.

**B. Cocaine** It can be highly dangerous and addictive. However it has also been used as an anesthetic. Cocaine has long been used by the people of South American to alleviate hunger. Cocaine derivatives are very dangerous which habitually used and can be deadly.

**C. Caffeine** While we use it to stay alert, it has protective properties for the plants it comes from cocoa coffee and tea seedlings of the coffee plant have a high concentration of caffeine. The high concentration is toxic and protects the seedlings from insects that want to shack on it. Toxic levels also have defense mechanism. It prevents the germination of any other plants in the area. This is referred to as allelopathy.

### 2. Terpenoids

Terpenoids are made of isoprene units and are found in all plants. They are the largest group of secondary metabolites and are very volatile, which means they evaporate easily.

1. **Isoprene** is a gas produced in the chloroplasts and released by the leaves. Isoprene is thought to protect the plants from heat.
2. **Essential oils** give plants their fragrance. We use essential oils for aromatherapy and medicine. In aromatherapy essential oils are thought to improve the mood and mental functioning.

Most of the time, essential oils are dangerous if consumed so they are usually applied topically or inhaled. They can be used for skin issue respiratory oilments and antiseptics.

3. **Taxol** which has become important in the medical field. It is used to treat ovarian and breast cancer. Taxol comes from the bark of the pacific yew tree. The bark produced very small amounts of taxol and the process killed the tree.
4. The final type of terpenoid is the one that is the most familiar to us rubber. It is the largest of the terpenoids because it contains over 400 isoprene units. Rubber is obtained from latex. Which is a fluid produced by Hevea brasilenis.

### **3. Phenols**

The phenols consist of a hydroxyl group (-OH) attached to an aromatic ring. Phenols are found in nearly all parts of the plant and in nearly every plant on the planet.

### **4. Flavonoid**

The first group of phenols is the flavonoids. Flavanoids are water-soluble pigments found in the vacuoles of plant cells. Flavanoids can be further divided into three groups.

(a) Anthocyanins range in color from red to blue and purple. The color depends on the pH of the environment. Anthocyanins are most commonly found in grapes, berries and have a wide range of health benefits. Anthocyanins are believed to protect against heart disease. The next two group have white or yellow pigments. They are called (b) flavones and (c) Flavonols. As a group, the phenols attract pollinators to the plants and even impact how plants act with one another.

### **5. Salicylic acid**

It is active ingredients in aspirin. It comes from the bark of the willow tree. It is used in numerous skincare products to treat acne, large pores and dermatitis.

### **6. Lignin**

It adds stiffness and strength to cell walls of the plant cells. Lignin is crucial to terrestrial plants because it supports the branches and size. It also allows the cell wall to be waterproof and protects the plant from fungal attacks.

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## 6.8 Germplasm Storage

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A germplasm is a collection of genetic resources for an organism. For plant, the germplasm may be stored as a seed collection (even a large seed bank) or for trees, in a nursery. Animal as well as plant genetics may be stored in a gene bank or cryobank.

- Germplasm conservation of any species opens many development methods to improving plant breeding so requires its conservation.
- Germplasm provided the genetic material of any species which uses for desire purposes to improving plant varieties.
- Conventionally germplasm stored as seed form at ambient temperature but many plants produced very short life seeds.

### 6.8.1 *ex-situ* conservation

In this germplasm preservation in gene banks it is most cheap way of conservation and easy to handling. Generally seeds or *in-vitro* maintained plant cells, tissues and organs are preserved under appropriate conditions for long-term storage as gene banks.

Conservation of plant genetic resources or germplasm by several national (National Bureau of Plant Genetic Resources = NBPGR) and international organizations. IBPGR now renamed as International Plant Genetic Resources Institute (IPGRI) has become a thrust area of biotechnology in recent years.

Germplasm can be stored in a variety of forms including seeds, buds, protoplasts, cells, tissue etc.

The technique involving storage of tissue in culture may use the germplasm in the growing stage and their growth may be suspended, by any one of the following methods.

- (i) Lowering the temperature
- (ii) Adding chemical retardant or hormones
- (iii) Reduction in oxygen concentration. Such methods where limited growth of cultures is allowed and remains effective for about a year. However the most popular and effective method for long term storage of cell cultures,

involves storage at a very low temperature using liquid nitrogen? This is described as cryopreservation, which allows storage for virtually indefinite periods.

### **6.8.2 *in-situ* conservation**

This method of conservation mainly aims at preservation of land races with wild relatives in which genetic diversity exists and or or in which the weedy or wild forms present hybridize with related cultivars. This mode of conservation has some limitations. However there is a risk of the material being lost due to environmental hazards. Further, the cost of maintaining a large proportion of available genotypes in nurseries or fields may be extremely high.

### **Methods of *in-vitro* conservation**

(1) Slow growth systems :

In this method cells & tissues can be stored at non-freezing condition in a slow growth state by a growth retardant, low temperature, high osmotic concentration of medium etc.

(2) Cryopreservation:

It is a procedure to preserve plant material in frozen and maintained at temperature of liquid N<sub>2</sub> (-196°c). At this temperature cells stay in completely inactive state.

(3) Freezing:

(A) **Slow freezing:** This method is mainly based on the physiochemical events occurring during the freezing this processes prevents the intracellular ice formation and consequently freezing injury is prevented.

(B) **Rapid freezing:** This method is unsuitable for the cryopreservation of cell cultures. It is employed to cryopreserve short tips.

(C) **Stepwise freezing** method is used for cryopreservation.

(D) **Dry freezing** method is used for cryopreservation.

(E) **Vitrification:** It is very simple method and does not require costly programmable freezing equipment. It is used for the cryostorage of cultured plant cell and organ is of recent origin.

**(F) Encapsulation:** This method is used in artificial or synthetic seed technology by coating somatic embryos in alginate beads. In this processes encapsulated material loses water by exo-osmosis further drying is carried out in laminar air flow for 2-6 hours and than transferred to liquid nitrogen.

### **Applications of Cryopreservation**

- (1) Conservation of genetic uniformity
- (2) Preservation of rare genomes
- (3) Freezing storage of cell cultures and cell lines.
- (4) Maintenance of disease free material
- (5) Cold acclimatization and frost resistance
- (6) Slow rate of metabolism and aging
- (7) Retention of morphogenetic potential in long term cultures.

### **Culture medium**

- It general MS required has been found satisfactory for most plant species.
- But for some species a much lower self concentration of MS medium may be adequate on even necessary since the high salt concentration of MS medium may be deleterious or even toxic e.g. blueberry  $\frac{1}{4}$  MS salt are best while full MS is after toxic.
- The GR requirement depends on the stage of culture process.
  - 1) Culture initiation
  - 2) Shoot multiplication
  - 3) Rooting of shots
  - 4) Transfer of plantlets to pot or soil

### **Culture initiations**

Consist of surface sterilization of expected establishing them *in-vitro* (for elimination control of contamination).

### **Shoot multiplication**

After 2-3 weeks, culture is transferred to a shoot multiplication medium designed to permote axillary branching.

Medium regularly certain cytokinin (usually 1-2 mg or 1 but up to 30 mg or 1 has been used).

BAP – Mostly used as cytokinin but some species like blueberry, garlic Z-ip is more effective.

- Auxin – NAA, IBA, IAA are used.
- GR combination should be determined to obtain optimum shoot multiplication ratio with the minimum risk of adventitious shoot bud.

### **Rooting of shoot**

For rooting auxin – NAA, IBA, IAA containing media are used.

In general the rooting medium has low salt, eg.  $\frac{1}{2}$  or even  $\frac{1}{4}$  salt of the MS medium is reduced sugar levels, reduced salt being essential for rooting in some species like Narcissius.

- Most species 0.1 – 1.0 mg or 1 NAA or IBA is required for rooting.
- In plant like citrus, however a pulse treatment with an auxin (10 mix with 100 mg or 1 NAA on IBA) gives optimum rooting.
- Rooting takes about 10-15 days, depending mainly on species, plantlets with 0.5 to 1 cm roots are usually transplanted into pots since longer roots tend to be damaged.
- The cut ends of shoots are treated with high relative humidity and low light intensity.
- This saves cost as rooting and soil transfer stages are combined and the rooting medium is eliminated.

### **Transfer of plantlets to soil**

- Roots shoots are removed from the medium
- Agar sticking to their roots washed with tap water, they are transplanted into plastic cups (pots).
- Plants are kept in high (90%) more humidity low light.
- High humidity can be attained beg.
  - (i) Fog (water drops 10  $\mu\text{m}$  or less)

- (ii) A clear plastic to cover individual or groups of plants.
- The humidity is gradually decreased to the ambient level about 7-15 days and light intensity is increased.

Plant are finally exposed to green house condition

Temp. – 25°C

Light – 1000 lux

Thermotherapy

Meristem culture

Viruses are eliminated by thermotherapy of whole plant in which plant are exposed to temperature between 35-40°C for few minutes to several weeks depending on the most virus contamination.

Thermotherapy is based on the fact that most viruses are killed at temperature much below those, which kills their host plant. Thermotherapy is usually effective against thread like viruses, micoplasma etc.

Thermotherapy is often combined profitably with meristem culture to obtain virus free plant. In general shoot tips are excised from heat treated plant environment cultured meristem may also be given thermotherapy, former is preferably since larger explant can be safely taken from heat treated plant (this ↑ the survival rate of explant).

Potato viruses as (PVS) and potato virus (X) PVX are not eliminated either by T.T. or meristemic culture alone but both these viruses have been eliminate by culturing meristem from heat treated plant.

The duration of treatment may vary from few to several days ex. 8-18 weeks for PVX, depending from the host virus combination this is perhaps most successful approach of virus elimination.

### **Cryotherapy**

More recently, prolonged exposure to a low temperature 5° followed by shoot tip culture has proved quite successful virus elimination. This is after called CT. eg. Shoot tip culture from.

*Crysanthemum* plant treated with 5°C for 4 month yield 67% plant free from *Crysanthemum* Stunt Virus (CSV). The frequency of virus free plant increases upto 73% in case of CSV after a 9-5 month exposure to 5°C.

Cold treatment is preferable to heat treatment as it is less injuries to the plant and often more effective in virus elimination.

### **Chemotherapy**

Some chemicals like virazol (ribavirin) actinomycin-D, cycloheximide etc. which interface with virus multiplication may be added into culture medium for curing the shoot tip of viruses is known as chemotherapy e.g. virazol was shown to irradicate Potato Virus (PVX) from shoot dried from tobacco protoplast. Virezol is effective against a range of animal DNA and RNA viruses.

### **Virus Indexing**

It may be emphasized that all the plants obtain than meristem culture, with or without chemo and thermotherapy are not virus free deference, such plant have to be tested for the presence of conserved virus this is called Virus Indexing. The simplest method for virus indexing is to score the test plant for the presence of specific symptoms produced by the relevant virus however the visible symptoms may take considerable time.

Alternatively the saps from test plant may be used to inoculate highly sensitive and healthy indicator plant. An indicator plant for a virus is the plant species or variety, which is highly susceptible to the virus and readily developed the symptoms.

### **Maintenance of virus free stocks**

Virus free plants are maintained in insect free glass house. A large scale multiplication of such plant can be done in area where during season the chances of infection by the virus are minimal.

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## **6.9 Summary**

This unit emphasizes the fundamental aspects for production of disease free plants and organic haploids embryo rescue, somaclonal variations, distant hybridization, production of artificial seed, production of secondary metabolites germplasm a storage. The aspects further will enrich the knowledge about the recent trends in biotechnology and principles and processes of genetic engineering.

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## 6.10 Glossary

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- **Disease free:** Free from any known disease
  - **Secondary metabolites:** Metabolites synthesized by plant besides primary metabolites, which are not essential for its survival.
  - **Somaclonal variation:** The variability generated by the use of a tissue culture cycle.
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## 6.11 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Define propagation.
2. What is clonal propagation?
3. Define germ plasm.
4. Define somaclonal variations.

### Section B : (Short Answer Type Questions)

1. What is androgenic haploid?
2. What are secondary metabolites?

### Section C : (Long Answer Type Questions)

1. Write note on production of secondary metabolites.
  2. Write an essay on applications of plant tissue culture.
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## 6.12 References

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## Unit -7

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# Germplasm Storage and Cryopreservation

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### Structure of the Unit:

- 7.0 Objectives
- 7.1 Introduction
- 7.2 Germplasm Storage
- 7.3 Cryopreservation
- 7.4 Cryopreservation and Regeneration of Plants
  - 7.4.1 Raising sterile tissue cultures
  - 7.4.2 Addition of cryo-protectant
  - 7.4.3 Freezing
  - 7.4.4 Thawing
  - 7.4.5 Washing and Reculturing
  - 7.4.6 Determination of Viability
  - 7.4.7 Plant Growth and Regeneration
- 7.5 Slow Growth Method
- 7.6 Advantages and disadvantages of Cryopreservation
- 7.7 Future Prospects
- 7.8 Summary
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- 7.10 Self-Learning Exercises
- 7.15 References

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### 7.0 Objectives

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After studing this unit you will able to understand about:

- Germplasm Storage
- Cryopreservation and method of Cryopreservation
- Advantages and future projects of Cryopreservation

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## 7.1 Introduction

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India is a major center of origin and diversity of crop plants. It occupies a special significance in gene rich countries because of its immensely rich landrace diversity in major agri-horticultural crops and their wild relatives.

Germplasm conservation technique provides a long term storage method for conservation of plant genetic resources which is not possible by conventional method.

The valuable plant genetic resources are however being lost at very fast rate because of their replacement by high yielding varieties or due to heavy pressure on their nature habitats. The *in-vitro* techniques have contributed significantly for the conservation of germplasm of elite varieties particularly in difficult species.

The conventional method of seed storage used to be the way of germplasm storage but it has limitations.

Conventionally, germplasm is conserved as seeds stored at ambient temperature, low temperature or ultra low temperature but sometimes it's not possible to store seeds because

- (i) some plants do not produce fertile seeds.
- (ii) some seeds do not remain viable for longer time
- (iii) some time seeds of certain species deteriorate rapidly due to seed borne pathogens.
- (iv) few seeds are very heterozygous and not suitable for maintaining true to type genotype.

On going research on high yielding varieties of crop plants that have resistance to pathogens and pests warrants the viability and maintenance of large collection of germplasm.

Recently Cryopreservation is being increasingly used to conserve endangered species and tropical rain forest trees.

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## 7.2 Germplasm Storage

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Modern agricultural practices and other developmental activities have caused a rapid decline in genetic variability which is caused **genetic erosion**. Because of genetic erosion, it is necessary to conserve germplasm. The sum total of all the genes present in crop and related species constitutes its "**Germplasm**".

Germplasm is represented by a collection of various strains and species. Germplasm is the raw material (gene) used by breeder to develop crop varieties. We can say, germplasm is basic indispensable ingradient of all breeding programme. So great emphasis has been place on collection, evaluation and conservation of germplasm.

Conventionally, germplasm is conserved as seeds, stored at ambient temperature, low temperature or ultra low temperature. But it has limitations because many crops produce recalcitrant or short lived seeds.

The Germplasm conservation technique provides a long term storage method of conservation of plant genetic resources which is not possible by conventional preservation method. In clonal crops, seeds are not ideal material to conserve because of genetic heterogeneity. Crops modified by genetic engineering may be unstable and should be conserved for future use.

***In-vitro*** techniques for germplasm storage hold great promise for clonally propagated crops and also for those crops having recalcitrant seeds or short lived seeds. The ***in-vitro*** technique provides an ideal tool for germplasm collection. There are two methods of germplasm conservation: (i) ***in-situ*** methodology (ii) ***ex-situ*** methodology

- (i) ***in-situ* methodology:** This methodology involves the conservation of genetic resources within their natural habitats as wild species or cultivated field in a farmer's field.
- (ii) ***ex-situ* methodology:** Genetic resources are conserved away form their place of origin. Seed bank, field gene bank, botanical gerdens, DNA or pollen bank falls under the ex-situ conservation. This *ex-situ* germ plasm conservation is often applied for plants which are on the verge of extinction or face the hazard of destruction.

Approaches of Germplasm Conservation are:

- (i) Freeze preservation or Cryo-preservation.
- (ii) Slow growth cultures.
- (iii) Dессicated somatic hybrids or artificial seeds
- (iv) DNA clones.

Cryopreservation of genetic resources is done at ultra temperature at  $-196^{\circ}\text{C}$  under the influence of liquid nitrogen. Very low temperature restricts cell division and metabolic activities so that plant material can be stored for long periods. The classic examples of *Cocos-nucifera* (coconut) and *Theobroma cacao* (cocoa) have well proven the role of in-vitro technology in germplasm conservation.

This storage procedure is increasingly being used to conserve crop plant germplasm. It can be applied to a wide range of organisms and biological tissues.

### 7.3 Cryopreservation

Cryopreservation is a procedure to preserve viable cells, tissues and organs at ultra low temperature. This storage procedure is increasingly being used to conserve crop plant's germplasm. It provides a long term storage method for the conservation of plant genetic resources.

Cryopreservation is a Greek word (kryos = frost). It means preservation in the frozen state. Generally it meant to be storage at very low temperatures e.g. over solid carbon dioxide ( $-79^{\circ}\text{C}$ ), at low temperature in deep freezers ( $-80^{\circ}$  or above) and in liquid nitrogen ( $-196^{\circ}\text{C}$ ).

Generally plant material is frozen and maintained at the temperature of liquid nitrogen ( $-196^{\circ}\text{C}$ ). At this temperature, cells stay in completely inactive state. During freeze preservation, water present in the cells, turns in to solid state. But generally pure water freezes or becomes ice at  $0^{\circ}\text{C}$ . But water inside the cell, needs much lower temperature to turn in to solid state (ice) because of freezing point depression by salts or organic molecules.

Freezing and storage of tissue cultures in liquid nitrogen at  $-196^{\circ}$  is of great value in the conservation of germplasm especially of those crops which normally do not produce seeds e.g. root and tuber crops or where seed storage is not desirable.

The preservation of cells, tissues and organ in liquid nitrogen is called **cryopreservation** and the science pertaining to this activity is known as **cryobiology**. Storage at a very low temperature slows down or halts metabolic processes and biological deterioration. Cryopreservation of plant cells, organs and tissues received widespread importance due to emphasis on ***in-vitro*** manipulations with cultured cells.

In practice, meaning of cryopreservation is storage at very low temperature, like

- (i) over solid carbon dioxide ( -79°C)
- (ii) at low temperature in deep freezers ( - 80°C and above)
- (iii) in vapour phase of nitrogen ( -150°C)
- (iv) In liquid nitrogenat (-196°C).

At this state, cells stay completely in inactive state. Very low temperature usually restricts the cell division and metabolic activities. So plant material can be stored for long periods of time.

There are following approaches for germplasm conservation:

- (i) Freeze preservation
- (ii) Slow growth cultures
- (iii) Desiccated somatic embryos, artificial seeds
- (iv) DNA clones

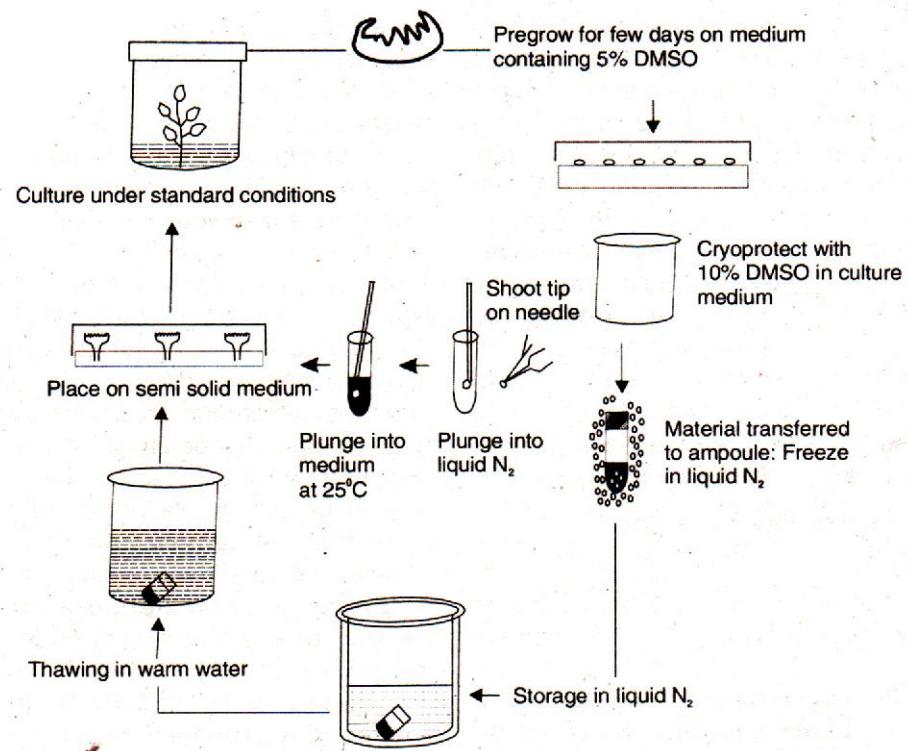
*in-vitro* conservation has a number of advantages over *in-vivo* conservation.

These Advantages are as follows:

- (i) *in-vitro* culture conserves that spp. which are in danger of being extinct.
- (ii) *in-vitro* storage of vegetatively propagated plants saves storage and time.
- (iii) Sterile plants can be maintained *in-vitro*.

Cryo-preservation is the only method available for long term storage of vegetatively propagated plant species, cells, protoplasts, shoot meristems, somatic or zygotic embryos and whole seeds at liquid nitrogen.

Cryopreservation offers a method to store the plant genetic resources in a limited space, under sterile conditions in a cost effective manner for unlimited periods of time. This procedure is gaining popularity for application for long term conservation of a wide variety of economically important plants, elite genotypes and germplasm of many endangered species.



**Fig. 7.1: Standard protocol for Cryopreservation of shoot tips**

Theoretical basis of freeze preservation is the transfer of water present in the cell to the solid state. As we know pure water becomes ice at 0°C but cell water needs much lower temperature because of freezing point depression by salts or organic molecules. There are two things which should be brought under consideration for freezing of specimens.

- I. degree of freeze tolerance shown by a given genotype to reduce temperatures.
- II. formation of ice crystals within the cell.

## 7.4 Cryopreservation and Regeneration of Plants

- (i) Raising sterile tissue cultures
- (ii) Pretreatment and addition of cryo-protectants
- (iii) Freezing
- (iv) Storage
- (v) Thawing

- (vi) Determination of survival or viability
- (vii) Plant growth and regeneration

#### **7.4.1 Raising sterile tissue cultures**

The morphological and physiological conditions of the plant material affect the ability of an explant to survive freezing at  $-196^{\circ}\text{C}$ . Generally small richly cytoplasmic meristematic cells survive better than large highly vacuolated cells. Cell suspension has been successfully frozen in number of species.

A rapidly growing stage of callus (one or two weeks after subculture in the medium) has been proved best for cryopreservation. Organized structures such as shoot apices, embryos or young plantlets are preferred for cryopreservation over the cultured cells. Old cells on the top of callus and blackened area should be avoided for cryopreservation.

Besides, apical meristem, lateral meristem, plant organs (embryo, endosperm, ovules, pollen or anther) seeds, somatic embryo, protoplast calluses are used for freezing.

Plant meristems are often preferred for preservation. It has been observed, water content of cells, tissues or plant organ for cryopreservation, and should be low because with low freezable water tissues can withstand extremely low temperature.

#### **7.4.2 Addition of cryo-protectant**

The tissue which is prepared for freezing is treated with various cryo-protectants. This helps to withstand the freezing conditions of  $-196^{\circ}\text{C}$ . The effect of temperature on plants depends on genotype environment and also on physiological condition. Genotype is unchangeable factor but other two factors can be altered.

Culturing of shoot apices for a brief period under optimum conditions before freezing has been proved beneficial. There are two reasons of cell damage during freeze preservation.

- I. Because of formation of large ice crystals in the cells leading to the rupture of organelles and the cell itself.
- II. Intra cellular concentration of solutes increases to toxic levels.

Cryo-protectant controls the formation of ice crystal in cells and protects the cells from the toxic solution effect. There are large number compound which have cryo-

protective properties. e.g. glycerol, dimethyl sulfoxide (DMSO) sucrose, mannose, ribose, glucose, acetamides glycals (ethylene, diethylene, Propylene), proline etc. Most frequently used are DMSO, Sucrose, glycerol and proline.

### **Role of Cryoprotectant**

Cryoprotectant depresses both the freezing point and super cooling point of water. DMSO has been proved an excellent cryo-protectant because of its good properties

- (i) It has low Molecular Weight
- (ii) It is easily miscible with the solvent
- (iii) Non toxic at low concentration
- (iv) Easily permeable in to cells and easily washable from cells

Generally DMSO at concentration of 5-10 % and Glycerol at (10-20%) is adequate for most material. Some times a mixture of cryo-protectant has been proved beneficial.

Sorbitol does not enter the cells which actually reduce cellular water content hence decrease the rate of initial ice crystal formation. DMSO enters the cells and reduces cellular dehydration during freezing. Thus initial ice crystal formation and subsequent dehydration during freezing are reduced when two different cryo-protectants are used.

In the practice, material suspended in the culture medium and treated with a suitable cryo-protectant is transferred to sterile polypropylene cryo-vials or ampoules with a screw cap.

A dilute solution of the cryo-protectant e.g. 5-10 % DMSO is added gradually or at intervals to the ampoules to which contain the tissue materials to prevent plasmolysis and protects cells against osmotic shocks.

There should be an interval of 20-30 minutes after the last addition of cryoprotectant and cooling.

The ampoules containing the cryoprotectants are then frozen.

### **7.4.3 Freezing**

The type of crystal water within the stored cells is very important for survival of the tissues. Three different type of freezing procedures have been developed (many

type of freezing units and cryostats are available by which different cooling rate can easily be regulated.

(i) **Rapid freezing:** Rapid freezing has been employed to cryopreserved shoot tips of carnation, Potato, strawberry and somatic embryos.

In this type of freezing, the plant material is placed in vials and plunged in to liquid nitrogen which has a cooling rate of – 300 to – 3000° C per minute or more.

This is a simple method technically. If the freezing is done quicker, then intra cellular ice crystals are smaller.

(ii) **Slow freezing:** In this method, tissue is slowly frozen with a temperature decrease of 0.1-10 °c or minute from 0°C to - 100°C and then transferring to liquid nitrogen. Slow freezing may have beneficial effect of dehydration which reduces the amount of water that freezes intra-cellularly.

Slow cooling permits the flow of water from the cells to outside. This phenomenon promotes extra cellular ice formation instead of lethal inter-cellular freezing. It has been observed that on extracellular freezing the cytoplasm will be effectively concentrated and plant cell will survive better when adequately dehydrated.

Example of cryopreservation of meristem includes diverse species as peas, strawberry, potato, cassava etc.

(iii) **Stepwise freezing:** This method includes the advantages of both rapid and slow methods. A slow freezing procedure initially to -20 to -40°C allows protective dehydration of the cells. An additional rapid freezing in liquid nitrogen prevents the growing of big ice crystals in the biochemically important structures. Stepwise freezing gives very good result in suspension cultures and in strawberry.

(iv) **Storage:** Storage of frozen material is also a crucial issue. It is as important as freezing.

- Frozen cells and tissues are immediately kept for storage at temperature ranging from -70°C to – 196°C.
- A liquid nitrogen refrigerator running at -150°C in the vapor phase or – 196°C in the liquid phase is ideal for this purpose.
- For long term storage of cells, the temperature should be sufficiently low.

- Low temperature during storage of cells stops all metabolic activity of cells and prevents biochemical injury.
- The best long term storage is done at -196° C so regular supply of liquid nitrogen should be maintained in liquid nitrogen refrigeration to maintain the frozen material.
- The injury in cells is generally caused when they are not stored at sufficiently low temperature.
- Popov (1968) stored the cultures of carrot cells for about 5 years at -196°C.

#### **7.4.4 Thawing**

It is the process of releasing the vials containing cultures or ampoule containing sample in a warm water bath (35-45°c) from frozen state. It should be done quickly without over heating. It is important for survival of the tissue that the tubes should not be left in the warm water bath, after the ice melts.

Just at the point of thawing, quickly transfer the vial or ampoule in bath tub maintained at room temperature (20-25°c) (popov, 1985) and continue the swirling action for 15 second to cool the warm walls of the tubes.

During the process of freezing and thawing, major biophysical changes occur in the cell. The freshly thawed cells need suitable nourishment because they are prone to further damage.

#### **7.4.5 Washing and Reculturing**

Washing of plant materials is done to remove the cryoprotectants. If the nontoxic or less toxic cryoprotectants are used, the cultures should not be washed. They can simply be recultured. If the Cryoprotectants are toxic, washing involves following steps:

(i) Dilution (ii) Resuspension (iii) Centrifugal and removal of cells.

However, it is possible that some cells die due to storage stress and the most stable ones survive. So determination of cell viability by culturing them on growth medium is essential.

#### 7.4.6 Determination of Viability

Regrowth of plants from stored tissues or cells is the only realistic test of survival of plant materials. Cells viability test can be done by using FDA staining and growth measurement.

The parameters of growth measurements are counting cell numbers, dry and fresh weight, mitotic index etc. Mitotic index is counted by using the following formulas.

**Number of cells destined to cell division (i.e. P + M+A+T)**

$$\text{MI} = \frac{\text{Number of cells destined to cell division (i.e. P + M+A+T)}}{\text{Total number of cells both dividing and undividing}}$$

P= Prophase

M= Metaphase

A= Anaphase

T= Telophase

Other staining methods are TTC (triphenyl tetrazolium chloride) method and Evan's blue method.

#### 7.4.7 Plant Growth and Regeneration

Regrowth of cryopreserved material would depend upon the manner in which specimens are handled e.g. plating of cells without washing away the cryoprotectants, Incorporation of special additives in re-growth medium and the physical environmental conditions, the cells are exposed to during the early stage of re-growth.

There are few examples where plant growth pattern was affected by washing the stored material e.g. in *Gossypium hirsutum*, washing of cell suspensions and embryogenic callus cultures prevented their growth.

In *Saccharum* – without washing of culture is deleterious.

Addition of certain compounds in the media during re-growth has shown increased surviving ability e.g. GA3 in the medium for freeze preserved shoot tips of tomato, activated charcoal for carrot.

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## 7.5 Slow Growth Method

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Cell and tissues can be stored at non freezing conditions in a slow growth state.

In this method, shoot cultures are maintained in a state of slow growth imposed by a growth retardant, low temperature and elevated osmotic concentration of the medium. Other factors like size of culture vessel, nutrient restriction and gas restriction also affect culture growth. In this method, water in the tissue is maintained in the liquid condition. Factors which make the growth of cultures slow are following---

### **Low temperature**

Lowering the surrounding temperature to 2-18°C retards the growth of cultures and they require infrequent sub culturing.

Temperate species can tolerate temperatures between 5 and 10°C. Tropical species show chilling damage at these temperatures. They show satisfactory growth at 15-20 °C.

### **Nutrient Restriction**

To make the growth of cultures slow, concentration of nutrients can be lowered. Altering the concentration of sugar in the medium may make the slow growth of culture.

In many species, reduction of MS salts to 75, 50 or 25 percent sugar of the full strength reduces growth of plantlets in many species.

In case of Papaya, substitution of 1 % fructose for 2% sucrose significantly reduced the growth rate of single node explants.

Another example is grape shoot splices grown on MS medium with 6% of ammonium nitrate showed 70 – 80% survival and greatly reduced the growth rate.

### **Growth Regulators or Use of growth retardants**

For delaying culture growth, some plant growth retardants can be used e.g. Cycocel (CCC), Abscisic acid (ABA) Tri iodobenzoic acid (TIBA) Paclolobutrazole, daminozide.

Absicic acid retards shoot growth and increase the shelf life of cultures. Under standard tissue culture condition, ABA is known gibberelins antagonist. TIBA Inhibits polar transport of auxins the growth regulator that promotes plant growth.

### **High Osmoticum**

Culturing of explants under high osmotic concentrations created by high levels of sucrose, mannitol or sorbitol prolongs the storage duration. For up to 30 months. Very high concentrations (100 and 171.2 g or l<sup>-1</sup>) of sucrose have been used for extended storage of cultures. Using high osmoticum virus free strawberry plants were maintained for 6 years at 4°C. Grape plants have been stored for 15 years at 9°C by yearly transfer the fresh medium. It has been concluded that when cost and risk of culture loss are considered, storage at 25°C under standard culture conditions with sub culture every 10-12 months is the most suitable protocol.

### **Culture vessel**

The size and type of culture vessel may markedly affect contamination risk and even culture survival. Storage of straw berry plantlets in-vitro in 5- section Polythene bags (having gelrite medium at 4 °c) showed higher survival rate in plastic boxes and culture tubes.

### **Restricted illumination**

Use of reduced light intensities or total darkness with reduced temperature helps in retarding culture growth. Besides, quality of light during storage also affects the quality of plantlets. White light is preferable to red and blue light.

### **Lower O<sub>2</sub> Concentration**

Growth of tissue cultures declines when O<sub>2</sub> partial Pressure decreases below 50 mm Hg. It has been observed that hypoxic conditions partially replace low temperature for culture storage.

### **DNA clones**

Germplasm can also be conserved in form of DNA segments cloned in a desirable vector e.g. Cosmids, Phasmids or YACS. The technique to conserve DNA segment is highly sophisticated, demanding and expensive. It is used to conserve valuable genes or DNA segments from threatened species.

### **Dessication**

Dessicated Somatic embryos and artificial seeds can be stored at low temp – ( $4^{\circ}\text{C}$ ) or ultra low ( $-20^{\circ}\text{C}$ ) temperature for prolonged periods like zygotic seeds. This approach involves complicated dessication procedures and it can be applied only to those species or genotypes where somatic embryo regeneration occurs in adequate frequency.

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## **7.6 Advantages & Disadvantages of Cryopreservation**

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### **Advantages**

The *in-vitro* techniques hold great promise for clonally propagated crops and also for those crops having recalcitrant or short lived seeds. The potential advantages of these techniques are as follows.

- (i) Requirement of relatively very small space
- (ii) Storage of germplasm free from diseases and other pathogens
- (iii) Storage over long period.
- (iv) Reduced risk of errors in labeling
- (v) Such materials are ideal for germplasm exchange.

### **Disadvantages**

- I. For DNA cloning and freeze preservation, sophisticated techniques are required.
- II. This technique demand greater skill in handling and maintenance.
- III. Cell or tissue may damage during cryo-preservation. DNA may also become damaged due to Cryopreservation under sub optimal condition.
- IV. Shoot tip derived plants may show genetic instability at least in some species.
- V. Cryopreservation procedures are genotype dependant.
- VI. Cost of maintenance of large collection is very high.
- VII. Slow growth cultures are vulnerable to contamination.

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## **7.7 Future Prospects**

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Loss of biodiversity is a matter of great concern as it has threatened the survival of mankind. Genetic erosion is a matter of concern all around the world.

The aim of Germplasm conservation is to ensure the availability of useful germplasm at any time. Plant genetic resources are a reservoir of genetic adaptability which acts as a buffer against potentially harmful environment and economic change.

Germplasm storage in-vitro is crucial for the future development and safety agriculture. Through Germplasm conservation, a large number of plant species can be maintained by cryopreservation of cultured embryos, tissues, cells or protoplasts. Pathogen free stocks of rare plant materials will be able to freeze, revive and propagated when there would be need or requirement. This cryopreservation technique would prove ideal approach for international exchange of such materials.

In future, through this technique large number of cultures of asexually propagated and woody crops can easily be maintained for example in NBPGR (National Bureau of Plant Genetic Resources) for maintenance of cultures, slow growth approaches are being used.

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## 7.8 Summary

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Germplasm is the collection of different strains and related species of the concerned crop. Germplasm conservation in-vitro is crucial for the future development and safety agriculture. Germplasm conservation is done by protecting areas of diversity, through seed bank and field collection. The in-vitro system is extremely suitable for storage of plant genetic material because it can be stored on small scale, disease free and under those conditions which limit growth.

The aim of germplasm conservation is to ensure the availability of useful germplasm at any time. In-situ and ex-situ are two methodologies by which conservation of genetic resources can be accomplished.

Storage in seed banks, field gene banks, botanical gardens, DNA or pollen banks fall under ex situ conservation. The in-vitro techniques have contributed significantly for the conservation of germplasm of elite varieties particularly in difficult species. The breeding programme heavily rely on germplasm. Genetic erosion due to various reasons has created aggravated of germplasm conservation.

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## 7.9 Glossary

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- **Germplasm:** The sum total of all the genes present in a crop and its related species constitute its germ plasm.
- **Cryo-preservation:** Storage and preservation of cell tissue and organ in liquid nitrogen at ultra low temperature of -196 °c is called Cryo-preservation.
- **Cryo-protectant:** An agent able to prevent freezing and thawing damage to cells. They prevent the formation of ice crystals in cells and protect from toxic solution. Agents like PEG, mannitol, sorbitol which maintains the osmotic potential of a nutrient medium equivalent to that of culture cells.
- **In situ.** In original or natural place.
- **Thawing:** The process in which frozen material is plunged in the vials in to water at 37- 40°c for 90 seconds. Then material is transferred in to ice bath till it is re-cultured.
- **DMSO:** Dimethyl Sulfoxide used as **cryoprotectant**.
- **CCC:** Chlormequat (chemical added as growth retardant)
- **NBPGR:** National Bureau of plant genetic resource.
- **TIBA:** Tri iodo benzoic acid.
- ***in-situ*** : In original or in natural place.

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## 7.10 Self-Learning Exercises

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### Section A : (Very Short Answer Type Questions)

1. Cryopreservation is carried out at an ultra low temperature of .....?
2. What do you understand with “cryopreservation”?
3. Which cryo-protectant is considered superior to other cryo-protectant?
4. Name four cryo-protectants.
5. What is “Plant genetic resources”?
6. Name two plant germplasm conservation centers of India.

7. What are the two approaches of conservation of plant genetic resources?
8. Name anti-stress agents used at the time of pre-treatment before cryo-preservation.

### **Section B : (Short Answer Type Questions)**

1. Define germplasm collection and genetic erosion. What are the causes of genetic erosion?
2. Discuss the use of plant tissue culture for germ plasm storage.
3. What is the need to conserve plant genetic resource?
4. What are different steps of Cryopreservation protocol? Explain in brief.
5. Write a note on “cell cryobank”.

### **Section C : (Long Answer Type Questions)**

1. Discuss the various techniques for Cryopreservation used for germ plasm conservation.
2. Write note on:  
(i)“Cryobiology” (ii) “Germplasm bank”
3. Discuss the difficulties associated with Cryopreservation of genetic stock.
4. Discuss achievements through cryo-preservation.

### **Answer key of section A**

1. 196 °C
2. Cryopreservation is a procedure to preserve viable, cells, tissues and organ at ultra low temperature.
3. DMSO = Dimethyl sulphoxide.
4. Cryo-protectant = PVP (Poly vinyl pyrrolidone) sorbitol, glycerine, mannitol, proline, alcohols, glycals.
5. Plant genetic resources are actually the genetic material of plant which determines the characteristics of plants hence their ability to adapt and survive.
6. (i) N B P G R (National Bureau of plant genetic resources ), New Delhi  
(i) C.I.M.A.P. Central Institute of medicinal and aromatic plant, Lucknow  
(iii) I.I.S.R. Calicut. Indian Institute of Spices Research, Calicut
7. ( i) Cryopreservation (ii) slow minimal growth

8. Anti-stress agent: proline, abscisicacid, trehalose.

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## Unit - 8

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# Genetic Engineering: An Introduction

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### Structure of the Unit:

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  - 8.1 Introduction
  - 8.2 Genetic Engineering Revolution
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    - 8.3.1 Restriction enzymes
    - 8.3.2 Vectors (The carriers of DNA molecules)
      - 8.3.2.1 Plasmid
      - 8.3.2.2 Bacteriophage
      - 8.3.2.3 Cosmids
      - 8.3.2.4 Phasmids
      - 8.3.2.5 Others Vectors
  - 8.4 Technique Involved in Genetic engineering
    - 8.4.1 Gene Cloning
    - 8.4.2 Southern Blotting
    - 8.4.3 Northern Blotting
    - 8.4.4 Replica Plating
    - 8.4.5 Polymerase Chain Reaction
    - 8.4.6 Advanced PCR Techniques
  - 8.5 Summary
  - 8.6 Glossary
  - 8.7 Self-Learning Exercise
  - 8.8 References
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## 8.0 Objectives

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After going through this unit you will be able to understand about :

- Origin and history of genetic engineering

- Basic principal of genetic engineering
  - Type and functioning of restriction endonuclease and their use in genetic engineering
  - Types of vector and their uses in genetic engineering
  - Various techniques used in genetic engineering such as gene cloning southern blotting northern blotting, replica plating and polymerase chain reaction (PCR)
- 

## 8.1 Introduction

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In the early 1970s, a scientific experiment changed the relationship of human kind to the fundamental processes of nature. For the first time, DNA—the essence of heredity—was purposefully transferred from one species of organism, *Xenopus laevis* (the African clawed frog) into another species, *Escherichia coli* (the common human intestinal bacterium). Nothing exciting happened. The bacteria grew normally, blithely replicating the piece of foreign DNA from another species that had been inserted into a carrier bacterial DNA plasmid in their cytoplasm. Although this experiment was in itself only an incremental extension of previous work, the workers in Stanley Cohen's and Herbert Boyer's laboratories had prepared the frog-bacteria plasmid in a test tube using isolated bacterial enzymes to cut and paste the DNA fragments together in a specific order. They had become genetic engineers, rearranging the DNA code for their own purpose. This simple demonstration ushered in the age of genetic engineering. Optimists foresaw that bacteria, yeast, plants, and animals could be modified to produce raw materials for industry, to improve food, to discover new medicines, to remove environmental contaminants, to recycle waste, and to provide permanent cures for inherited diseases. But there was a cloud over this vision. To some people's minds, genetic engineering changed the world's natural order by mixing genes from one species with those of another species. Ahead lay catastrophic disruption of the earth's ecosystem, uncontrolled spread of microorganism antibiotic resistance with attendant new plagues, and corruption of the ideal of the sanctity of life itself.

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## 8.2 Genetic Engineering Revolution

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Genetic engineering is normally taken to mean recombinant DNA technology means altering the genes in a living organism to produce a new genotype or the artificial addition, deletion or rearrangement of sequences of bases in DNA in order to alter the observable form and function of an organism. However, genetic engineering is sometimes referred to as biotechnology and biotechnologists have been quick to point out that mankind has been doing biotechnology ever since the first farmers some 10,000 years ago started to domesticate wild species by selecting seeds of wild plants for cultivation or selectively breeding wild animals. In this history, therefore, we include any artificial intervention in the reproductive process as well as some landmarks in the development of genetics which has made genetic engineering possible. This will include selection procedures old and new which could influence the genetic outcome of a reproductive event, gene therapy and diagnosis as well as cloning, vegetative reproduction and *in vitro* (test-tube) reproduction procedures.

**1869:** Friedrich Miescher discovered

**1960:** Stewart Linn & Werner Arber discovered restriction enzymes in *E. coli*. These enzymes known as endonucleases 'cut' DNA at specific sites determined by the adjacent base sequences. Since then hundreds of these enzymes have been discovered in different micro-organisms and together they comprise one of the most important tools in the genetic engineer's toolbox. The property of restriction enzymes to create 'sticky ends' in the 'cut' DNA allows fragments of DNA of known properties and composition to be 'spliced' into the DNA 'chain'.

**1971:** Takebe et al. regenerated Tobacco from protoplasts

**1972:** Paul Berg made the first recombinant DNA *in vitro*.

**1973:** Herbert Boyer & Stanley Cohen performed the first recombinant DNA cloning experiment using restriction enzymes discovered only a few years before to insert DNA into a plasmid and use the transgenic plasmid (a circular bacterial 'chromosome') to transform bacteria.

**1975:** The "Council of Asilomar", a conference in the USA at which the "molecular bishops" met to discuss how the newly discovered and potentially very dangerous recombinant DNA technology (genetic engineering) should proceed.

J. B. Gurdon and others: cloned frog keratinocytes (skin cells) survived to the tadpole stage.

O' Farrel developed 2D gel electrophoresis that led the development of Proteomics  
The British biologist Edwin Southern discovered southern blotting for detection of a specific DNA sequence in DNA samples.

1976: Chien et al. isolated a thermostable DNA polymerase *Taq* polymerase from bacterium *Thermus aquaticus* and it is frequently used in polymerase chain reaction (PCR).

**1977:** Walter Gilbert and Frederick Sanger worked out methods to determine the sequence of bases in DNA.

Frederick Sanger determined the base sequence of an entire viral genome (f X174).

Phillip Sharp, Richard Roberts, and others discovered interruptions (introns) in genes.

The northern blot technique was developed by James Alwine, David Kemp, and George Stark at Stanford University to study gene expression by detection of RNA (or isolated mRNA) in a sample.

**1978:** Birth of Louise Brown (25<sup>th</sup> July) -- the first 'test-tube' baby born from *in-vitro* fertilization (IVF).

**1979:** First production of insulin through genetic engineering.

**1980:** First transgenic (genetically modified) mouse.

**1983:** Kary Mullis, a biochemist invented the polymerase chain reaction or polymerase chain reaction which is a technique enabling scientists to reproduce bits of DNA faster than ever before. (Mullis was awarded the Nobel Prize for this in 1993)

**1984:** Development of genetic fingerprinting by Alec Jeffreys, a technique that has greatly helped the police force in finding and identifying criminals.

De block et al and Horsch et al. developed Transgenic Tobacco by using Agrobacterium

**1990:** Williams et al., Welsh and McClelland developed RAPD

**1995:** Vos et al. developed AFLP technique

**1996** - Ian Wilmut and Keith Campbell cloned the first animal from adult cells. Dolly the sheep, born on July 5, 1996, was created using the so-called Roslin

Technique (see "How was Dolly clone?" section of this webpage). The cloning of Dolly is one of the most important milestones in the history of animal cloning, as it proves that cloning of adult animals is possible.

**1997:** Sequencing of E coli genome (Blattner *et al.*)

### **8.3 Basic Concept and Principal of Genetic Engineering**

Genetic engineering or recombinant DNA (rDNA) technology involves artificial transfer of genes or gene fragments from one organism to another to produce novel traits in the recipient living organism. The development of Genetic Engineering permitting the transfer of genetic material between widely divergent species has opened a new era of research into the structure and function of the genome. The Genetic Engineering is defined as "the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation.

Various kinds of genetic modification are possible:

- inserting a foreign gene from one species into another
- altering an existing gene so that its product is changed
- changing gene expression so that it is translated more often or not at all

The ability to manipulate DNA in vitro depends entirely on the availability of purified enzymes that can cleave, modify and join the DNA molecule in specific ways. At present, no chemical method can achieve the ability to manipulate the DNA in vitro in a predictable way. Only enzymes are able to carry out the function of manipulating the DNA. Each enzyme has a vital role to play in the process of genetic engineering. The various enzymes such as Restriction enzymes; Nucleases; DNA ligase; Kinase; Phosphatase; Reverse transcriptase; Terminal Deoxynucleotide Transferase; RNaseP etc. are widely used in genetic engineering.

The functions of the various enzymes used in genetic engineering are depicted below:

### 8.3.1 Restriction Enzymes

It is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites. In bacteria, restriction enzymes form a combined system with modification enzymes that cause methylation of the bacterial DNA (restriction + modification system). The methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from restriction enzyme that cleaved the recognition sites.

There are two different kinds of restriction enzymes:

(1) **Exonucleases:** It catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5' to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.

(2) **Endonucleases:** In 1970 the first restriction endonuclease enzyme HindII was isolated. For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978 Daniel Nathans, Werner Arber, and Hamilton O. Smith awarded for Nobel Prize for Physiology or Medicine. Since then, restriction enzymes have been used as an essential tool in recombinant DNA technology. Endonucleases can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: EcoRI, Hind III, BamHI etc.

### 8.3.2 Vectors (The carriers of DNA molecules)

A vector is a DNA molecule which can replicate in a suitable host organism, and into which a fragment of foreign DNA can be introduced. Most vectors used in molecular biology are based on bacterial plasmids and bacteriophages (bacteria-infecting viruses). In order to perform its function, a vector must possess the following properties:

- They should be capable of autonomous replication in at least one host organism.
- They should be of small size, since this aids the preparation vector DNA and reduces the complexity of analyzing recombinant molecules. They should be capable of amplifying the cloned sequence by occurring in multiple copies. High copy number facilitates in maximizing expression of cloned genes.

- There should be a unique cleavage site for a range of restriction endonucleases. Occurrence of multiple cleavage sites reduces the likelihood of functional recombinant DNA formation.
- They should possess one or more genetic markers enabling easy selection of cloned molecules.
- They should permit detection by simple genetic tests, of the presence of passenger DNA inserted at cloning site.
- They should have appropriate transcriptional and translational signals located adjacent to cloning sites for better expression of cloned DNA sequences.
- They should have host specificity when there is biological containment for a vector.

A variety of different cloning vectors such as plasmids, phages, cosmids, phasmids, shuttle vectors, expression vectors etc. are widely used in genetic engineering.

### 8.3.2.1 Plasmids

Plasmids are circular, double-stranded DNA molecules that are independent from a cell's chromosomal DNA. These extrachromosomal DNAs occur naturally in bacteria and in the nuclei of yeast and some higher eukaryotic cells, existing in a parasitic or symbiotic relationship with their host cell. Most naturally occurring plasmids contain genes that provide some benefit to the host cell, fulfilling the plasmid's portion of a symbiotic relationship. Some bacterial plasmids, for example, encode enzymes that inactivate antibiotics. Therefore, a bacterial cell containing such a plasmid is resistant to the antibiotic, whereas the same type of bacterium lacking the plasmid is killed. The plasmids range in size from a few thousand bps to more than 100 kilobases (kb). The plasmids most frequently used in recombinant DNA technology are derived from and replicate in *E. coli*. In general, these plasmids have been modified to optimize their use as vectors in DNA cloning. One such modification, for example, is the reduction in size to approximately 3 kb, which is much smaller than that of naturally occurring *E. coli* plasmids. In addition, most plasmids contain a multiple cloning site (MCS), a short sequence of DNA containing many restriction enzyme sites close together. Thus,

many different restriction enzymes can be used for the insertion of foreign DNA fragments. In addition to antibiotic resistance genes, many modern plasmid vectors also contain a system for detecting the presence of a recombinant insert, such as the blue/white  $\beta$ -galactosidase system that allows simple visual screening of bacterial clones.

### **pBR322 plasmid**

Plasmid pBR322 is the one of the best studied and most often used "general purpose" plasmids. The BR of the pBR322 recognizes the work of the researchers F. Bolivar and R. Rodriguez, who created the plasmid and 322 is a numerical designation that has relevance to these workers. pBR322 is 4362 base pair long and completely sequenced. pBR322 carries two antibiotic resistance genes. One confers resistance to ampicillin (*Ampr*) and the other confers resistance to tetracycline (*Tetr*). There are eleven known enzymes which cleave pBR 322 at unique sites. For three of the enzymes, *Hind* III, *Bam* HI and *Sal* I, the target site lies within the *Tetr* genes and for another two, *Pst* I and *Pru* I, they lie in *Ampr* genes. Thus cloning in pBR 322 with the aid of these enzymes results in insertional inactivation where the inserted DNA disrupts the function of the gene containing the cloning site. Whereas the cloning site is within in an antibiotic resistance gene, such insertional inactivation results in transformants sensitive to the appropriate antibiotic. Thus, insertional inactivation helps in the selection of recombinants.

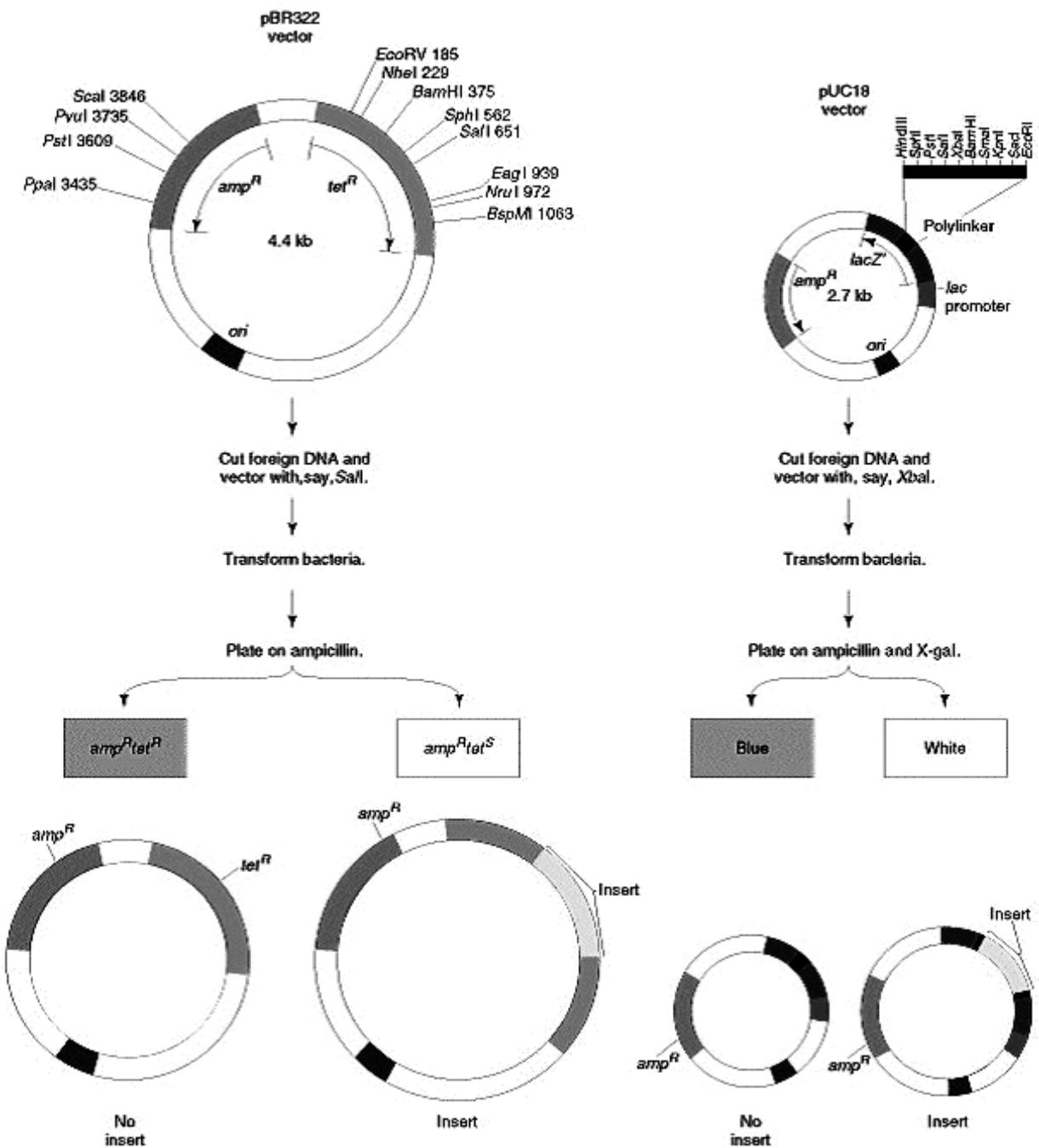


Fig. 8.2: Cloning of gene by using plasmid

### pUC19 plasmid

Plasmid pUC19 is 2686 bp long and contains an ampicillin resistance (*Ampr*) gene, a regulatable segment of  $\alpha$ - galactosidase gene (*lacZ*) of the lactose operon of *E. coli*, *lac I* gene that produces a repressor protein that regulates the expression of *lacZ* gene, a short sequence with multiple cloning sites (*EcoRI*, *SacI*, *KpnI*, *XmaI*, *SmaI*, *BamHI*, *XbaI*, *SalI*, *HincII*, *AccI*, *BspMI*, *PstI*, *SphI* and *HindIII*) and the origin of replication from pBR322. The presence of *lac Z* and *lacI* genes allows selecting the recombinants based on the  $\alpha$ - galactosidase production in the presence of isopropyl-  $\beta$  - D-thiogalactopyranoside (IPTG), an inducer of the *lac* operon. (UC in pUC stands for University of California).

#### 8.3.2.2 Bacteriophages

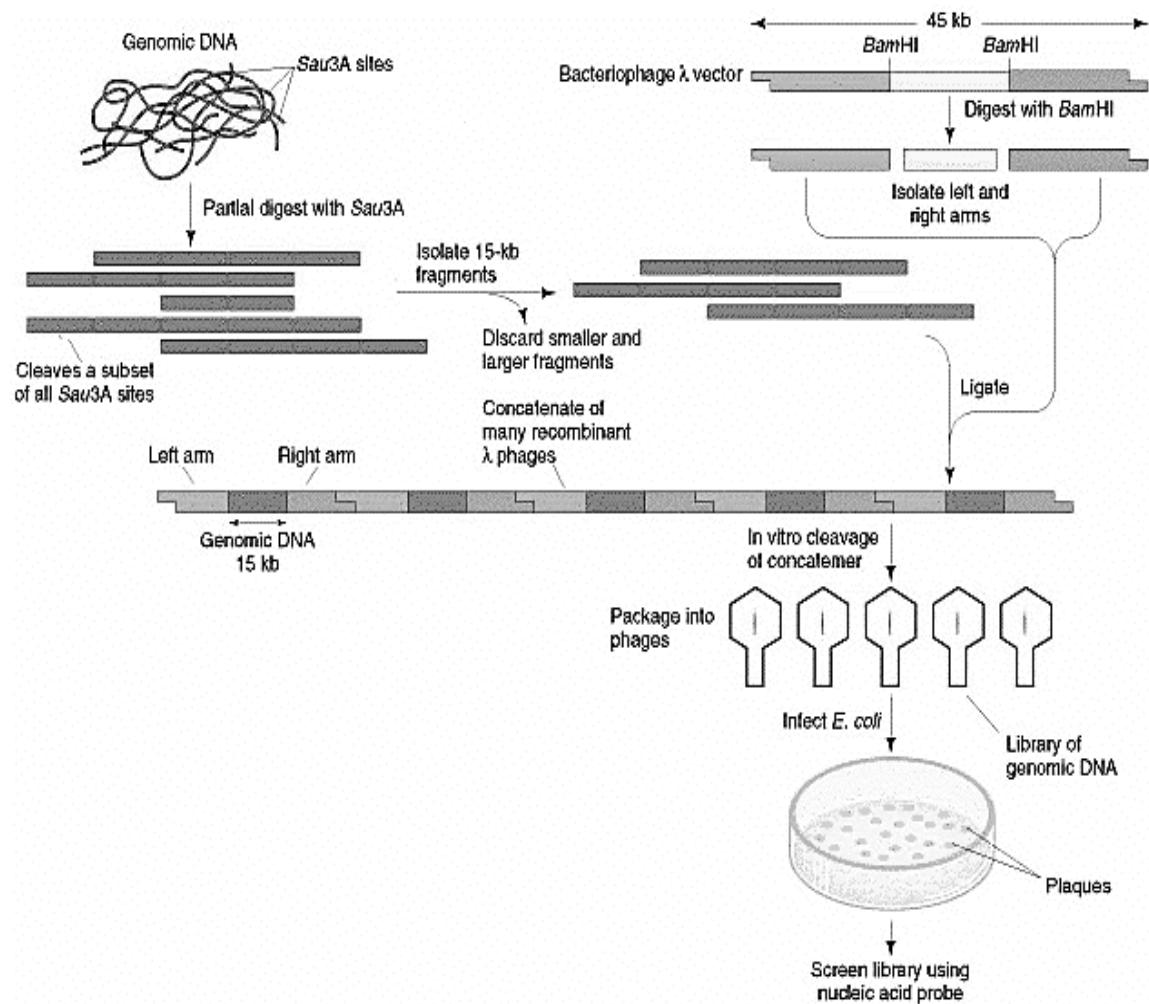
Bacteriophages, or phages, are viruses that infect bacteria. They can display either lytic life cycles, leading to the death of the host bacterium and release of new phage particles, or more complex lysogenic cycles during which the phage genome is integrated into the bacterial genome. Wild type phage DNA itself cannot be used as a vector since it contains too many restriction sites. Further, these sites are often located within the essential regions for phage's growth and development. From these wild phages, derivatives with single target sites and two target sites have been synthesized. Phage vectors which contain single site for the insertion of foreign DNA have been designated as insertional vectors; vectors with two cleavage sites, which allow foreign DNA to be substituted for the DNA sequences between those sites, are known as replacement vectors. Apparently if too much non-essential DNA is deleted from the genome it cannot be packaged into phage particles efficiently. For both types of vector, the final recombinant genome must be between 39 and 52 kb of the wild type phage genome, if they are to be packaged into infectious particles. Insertion vectors must therefore be at least 39 kb in length to maintain their viability. This places an upper limit of about 12 kb for the size of foreign DNA fragments which can be inserted. Replacement vectors have a larger capacity because the entire non-essential region can be replaced, allowing the cloning of the fragments upto 22 kb. Several types of vectors have been developed

which allow direct screening for recombinant phages and are useful for cloning specific DNA fragments.

One of the best studied phages is bacteriophage  $\lambda$  (Lambda) whose derivatives are commonly used as cloning vectors. The  $\lambda$  phage particle consists of a head containing the 48.5 kb double-stranded DNA genome, and a long flexible tail. During infection, the phage binds to certain receptors on the outer membrane of *E. coli* and subsequently injects its genome into the host cell through its tail. The phage genome is linear and contains single-stranded ends that are complementary to each other (the so called cos ends). Due to the complementarity, the cos ends rapidly bind to each other upon entry into the host cell, resulting in a nicked circular genome. The nicks are subsequently repaired by the cellular enzyme DNA ligase. A large part of the central region of the phage genome (15 kb) which is not required for replication or formation of progeny phage in *E. coli*, and it can be replaced by unrelated DNA sequence. The limit to the size of DNA fragments which can be incorporated into a  $\lambda$  particle is 15 kb, which is significantly larger than fragments suitable for plasmids (around 10 kb maximum). A further advantage of  $\lambda$ -based vectors is that each phage particle containing recombinant DNA will infect a single cell. The infection process is about a thousand times more efficient than transformation of bacterial cells with plasmid vectors.

This can be done as follows:

1. Mix RE cut donor DNA and lambda DNA in test tube
2. Ligation between donor DNA and lambda DNA
3. Use *in vitro* packaging mix that will assemble progeny phage carrying the foreign DN
4. Infect *E. coli* with the phage to amplify



**Fig. 8.3: Cloning through lambda phage**

(source: <http://www.bio.miami.edu/dana/pix/lambda.jpg>)

### 8.3.2.3 Cosmids

Both  $\lambda$  phage and *E. coli* plasmid vectors are useful for cloning only relatively small DNA fragments. However, several other vectors have been developed for cloning larger fragments of DNA. One common method for cloning large fragments makes use of elements of both plasmid and  $\lambda$ -phage cloning. In this method, called cosmid cloning, recombinant plasmids containing inserted fragments up to a length of 45 kb can be efficiently introduced into *E. coli* cells. A cosmid vector is produced by inserting the cos sequence from  $\lambda$ -phage DNA into a small *E. coli* plasmid vector about 5 kb long. Cosmid vectors contain all the

essential components found in plasmids. The cosmid can incorporate foreign DNA inserts that are between 35 and 45 kb in length. Such recombinant molecules can be packaged and used to transform *E. coli*. Since the injected DNA does not encode any  $\lambda$ -phage proteins, no viral particles form in infected cells and likewise the cells are not killed. Rather, the injected DNA circularizes, forming in each host cell a large plasmid containing the cosmid vector and the inserted DNA fragment. Cells containing cosmid molecules can be selected using antibiotics as described for ordinary plasmid cloning. A recently developed approach similar to cosmid cloning makes use of larger *E. coli* viruses such as bacteriophage P1. Recombinant plasmids containing DNA fragments of up to  $\approx 100$  kb can be packaged *in vitro* with the P1 system.

Cosmids have advantages of both plasmids and phage vectors they can be delivered to the host by the more efficient infection procedures rather than by transformation. Cloning with cosmid vectors has widened the scope of plasmid cloning in the following ways.

- The infectivity of plasmid DNA packaged in phage head is at least three orders of magnitude higher than that of pure plasmids DNA.
- The process almost exclusively yields hybrid clones so that a subsequent selection for recombinant DNA becomes unnecessary.
- In contrast to normal plasmid transformations, the system strongly selects for clones containing large DNA inserts. It is therefore, suited for generating genomic libraries.

#### 8.3.2.4 Phasmids

Phasmids, also called as phagemids, are hybrids formed between small multicopy plasmids and bacteriophages. A phasmid can be propagated as a plasmid or lytically as a phage. Lytic functions of phasmid can be switched off by propagation in the appropriate lysogene where the plasmid origin of replication is used for maintenance. The phasmid may replicate as phage if propagated in a non-lysogenic strain. In the case of phasmids based on  $\lambda$ , such as  $\lambda$ 1130, the temperature sensitive gene, *cI857* carried by the vector may be used to switch between replication modes, simply by growing the host at the permissive (plasmid mode) or restrictive (phage mode) temperature. Phasmids are particularly useful in the generation and

analysis of mutations exhibiting non-selectable or lethal phenotypes, such as those affecting the replication of plasmids. Phasmids may also be used as phage replacement vectors and for directing the high level expression of protein from cloned sequences by replication in the phage mode.

### **8.3.2.5 Others Vectors**

#### **Yeast artificial chromosomes (YAC)**

**YAC** s are constructed by ligating the components required for replication and segregation of natural yeast chromosomes to very large fragments of target DNA, which may be more than 1 Mb in length. They consist of:

1. Telomeres, which are ends of chromosomes involved in the replication and stability of linear DNA.
2. Origin of replication sequences necessary for the replication in yeast cells.
3. A yeast centromere, which is a specialized chromosomal region where spindle fibers attach during mitosis.
4. A selectable marker for identification in yeast cells.
5. Ampicillin resistance gene for selective amplification.
6. Recognition sites for restriction enzymes.

#### **Bacterial artificial chromosomes (BAC)**

**BAC** vectors were developed to avoid problems that were encountered with YACs to clone large genomic DNA fragments. Although YACs can accommodate very large DNA fragments they may be unstable, i.e. they often lose parts of the fragments during propagation in yeast. In general, BACs can contain up to 300–350 kb of insert sequence. In addition, they are stably propagated and replicated in *E. coli*, are easily introduced into their host cell by transformation, large amounts can be produced in a short time due to the fast growth of *E. coli*, and they are simple to purify. The vectors are based on the naturally occurring plasmid F factor of *E. coli*, which encodes its own DNA polymerase and is maintained in the cell at a level of one or two copies. A BAC vector consists of the genes essential for replication and maintenance of the F factor, a selectable marker gene (SMG) and a cloning site for the insertion of target fragment DNA.

#### **Shuttle vectors**

Shuttle vectors normally comprise an *E. coli* plasmid or part of such plasmid (*e.g.*, Pbr 322), ligated *in vitro* to a plasmid or virus replicon from another species.

Shuttle vectors can be made, for example, for *E. coli/B. suBtilis*, *E. coli/yeast* or *E. coli/mammalian* cells. The shuttle vector strategy permits the exploitation of the many manipulative procedures, such as amplification, available in *E. coli* (or other genetically well characterized species such as *B. suBtilis* or *S. cerevisiae*) backgrounds. The ability to transfer cloned genes across species boundaries is of potential value in the genetic manipulations of industrially important species and this can be achieved by using shuttle vectors.

### **Expression vectors**

In DNA cloning experiments all the genes cloned are not expressed fully because of weak promoters in vector DNA. This can be dramatically improved by placing such genes downstream of strong promoters. An additional problem in maximizing expression of cloned genes in *E. coli* which is frequently encountered with genes from a heterologous source is that the gene carries no translation start signal which can be efficiently recognized by the *E. coli* translation system. This problem may arise for heterologous genes cloned into any host. Thus, even though the gene can be transcribed from a promoter within the vector, the resulting mRNA is poorly translated and little or no protein product will be synthesized. In such cases alternative strategies available are fusing the gene to amino terminal region of vector gene that is efficiently translated in the host or coupling the gene to a DNA fragment carrying both strong promoter and a ribosomal binding site. Vectors with this additional feature are called expression vectors.

Now we can summarize, that cloning vectors are DNA molecules that can incorporate foreign DNA fragments and replicate in a suitable host, producing large quantities of the desired DNA fragment. Such methods are highly important for a variety of molecular biology applications and are the basis of recombinant DNA technology. However, for the production of transgenic organisms and related biotechnological applications, such vectors need to possess additional sequence elements and properties that allow targeted transfer of specific genes and controlled expression of these genes in a host organism. The necessary features to accomplish these tasks will be discussed in the following paragraphs.

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## **8.4 Technique Involved in Genetic Engineering**

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### **8.4.1 Gene Cloning**

In short, gene cloning is essentially the insertion of a specific piece of desired DNA into a host cell in such a way that the inserted DNA is replicated and handed onto daughter cells during cell division. Sequential steps involved in DNA cloning using plasmid DNA as vector are the as follow:

**Step 1: Isolation of DNA (gene of interest and vector)**

The first initial step in cloning a DNA fragment is to isolate foreign DNA containing gene of interest and bacterial plasmid. If the sequence of the gene of interest is known it is isolated by PCR amplification using gene specific primers which include restriction sites selected from the multiple cloning site of the plasmid selected for cloning. When the sequence of the gene is not known degenerate primers are used for PCR amplification. Most of the time people generate genomic DNA library and screen for the gene using southern hybridization technique. According to the result of southern hybridization, the DNA is sequenced and the gene was confirmed by BLAST analysis. Now the gene is amplified by PCR and cloned. There are many plasmids available commercially for cloning.

Isolation of DNA as follow:

1. Crude isolation of donor (foreign) DNA is accomplished by isolating cells, than membrane of isolated cell can be disrupted with detergents. The resultant, produced cell extracts contain protein, RNA and DNA. The proteins in the extract can be removed by treatment of with phenol or proteases while RNA can be removed by treatment of RNase. Get the crude DNA
2. Crude isolation of plasmid vector DNA is accomplished by an alkaline lysis procedure or by boiling cells which removes bacterial chromosomal DNA from plasmid DNA.
3. To get purer DNA from either (1) or (2), crude DNA is
  - a) Fractionated on a CsCl<sub>2</sub> gradient
  - b) Precipitated with ethanol
  - c) Poured over a resin column that specifically binds DNA

**Step 2: Treatment of plasmid and foreign DNA with the same restriction enzyme and ligation:**

The gene of interest and the plasmid are modified using same restriction enzymes. Plasmid vectors are engineered to contain a specific antibiotic resistance gene and a multiple cloning site (also called the polylinker region) which contain many unique target sites for restriction endonucleases. When the circular plasmid is cut with one of the restriction enzyme whose restriction site is present in the plasmid, it results the linearization of plasmid. A variety of RE have been isolated and are commercially available. Most cut at specific palindromic sites in the DNA (sequence that is the same on both antiparallel DNA strands). These cuts can be a staggered which generates “sticky or overhanging ends” or a blunt which generate flush ends. A fragment of DNA molecule, referred to as the “insert,” is treated with the same restriction enzyme, and then can be joined to the plasmid DNA in a ligation reaction. The chance for recombinant clones in ligations of the insert to vector will not be 100% as there is more possibility of self-ligation of two ends of the plasmid. To decrease the degree of self-ligation, enzyme phosphatase is used which removes the terminal 5'-phosphate and prevents self-ligation. Another strategy to overcome self-ligation is by using two different restriction enzymes cutting sites with non- complementary sticky ends. In this way self-ligation is inhibited and also promotes correct orientation of the insert DNA within the plasmid. The ligation of the digested insert and the plasmid is performed by pooling both in a single reaction tube and adding DNA ligase enzyme which catalyses the formation of phosphodiester bond between insert and plasmid DNAs, thereby forming the recombinant DNA molecule.

**Step 3: Transformation: transfer of recombinant plasmid DNA to a suitable host**

The ligation reaction mixture of recombinant DNA described in the step 2 is introduced into bacterial cells in a process called transformation. The traditional method to prepare cells for transformation process is to incubate the cells in a concentrated calcium salt solution to neutralize the negative charge of membrane (due to salicylic acid), so that the negatively charged DNA molecules can come close to bacterial membrane and during heat shock (method of transformation) can easily enter in the cells. These “competent” cells are then mixed with ligation product to allow entry of the DNA into the bacterial cell. An alternative mode of transformation is electroporation method which is used to drive DNA

(comparatively larger size) into cells by a strong electric current. This method is not very common due to less percentage of survival of transformed cells. As mentioned earlier bacterial species use restriction enzymes to degrade foreign DNA lacking the methylation pattern, including the plasmids, then why don't they degrade the transformed recombinant DNA. The answer is that molecular biologist have cleverly engineered and developed the bacterial strains that lack restriction-modification system. The best example is common lab strain *E.coli* DH5 $\alpha$ . A transformed bacterial cell may carry either recombinant or non-recombinant plasmid DNA. The plasmid DNA multiplies within each transformed bacterial cell. Each transformed bacterial cell when plated to the solid agar media (nutrient media) can multiply to form a visible colony made of millions of identical cells. As the transformed cell divides, the plasmids are passed on to progeny, where they continue to replicate. Single transformed bacteria undergo numerous cell divisions results in clones of a cell (single bacterial colony) from a single parental cell. From this step the name "cloning" is given. From the colony of bacterial cells the cloned DNA can now be isolated.

#### **Step 4: Screening for transformed cells**

To avoid the growth of the untransformed bacterial cells, plasmid vectors are engineered with selectable marker gene for resistance to the antibiotics (Table 1). The media in which the transformed bacterial cells are grown is supplied with that antibiotic whose resistance gene is present in the plasmid. Due to this only transformed cells show antibiotic resistance will grow in the media supplied with antibiotic and untransformed cells cannot grow as they do not carry antibiotic resistance gene. Transformed bacterial cells may contain either recombinant plasmid DNA (vector containing foreign DNA insert) or non-recombinant plasmid DNA (self ligated vector only). Both type of transformed bacterial cells will show antibiotic resistance and grow on the agar media plate.

Blue-white screening or "*lac* selection" (also called  $\alpha$ -complementation) can be used to distinguish between recombinant transformants and non-recombinant transformants. Bacterial colonies are allowed to grow on selective media containing antibiotic and X-gal (5-bromo-4-chloro-indolyl- $\beta$ -D-galactopyranoside), a colorless chromogenic compound. Not all plasmid vectors are engineered for "*lac* selection"; the plasmid that are engineered for blue-white

screening carry a MCS site in between gene that encodes for amino acids for enzyme  $\beta$ -galactosidase which cleaves  $\beta$ -glycosidic bond in D-lactose. X-gal mimic D-lactose and  $\beta$ -galactosidase enzyme acts on X-gal and produces a blue color complex.

#### 1. Construction of a recombinant DNA molecule

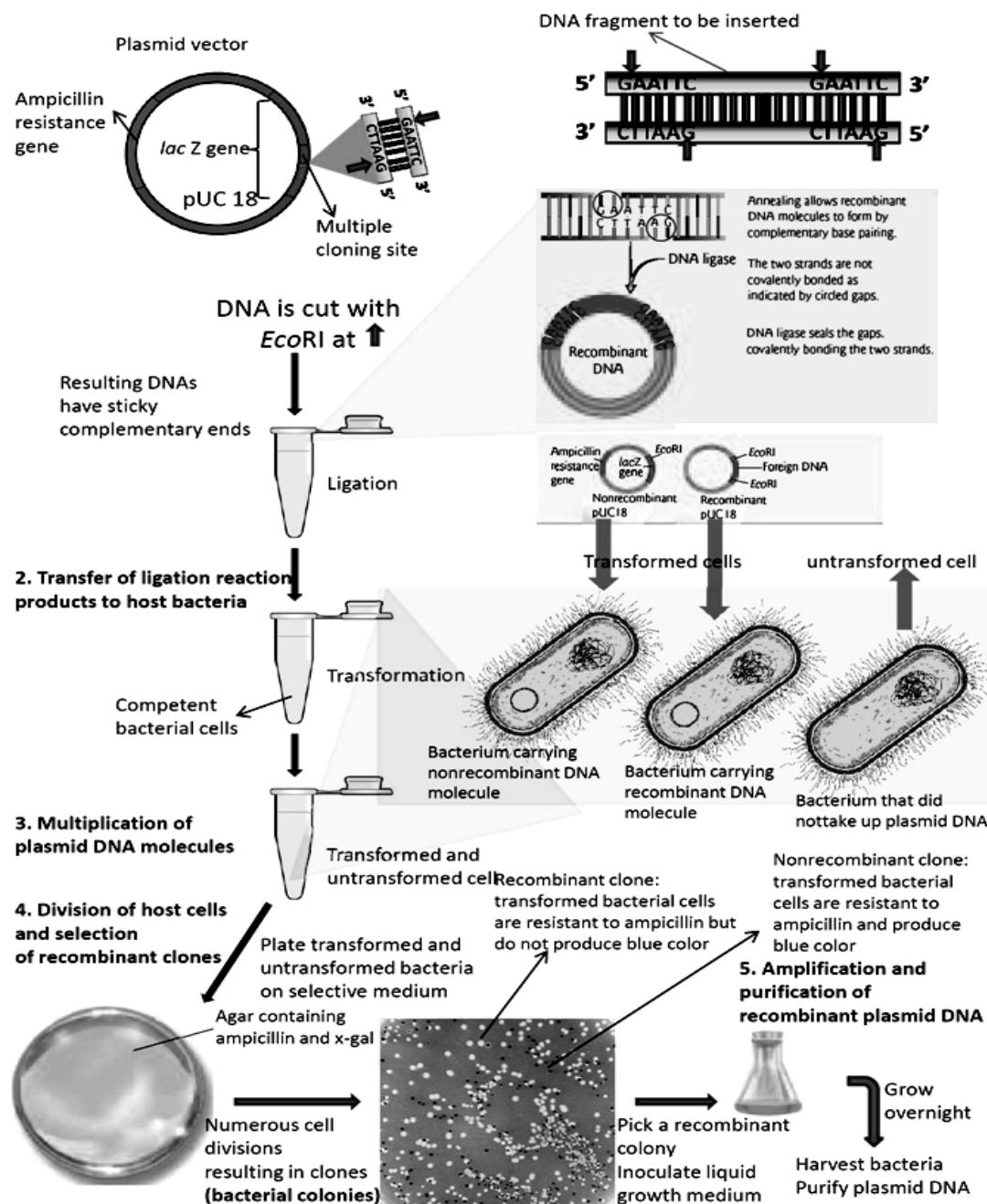


Fig. 8.4: Cloning of Gene and their various steps

A successful ligation of the desired gene disrupts the *lac Z* gene, hence no functional  $\beta$ -galactosidase is produced resulting in white colonies. Hence successful recombinant transformed colonies can be easily identified by its white coloration from unsuccessful blue ones.

### **Step 5: Amplification and purification of recombinant plasmid DNA**

The final step in DNA cloning is the isolation of the cloned recombinant DNA. A positive colony containing recombinant plasmid is identified and it is aseptically transferred to liquid medium and cells are allowed to grow exponentially overnight. A fully grown culture contains trillions of identical cells, which is harvested for the isolation of the plasmid DNA. The plasmid DNA is purified from harvested bacterial cell lysates. The purified plasmid DNA is dissolved in an appropriate buffer solution and can be used for further confirmation of the clone by restriction digestion and sequencing the plasmid DNA.

#### **8.4.2 Southern Blotting**

A **Southern blot** is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

The method is named after its inventor, the British biologist Edwin Southern.

Steps:

1. Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments.
2. The DNA fragments are then electrophoresed on an agarose gel to separate them by size.
3. If some of the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl. This depurinates the DNA fragments, breaking the DNA into smaller pieces, thereby allowing more efficient transfer from the gel to membrane.
4. If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (typically containing sodium hydroxide) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged thymine residues of DNA to a positively

charged amino groups of membrane, separating it into single DNA strands for later hybridization to the probe (see below), and destroys any residual RNA that may still be present in the DNA. The choice of alkaline over neutral transfer methods, however, is often empirical and may result in equivalent results.

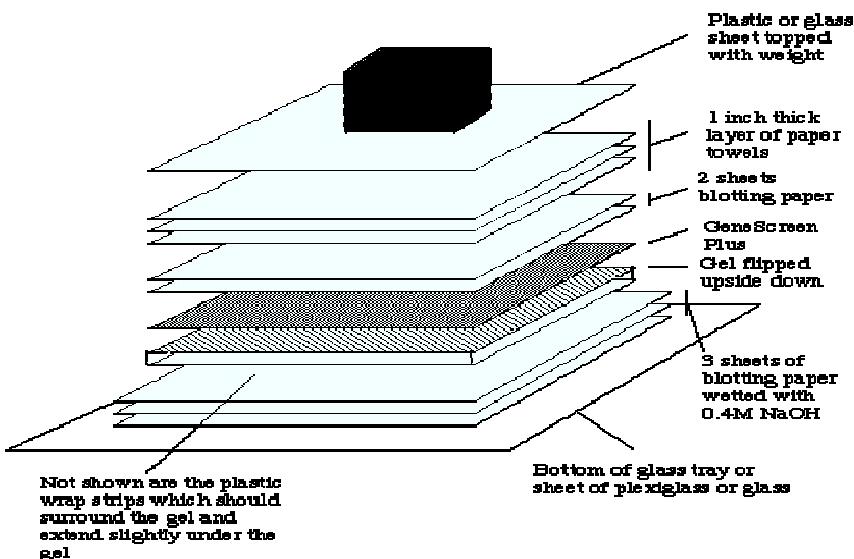
5. A sheet of nitrocellulose (or, alternatively, nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. If transferring by suction, 20X SSC buffer is used to ensure a seal and prevent drying of the gel. Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is then used to move the DNA from the gel onto the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
6. The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours (standard conditions; nitrocellulose or nylon membrane) or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.
7. The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. In some cases, the hybridization probe may be made from RNA, rather than DNA. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use salmon or herring sperm DNA for blocking of the membrane surface and target DNA, deionized formamide, and detergents such as SDS to reduce non-specific binding of the probe.

After hybridization, excess probe is washed from the membrane (typically using SSC buffer), and the pattern of hybridization is visualized on X-ray film

by autoradiography in the case of a radioactive or fluorescent probe, or by development of color on the membrane if a chromogenic detection.

## Results

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. It also allows for the fixation of the target-probe hybrids, required for analysis by autoradiography or other detection methods. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome. A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (for example, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.



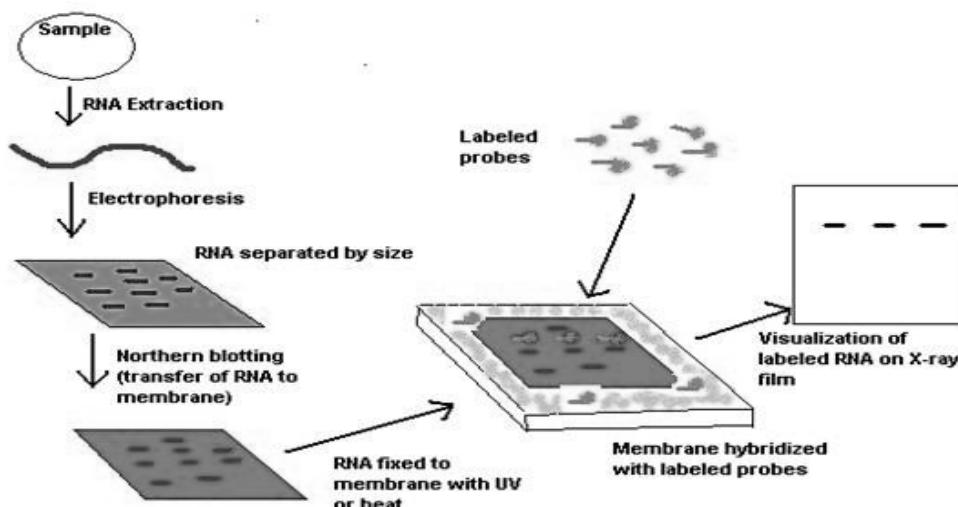
**Fig. 8.5: Stack of component for southern blot**

(source: [http://www.personal.psu.edu/dsg11/labmanual/Transformation/Southern\\_blotting.htm](http://www.personal.psu.edu/dsg11/labmanual/Transformation/Southern_blotting.htm))

### 8.4.3 Northern Blotting

The Northern blot procedure essentially identical to that of southern blotting except that here RNA are separated by gel electrophoresis. Northern blots allow investigators to determine the molecular weight of an mRNA and to measure relative amounts of the mRNA present in different samples.

- RNA (either total RNA or just mRNA) is separated by gel electrophoresis, usually an agarose gel. Because there are so many different RNA molecules on the gel, it usually appears as a smear rather than discrete bands.
- The RNA is transferred to a sheet of special blotting paper called nitrocellulose, though other types of paper, or membranes, can be used. The RNA molecules retain the same pattern of separation they had on the gel.
- The blot is incubated with a probe which is single-stranded DNA. This probe will form base pairs with its complementary RNA sequence and bind to form a double-stranded RNA-DNA molecule. The probe cannot be seen but it is either radioactive or has an enzyme bound to it (e.g. alkaline phosphatase or horseradish peroxidase).
- The location of the probe is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product that can be seen or gives off light which will expose X-ray film. If the probe was labeled with radioactivity, it can expose X-ray film directly.

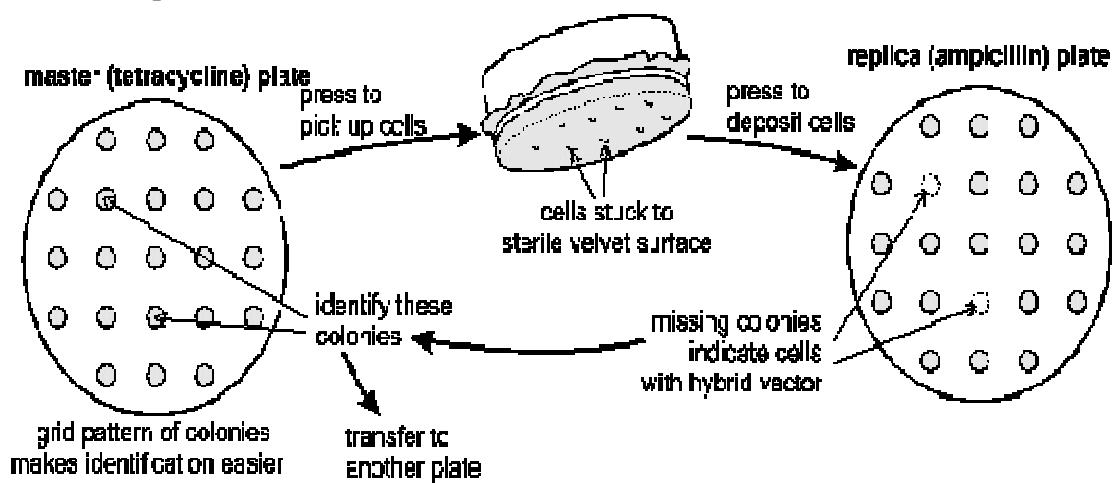


**Fig. 8.6 : Northern Blot Technique**

#### 8.4.4 Replica Plating

It is a simple technique for making an exact copy of an agar plate. A pad of sterile cloth the same size as the plate is pressed on the surface of an agar plate with bacteria growing on it. Some cells from each colony will stick to the cloth. If the cloth is then pressed onto a new agar plate, some cells will be deposited and colonies will grow in exactly the same positions on the new plate. This technique has a number of uses, but the most common use in genetic engineering is to help solve another problem in identifying transformed cells. This problem is to distinguish those cells that have taken up a hybrid plasmid vector (with a foreign gene in it) from those cells that have taken up plasmids without the gene. This is where the second marker gene (for resistance to ampicillin) is used. If the foreign gene is inserted into the middle of this marker gene, the marker gene is disrupted and won't make its proper gene product. So cells with the hybrid plasmid will be killed by ampicillin, while cells with the normal plasmid will be immune to ampicillin. Since this method of identification involves killing the cells we want, we must first make a master agar plate and then make a replica plate of this to test for ampicillin resistance.

Once the colonies of cells containing the correct hybrid plasmid vector have been identified, the appropriate colonies on the master plate can be selected and grown on another plate.



**Fig. 8.7: Replica Plating Method**

(Source: <http://www.biologymad.com/master.html>)

### 8.4.5 Polymerase Chain Reaction (PCR)

This is an in vitro method for making many copies of a specific section of DNA, without the need for vectors or host cells. The DNA to be copied – the template DNA – is mixed with forward and reverse primers complementary to the end of the template DNA, nucleotides, and a version of DNA polymerase known as Taq polymerase. (This enzyme is stable under high temperatures, and is obtained from the thermophilic bacterium *Thermus aquaticus*.) The process involves the repetition of three steps:

1. Denaturation, which separates the two nucleotide strands of the DNA molecule
2. Primer annealing, in which the primers bind to the single-stranded DNA
3. Extension, in which nucleotides are added to the primers – in the 5' to 3' direction – to form a double-stranded copy of the target DNA.

After repeated cycles, the amount of DNA sequence between the two primers increases exponentially. First 2 strands, then 4, 8, 16, up to about a million. Thus, in a couple of hours, you can get million-fold amplification of a DNA sequence.

Each cycle takes a few minutes, and repeated cycles can produce large amounts of a specific DNA sequence in a matter of hours rather than days. However, this cloning method does require knowledge of some details about the nucleotide sequence to be copied, and the technique is very sensitive to small amounts of contamination.

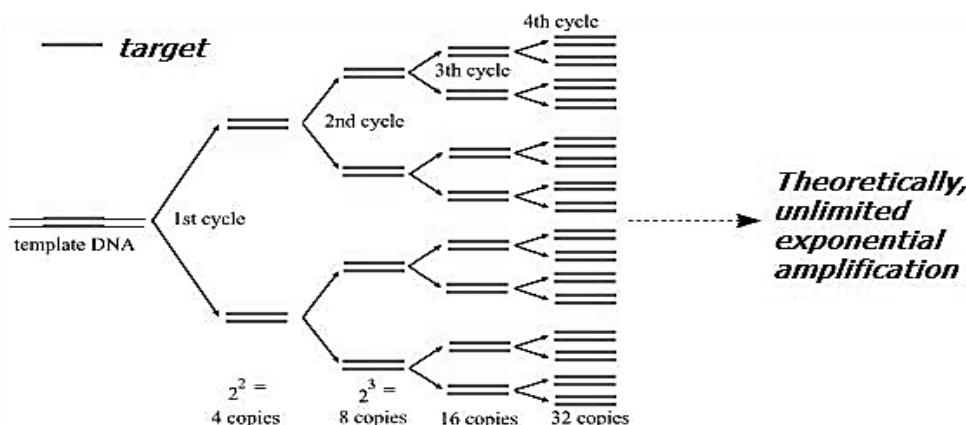


Fig. 8.7: Gene amplification by using PCR

## Applications of PCR

PCR has replaced cloning for many purposes, particularly the sequencing of DNA. It is faster and requires no vectors, which can mutate as they reproduce. It can be used forensically, to amplify tiny amounts of DNA from criminal evidence; or clinically, to detect DNA sequences linked to inherited disorders.

### The main limitations of PCR

- Only relatively short sequences can be amplified reliably. Anything more than 10,000 base pairs are unlikely to be amplified.
- You need to know the right primer sequences to use, at both ends of the sequence you want to amplify. If two related genes have the same end sequences, you might amplify the wrong gene.
- You only obtain a DNA fragment. To see this DNA at work inside a living organism, some type of cloning has to be done.

### 8.4.6 Advanced PCR Techniques

#### Reverse Transcription PCR (RT-PCR)

Frequently, one wants to get the information of the expression of a gene or characterizing an RNA transcript. In these cases, Reverse Transcription PCR is a convenient tool. The main difference of this between the normal basic PCR techniques above is this one starts with an mRNA instead of a double-stranded DNA. The RNA to DNA step can be done with reverse transcriptase and reverse primer. Once the single stranded DNA is obtained, a forward primer can be used to convert it to double stranded one. Then the same PCR process is operated with these two primers until the desired amount of DNA is obtained.

#### Real-Time PCR (RT-PCR)

Real-Time PCR is a way of quantifying the amplification of a DNA as it occurs—that is, in real time. As illustrated in the following graph: after separation of two DNA strands, they are annealed, not only to the forward and reverse primers, but also to a fluorescent-tagged oligonucleotide that is complementary to part of one of the DNA strands and serves as a reporter probe. This probe has a fluorescent tag at its 5' end and fluorescence quenching tag at its 3' end. During the polymerization step, the polymerase extends the forward primer and then encounters the reporter probe. When that happens, the polymerase begins degrading the reporter probe. As

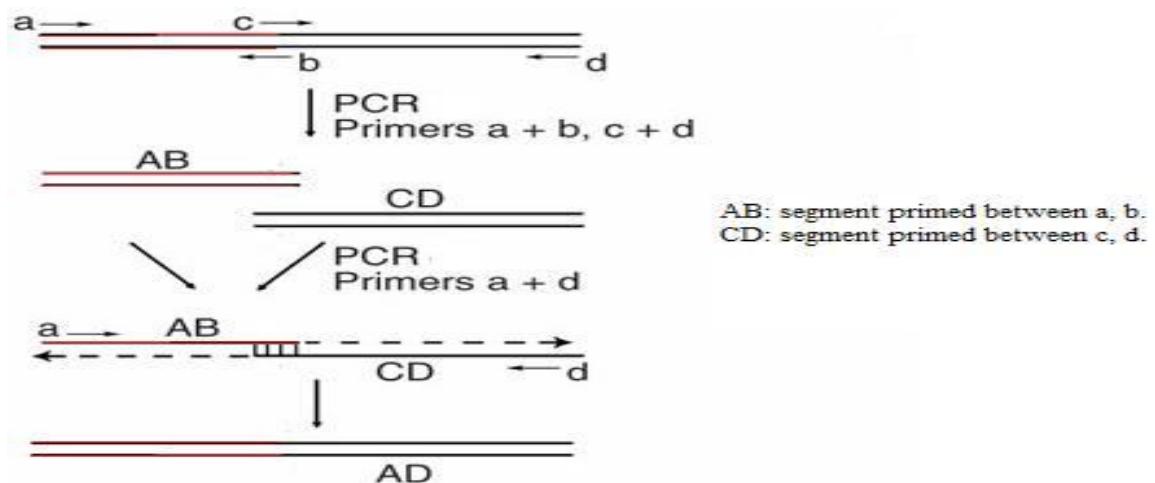
the probe is degraded, the fluorescent tag is separated from the quenching tag, so its fluorescence increases dramatically. A fluorimeter can measure the intensity along the PCR reaction course, which in turn, gives the real time progress.

### Overlapping PCR

This technique is to insert mutations at specific points or to join the spliced smaller DNA fragments into a larger one. As can be seen from the following graph, this PCR consists of two stages:

On the first stage, it is normal PCR amplifying the two segments subjected for joint. Specifically, primers for the joining position are designed in a way that they overlap with each other. Mutations can be inserted into these primers if needed.

On the second stage, the products from the first stage, the individual segments waiting for joining is denatured and annealed with each other. This can be done because of the joining primers on the first stage can hybridize with one another. Then, by applying only the end primers (primers that prime the ends of the longer joint final product), another PCR is performed. The final longer segment is thus formed and amplified.



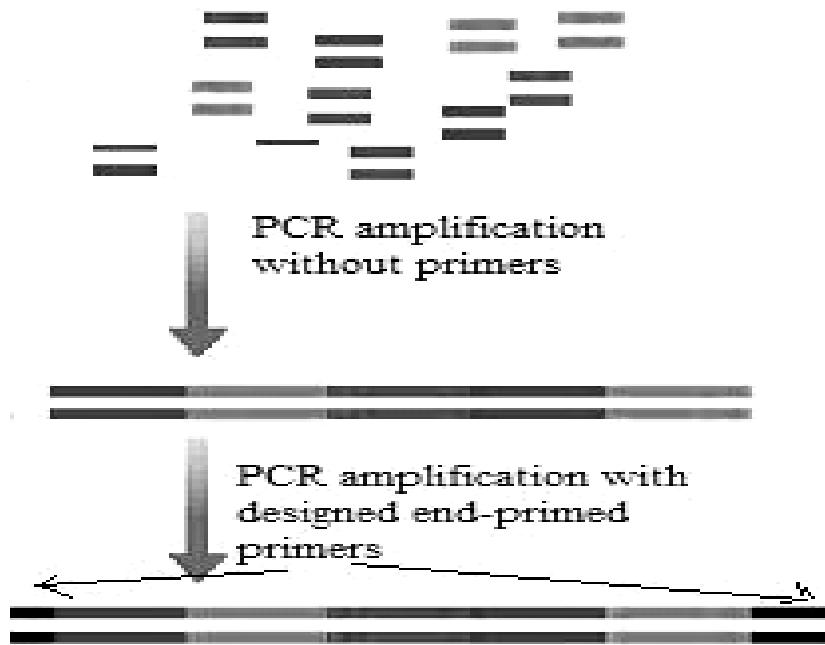
**Fig. 8.8 : Overlapping PCR technique**

### PCR mediated DNA shuffling

DNA shuffling is an important tool in *In vitro* directed evolution, which uses the protein engineering principles to harness the power of natural selection to evolve

proteins or RNA with desirable properties. Traditionally, DNA shuffling is done through a restriction enzyme way: gene members from a family are digested with restriction enzymes and are further ligated to produce hybrids for subsequent screening. However, a PCR mediated way, as illustrated by the following graph is of more efficiency.

This technique begins by fragmenting a pool of double-stranded parent genes with DNaseI. Then small fragments are selected by size fractionation to maximize the probability of multiple recombination events. After this, the first round of PCR is performed with no added primers, instead, small fragments that overlap with each other cross-primed at this stage. After filling the space of a long recombined product from the fragments, another round of PCR is performed to amplify the recombined full-length product using terminal primers. However, there is a drawback to this diversification: Most DNA shuffles on average of just 4 crossovers per round, limiting the potential diversity. Furthermore, crossovers in regions of identity shorter than 15 bases are rare, resulting in lengthy tracts of sequence still contains up to 100% unshuffled parental wild-type clones after the shuffling attempt. A new technique called RACHITT (Random Chimeragenesis on Transient Templates) can overcome some of these to some extent. RACHITT begins with fragmentation and size fractionation of single-stranded DNA, and hybridization without polymerase to a complementary single-stranded scaffold of poly uracil. Any overlapping fragments with overhangs will be trimmed down (Mismatch or overlapping). The gaps between fragments are filled in by complementarity; the fragments are then ligated to get a full-length, diversified single strands hybridized to the scaffold. The poly-uracil scaffold can be efficiently degraded to preclude its amplification. With PCR, it is replaced by a new strand complementary to the recombined product, and the whole is amplified for subsequent screening test.



**Fig. 8.9 : PCR mediated DNA shuffling**

### Reverse PCR

PCR is applied usually with known sequence. And in this way, primers can be specifically used to prime the target sequence with it being amplified. However, it can also be used in cases when we want to explore the unknown flanking regions, as shown by the following graph:

In this technique, as can be seen from the graph, restriction enzyme site is being "inserted" into the flanking regions, providing a basis for future ligation of the linear sequence into a plasmid. Once this plasmid is formed, primers from the known sequence can be used for PCR of this plasmid in a normal way. A way, if seen from a prospective of the previous linear segment, is to amplify the unknown flanking regions.

The "insertion" of the restriction enzyme cutting site is not actually inserted by primer design, since the flanking sequence is unknown. Generally, this is implemented through a genomic cutting by only one type of restriction enzymes (so that the cohesive ends can join with the same complementarity).

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## 8.5 Summary

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The Genetic engineering involves artificial transfer of genes or gene fragments from one organism to another to produce novel traits in the recipient living organism. The various enzymes such as Restriction enzymes; Nucleases; DNA ligase; Kinase; Phosphatase; Reverse transcriptase; Terminal Deoxynucleotide Transferase; RNaseP etc. are widely used in genetic engineering. Vector is also very important tool for genetic engineering which carries the target DNA into recipient organism. Gene cloning starts from isolation of desired DNA sequence to finally the selection of transformant cells. Southern and northern blotting used for detection of particular DNA/RNA in nucleotides samples. Other techniques such as PCR, replica plating etc. are also very important for genetic engineering.

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## 8.6 Glossary

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- **Genetic engineering:** It involves artificial transfer of genes or gene fragments from one organism to another to produce novel traits in the recipient living organism.
- **Plasmid:** It is a circular, double-stranded DNA molecules that are independent from a cell's chromosomal DNA.
- **Phasmids:** Also called as phagemids, are hybrids formed between small multicopy plasmids and bacteriophages.
- **Northern blot:** It is a method used in molecular biology for detection of a specific RNA sequence in nucleotides samples.
- **PCR (polymerase chain reaction):** It is an in vitro method for making many copies of a specific section of DNA, without the need for vectors or host cells.
- **Replica plating:** It is a simple technique for making an exact copy of an agar plate.
- **Restriction enzyme:** It is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites.
- **Southern blot:** It is a method used in molecular biology for detection of a specific DNA sequence in DNA samples.

- **Vector:** A vector is a DNA molecule which can replicate in a suitable host organism, and into which a fragment of foreign DNA can be introduced.

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## 8.7 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Name the any two restriction enzymes, producing sticky end.
2. Who discovered the restriction enzymes?
3. Name the antibiotic resistance gene present in plasmid p<sup>BR322</sup>.
4. Who discovered the PCR?
5. Who discovered the AFLP?

### Section B : (Short Answer Type Questions)

1. Define the term genetic engineering.
2. Define the vector
3. Write difference between plasmid p<sup>BR322</sup> and p<sup>UC19</sup>.
4. Mention the step of gene cloning.
5. What is real time PCR?

### Section C : (Long Answer Type Questions)

1. Give a detail account on restriction endonuclease.
2. Write an explanatory note on vector.
3. Describe the gene cloning in detail.
4. Explain the PCR and its application.

### Answer Key of Section-A

1. EcoR1, BamH1
2. Stewart Linn & Werner Arber
3. *amp*<sup>R</sup> and *tet*<sup>R</sup>
4. Kary Mullis
5. Vos et al.

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## 8.8 References

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- [http://openi.nlm.nih.gov/detailedresult.php?img=3071783\\_1423-0127-18-23-3&req=4](http://openi.nlm.nih.gov/detailedresult.php?img=3071783_1423-0127-18-23-3&req=4)

## Unit - 9

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# Recombinant Technology-I: Restriction Enzymes

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### Structure of the Unit:

- 9.0 Objectives
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- 9.2 Restriction Endonucleases (RE)
  - 9.2.1 Classification of Restriction Endonucleases
    - 9.2.1.1 Type I Restriction Endonucleases
    - 9.2.1.2 Type II Restriction Endonucleases
    - 9.2.1.3 Type III Restriction Endonucleases
  - 9.2.2 Restriction Endonucleases Nomenclature
  - 9.2.3 Cleavage Pattern of Restriction Endonucleases
  - 9.2.4 Selection of Restriction Enzyme
  - 9.2.5 Applications
- 9.3 Ligase
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  - 9.3.2 Applications
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  - 9.4.2 Alkaline Phosphatase
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  - 9.8.4 Mung Bean Nuclease
- 9.9 Rec A Protein
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  - 9.9.3 DNA Binding region of Rec A
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  - 9.9.5 Role of Rec A in Natural Transformation
- 9.10 Summary
- 9.11 Glossary
- 9.12 Self-Learning Exercise

### 9.13 References

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## 9.0 Objectives

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After going through this unit you will be able to understand:

- Restriction Endonuclease
  - Ligase
  - Phosphatase
  - Kinas
  - Transferase
  - Polymerase
  - Nucleases
  - Rec A Protein
- 

### 9.1 Introduction

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The discovery of restriction enzymes made genetic engineering possible but there are other enzymes also with are involved in Recombinant Technology & plays important roles like Ligase, Phosphatase, Kinas, Transferase, Polymerase , Nucleases, Rec A Protein . Restriction enzymes first made it possible to work with small, defined pieces of DNA. Chromosomes are huge molecules that usually contain many genes. Before restriction enzymes were discovered, a scientist might be able to tell that a chromosome contained a gene for an enzyme required to ferment lactose because he knew that the bacterium could ferment lactose and he could purify the protein from bacterial cells.

Genetic engineering is possible because of special enzymes that cut DNA. These enzymes are called restriction enzymes. A restriction enzyme is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites. Restriction enzymes are proteins produced by bacteria to prevent or restrict invasion by foreign DNA. They act as DNA scissors, cutting the foreign DNA into pieces so that it cannot function. A nuclease is any enzyme that cuts the phosphodiester bonds of the DNA backbone, and an endonuclease is an enzyme that cuts somewhere within a DNA molecule. In contrast, an exonuclease cuts phosphodiester bonds by starting from a free end of the DNA and working inward. In bacteria, restriction enzymes form a combined system (restriction +

modification system) with modification enzymes that methylate the bacterial DNA. Methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from being cleaved by restriction enzyme. Restriction enzymes were originally discovered through their ability to break down, or restrict, foreign DNA. Restriction enzymes can distinguish between the DNA normally present in the cell and foreign DNA, such as infecting bacteriophage DNA. They defend the cell from invasion by cutting foreign DNA into pieces and thereby rendering it nonfunctional. Restriction enzymes appear to be made exclusively by prokaryotes.

### History

The discovery of restriction enzymes gave scientists a way to cut DNA into defined pieces. In 1970 the first restriction endonuclease enzyme *HindII* was isolated. For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978 Daniel Nathans, Werner Arber, and Hamilton O. Smith awarded for Nobel Prize for Physiology or Medicine. Since then, restriction enzymes have been used as an essential tool in recombinant DNA technology.

There are two different kinds of restriction enzymes:

- (1) **Exonucleases** catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5' to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.
- (2) **Endonucleases** can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: *EcoRI*, *HindIII*, *BamHI* etc.

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## 9.2 Restriction Endonucleases(RE)

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Restriction endonucleases are enzymes that cleave the sugar-phosphate backbone of DNA strands. The vast majority of these enzymes have been isolated from bacteria, where they carry out a host-defence function for the cell. These enzymes recognize a specific DNA base sequence and cleave both strands of a double-stranded DNA molecule at or near the recognition site. Each endonuclease enzyme (and there are hundreds, made by many different bacteria) has its own type of site. In general, a restriction site is a 4- or 6-base-pair sequence that is a palindrome. A

DNA palindrome is a sequence in which the “top” strand read from 5' to 3' is the same as the “bottom” strand read from 5' to 3'.

### **9.2.1 Classification of Restriction Endonucleases**

There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

#### **9.2.2.1 Type I Restriction Endonucleases**

These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA. The cleavage occurs approximately 1000 bp away from the recognition site. The recognition site is asymmetrical and is composed of two specific portions in which one portion contain 3–4 nucleotides while another portion contain 4–5 nucleotides.

#### **9.2.1.2 Type II Restriction Endonucleases**

Restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other. The Cleavage of nucleotide sequence occurs at the restriction site. These enzymes are used to recognize rotationally symmetrical sequence which is often referred as palindromic sequence. These palindromic binding sites may either be interrupted or continuous. They require only Mg<sup>2+</sup> as a cofactor and ATP is not needed for their activity. Type II endonucleases are widely used for mapping and reconstructing DNA *in vitro* because they recognize specific sites and cleave just at these sites.

#### **9.2.1.3 Type III Restriction Endonucleases**

These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away. They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and other subunit (R) has nuclease action. Mg<sub>2</sub><sup>+</sup> ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM. This enzyme make a cleave only in one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

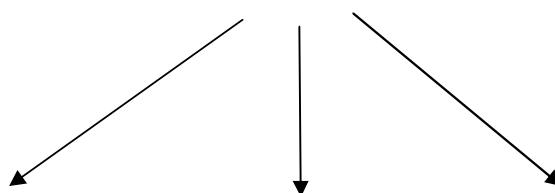
**Table 9.1: Difference between Type I, II and III Restriction Enzyme**

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non-specific location >	Cut both strands at a specific, usually	Cleavage of one strand, only 24-26
Restriction	Single	Separate nuclease	Separate enzymes
Nuclease	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg <sup>2+</sup> , SAM	Mg <sup>2+</sup>	Mg <sup>2+</sup> (SAM)
DNA	Two recognition	Single recognition	Two recognition
Enzymatic	No	Yes	Yes
DNA	Yes	No	No
Site of	At recognition site	At recognition site	At recognition site

### 9.2.2 Restriction Endonuclease Nomenclature

Restriction endonucleases are named according to the organism in which they were discovered, using a system of letters and numbers. For example, *HindIII* (pronounced “hindee-three”) was discovered in *Haemophilus influenza* (strain d). The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes indicating the order in which restriction enzymes were discovered in a particular strain.

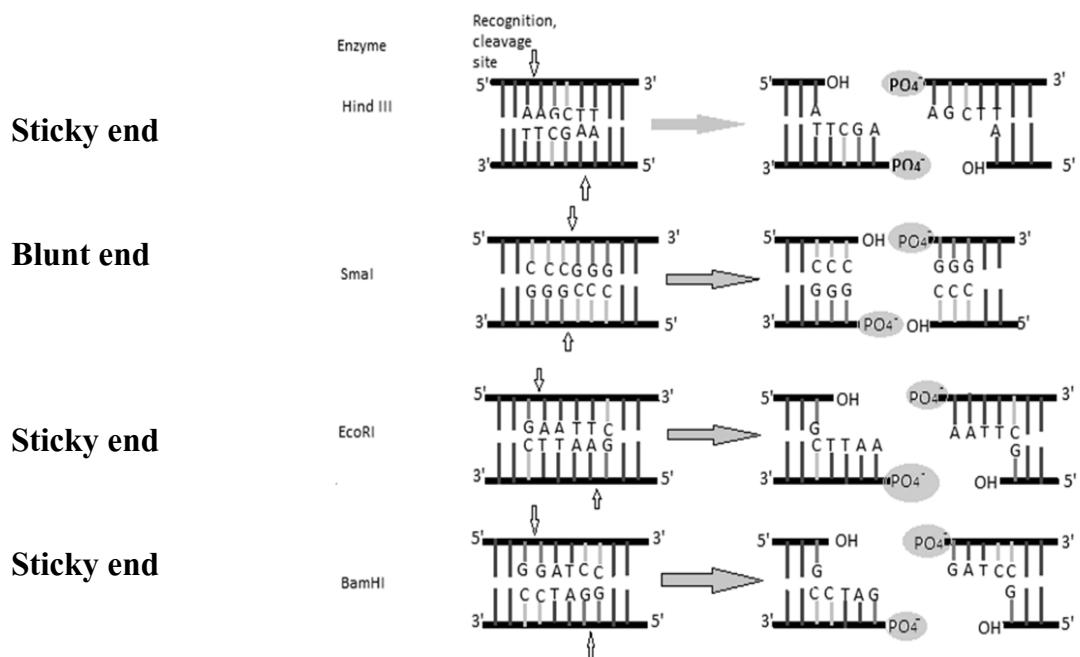
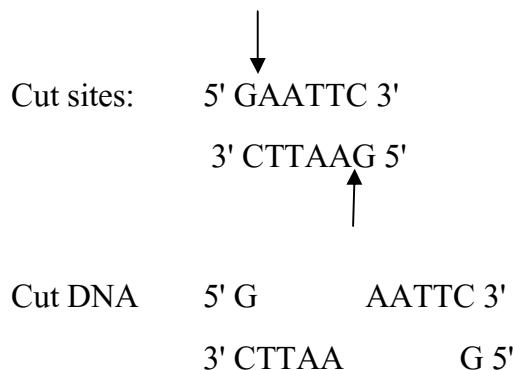
***HindIII***



First letter of the genus name ‘d’ is the  
(*Haemophilus*) and the first strain type  
two letter of the species ‘III’ is for the 3<sup>rd</sup> enzyme  
name (*influenza*) discovered in that organism

### 9.2.3 Cleavage Patterns of Restriction Endonucleases

The recognition and cleavage sites and cleavage patterns of *HindIII*, *SmaI*, *EcoRI*, and *BamHI* are shown. Cleavage by an endonuclease creates DNA sequence with either a sticky end or blunt end. The blunt ended fragments can be joined to any other DNA fragment with blunt ends using linkers or adapters, making these enzymes useful for certain types of DNA cloning experiments. *EcoRI* makes one cut between the G and A in each of the DNA strands. After the cuts are made, the DNA is held together only by the hydrogen bonds between the four bases in the middle. Hydrogen bonds are weak, and the DNA comes apart.



**Fig. 9.1 : Cleavage Patterns of *HindIII*, *SmaI*, *EcoRI* and *BamHI***

#### **9.2.4 Selection of Restriction Enzymes**

Restriction enzymes are enzymes that cut both single- and double-stranded DNA. Each restriction enzyme has a specific nucleotide sequence, called a restriction site, that it recognizes and cuts. Restriction enzymes are used for DNA sequencing, mutational analysis, and cloning and amplification of DNA. Scientists use restriction enzymes to insert genes of interest into expression vectors, DNA molecules that can replicate separately from chromosomal DNA. The vector containing the gene of interest can then be introduced into a bacterial strain for expression and characterization of proteins.

##### **Step 1**

Identify restriction enzyme sites on your vector by looking at a restriction map. The restriction map will tell you which enzymes will cut your vector, and where.

##### **Step 2**

Choose a restriction enzyme that also has a site present on your gene insert, by looking at the sequence of the insert. Ensure the restriction site is at a position on your insert that is outside the gene of interest, so you do not lose any part of the gene.

##### **Step 3**

Ensure that there are no duplicates of the restriction site anywhere in your gene insert or vector. This will cause multiple cuts in your DNA and give you misleading data.

##### **Step 4**

Try to choose restriction enzymes that cut with sticky ends, rather than blunt ends. Sticky ends occur when the enzyme cuts double stranded DNA in a staggered manner, leaving a single stranded overhang that facilitates attachment with an insert cut in the opposite manner. Blunt ends occur when the double-stranded DNA is cut in a smooth manner, and these are more difficult to attach.

**Step 5**

Choose a different restriction enzyme for both ends of your insert to ensure it is inserted into the vector in the proper orientation and to ensure the vector does not re-attach to itself.

**Step 6**

Try to choose two restriction enzymes which function well in the same buffer system and temperature. If this is not possible, run each digestion separately.

**9.2.5 Applications**

In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments. Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.

**9.3 Ligase**

Ligase (from the Latin verb *ligare* - "to bind" or "to glue together") is an enzyme that can catalyze the joining of two large molecules by forming a phosphodiester bond between two deoxynucleotide residues of two DNA strands, usually with accompanying hydrolysis of a small chemical group dependent to one of the larger molecules or the enzyme catalyzing the linking together of two compounds. DNA ligase enzyme requires a free hydroxyl group at the 3' -end of one DNA chain and a phosphate group at the 5'-end of the other and requires energy in the process. *E.coli* and other bacterial DNA ligase utilize NAD<sup>+</sup> as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor. The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the Okazaki fragments. This joining process

is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break are repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.

DNA ligases close nicks in the phosphodiester backbone of DNA. Biologically, DNA ligases are essential for the joining of Okazaki fragments during replication, and for completing short-patch DNA synthesis occurring in DNA repair process. There are two classes of DNA ligases. The first uses NAD<sup>+</sup> as a cofactor and only found in bacteria. The second uses ATP as a cofactor and found in eukaryotes, viruses and bacteriophages. The smallest known ATP-dependent DNA ligase is the one from the bacteriophage T7 (at 41KdA). Eukaryotic DNA ligases may be much larger (human DNA ligase I is >100KDA) but they all appear to share some common sequences and probably structural motifs.

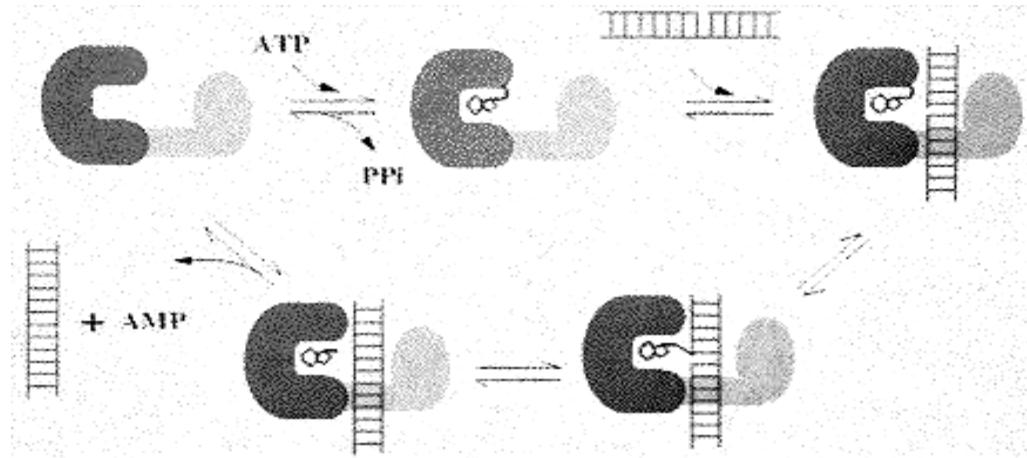
### **9.3.1 DNA Ligase Mechanism**

ATP, or NAD<sup>+</sup>, reacts with the ligase enzyme to form a covalent enzyme-AMP complex in which the AMP is linked to ε-amino group of a lysine residue in the active site of the enzyme through a phosphoamide bond. The AMP moiety activates the phosphate group at the 5'-end of the DNA molecule to be joined. It is called as the donor. The final step is a nucleophilic attack by the 3'-hydroxyl group on this activated phosphorus atom which acts as the acceptor. A phosphodiester bond is formed and AMP is released. The reaction is driven by the hydrolysis of the pyrophosphate released during the formation of the enzyme-adenylate complex. Two high-energy phosphate bonds are spent in forming a phosphodiester bond in the DNA backbone with ATP serving as energy source. The temperature optimum for T4 DNA ligase mediated ligation *in vitro* is 16°C. However ligation is also achieved by incubation at 4°C by incubating over night or at room temperature condition by incubating for 30 minutes.

The reaction occurs in three stages in all DNA ligases:

1. Formation of a covalent enzyme-AMP intermediate linked to a lysine side-chain in the enzyme.

2. Transfer of the AMP nucleotide to the 5' phosphate of the nicked DNA strand.
3. Attack on the AMP-DNA bond by the 3'-OH of the nicked DNA sealing the phosphate backbone and resealing AMP.

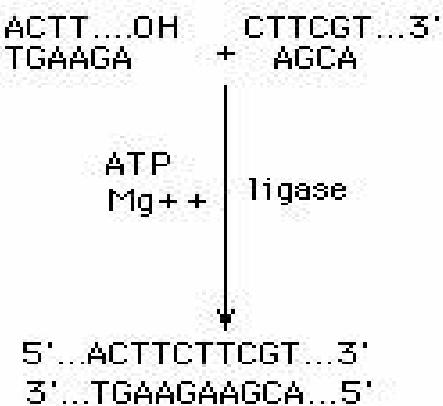


**Fig. 9.2 : Three Reaction Stages of Ligase**

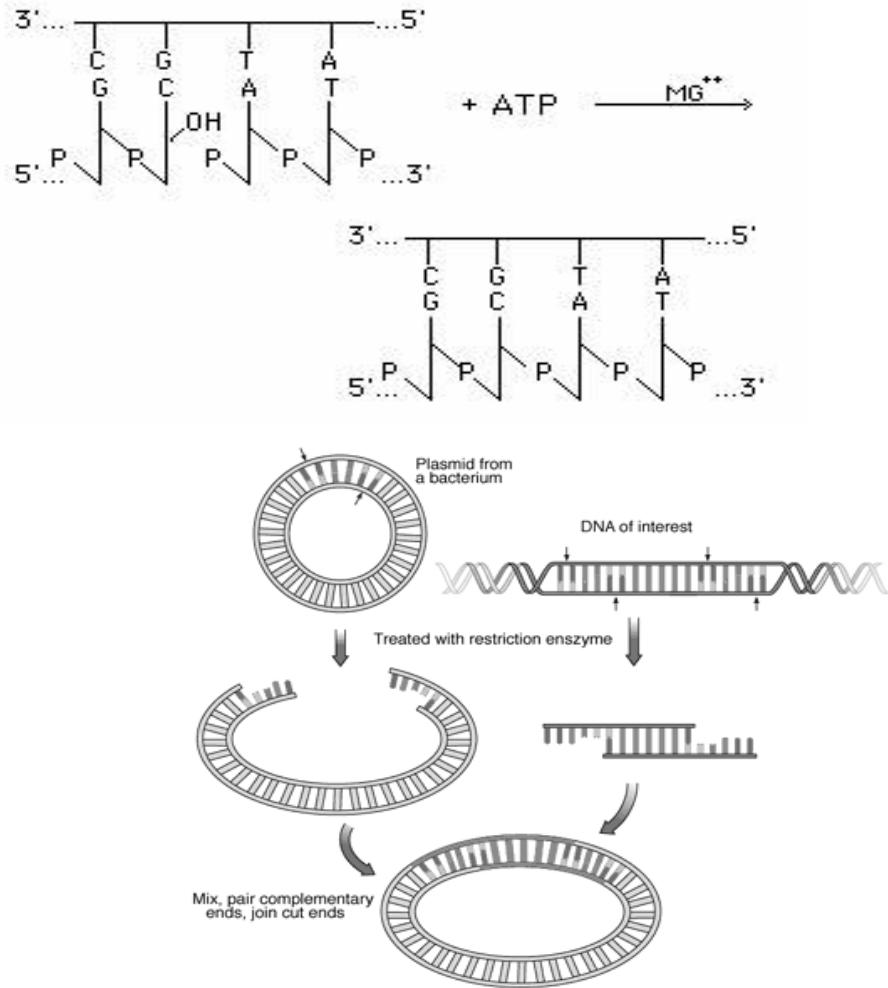
### DNA Ligase, T4

The catalytic activity of the enzyme requires the presence of ATP and Mg<sup>++</sup>. DNAs that lack the required phosphate residues can be rendered capable of ligation by phosphorylation with T4 polynucleotide kinase. The enzyme also catalyzes an addition reaction of phosphate between pyrophosphate and ATP. The ligation and the repair catalyzed reactions of T4 DNA ligases are illustrated in the following:

#### 1- Ligation of DNA with complementary cohesive termini



## 2- Repair reaction



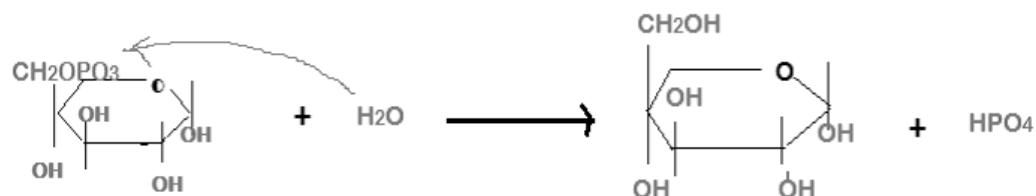
**Fig.-9.3 : Diagrammatic Presentation of Ligase Action**

### 9.3.2 Applications

- DNA ligase enzyme is used by cells to join the “okazaki fragments” during DNA replication process. In molecular cloning, ligase enzyme has been routinely used to construct a recombinant DNA. Followings are some of the examples of application of ligase enzyme in molecular cloning. Joining of adapters and linkers to blunt end DNA molecule.
- Cloning of restricted DNA to vector to construct recombinant vector.

## 9.4 Phosphatase

A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a freehydroxyl group. This action is directly opposite to that of phosphorylases and kinases, which attach phosphate groups to their substrates by using energetic molecules like ATP. A common phosphatase in many organisms is alkaline phosphatase. Another large group of proteins present in archaea, bacteria, and eukaryoteexhibits deoxyribonucleotideand ribonucleotide phosphatase or pyrophosphatase activities that catalysethedecompositionof dNTP/NTP into dNDP/NDP and a free phosphate ion or dNMP/NMP and a free pyrophosphate ion. Phosphatases act in opposition to kinases/phosphorylases, which add phosphate groups to proteins. The addition of a phosphate group may activate or de-activate an enzyme or enable a protein-protein interaction to occur; therefore phosphatases are integral to many signal transductionpathways. It should be noted that phosphate addition and removal do not necessarily correspond to enzyme activation or inhibition, and that several enzymes have separate phosphorylation sites for activating or inhibiting functional regulation.

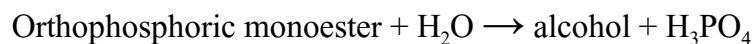


**Fig. 9.4 : Hydrolytic Cleavage of Phosphate Group (-PO<sub>4</sub>)**

### 9.4.1 Acid Phosphatase

Acid phosphatase is a phosphatase, a type of enzyme, used to free attached phosphate groups from other molecules during digestion. It is basically a phosphomonoesterase. It is stored in lysosomesand functions when these fuse with endosomes, which are acidified while they function; therefore, it has an acid pH optimum. It shows its optimal activity at pH between 3 and 6.

Different forms of acid phosphatase are found in different organs, and their serum levels are used to evaluate the success of the surgical treatment of prostate cancer. In the past, they were also used to diagnose this type of cancer. Acid phosphatase catalyzes the following reaction at an optimal pH below 7-



Phosphatase enzymes are also used by soil microorganisms to access organically bound phosphate nutrients. An assay on the rates of activity of these enzymes may be used to ascertain biological demand for phosphates in the soil. Some plant roots, especially cluster roots, exude carboxylates that perform acid phosphatase activity, helping to mobilise phosphorus in nutrient-deficient soils.

#### **9.4.2 Alkaline Phosphatase**

Alkaline phosphatase (ALP, ALKP, ALPase, Alk Phos) is hydrolyze enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called *dephosphorylation*. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase. They show their optimal activity at pH of about 10. Alkaline phosphatase was the first zinc enzyme discovered having three closed spaced metal ion. Two  $\text{Zn}^{++}$  ions and one  $\text{Mg}^{++}$  ion, in which  $\text{Zn}^{++}$  ions are bridges by *Asp* 51. The mechanism of action is based on reaction where a covalent serine – phosphate intermediate is formed to produce inorganic phosphate and an alcohol. In human body it is present in four isoforms, in which three are tissue specific isoform i.e. placental, germ cell, intestinal and one is non tissue specific isoform. The genes that encode for tissue specific isoforms are present on chromosome -2 p37-q37, while the genes for one non tissue specific are present on chromosome -1 p34- p36.1. During post-translational modification, alkaline phosphatase is modified by N-glycosylation. It undergoes a modification through which uptake of two  $\text{Zn}^{++}$  ion and one  $\text{Mg}^{++}$ ion occurs which is important in forming active site of that enzyme. Alkaline phosphatases are isolated from various sources like microorganisms, tissue of different organs, connective tissue of invertebrate and vertebrate, and human body.

### 9.4.3 Types of Alkaline Phosphate (AP)

There are several AP that are used in gene manipulation:

**9.4.3.1 Bacterial Alkaline Phosphatase (BAP)** - Bacterial alkaline phosphate is a phosphomonoester that hydrolyzes 3' and 5' phosphate from nucleic acid (DNA/ RNA). It more suitably removes phosphate group before end labeling and remove phosphate from vector prior to insert ligation. BAP generally shows optimum activity at temperature 65°C. BAP is sensitive to inorganic phosphate so in presence of inorganic phosphates activity may reduce.

There are several AP that are used in gene manipulation-

**9.4.3.2 Calf Intestinal Alkaline Phosphatase (CIAP)** – It is isolated from calf intestine, which catalyzes the removal of phosphate group from 5' end of DNA as well as RNA. This enzyme is highly used in gene cloning experiments, as to make a construct that could not undergo self-ligation. Hence after the treatment with CIAP, without having a phosphate group at 5' ends a vector cannot self ligate and recircularise. This step improves the efficiency of vector containing desired insert.

**9.4.3.3 Shrimp Alkaline Phosphatase (SAP)** - Shrimp alkaline phosphatase is highly specific, heat labile phosphatase enzyme isolated from arctic shrimp (*Pandalus borealis*). It removes 5' phosphate group from DNA, RNA, dNTPs and proteins. SAP has similar specificity as CIP but unlike CIP, it can be irreversibly inactivated by heat treatment at 65°C for 15mins. SAP is used for 5' dephosphorylation during cloning experiments for various applications as follows:

- Dephosphorylate 5'-phosphate group of DNA or RNA for subsequent labeling of the ends.
- To prevent self-ligation of the linearized plasmid.
- To prepare PCR product for sequencing.
- To inactivate remaining dNTPs from PCR product (for downstream sequencing application).

### Uses of Alkaline Phosphatase in DNA Modification

It removes 5' phosphate from different vector like plasmid, bacteriophage after treating with restriction enzyme. This treatment prevents self ligation because unavailability of phosphate group at end. So, this treatment greatly enhances the ligation of desired insert. During ligation of desired insert, the complementary ends of the insert and vector will come to proximity of each other (only for sticky ends but not for blunt ends). One strand of the insert having 5'-phosphate will ligate with the 3'OH of the vector and the remaining strand will have a nick. This nick will be sealed in the next step by ligase enzyme in the presence of ATP. It is used to remove 5' phosphate from fragment of DNA prior to labeling with radioactive phosphate.

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### 9.5 Kinase

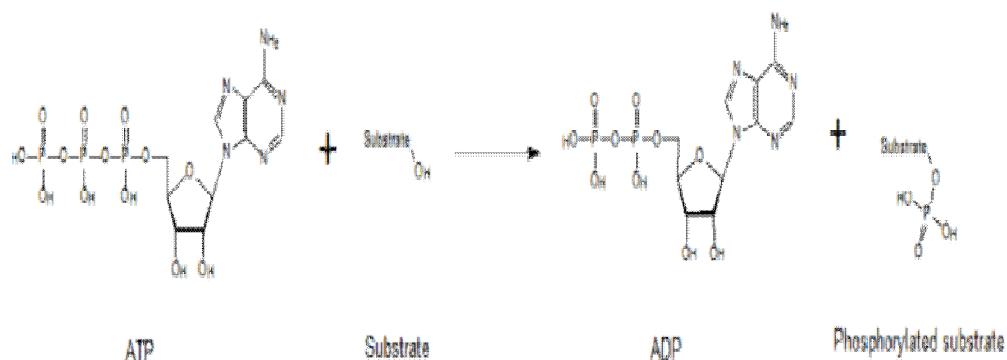
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In biochemistry, a kinase is a type of enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates. This process is known as phosphorylation when the substrate gains a phosphate group and the high energy molecule of ATP donates a phosphate group (producing a phosphorylated substrate and ADP). Conversely, it is referred to as dephosphorylation when the phosphorylated substrate donates a phosphate group and ADP gains a phosphate group (producing a dephosphorylated substrate and the high energy molecule of ATP). These two processes, phosphorylation and dephosphorylation, occurs four times during glycolysis. Kinases are part of the larger family of phosphotransferases. Kinases are not to be confused with phosphorylases, which catalyze the addition of inorganic phosphate groups to an acceptor, nor with phosphatases, which remove phosphate groups. The phosphorylation state of a molecule, whether it is a protein, lipid, or carbohydrate, can affect its activity, reactivity, and its ability to bind other molecules. Therefore, kinases are critical in metabolism, cell signalling, protein regulation, cellular transport, secretory, and countless other cellular pathways.

Kinases mediate the transfer of a phosphate moiety from a high energy molecule (such as ATP) to their substrate molecule, as seen in the Fig. below. Kinases are needed to stabilize this reaction because the phosphoanhydride bond contains a high level of energy. Kinases properly orient their substrate and the phosphoryl group

within their active sites, which increases the rate of the reaction. Additionally, they commonly use positively charged amino acid residues, which electro-statically stabilize the transition state by interacting with the negatively charged phosphate groups. Alternatively, some kinases utilize bound metal cofactors in their active sites to coordinate the phosphate groups.

Kinases are used extensively to transmit signals and regulate complex processes in cells. Phosphorylation of molecules can enhance or inhibit their activity and modulate their ability to interact with other molecules. The addition and removal of phosphoryl groups provides the cell with a means of control because various kinases can respond to different conditions or signals. Mutations in kinases that lead to a loss-of-function or gain-of-function can cause cancer and disease in humans, including certain types of leukemia and neuroblastomas, glioblastoma, spinocerebellar ataxia (type 14), forms of agammaglobulinaemia, and many others.



**Fig.-9.5 : General Reaction of Kinase**

### 9.5.1 History and Classification

The first protein to be recognized as catalyzing the phosphorylation of another protein using ATP was observed in 1954 by Gene Kennedy at which time he described a liver enzyme that catalyzed the phosphorylation of casein. In 1956, Edmond Fischer and Edwin Krebs discovered that the interconversion between phosphorylase-a and phosphorylase-b was mediated by phosphorylation and dephosphorylation. The kinase that transferred a phosphoryl group to Phosphorylase b, converting it to Phosphorylase a, was named Phosphorylase Kinase. Years later, the first example of a kinase cascade was identified, whereby Protein Kinase A (PKA) phosphorylates Phosphorylase Kinase. At the same time,

it was found that PKA inhibited glycogen synthase, which was the first example of a phosphorylation event that resulted in inhibition. In 1969, Lester Reed discovered that pyruvate dehydrogenase was inactivated by phosphorylation, and this discovery was the first clue that phosphorylation might serve as a means of regulation in other metabolic pathways besides glycogen metabolism. In the same year, Tom Langan discovered that PKA phosphorylates histone H1, which suggested phosphorylation might regulate nonenzymatic proteins. The 1970s included the discovery of calmodulin-dependent protein kinases and the finding that proteins can be phosphorylated on more than one amino acid residue. The 1990s may be described as the "decade of protein kinase cascades". During this time, the MAPK/ERK pathway, the JAK kinases (a family of protein tyrosine kinases), and the PIP3-dependent kinase cascade were discovered.

Kinases are classified into broad groups by the substrate they act upon: protein kinases, lipid kinases, carbohydrate kinases. Kinases can be found in a variety of species, from bacteria to mold to worms to mammals. More than five hundred different kinases have been identified in humans. Their diversity and their role in signaling make them an interesting object of study. Various other kinases act on small molecules such as lipids, carbohydrates, amino acids, and nucleotides, either for signaling or to prime them for metabolic pathways. Specific kinases are often named after their substrates. Protein kinases often have multiple substrates, and proteins can serve as substrates for more than one specific kinase. For this reason protein kinases are named based on what regulates their activity (i.e. Calmodulin-dependent protein kinases). Sometimes they are further subdivided into categories because there are several isoenzymatic forms. For example, type I and type II cyclic-AMP dependent protein kinases have identical catalytic subunits but different regulatory subunits that bind cyclic AMP

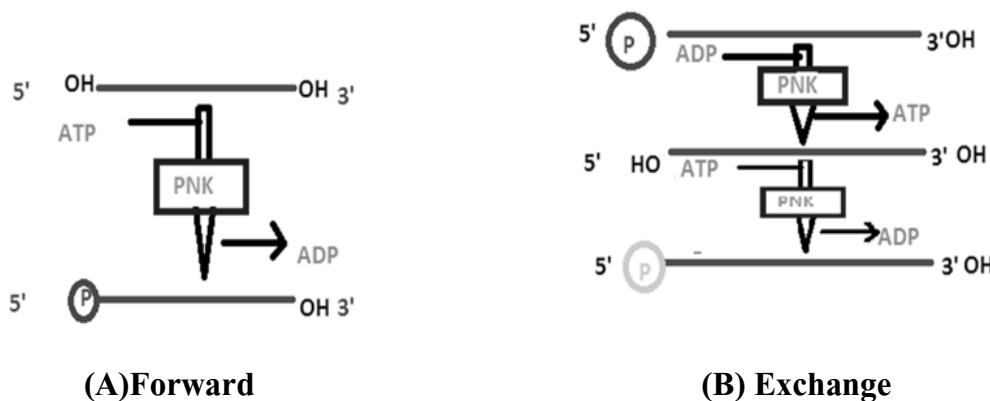
### 9.5.2 Enzymatic Activity of Kinase

Kinase carries out two types of enzymatic activity-

**Forward Reaction:**  $\gamma$ -phosphate is transferred from ATP to the 5' end of a polynucleotide (DNA or RNA). 5' phosphate is not present either due to chemical synthesis or dephosphorylation. The 5' OH nucleophile is activated by abstraction

of the proton. Asp35 of PNK forms the co-ordinate bond with 5'OH and attacks  $\gamma$  phosphorus forming an intermediate.

**Exchange Reaction:** target DNA or RNA having a 5' phosphate is incubated with an excess of ADP - where PNK transfers the phosphate from the nucleic acid to an ADP, forming ATP. PNK then performs a forward reaction and transfer a phosphate from ATP to the target nucleic acid. Exchange reaction is used to label with radioactive phosphate group.



**Fig. 9.6 : Polynucleotide Kinase Reaction**

### 9.5.3 Types of Kinase

On the basis of enzyme action kinase can be divided in following types-

#### 1. Protein Kinases

Protein kinases act on proteins, phosphorylating them on their serine, threonine, tyrosine, or histidine residues. Phosphorylation can modify the function of a protein in many ways. It can increase or decrease a protein's activity, stabilize it or mark it for destruction, localize it within a specific cellular compartment, and it can initiate or disrupt its interaction with other proteins. The protein kinases make up the majority of all kinases and are widely studied. These kinases, in conjunction with phosphatases, play a major role in protein and enzymeregulation as well as signalling in the cell.

A common point of confusion arises when thinking about the different ways a cell achieves biological regulation. There are countless examples of covalent

modifications that cellular proteins can undergo; however, phosphorylation is one of the few reversible covalent modifications. This provided the rationale that phosphorylation of proteins is regulatory. The potential to regulate protein function is enormous given that there are many ways to covalently modify a protein in addition to regulation provided by allosteric control. In his Hopkins Memorial Lecture, Edwin Krebs asserted that allosteric control evolved to respond to signals arising from inside the cell whereas phosphorylation evolved to respond to signals outside of the cell. This idea is consistent with the fact that phosphorylation of proteins occurs much more frequently in eukaryotic cells in comparison to prokaryotic cells because the more complex cell type evolved to respond to a wider array of signals.

## **2. Cyclin Dependent Kinase**

Cyclin dependent kinases (CDKs) are a group of several different kinases involved in regulation of the cell cycle. They phosphorylate other proteins on their serine or threonine residues, but CDKs must first bind to a cyclin protein in order to be active. Different combinations of specific CDKs and cyclins mark different parts of the cell cycle. Additionally, the phosphorylation state of CDKs is also critical to their activity, as they are subject to regulation by other kinases (such as CDK-activating kinase) and phosphatases (such as Cdc25). Once the CDKs are active, they phosphorylate other proteins to change their activity, which leads to events necessary for the next stage of the cell cycle. While they are most known for their function in cell cycle control, CDKs also have roles in transcription, metabolism, and other cellular events.

Because of their key role in the controlling cell division, mutations in CDKs are often found in cancerous cells. These mutations lead to uncontrolled growth of the cells, where they are rapidly going through the whole cell cycle repeatedly. CDK mutations can be found in lymphomas, breast cancer, pancreatic tumors, and lung cancer. Therefore, inhibitors of CDK have been developed as treatments for some types of cancer.

## **3. Mitogen-activated Protein Kinase**

Mitogen-activated Protein Kinases (MAPKs) are a family of serine/threonine kinases that respond to a variety of extracellular growth signals. For example,

growth hormone, epidermal growth factor, platelet-derived growth factor, and insulin are all considered mitogenic stimuli that can engage the MAPK pathway. Activation of this pathway at the level of the receptor initiates a signaling cascade whereby the Ras GTPase exchanges GDP for GTP. Next, Ras activates Raf Kinase (also known as MAPKKK), which activates MEK (MAPKK). MEK activates MAPK (also known as ERK), which can go on to regulate transcription and translation. Whereas RAF and MAPK are both serine/threonine kinases, MAPKK is a tyrosine/threonine kinase. MAPK can regulate transcription factors directly or indirectly. Its major transcriptional targets include ATF-2, Chop, c-Jun, c-Myc, DPC4, Elk-1, Ets1, Max, MEF2C, NFAT4, Sap1a, STATs, Tal, p53, CREB, and Myc. MAPK can also regulate translation by phosphorylating the S6 kinase in the large ribosomal subunit. It can also phosphorylate components in the upstream portion of the MAPK signalling cascade including Ras, SOS, and the EGF receptor itself.

#### **4. Lipid Kinases**

Lipid kinases phosphorylate lipids in the cell, both on the plasma membrane as well as on the membranes of the organelles. The addition of phosphate groups can change the reactivity and localization of the lipid and can be used in signal transmission.

#### **5. Carbohydrate Kinases**

For many mammals, carbohydrates provide a large portion of the daily caloric requirement. To harvest energy from oligosaccharides, they must first be broken down into monosaccharides so they can enter metabolism. Kinases play an important role in almost all metabolic pathways. The anhydride linkage in 1,3 bisphosphoglycerate is unstable and has a high energy. 1,3-bisphosphoglycerate kinase requires ADP to carry out its reaction yielding 3-phosphoglycerate and ATP. In the final step of glycolysis, pyruvate kinase transfers a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate.

Hexokinase is the most common enzyme that makes use of glucose when it first enters the cell. It converts D-glucose to glucose-6-phosphate by transferring the gamma phosphate of an ATP to the C6 position. This is an important step in glycolysis because it traps glucose inside the cell due to the negative charge. In its

dephosphorylated form, glucose can move back and forth across the membrane very easily. Mutations in the hexokinase gene can lead to a hexokinase deficiency which can cause nonspherocytic hemolytic anemia.

## **6. Riboflavin Kinase**

Riboflavin kinase catalyzes the phosphorylation of riboflavin to create flavin mononucleotide (FMN). It has an ordered binding mechanism where riboflavin must bind to the kinase before it binds to the ATP molecule. Divalent cations help coordinate the nucleotide.

## **7. Thymidine Kinase**

Thymidine kinase is one of the many nucleoside kinases that are responsible for nucleoside phosphorylation. It phosphorylates thymidine to create thymidine monophosphate (dTMP). This kinase uses an ATP molecule to supply the phosphate to thymidine, as shown below. This transfer of a phosphate from one nucleotide to another by thymidine kinase, as well as other nucleoside and nucleotide kinases, functions to help control the level of each of the different nucleotides.

### **9.5.4 Uses of Kinase**

1. The linkers and adopters are phosphorylated along with the fragments of DNA before ligation, which requires a 5' phosphate. This includes products of polymerase chain reaction, which are generated by using non-phosphorylated primers.
2. Kinase is also used for radio labelling oligonucleotides, generally with  $^{32}\text{P}$  for preparing hybridization probes.

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## **9.6 Transferases**

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Transferase is the general name for the class of enzymes that enact the transfer of specific functional groups (e.g. a methyl or glycosyl group) from one molecule (called the donor) to another (called the acceptor). They are involved in hundreds of different biochemical pathways throughout biology, and are integral to some of life's most important processes.

Transferases are involved in myriad reactions in the cell. Some examples of these reactions include the activity of Co-Atransferase, which transfers thiol esters, the action of N-acetyltransferase is part of the pathway that metabolizes tryptophan, and also includes the regulation of PDH, which converts pyruvate to Acetyl CoA. Transferases are also utilized during translation. In this case, an amino acid chain is the functional group transferred by a Peptidyl transferase. The transfer involves the removal of the growing amino acid chain from the tRNA molecule in the A-site of the ribosome and its subsequent addition to the amino acid attached to the tRNA in the P-site.

Mechanistically, an enzyme that catalyzed the following reaction would be a transferase-



In the above reaction, X would be the donor, and Y would be the acceptor. "Group" would be the functional group transferred as a result of transferase activity. The donor is often a coenzyme.

### 9.6.1 History

Some of the most important discoveries relating to transferases occurred as early as the 1930s. Earliest discoveries of transferase activity occurred in other classifications of enzymes, including Beta-galactosidase, protease, and acid/base phosphatase. Prior to the realization that individual enzymes were capable of such a task, it was believed that two or more enzymes enacted functional group transfers.

Transamination, or the transfer of an amine (or NH<sub>2</sub>) group from an amino acid to a keto acid by an aminotransferase (also known as a "transaminase"), was first noted in 1930 by D. M. Needham, after observing the disappearance of glutamic acid added to pigeon breast muscle. This observance was later verified by the discovery of its reaction mechanism by Braunstein and Kitzmann in 1937. Their analysis showed that this reversible reaction could be applied to other tissues. This assertion was validated by Rudolf Schoenheimer's work with radioisotopes as tracers in 1937. This in turn would pave the way for the

possibility that similar transfers were a primary means of producing most amino acids via amino transfer.

Another such example of early transferase research and later reclassification involved the discovery of uridyl transferase. In 1953, the enzyme UDP-glucose pyrophosphorylase was shown to be a transferase, when it was found that it could reversibly produce UTP and G1P from UDP-glucose and an organic pyrophosphate.

Classification of transferases continues to this day, with new ones being discovered frequently. An example of this is Pipe, a sulfotransferase involved in the dorsal-ventral patterning of *Drosophila*. Initially, the exact mechanism of Pipe was unknown, due to a lack of information on its substrate. Research into Pipe's catalytic activity eliminated the likelihood of it being a heparan sulfate glycosaminoglycan. Further research has shown that Pipe targets the ovarian structures for sulfation. Pipe is currently classified as a *Drosophila* heparan sulfate 2-O-sulfotransferase.

### 9.6.2 Nomenclature

Systematic names of transferases are constructed in the form of "donor: acceptor grouptransferase." For example, methylamine:L-glutamate N-methyltransferase would be the standard naming convention for the transferase methylamine-glutamate N-methyltransferase, where methylamine is the donor, L-glutamate is the acceptor, andmethyltransferase is the EC category grouping. This same action by the transferase can be illustrated as follows-



However, other accepted names are more frequently used for transferases, and are often formed as "acceptor grouptransferase" or "donor grouptransferase." For example, aDNA methyltransferase is a transferase that catalyzes the transfer of a methyl group to a DNA acceptor. In practice, many molecules are not referred to using this terminology due to more prevalent common names. For example, RNA Polymerase is the modern common name for what was formerly known as RNA nucleotidyltransferase, a kind ofnucleotidyl transferase that transfers nucleotides to the 3' end of a growing RNA strand. In the EC system of classification, the accepted name for RNA Polymerase is DNA-directed RNA polymerase.

### **9.6.3 Types of Transferases**

Transferase can be divided in following types on the basis of action-

#### **1. Terminal Transferases**

Terminal transferases can be used to label DNA or to produce plasmid vectors. It accomplishes both of these tasks by adding deoxynucleotides in the form of a template to the downstream end or 3' end of an existing DNA molecule. Terminal transferase is one of the few DNA polymerases that can function without an RNA primer.

#### **2. Glutathione Transferases**

The family of glutathione transferases is extremely diverse, and therefore can be used for a number of biotechnological purposes. Plants use glutathione transferases as a means to segregate toxic metals from the rest of the cell. These glutathione transferases can be used to create biosensors to detect contaminants such as herbicides and insecticides. Glutathione transferases are also used in transgenic plants to increase resistance to both biotic and abiotic stress. Glutathione transferases are currently being explored as targets for anti-cancer medications due to their role in drug resistance. Further, glutathione transferase genes have been investigated due to their ability to prevent oxidative damage and have shown improved resistance in transgenic cultigens.

#### **3. Rubber Transferases**

Currently the only available commercial source of natural rubber is the Hevea plant (*Hevea brasiliensis*). Natural rubber is superior to synthetic rubber in a number of commercial uses. Efforts are being made to produce transgenic plants capable of synthesizing natural rubber, including tobacco and sunflower. These efforts are focused on sequencing the subunits of the rubber transferase enzyme complex in order to transfet these genes into other plants.

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### **9.7 Polymerase**

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A polymerase is an enzyme that synthesizes long chains or polymers of nucleic acids. DNA polymerase and RNA polymerase are used to assembleDNAandRNA

molecules, respectively, by copying a DNA or RNA template strand using base-pairing interactions.

It is an accident of history that the enzymes responsible for the generation of other biopolymers are not also referred to as polymerases. For example, the enzymatic complex that assembles amino acids into proteins is termed the ribosome, rather than "protein polymerase".

A polymerase from the thermophilic bacterium, *Thermus aquaticus* (*Taq*) is used in the polymerase chain reaction, an important technique of molecular biology.

### 9.7.1 DNA Polymerase

The DNA polymerases are enzymes that creates DNA molecules by assembling nucleotides, the building blocks of DNA. These enzymes are essential to DNA replication and usually work in pairs to create two identical DNA strands from a single original DNA molecule. During this process, DNA polymerase "reads" the existing DNA strands to create two new strands that match the existing ones.

Every time a cell divides, DNA polymerase is required to help duplicate the cell's DNA, so that a copy of the original DNA molecule can be passed to each of the daughter cells. In this way, genetic information is transmitted from generation to generation.

Before replication can take place, an enzyme called helicase unwinds the DNA molecule from its tightly woven form. This opens up or "unzips" the double-stranded DNA to give two single strands of DNA that can be used as templates for replication.

#### 9.7.1.1 History

In 1956, Arthur Kornberg and colleagues discovered the enzyme DNA polymerase I, also known as Pol I, in *Escherichia coli*. They described the DNA replication process by which DNA polymerase copies the base sequence of a template DNA strand. Subsequently, in 1959, Kornberg was awarded the Nobel Prize in Physiology or Medicine for this work. DNA polymerase II was also discovered by Kornberg and Malcolm E. Gefter in 1970 while further elucidating the role of Pol I in *E. coli* DNA replication.

### 9.7.1.2 Structure

The known DNA polymerases have highly conserved structure, which means that their overall catalytic subunits vary very little from species to species, independent of their domain structures. The shape can be described as resembling a right hand with thumb, finger, and palm domains. The palm domain appears to function in catalyzing the transfer of phosphoryl groups in the phosphoryl transfer reaction. DNA is palm when the enzyme is active. This reaction is believed to be catalyzed by a two metal ion mechanism. The finger domain functions to bind the nucleotide triphosphate with the template base. The thumb domain plays a potential role in the processivity, translocation, and positioning of the DNA. Conserved structures usually indicate important, irreplaceable functions of the cell, the maintenance of which provides evolutionary advantages.

### 9.7.1.3 Functions

The main function of DNA polymerase is to make DNA from nucleotides, the building blocks of DNA. The DNA copies are created by the pairing of nucleotides to bases present on each strand of the original DNA molecule. This pairing always occurs in specific combinations, with cytosine and guanine and thymine and adenine forming two separate pairs respectively.

When creating DNA, DNA polymerase can add free nucleotides only to the 3' end of the newly forming strand. This results in elongation of the newly forming strand in a 5'-3' direction. No known DNA polymerase is able to begin a new chain (*de novo*); it can only add a nucleotide onto a pre-existing 3'-OH group, and therefore needs a primer at which it can add the first nucleotide. Primers consist of RNA or DNA bases (or both). In DNA replication, the first two bases are always RNA, and are synthesized by another enzyme called primase. An enzyme known as a helicase is required to unwind DNA from a double-strand structure to a single-strand structure to facilitate replication of each strand consistent with the semiconservative model of DNA replication.

It is important to note that the directionality of the newly forming strand (the daughter strand) is opposite to the direction in which DNA polymerase moves along the template strand. Since DNA polymerase requires a free 3' OH group for

initiation of synthesis, it can synthesize in only one direction by extending the 3' end of the preexisting nucleotide chain. Hence, DNA polymerase moves along the template strand in a 3'-5' direction, and the daughter strand is formed in a 5'-3' direction. This difference enables the resultant double-strand DNA formed to be composed of two DNA strands that are antiparallel to each other.

### **9.7.2 Prokaryotic DNA Polymerase**

#### ***1. Polymerase I (Pol I)***

Prokaryotic Family A polymerases include the DNA polymerase I (Pol I) enzyme, which is encoded by the *polA* gene and ubiquitous among prokaryotes. This repair polymerase is involved in excision repair with 3'-5' and 5'-3' exonuclease activity and processing of Okazaki fragments generated during lagging strand synthesis. Pol I is the most abundant polymerase accounting for >95% of polymerase activity in *E. coli*, yet cells lacking Pol I have been found suggesting Pol I activity can be replaced by the other four polymerases. Pol I adds ~15-20 nucleotides per second, thus showing poor processivity. Instead, Pol I starts adding nucleotides at the RNA primer: template junction known as the origin of replication (*ori*). Approximately 400 bp downstream from the origin, the Pol III holoenzyme is assembled and takes over replication at a highly processive speed and nature.

#### ***2. Polymerase II (Pol II)***

DNA polymerase II, a Family B polymerase, is a *polB* gene product also known as DinA. Pol II has 3'-5' exonuclease activity and participates in DNA repair, replication restart to bypass lesions, and its cell presence can jump from ~30-50 copies per cell to ~200-300 during SOS induction. Pol II is also thought to be a backup to Pol III as it can interact with holoenzyme proteins and assume a high level of processivity. The main role of Pol II is thought to be the ability to direct polymerase activity at the replication fork and help stalled Pol III bypass terminal mismatches.

#### ***3. Polymerase III (Pol III)***

DNA polymerase III holoenzyme is the primary enzyme involved in DNA replication in *E. coli* and belongs to Family C polymerases. It consists of three

assemblies: the pol III core, the beta sliding clamp processivity factor and the clamp-loading complex. The core consists of three subunits - **α**, the polymerase activity hub, **ε**, exonucleolytic proofreader, and **θ**, which may act as a stabilizer for **ε**. The holoenzyme contains two cores, one for each strand, the lagging and leading. The beta sliding clamp processivity factor is also present in duplicate, one for each core, to create a clamp that encloses DNA allowing for high processivity. The third assembly is a seven-subunit ( $T_2\gamma\delta\delta'\chi\Psi$ ) clamp loader complex. Recent research has classified Family C polymerases as a subcategory of Family X with no eukaryotic equivalents.

### **9.7.3 RNA Polymerase**

RNA polymerase (RNAP or RNAPol) also known as DNA-dependent RNA polymerase, is an enzyme that produces primary transcript RNA. In cells, RNAP is necessary for constructing RNA chains using DNA genes as templates, a process called transcription. RNA polymerase enzymes are essential to life and are found in all organisms and many viruses. In chemical terms, RNAP is a nucleotidyl transferase that polymerizes ribonucleotides at the 3' end of an RNA transcript.

#### **9.7.3.1 History**

RNAP was discovered independently by Charles Loe, Audrey Stevens, and Jerard Hurwitz in 1960. By this time, one half of the 1959 Nobel Prize in Medicine had been awarded to Severo Ochoa for the discovery of what was believed to be RNAP, but instead turned out to be polynucleotide phosphorylase. The 2006 Nobel Prize in Chemistry was awarded to Roger D. Kornberg for creating detailed molecular images of RNA polymerase during various stages of the transcription process.

#### **9.7.3.2 Action of RNAP**

RNA polymerase binding in bacteria involves the sigma factor recognizing the core promoter region containing the -35 and -10 elements and also, at some promoters, the **α** subunit C-terminal domain recognizing promoter upstream elements. There are multiple interchangeable sigma factors, each of which

recognizes a distinct set of promoters. For example, in *E. coli*,  $\Sigma^{70}$  is expressed under normal conditions and recognizes promoters for genes required under normal conditions ("housekeeping genes"), while  $\Sigma^{32}$  recognizes promoters for genes required at high temperatures ("heat-shock genes").

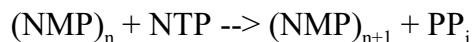
After binding to the DNA, the RNA polymerase switches from a closed complex to an open complex. This change involves the separation of the DNA strands to form an unwound section of DNA of approximately 13 bp, referred to as the transcription bubble. Ribonucleotides are base-paired to the template DNA strand, according to Watson-Crick base-pairing interactions. Supercoiling plays an important part in polymerase activity because of the unwinding and rewinding of DNA. Because regions of DNA in front of RNAP are unwound, there is a compensatory positive supercoil. Regions behind RNAP are rewound and negative supercoils are present.

### **9.7.3.3 Elongation Mechanism by RNAP**

Transcription elongation involves the further addition of ribonucleotides and the change of the open complex to the transcriptional complex. RNAP cannot start forming full length transcripts because of its strong binding to the promoter. Transcription at this stage primarily results in short RNA fragments of around 9 bp in a process known as abortive transcription. Once the RNAP starts forming longer transcripts it clears the promoter. At this point, the contacts with the -10 and -35 elements are disrupted, and the  $\Sigma$  factor falls off RNAP. This allows the rest of the RNAP complex to move forward, as the  $\Sigma$  factor held the RNAP complex in place. The 17-bp transcriptional complex has an 8-bp DNA-RNA hybrid, that is, 8 base-pairs involve the RNA transcript bound to the DNA template strand. As transcription progresses, ribonucleotides are added to the 3' end of the RNA transcript and the RNAP complex moves along the DNA. Although RNAP does not seem to have the 3' exonuclease activity that characterizes the proofreading activity found in DNA polymerase, there is evidence of that RNAP will halt at mismatched base-pairs and correct it.

Aspartyl (asp) residues in the RNAP will hold on to  $Mg^{++}$  ions, which will, in turn, coordinate the phosphates of the ribonucleotides. The first  $Mg^{++}$  will hold on to the  $\alpha$ -phosphate of the NTP to be added. This allows the nucleophilic attack of the

3'OH from the RNA transcript, adding another NTP to the chain. The second Mg<sup>++</sup> will hold on to the pyrophosphate of the NTP. The overall reaction equation is-



#### **9.7.3.4 Types of RNAP**

Eukaryotes have multiple types of nuclear RNAP, each responsible for synthesis of a distinct subset of RNA. All are structurally and mechanistically related to each other and to bacterial RNAP:

RNA polymerase I synthesizes a pre-rRNA 45S (35S in yeast), which matures into 28S, 18S and 5.8S rRNAs which will form the major RNA sections of the ribosome.

RNA polymerase II synthesizes precursors of mRNAs and mostsnRNA andmicroRNAs. This is the most studied type, and, due to the high level of control required over transcription, a range of transcription factors are required for its binding to promoters.

RNA polymerase III synthesizes tRNAs, rRNA 5S and other small RNAs found in the nucleus and cytosol.

## **9.8 Nucleases**

A nuclease is an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids. Older publications may use terms such as "polynucleotidase" or "nucleodepolymerase".

Nucleases are usually further divided intoendonucleases and exonucleases, although some of the enzymes may fall in both categories. Well known nucleases are deoxyribonuclease and ribonuclease.

#### **9.8.1 Introduction**

In the late 1960s, scientists Stuart Linn and Werner Arber isolated examples of the two types of enzymes responsible for phage growth restriction in Escherichia coli (E. coli) bacteria. One of these enzymes added a methyl group to the DNA, generating methylated DNA, while the other cleaved unmethylated DNA at a wide

variety of locations along the length of the molecule. The first type of enzyme was called a "methylase" and the other a "restriction nuclease". These enzymatic tools were important to scientists who were gathering the tools needed to "cut and paste" DNA molecules. What was then needed was a tool that would cut DNA at specific sites, rather than at random sites along the length of the molecule, so that scientists could cut DNA molecules in a predictable and reproducible way.

### **9.8.2 Ribonuclease (RNase)**

Nucleases that can catalyze hydrolysis of ribonucleotides from either single stranded or double stranded RNA sequence are called ribonucleotides (RNase). They are classified into two types depending on position of cleavage, i.e. endoribonuclease (cleave internal bond) and exoribonuclease (cleave terminal bond). RNase is important for RNA maturation and processing. Two types of ribonuclease i.e. RNaseA and RNaseH play important role in initial defence mechanism against RNA viral infection.

#### **9.8.2.1 RibonucleaseA (RNaseA)**

It is an endo-ribonuclease that cleaves specifically single-stranded RNA at the 3' end of pyrimidine residues. The RNA is degraded into 3'-phosphorylated mononucleotides C and U residues and oligonucleotides in the form of 2', 3'-cyclic monophosphate intermediates. Optimal temperature for RNaseA is 60°C (activity range 15-70°C) and optimal pH is 7.6.

RNaseA has two histidine residues in its active site (His12 and His119). In the first step, His12 acts as a base; accepting proton forming a nucleophile which then attacks positively charged phosphorus atom. His119 acts as an acid in this case, donates a proton to oxygenated P-O-R' bond. The imidazole side chain acts as base in His 12 here. The side chain of Lys41 and Phe120 further stabilize the transition state. Nitrogen of the main chain of Phe120 donates hydrogen, thus bonding with the unbound oxygen atom.

In the second step the acid base activities get reversed and His119 accepts proton from water causing hydroxyl attack on cyclic intermediate. Activity of RNaseA can be inhibited by alkylation of His12 and His119 residue essential for activity of the enzyme.

### 9.8.2.2 RibonucleaseH (RNaseH)

RNase H is a ribonuclease that cleaves the RNA in a DNA/RNA duplex to produce ssDNA. RNase H is a non-specific endonuclease and catalyzes the cleavage of RNA via a hydrolytic mechanism; aided by an enzyme-bound divalent metal ion. Members of the RNase H family can be found in nearly all organisms, from bacteria to archaea to eukaryotes. RNase H is ribonuclease activity cleaves the 3'-O-P bond of RNA in a DNA/RNA duplex substrate to produce 3'-hydroxyl and 5'-phosphate terminated products. In DNA replication, RNase H is responsible for removing the RNA primer, allowing completion of the newly synthesized DNA. The 3-D structure of RNase H commonly consists of a 5-stranded  $\beta$ -sheet surrounded by a distribution of  $\alpha$ -helices. In some RNase H, such as the one found in HIV-1, the enzyme is missing one of the helices known as the C-helix, a positively charged  $\alpha$ -helix whose protrusive shape increases substrate binding capacity. The active site of the enzyme is centered on a conserved DEDD motif (composed of residues: D443, E478, D498, and D549) which performs the hydrolysis of the RNA substrate. Amagnesium ion is commonly used as a cofactor during the hydrolysis step. It is also a potential but unconfirmed mechanism in which multiple ions are necessary to perform the hydrolysis. The enzyme also contains a nucleic acid binding cleft about 60 Å in length that can encompass a region of 18 bound RNA/DNA base pairs.

Because RNase H specifically degrades only the RNA in RNA:DNA hybrids, it is commonly used in molecular biology to destroy the RNA template after first-strand complementary DNA (cDNA) synthesis by reverse transcription, as well as in procedures such as nuclease protection assays. RNase H can also be used to degrade specific RNA strands when the cDNA oligonucleotide is hybridized, such as the removal of the polyadenine tail from mRNA hybridized to oligo(dT), or the destruction of a chosen non-coding RNA inside or outside the living cell. To terminate the reaction, a chelator, such as EDTA, is often added to sequester the required metal ions in the reaction mixture.

### 9.8.2.3 Applications

- It is used to remove RNA contamination from DNA sample.

- Non-specific endoribonuclease that degrades RNA by hydrolytic mechanism from DNA/RNA duplex resulting in single stranded DNA.
- Enzyme bound divalent metal ion is a cofactor here. The product formed is 5' phosphorylated ssDNA.
- During cDNA library preparation from RNA sample, RNaseH enzyme is used to cleave RNA strand of DNA-RNA duplex.

### **9.8.3 Deoxyribonuclease (DNase)**

A nuclease enzyme that can catalyze the hydrolytic cleavage of phosphodiester bonds in the DNA backbone are known as deoxyribonuclease (DNase). Based on the position of action, these enzymes are broadly classified as endodeoxyribonuclease (cleave DNA sequence internally) and exodeoxyribonuclease (cleave the terminal nucleotides).

Unlike restriction enzymes, DNase does not have any specific recognition/restriction site and cleave DNA sequence at random locations. There is a wide variety of deoxyribonucleases known which have different substrate specificities, chemical mechanisms, and biological functions.

#### **9.8.3.1 Deoxyribonuclease I (DNaseI)**

An endonuclease which cleaves double-stranded DNA or single stranded DNA. The cleavage preferentially occurs adjacent to pyrimidine (C or T) residues. The major products are 5'-phosphorylated bi-, tri- and tetranucleotides. It requires divalent ions ( $\text{Ca}_2^+$  and  $\text{Mn}_2^+/\text{Mg}_2^+$ ) for its activity and creates blunt ends or 1-2 overhang sequences. DNaseI is the most widely used enzyme in cloning experiments to remove DNA contamination from mRNA preparation (to be used for cDNA library preparation, northern hybridization, RT-PCR etc). The mode of action of DNaseI varies according to the divalent cation used. In the presence of magnesium ions ( $\text{Mg}^{++}$ ), DNaseI hydrolyzes each strand of duplex DNA producing single stranded nicks in the DNA backbone, generating various random cleavages.

On the other hand, in the presence of manganese ions ( $\text{Mn}^{++}$ ), DNaseI cleaves both strands of a double stranded DNA at approximately the same site, producing blunt

ended DNA fragments or with 1-2 base overhangs. Some applications of DNase I are as follows-

- Eliminating DNA contamination (e.g. plasmid) from preparations of RNA.
- Analyzing the DNA-protein interactions via DNA footprinting.
- Nicking DNA prior to radio-labeling by nick translation.

#### **9.8.3.2 DeoxyribonucleaseII (DNaseII)**

It is a non-specific endonuclease with optimal activity at acidic pH (4.5-5.5) and conserved from human to *C.elegans*. It does not require any divalent cation for its activity. DNaseII initially introduces multiple single stranded nicks in DNA backbone and finally generates 3' phosphate groups by hydrolyzing phosphodiester linkages.

This enzyme releases 3'phosphate groups by hydrolyzing phosphodiester linkage and creating nicks in the DNA backbone. DNaseII acts by generating multiple single stranded nicks followed by production of acid soluble nucleotides and oligonucleotides.

#### **9.8.4 Mung Bean Nuclease**

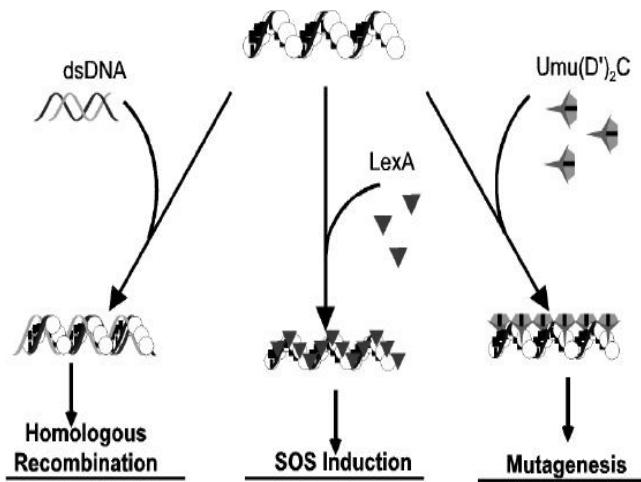
As the name suggest, this nuclease enzyme is isolated from mung bean sprouts (*Vigna radiata*). Mung bean nuclease enzymes can degrade single stranded DNA as well RNA. Under high enzyme concentration, they can degrade double stranded DNA, RNA or even DNA/RNA hybrids.

Mung bean nuclease can cleave single stranded DNA or RNA to produce 5'-phosphoryl mono and oligonucleotides. It requires  $Zn^{++}$  ion for its activity and shows optimal activity at 37°C. The enzyme works in low salt concentration (25mM ammonium acetate) and acidic pH (pH 5.0). Treatment with EDTA or SDS results in irreversible inactivation of the enzyme. Mung bean nuclease is less robust than S1 nuclease and easier to handle. It has been used to create blunt end DNA by cleaving protruding ends from 5' ends. This enzyme cannot produce nicks in a double stranded DNA but at higher concentration, it can generate nicks and cleave double stranded DNA.

## 9.9 Rec A Protein

Recombination plays a critical role in DNA repair and genome maintenance. However, it is equally critical to regulate where, when, and how recombination takes place. When unregulated, recombination can lead to genome instability and carcinogenesis. Meiotic recombination in eukaryotes is under tight regulation, ensuring the proper spacing and complete chromosomal coverage of the recombination events that are needed for proper chromosome segregation. Defects in many recombination functions result not only in DNA repair defects, but also in more general genomic instability. Much of this is associated with stalled replication forks.

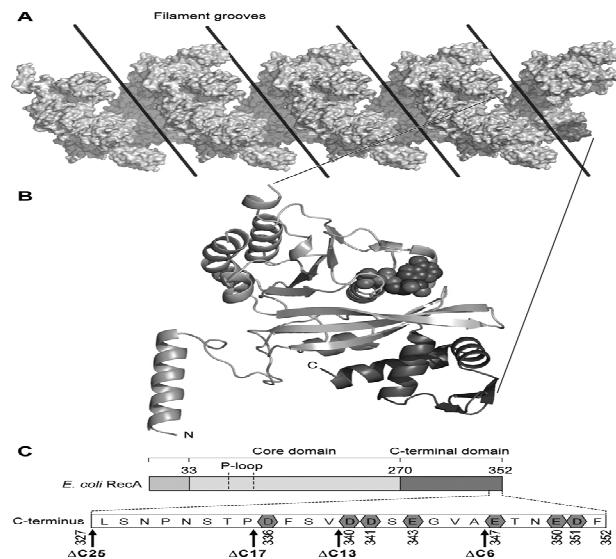
RecA is a 38 kilodalton protein essential for the repair and maintenance of DNA. A RecA structural and functional homolog has been found in every species in which one has been seriously sought and serves as an archetype for this class of homologous DNA repair proteins. The RecA protein of *Escherichia coli* (EcRecA) is the prototype of this class of protein and it has three very different roles in the cell. First, it promotes the central steps of recombination, aligning and pairing two DNA molecules, and then promoting a strand switch followed by branch migration. *In vitro*, the RecA protein catalyzes a DNA strand exchange reaction that serves as the major experimental model for its recombination activities. Second, RecA itself has a regulatory function. RecA has an activity generally referred to as its coprotease function, facilitating the autocatalytic cleavage of the LexA repressor as required for induction of the SOS response. Third, RecA functions to directly facilitate replicative bypass of DNA lesions by DNA polymerase V during SOS, utilizing a mechanism that is gradually yielding to investigation.



**Fig.-9.7 : Roles of the RecA Nucleoprotein Filament in DNA Metabolism**

### 9.9.1 Structure

The RecA monomer consists of three domains, a large, central domain, surrounded by relatively small amino and carboxydomains. The central domain, involved in DNA and ATP binding, consists primarily of a twisted beta sheet with 8-strands, bounded by 8-helices. The amino domain contains a large-helix and short-strand, this structure being important in formation of the RecA polymer. Three-helices and a three-stranded-sheet are found in the carboxy domain, which facilitates inter-filament associations.



**Fig. 9.8 : RecA Protein Structure**

### 9.9.2 RecA Filament Structure

Catalysis of homologous recombination by RecA begins with the formation of a filament composed of RecA monomers around ssDNA. The RecA filament wraps around the DNA helically, with 6 monomers per revolution. The RecA helix is approximately 120 Å wide, with a central diameter of 25 Å. The carboxy termini of each monomer, which are believed to be important in interfilament interactions, project outward from the RecA helix. ATP is bound near the center of the helix. The amino domain of each RecA monomer is involved in maintaining the RecA polymer bonds. As described in the structural overview section, this region of the monomer contains a protruding unit.

The polymerization of RecA monomers into filaments involves extensive association of the amino domain of one monomer and the central domain of the next monomer in the filament (with a loss of 2,890 Å<sup>2</sup> of solvent-accessible surface area/monomer). This association can be visualized in a RecA dimer. Part of the subunit interface involves the packing of the amino helix of one monomer between a complementary helix and sheet in the central domain of a neighboring monomer. Thus, the RecA filament has an amino domain-to-central domain polarity. The monomers are held together by a combination of hydrophobic and electrostatic interactions.

Experimental evidence supports the crystallographic data. Filament formation, for example, is severely inhibited among RecA monomers in which the amino terminal has been enzymatically removed. Similarly, proteins consisting only of the amino portion of RecA prevent polymerization via competitive inhibition of the central domain binding region. Mutation analyses have been used to identify residues at the subunit interface critical for RecA polymerization. Monomers in which lysine, phenylalanine, or arginine are replaced by other amino acids are unable to polymerize.

### 9.9.3 DNA Binding Regions of RecA

The RecA monomer contains two DNA binding sites in the large central domain, one for binding ssDNA, and the other for binding duplex DNA. Both DNA binding regions include disordered loops (L1 & L2), containing residues with low electron

density in the crystal. These loops, not shown in the structure, lie close to the filament axis, and therefore are juxtaposed with DNA. In the views that follow, the loops would project towards the viewer, i.e. towards the DNA in a RecA-DNA filament.

The putative ssDNA binding region includes alpha helix G as well as L2 (not shown), between glu and thr. The putative binding site for duplex DNA is found in another disordered region, L1, located between glu and gly.

Phylogenetic analyses have supported the conclusion that the regions containing L1 and L2 represent DNA binding regions. Because DNA binding is an essential function of RecA, the regions of the protein involved in this process should be highly conserved among bacterial species. Indeed, 10 of the 23 amino acids that compose the disordered loops are invariant in 16 different RecA proteins. Alpha helix G, located on the carboxy side of L2, is the most highly conserved region in the RecA monomer. At the boundary between alpha helix G and L2 are two invariant glycine residues, which, due to their small size, could allow maximal interaction between the negatively-charged sugar-phosphate backbone of the DNA molecule and the positively-charged amine groups of the helix.

#### **9.9.4 Regulation of RecA Function**

The activity of RecA protein, and presumably all related recombinases, is regulated on at least three levels. First, *recA* gene expression is controlled within the SOS regulon. This aspect of RecA function will not be considered here. Second, RecA protein is subject to autoregulation. Its activities are suppressed, to degrees that vary with conditions, by the C-terminus (and perhaps other parts) of the protein. Third, the activity of RecA protein is modulated by a growing array of other proteins.

#### **9.9.5 Role of RecA in Natural Transformation**

Based on analysis of the molecular properties of the RecA system, it is concluded that the data “provide compelling evidence that the primary mission of RecA protein is DNA repair.” In a further essay on the function of the RecA protein, Cox summarized data demonstrating that “RecA protein evolved as the

central component of a recombinational DNA repair system, with the generation of genetic diversity as a sometimes useful byproduct.”

Natural bacterial transformation involves the transfer of DNA from one bacterium to another (ordinarily of the same species) and the integration of the donor DNA into the recipient chromosome by homologous recombination, a process mediated by the RecA protein. Transformation, in which RecA plays a central role, depends on expression of numerous additional gene products (e.g. about 40 gene products in *Bacillus suBtilis*) that specifically interact to carry out this process indicating that it is an evolved adaptation for DNA transfer. In *B. suBtilis* the length of the transferred DNA can be as great as a third and up to the size of the whole chromosome. In order for a bacterium to bind, take up and recombine exogenous DNA into its chromosome, it must first enter a special physiological state termed “competence”. Transformation is common in the prokaryotic world, and thus far 67 species are known to be competent for transformation.

One of the most well studied transformation systems is that of *B. suBtilis*. In this bacterium, the RecA protein interacts with the incoming single-stranded DNA (ssDNA) to form striking filamentous structures. These RecA/ssDNA filaments emanate from the cell pole containing the competence machinery and extend into the cytosol. The RecA/ssDNA filamentous threads are considered to be dynamic nucleofilaments that scan the resident chromosome for regions of homology. This process brings the incoming DNA to the corresponding site in the *B. suBtilis* chromosome where informational exchange occurs.

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## 9.10 Summary

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Restriction endonucleases (or restriction enzymes) are bacterial enzymes that act as defence mechanisms in these organisms. Restriction endonucleases cleave double-stranded DNA internally, cutting both strands at regions of specific nucleotide sequences that vary from one enzyme to another. The sequence cut by a restriction endonuclease is its target site (also called its recognition site). When foreign DNA, such as viral DNA, is introduced into a bacterial cell, a restriction endonuclease cuts the foreign DNA into shorter pieces, thereby interrupting most of the foreign genes. This helps defend the cell against invasion by and expression of genes that could be harmful to the organism. A bacterium protects its own DNA against

digestion by its own restriction enzymes by chemically modifying its DNA soon after DNA replication, usually by adding methyl groups to bases within the target site of the endonuclease. The enzyme responsible for protection of the cell's DNA in this way is a DNA methylase.

In molecular biology, restriction enzymes are used in several ways to modify and manipulate DNA molecules. One common use is to prepare fragments of DNA from one source to be combined with fragments of DNA from another source – to construct recombinant DNA. Another use is to prepare small fragments suitable for nucleotide sequence analysis.

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## 9.11 Glossary

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- **Exonucleases** : Exonucleases catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA.
- **Endonucleases** : Endonucleases can recognize specific base sequence (restriction site) within DNA or RNA.
- **Ligase** : Ligase can catalyze the joining of two large molecules by forming a phosphodiester bond between two DNA strands.
- **Phosphatase** : A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric acid monoesters into a phosphate ion.
- **Transferase** : Transferase is the general name for the class of enzymes that enact the transfer of specific functional groups from one molecule to another.
- **Nuclease** : A nuclease is an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids.
- **RecA** : RecA is a 38 kilodalton protein essential for the repair and maintenance of DNA.
- **CIAP** : Calf Intestinal Alkaline Phosphatase.
- **BAP** : Bacterial Alkaline Phosphatase.

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## 9.12 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Give the name of first isolated restriction enzyme.
2. What is the meaning of ligase?
3. What is full form of SAP?

### Section B : (Short Answer Type Questions)

1. What is difference between exonuclease and endonuclease?
2. Describe mechanism of ligase action.
3. Write the enzymatic activities of kinase.
4. What is DNA polymerase?
5. What are nucleases? Give description of ribonucleaseH.

### Section C : (Long Answer Type Questions)

1. Give the classification and nomenclature of restriction endonucleases.
2. Give detail description about phosphatase.
3. Write history, types and nomenclature of transferases.
4. Describe RNA polymerases.
5. Give detail description of RecA protein.

### Answer key of section-A

1. *HindII*
2. To bind
3. Shrimp Alkaline Phosphatase

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## 9.13 References

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## Unit - 10

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# Recombinant Technology-II: Cloning Vectors

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### Structure of the Unit:

- 10.0 Objectives
- 10.1 Introduction
- 10.2 Plasmid
- 10.3 Cosmids
  - 10.3.1 Cos Sequence
  - 10.3.2 Features and Uses of Cosmids
- 10.4 Transposons
  - 10.4.1 Discovery
  - 10.4.2 Properties and Effects of Transposable Elements
  - 10.4.3 Classification
  - 10.4.4 Transposons and Antibiotic Resistance
  - 10.4.5 Transposons and Diseases
  - 10.4.6 Applications
- 10.5 Bacteriophage
  - 10.5.1  $\lambda$  phage Vector
  - 10.5.2 Phage M13 Vector
- 10.6 Yeast Artificial Chromosomes (YACs)
  - 10.6.1 Features of YACs
  - 10.6.2 Construction of YACs
  - 10.6.3 Uses of YACs
- 10.7 Bacterial Artificial Chromosomes (BAC)
- 10.8 Summary
- 10.9 Glossary
- 10.10 Self-Learning Exercise
- 10.11 References

## 10.0 Objectives

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After going through this unit you will be able to understand:

- Cloning Vectors
  - Plasmid as Vector
  - Cosmid
  - Transposons
  - Bacteriophage as vectors
  - M13 Vector
  - Yeast Artificial Chromosomes
  - Bacterial Artificial Chromosomes
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### 10.1 Cloning Vectors: Introduction

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One of the most important elements in gene cloning is the vector, which in conjunction with the passenger DNA forms the recombinant DNA which can be propagated in suitable host cells. A cloning vector is a small piece of DNA, taken from a virus, a plasmid, or the cell of a higher organism, that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes. A vector is an autonomously replicating DNA into which a foreign DNA is inserted for transfer or propagation in an organism. In general, a vector should have an autonomous specialized and designed to perform a specific function.

#### Properties of a Good Vector

In order to perform its function, a vector must possess the following properties:

- They should be capable of autonomous replication in at least one host organism.
- They should be of small size, since this aids the preparation vector DNA and reduces the complexity of analyzing recombinant molecules. They should be

capable of amplifying the cloned sequence by occurring in multiple copies. High copy number facilitates in maximizing expression of cloned genes.

- There should be a unique cleavage site for a range of restriction endonucleases.
- Occurrence of multiple cleavage sites reduces the likelihood of functional recombinant DNA formation.
- They should possess one or more genetic markers enabling easy selection of cloned molecules.
- They should permit detection by simple genetic tests, of the presence of passenger DNA inserted at cloning site.
- They should have appropriate transcriptional and translational signals located adjacent to cloning sites for better expression of cloned DNA sequences.
- They should have host specificity when there is biological containment for a vector.

It should be kept in mind that (1) the DNA molecules used as vectors have coevolved with their specific natural host species, and hence are adapted to function well in them and in their closely related species. Therefore, the choice of vector depends largely on the host species into which the DNA insert or gene is to be cloned. In addition, (2) most naturally occurring vectors do not have all the required functions; therefore, useful vectors have been created by joining together segments performing specific functions (called modules) from two or more natural entities.

Cloning vector - a DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial (or yeast) cell and produces many copies of itself and the foreign DNA. The cloned genes in these vectors are not expected to express themselves at transcription or translation level. These vectors are used for creating genomic libraries or for preparing the probes or in genetic engineering experiments or other basic studies. Selection of cloning vectors depends on the objective of cloning experiment, ease of working, knowledge existing about the vector, suitability and reliability. A variety of small, autonomously replicating molecules are used as cloning vectors.

### **Cloning Site**

All cloning vectors have features that allow a gene to be conveniently inserted into the vector or removed from it. This may be a multiple cloning site (MCS) which contains many unique restriction sites. The restriction sites in the MCS are first cleaved by restriction enzymes, and a PCR-amplified target gene, also digested with the same enzymes, is then ligated into the vector using DNA ligase. The target DNA sequence can be inserted into the vector in a specific direction if so desired. The restriction sites may be further used for sub-cloning into another vector if necessary.

Other cloning vectors may use topoisomerase instead of ligase and cloning may be done more rapidly without the need for restriction digest of the vector or insert. In this TOPO cloning method a linearized vector is activated by attaching topoisomerase I to its ends, and this "TOPO-activated" vector may then accept a PCR product by ligating both the 5' ends of the PCR product, releasing the topoisomerase and forming a circular vector in the process. Another method of cloning without the use of DNA digest and ligase is by DNA recombination, for example as used in the Gateway cloning system. The gene, once cloned into the cloning vector (called entry clone in this method), may be conveniently introduced into a variety of expression vectors by recombination.

### **Selectable Marker**

A selectable marker is carried by the vector to allow the selection of positively transformed cells. Antibiotic resistance is often used as marker; an example is the beta-lactamase gene which confers resistance to the penicillin group of beta-lactam antibiotics like ampicillin. Some vectors contain two selectable markers, for example the plasmid pACYC177 has both ampicillin and kanamycin resistance gene. Shuttle vector which is designed to be maintained in two different organisms may also require two selectable markers, although some selectable markers such as resistance to zeocin and hygromycin B are effective in different cell types. Auxotrophic selection markers that allow an auxotrophic organism to grow in minimal growth medium may also be used; examples of these are *LEU2* and *URA3* which are used with their corresponding auxotrophic strains of yeast.

## Vectors and Gene Cloning

Gene cloning is a method for making many identical copies of a gene by inserting the gene into a living host cell. Under appropriate conditions, the host cell will replicate the “foreign” DNA along with its own DNA whenever it divides. With each cell division, the number of copies of the “foreign” gene doubles. This technique permits biologists to produce large quantities of a single gene so that they can study the gene in detail. Biologists can also induce cells containing cloned genes to produce large quantities of the protein coded for by the gene. By making large amounts of “proteins to order” in this way, scientists can study the protein’s structure and function. They can also produce large quantities of a protein for commercial or medical use. For example, scientists can produce proteins that are used as enzymes in commercial food processing techniques or proteins that can be used to treat disease. Vectors are tools used by molecular biologists to insert genes or pieces of foreign DNA into host cells. Two naturally occurring vectors that are used to insert foreign DNA into bacterial cells are bacteriophages and plasmids. Bacteriophages (or phages, for short) are viruses that infect bacterial cells by injecting their genetic material into the bacterial cell. Plasmids are small circles of DNA that are sometimes present in bacteria in addition to the larger circle of DNA that constitutes the main bacterial genome. Many bacteria readily absorb plasmids from the environment under appropriate conditions. In order to use a phage or plasmid as a vector, scientists combine the DNA they wish to clone with the DNA of the vector. When DNA from 2 different sources is combined, the resulting DNA is called recombinant DNA. If recombinant DNA gets inside a host cell, it can replicate along with the DNA of the host cell.

### Types of Cloning Vectors

A large number of cloning vectors are available, and choosing the vector may depend on a number of factors, such as the size of the insert, copy number and cloning method. Large insert may not be stably maintained in a general cloning vector, especially for those with a high copy number, therefore cloning large fragments may require more specialized cloning vector. They are as follows: plasmids, phages, cosmids, phasmids, shuttle vectors, transposons, expression vectors and single stranded DNA.

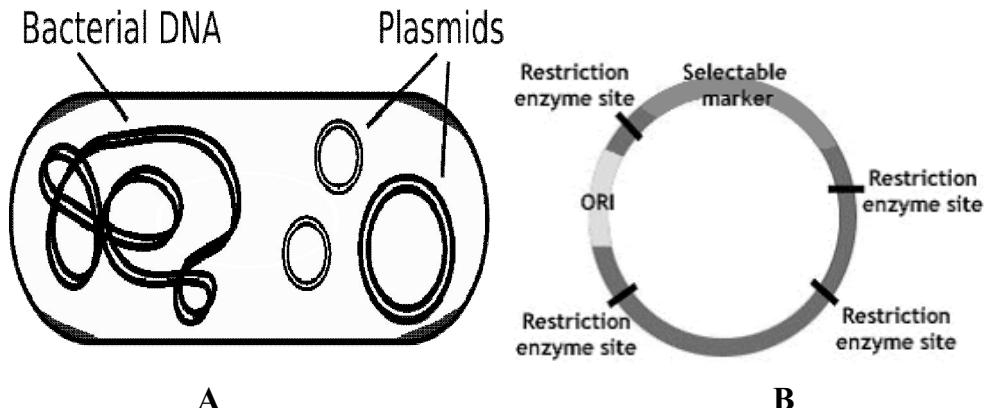
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## 10.2 Plasmid

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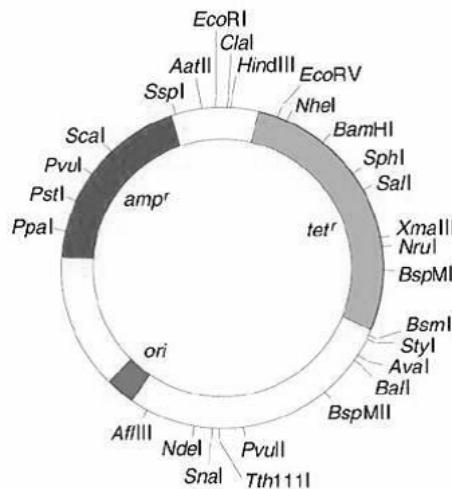
A plasmid is a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently. They are most commonly found in bacteria as small, circular, double-stranded DNA molecules; however, plasmids are sometimes present in archaea and eukaryotic organisms. Plasmids are widely distributed throughout the prokaryotes, vary in size from less than  $1 \times 10^6$  to greater than  $200 \times 10^6$  Ad and are generally dispensable. In nature, plasmids often carry genes that may benefit the survival of the organism, for example antibiotic resistance. While the chromosomes are big and contain all the essential information for living (an adequate analogy is the hard-drive of a computer), plasmids usually are very small and contain additional information (in this analogy, plasmids are the USB flash drives). Artificial plasmids are widely used as vectors in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms.

Plasmids can be grouped into two major types: conjugative and nonconjugative. In conjugative plasmids transfer genes (*tra*) and mobilizing genes (*mob*)are present whereas in non-conjugative plasmids *tra* genes absent. The non-conjugative plasmids can be mobilized by another conjugative plasmid present in the same cell, if the *mob* gene is intact. Non-conjugative differ from conjugative plasmids by the absence of *tra* gene Plasmids can also be categorized on the basis of their being maintained as multiple copies per cell(relaxed plasmids or high copy number plasmids) or as limited copies per cell(stringent plasmids or low copy number plasmids). The replication of stringent plasmids is coupled to chromosome replication, hence their low copy number. Generally conjugative plasmids are of low molecular weight and present in multiple copies per cell. An exception is the conjugative plasmids RBK which has a molecular weight of  $25 \times 10^6$ daltons and is maintained as relaxed plasmid.

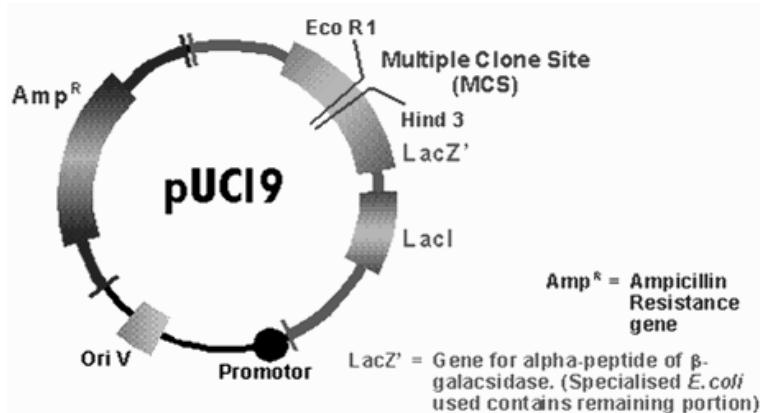


**Fig. 10.1 : A Plasmid in Bacterial Cell 10.1 B -Structural Map of Plasmid**

In general plasmid cloning vectors are designated by a lowercase 'p' which stands for plasmid, and some abbreviations that may be descriptive.pBR322 plasmids one of the best studied and most often used ““general purpose”” plasmids. The BR of the pBR322 recognizes the work of the researchers F. Bolivar and R. Rodriguez, who created the plasmid and 322 is a numerical designation that has relevance to these workers. pBR322 is 4362 base pair long and completely sequenced.pBR322 carries two antibiotic resistance genes. One confers resistance to ampicillin (*Ampr*) and the other confers resistance to tetracycline (*Tetr*). There are eleven known enzymes which cleave pBR 322 at unique sites. For three of the enzymes, *Hind* III, *Bam* HI and *Sal* I, the target site lies within the *Tetr* genes and for another two, *Pst* I and *Pru* I, they lie in *Ampr* genes. Thus cloning in pBR 322 with the aid of these enzymes results in insertional inactivation where the inserted DNA disrupts the function of the gene containing the cloning site. Where the cloning site is within in an antibiotic resistance gene, insertional inactivation results in transformants sensitive to the appropriate antibiotic. Thus, insertional inactivation helps in the selection of recombinants.

**Fig. 10.2 : Plasmid pBR322 and its Various Features**

Plasmid pUC19 is 2686 bp long and contains an ampicillin resistance (*Ampr*) gene, a regulatable segment of  $\alpha$ - galactosidase gene (*lacZ*) of the lactose operon of *E. coli*, *lac I* gene that produces a repressor protein that regulates the expression of *lacZ* gene, a short sequence with multiple cloning sites (*EcoRI*, *SacI*, *KpnI*, *XmaI*, *SmaI*, *BamHI*, *XbaI*, *SalI*, *HincII*, *AccI*, *BspMI*, *PstI*, *SphI* and *HindIII*) and the origin of replication from pBR322. The presence of *lac Z* and *lacI* genes allows to select there combinants based on the  $\alpha$ - galactosidase production in the presence of isopropyl-  $\alpha$ -D-thiogalactopyranoside (IPTG), an inducer of the *lac* operon. (UC in pUC stands for University of California).

**Fig. 10.3 : pUC19 Plasmid with Various Features**

## 10.3 Cosmids

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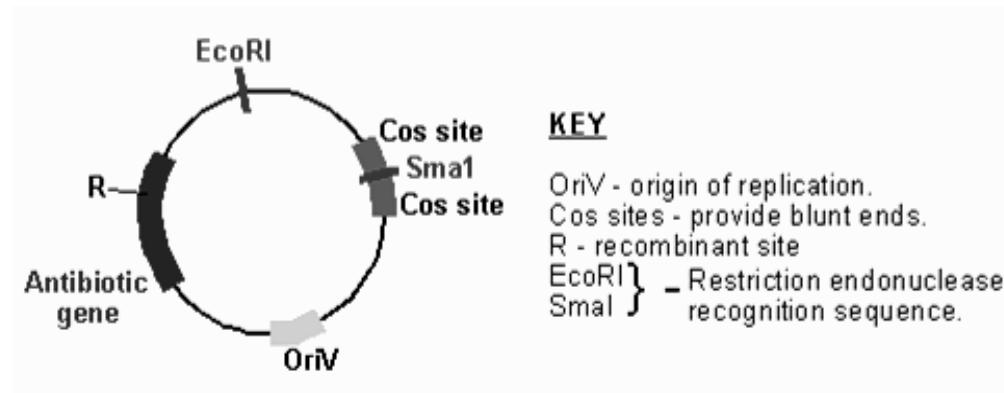
A cosmid is a type of hybrid plasmid that contains a Lambda phage *cos sequence*. Cosmids' (*cos sites + plasmid = cosmids*) DNA sequences are originally from the lambda phage. They are often used as a cloning vector in genetic engineering. Cosmids can be used to build genomic libraries. They were first described by Collins and Hohn in 1978.

Cosmids can contain 37 to 52 (normally 45) kb of DNA, limits based on the normal bacteriophage packaging size. They can replicate as plasmids if they have a suitable origin of replication: for example SV40 *ori* in mammalian cells, ColE1 *ori* for double-stranded DNA replication or f1 *ori* for single-stranded DNA replication in prokaryotes. They frequently also contain a gene for selection such as antibiotic resistance, so that the transformed cells can be identified by plating on a medium containing the antibiotic. Those cells which did not take up the cosmid would be unable to grow.

Unlike plasmids, they can also be packaged in phage capsid, which allows the foreign genes to be transferred into or between cells by transduction. Plasmids become unstable after a certain amount of DNA has been inserted into them, because their increased size is more conducive to recombination. To circumvent this, phage transduction is used instead. This is made possible by the *cohesive ends*, also known as *cos sites*. In this way, they are similar to using the lambda phage as a vector, but only that *all* the lambda genes have been deleted with the exception of the *cos* sequence.

### 10.3.1 Cos Sequences

*Cos* sequences are ~200 base pairs long and essential for packaging. They contain a *cosN* site where DNA is nicked at each strand, 12 bp apart, by terminase. This causes linearization of the circular cosmid with two "cohesive" or "sticky ends" of 12bp. (The DNA must be linear to fit into a phage head.) The *cosB* site holds the terminase while it is nicking and separating the strands. The *cosQ* site of next cosmid (as rolling circle replication often results in linear concatemers) is held by the terminase after the previous cosmid has been packaged, to prevent degradation by cellular DNases.



**Fig. 10.4 : Basic Features of Cosmid**

### 10.3.2 Features and Uses of Cosmids

Cosmids are predominantly plasmids with a bacterial *oriV*, an antibiotic selection marker and a cloning site, but they carry one, or more recently two, cos sites derived from bacteriophage lambda. Depending on the particular aim of the experiment broad host range cosmids, shuttle cosmids are available. The loading capacity of cosmids varies depending on the size of the vector itself but usually lies around 40–45 kb. The cloning procedure involves the generation of two vector arms which are then joined to the foreign DNA. Selection against wild type cosmid DNA is simply done via size exclusion. Cosmids, however, always form colonies and not plaques. Also the clone density is much lower with around  $10^5$  -  $10^6$  CFU per  $\mu\text{g}$  of ligated DNA.

After the construction of recombinant lambda or cosmid libraries the total DNA is transferred into an appropriate *E. coli* host via a technique called *in vitro* packaging. The necessary packaging extracts are derived from *E. coli* CI857 lysogens (red- gam- Sam and Dam (head assembly) and Eam (tail assembly) respectively. These extracts will recognize and package the recombinant molecules *in vitro*, generating either mature phage particles (lambda-based vectors) or recombinant plasmids contained in phage shells. These differences are reflected in the different infection frequencies seen in favor of lambda-replacement vectors. This compensates for their slightly lower loading capacity. Phage library are also stored and screened easier than cosmid (colonies) libraries.

Target DNA: the genomic DNA to be cloned has to be cut into the appropriate size range of restriction fragments. This is usually done by partial restriction followed by either size fractionation or dephosphorylation to avoid chromosome scrambling, i.e. the ligation of physically unlinked fragments.

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## 10.4 Trasnposons

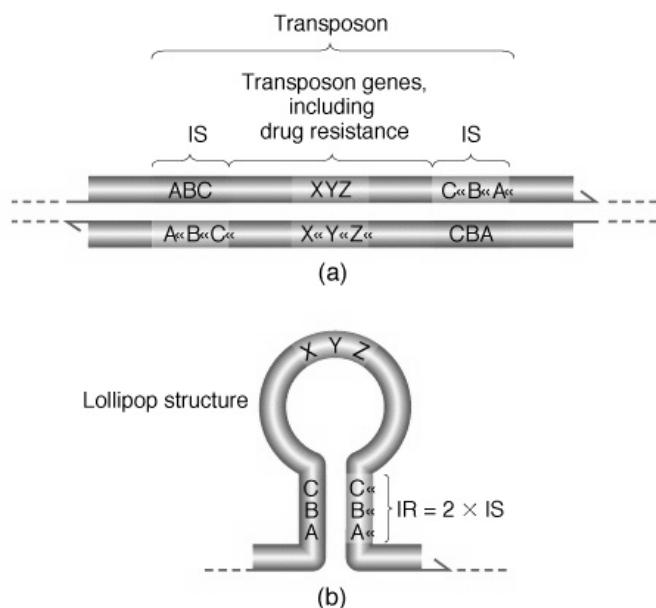
A transposable element (TE or transposon) is a DNA sequence that can change its position within the genome, sometimes creating or reversing mutations and altering the cell's genome size. Transposition often results in duplication of the TE.

TEs make up a large fraction of the C-value of eukaryotic cells. They are generally considered non-coding DNA, although it has been shown that TEs are important in genome function and evolution. In *Oxytricha*, which has a unique genetic system, they play a critical role in development. They are also very useful to researchers as a means to alter DNA inside a living organism.

### 10.4.1 Discovery

Barbara McClintock discovered the first TEs in maize, *Zea mays*, at the Cold Spring Harbor Laboratory and earned her a Nobel Prize in 1983. McClintock was experimenting with maize plants that had broken chromosomes. In the winter of 1944–1945 McClintock planted corn kernels that were self-pollinated, meaning that the flowers were pollinated by the silk of their own plant. These kernels came from a long line of plants that had been self-pollinated, causing broken arms on the end of their ninth chromosome. As the maize plants began to grow, McClintock noted unusual color patterns on the leaves. For example, one leaf had two albino patches of almost identical size, located side by side on the leaf. McClintock hypothesized that during cell division certain cells lost genetic material, while others gained what they had lost. However, when comparing the chromosomes of the current generation of plants and their parent generation, she found certain parts of the chromosomes had switched positions on the chromosome. She disproved the popular genetic theory of the time that genes were fixed in their position on a chromosome. McClintock found that genes could not only move, but they could also be turned on or off due to certain environmental conditions or during different stages of cell development. McClintock also showed that gene mutations could be reversed. McClintock presented her report on her findings in 1951, and published

an article on her discoveries in *Genetics* in November 1953 entitled, "Induction of Instability at Selected Loci in Maize." Her work would be largely dismissed and ignored until the late 1960s-1970s when it would be rediscovered after TEs were found in bacteria. She was awarded a Nobel Prize in Medicine or Physiology in 1983 for her discovery of TEs, more than thirty years after her research and initial discovery. TEs are more common than usually thought. Approximately 90% of maize genome is made up of TEs, and 50% in the human genome.



**Fig. 10.5 : Structure of Transposons**

#### 10.4.2 Properties and Effects of Transposable Elements

The defining property of transposable elements is their mobility; i.e. they are genetic elements that can move from one position to another in the genome. Beyond the common property of mobility, transposable elements show considerable diversity. Some move by DNA intermediates, and others move by RNA intermediates. Much of the mechanism of transposition is distinctive for these two classes, but all transposable elements effectively insert at staggered breaks in chromosomes. Some transposable elements move in a replicative manner, whereas others are non-replicative, i.e. they move without making a copy of themselves. Transposable elements are major forces in the evolution and rearrangement of genomes. Some transposition events inactivate genes, since the

coding potential or expression of gene is disrupted by insertion of the transposable element. A classic example is the *r* allele (*rugosus*) of the gene encoding a starch branching enzyme in peas is non-functional due to the insertion of a transposable element. This allele causes the wrinkled pea phenotype in homozygote originally studied by Mendel. In other cases, transposition can activate nearby genes by bringing an enhancer of transcription (within the transposable element) close enough to a gene to stimulate its expression. If the target gene is not usually expressed in a certain cell type, this activation can lead to pathology, such as activation of a proto-oncogene causing a cell to become cancerous. In other cases, no obvious phenotype results from the transposition. A particular type of transposable element can activate, inactivate or have no effect on nearby genes, depending on exactly where it inserts its orientation and other factors.

#### 10.4.3 Classification

Transposable elements (TEs) represent one of several types of mobile genetic elements. TEs are assigned to one of two classes according to their mechanism of transposition, which can be described as both copy and paste (class I TEs), or cut and paste (class II TEs).

##### Class I: Retrotransposons

Class I TEs are copied in two stages: first they are transcribed from DNA to RNA, and the RNA produced is then reverse transcribed to DNA. This copied DNA is then inserted at a new position into the genome. The reverse transcription step is catalyzed by a reverse transcriptase, which is often encoded by the TE itself. The characteristics of retrotransposons are similar to retroviruses, such as HIV.

Retrotransposons are commonly grouped into three main orders:

1. TEs with long terminal repeats (LTRs): encode reverse transcriptase, similar to retroviruses
2. LINEs (LINE-1s or L1s): encode reverse transcriptase, lack LTRs, and are transcribed by RNA polymerase II
3. SINEs: do not encode reverse transcriptase and are transcribed by RNA polymerase III.

Retroviruses can also be considered TEs. For example, after entering a host cell and conversion of the retroviral RNA into DNA, the newly produced retroviral DNA is integrated into the genome of the host cell. These integrated DNAs represent a provirus of the retrovirus. The provirus is a specialized form of eukaryotic retrotransposon, which can produce RNA intermediates that may leave the host cell and infect other cells. The transposition cycle of retroviruses has similarities to that of prokaryotic TEs, suggesting a distant relationship between these two TE types.

### **Class II: DNA Transposons**

The cut-and-paste transposition mechanism of class II TEs does not involve an RNA intermediate. The transpositions are catalyzed by several transposase enzymes. Some transposases non-specifically bind to any target site in DNA, whereas others bind to specific DNA sequence targets. The transposase makes a staggered cut at the target site resulting in single-strand 5' or 3' DNA overhangs (sticky ends). This step cuts out the DNA transposon, which is then ligated into a new target site; this process involves activity of a DNA polymerase that fills in gaps and of a DNA ligase that closes the sugar-phosphate backbone. This results in duplication of the target site. The insertion sites of DNA transposons may be identified by short direct repeats (created by the staggered cut in the target DNA and filling in by DNA polymerase) followed by a series of inverted repeats important for the TE excision by transposase. Cut-and-paste TEs maybe duplicated if their transposition takes place during S phase of the cell cycle when a donor site has already been replicated, but a target site has not yet been replicated. Such duplications at the target site can result in gene duplication, which plays an important role in evolution. Not all DNA transposons transpose through the cut-and-paste mechanism. In some cases, a replicative transposition is observed in which a transposon replicates itself to a new target site.

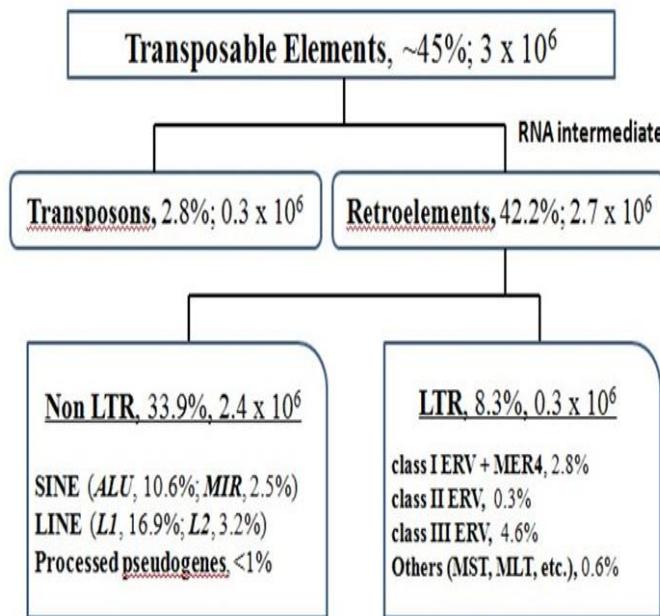
Class II TEs make less than 2% of the human genome, making the rest Class I.

### **Autonomous and Non-autonomous TEs**

Transposition can be also classified as either "autonomous" or "non-autonomous" in both Class I and Class II TEs. Autonomous TEs can move by themselves while non-autonomous TEs require the presence of another TE to move. This is often

because non-autonomous TEs lack transposase (for class II) or reverse transcriptase (for class I).

Activator element (*Ac*) is an example of an autonomous TE, and dissociation element (*Ds*) is an example of non-autonomous TE. Without *Ac*, *Ds* is not able to transpose.



**Fig. 10.6 : Classification of Transposable Elements in Human Genome**

#### 10.4.4 Transposons and Antibiotic Resistance

The simplest kinds of transposons merely contain a copy of the transposase with no additional genes. They behave as parasitic elements and usually have no known associated function that is advantageous to the host. More often, transposable elements have additional genes associated with them—for example, antibiotic resistance factors. Antibiotic resistance typically occurs when an infecting bacterium acquires a plasmid that carries a gene encoding resistance to one or more antibiotics. Typically, these resistance genes are carried on transposable elements that have moved into plasmids and are easily transferred from one organism to another. Once a bacterium picks up such a gene, it enjoys a great selective advantage because it can grow in the presence of the antibiotic.

Indiscriminate use of antibiotics actually promotes the buildup of these drug-resistant plasmids and strains.

#### **10.4.5 Transposons and Diseases**

The functions of transposons remain unclear. They have long been referred to as “junk” DNA because they appear to serve little or no purpose or as “selfish” DNA because they serve only to copy and amplify themselves within genomes. In rare cases, however, transposons are associated with genetic mutations or chromosomal rearrangements that cause disease in humans. Disease typically arises from the insertion of transposons into particular regions of genes that are involved in regulating gene activity. For example, insertions near promoter regions, which are short segments of DNA that are used to initiate gene transcription (the synthesis of RNA from DNA), can lead to over activity of genes. In some cases this can give rise to cancer. In other cases the site where a class II element is cut out of the genome is not repaired correctly, resulting in mutations that interfere with gene regulation and thereby cause cell dysfunction. There are also several diseases, including hemophilia and Duchenne muscular dystrophy, which are associated with repetitive DNA arising from retrotransposons.

#### **10.4.6 Applications**

The first TE was discovered in the plant maize (*Zea mays*, corn species), and is named dissociator (Ds). Likewise, the first TE to be molecularly isolated was from a plant (Snapdragon). Appropriately, TEs have been an especially useful tool in plant molecular biology. Researchers use them as a means of mutagenesis. In this context, a TE jumps into a gene and produces a mutation. The presence of such a TE provides a straightforward means of identifying the mutant allele, relative to chemical mutagenesis methods.

Sometimes the insertion of a TE into a gene can disrupt that gene's function in a reversible manner, in a process called insertional mutagenesis; transposase-mediated excision of the DNA transposon restores gene function. This produces plants in which neighboring cells have different genotypes. This feature allows researchers to distinguish between genes that must be present inside of a cell in

order to function (cell-autonomous) and genes that produce observable effects in cells other than those where the gene is expressed.

TEs are also a widely used tool for mutagenesis of most experimentally tractable organisms. The Sleeping Beauty transposon system has been used extensively as an insertional tag for identifying cancer genes.

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## 10.5 Bacteriophage as Vectors

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Bacteriophages were first described by Frederick Twort in 1915 and Felix d'Hérelle in 1917. D'Hérelle named them bacteriophages because they could lyses bacteria on the surface of agar plates (phage: from the Greek, "to eat"). There turn out to be a wide variety of phages with different shapes, host ranges and genetic composition -- much like mammalian viruses. Bacteriophages are viruses that attack bacteria. Most phages lyses the bacterial cells they infect (lytic phages). But many others can choose to follow either a lytic or a lysogenic cycle; in the latter situation, the phage chromosome integrates into the bacterial chromosome and multiplies with the latter as prophage (temperate or lysogenic phages). The prophage may dissociate from the bacterial chromosome and follow the lytic cycle. Bacteriophages are viruses that attack bacteria. Most phages lyses the bacterial cells they infect (lyticphages). But many others can choose to follow either a lytic or a lysogenic cycle; in the latter situation, the phage chromosome integrates into the bacterial chromosome and multiplies with the latter as prophage (temperate or lysogenic phages). The prophage may dissociate from the bacterial chromosome and follow the lytic cycle.

### 10.5.1 $\lambda$ Phage Vectors

The  $\lambda$  genome (size, 48,502 bp) contains an origin of replication; genes for head and tail proteins, and for enzymes for DNA replication, lysis and lysogeny; and single-stranded protruding cohesive ends of 12 bases (5'GGGC GGCGACCT; the other end is complementary to it, i.e., CCCGCCGCTGGA5). The  $\lambda$  genome remains linear in the phage head, but within E. coli cells the two cohesive ends anneal to form a circular molecule necessary for replication. The sealed cohesive

ends are called COS sites, which are the sites of cleavage during and are necessary for packaging of the mature phage DNA into phage heads.

The use of wild type  $\lambda$  genome as a vector presents two serious problems. The first problem concerns the size of DNA inserts. The X DNA must be larger than 38 kb and smaller than 52 kb to be packaged into phage particles. Thus X genome can accommodate only -3 kb DNA insert.

This problem is resolved by deleting the central region of  $\lambda$  genome. Most of the genes for lysogeny are located in the segment between 20 and 35 kb; this region is called the ‘nonessential’ region since it can be removed without affecting the ability of phage to infect *E. coli* cells. The whole or a part of this segment is deleted to create X vectors that (1) accommodate larger DNA inserts and (2) ensure the recombinant phage to be always lytic.

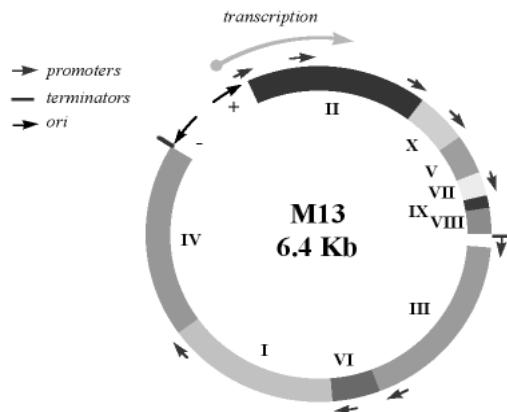
The second difficulty arises from the fact that the A, genome is so large that it contains more than one recognition site for virtually every restriction enzyme. As a result, cleavage by restriction enzymes cannot be used to integrate the DNA insert into the  $\lambda$  genome. A process of natural selection was used to recover  $\lambda$  genomes lacking recognition sites for specific restriction enzymes.

### 10.5.2 Phage M13 Vectors

These vectors are used for obtaining single-strand copies of cloned DNA, which are especially suited for DNA sequencing. They are derived from the 6.4 kb genome of *E. coli* filamentous bacteriophage M13 or f<sub>1</sub>. This phage has a single-stranded linear DNA genome in phage particles, which becomes converted into a double-stranded circular replicative intermediate within the host cells.

M13 infects only F<sup>+</sup> and F' cells; it injects its genome of only 6,407 nucleotides into the host cell through the F-pili of these cells. The double-stranded replicative form of M13 genome replicates by rolling circle mechanism. Each infected *E. coli* cell has ~100 copies of M13 genome, and about 1,000 new phage particles are produced (released into the culture medium) during each generation of an infected cell. Phage M13 does not lyse the infected host cells, but it forms turbid plaques due to growth retardation of these cells.

Ordinarily, the double-stranded form of M13 genome is used to produce recombinant molecules since single-stranded DNAs are not cleaved by type II restriction endonucleases; this form is readily isolated from M13-infected *E. coli* cells. However, the single-stranded form of M13 is used to recover single-stranded copies of the DNA inserts; this form of vector is available from the phage particles that are abundant in the growth medium.



**Fig. 10.7 : Double-stranded Circular Version of M13mp8**

### Properties of M13 Vectors

M13 genome has been used to produce M13mp series of vectors, e.g., M13mp8, M13mp9, etc. The first vector of M13mp series, viz., M13mpl, was constructed by placing the lacZ $\alpha$  gene within the noncoding region of M13 genome on one side of the origin. The lacZ $\alpha$  sequence does not possess any unique restriction site.

In vitro mutagenesis was used to change the hexanucleotide sequence GGATTC at the beginning of lacZ $\alpha$  to the EcoRI site GAATTC (second G of GGATTC was changed to A). This gave rise to M13mp2 vector, which permitted blue/white selection on plates containing X-gal and IPTG. The creation of EcoRI site modifies the sixth codon of lacZ $\alpha$  so that it now encodes asparagine in place of the original aspartic acid; this change, however, does not affect the function of the polypeptide encoded by the gene.

Additional unique restriction sites were created in the lacZ $\alpha$  gene by placing within it a synthetic polylinker sequence that has sticky EcoRI ends. The polylinker sequence is placed within the EcoRI site of M13mp2 to give rise to M13mp7, which has cloning sites for EcoRI, BamHI, Sait and PstI.

The polylinker sequence is designed to achieve the following-

- (1) Presence of several unique restriction sites,
- (2) Maintenance of the correct reading frame of the lacZ $\alpha$  sequence through the polylinker so that
- (3) lacZ $\alpha$  still produces a functional polypeptide although its amino acid sequence is altered. The latest M13 vectors (Fig. 2.17) have more complex polylinkers. Further, these vectors have the same polylinkers as the corresponding pUC series vectors, e.g., M13mp8 has the same polylinker as pUCS vector. The M13 vectors come in pairs, e.g., M13mp8/9, M13mp10/11, M13mp18/19; all these vectors are similar, except for their polylinkers.

The two vectors of a pair, e.g., M13mp8 and M13mp9, differ only in the orientation of their polylinkers. As a result, a DNA insert cloned in M13mp8, if excised by double restriction, will integrate in M13mp9 in the reverse orientation: this is important in DNA sequencing.

**The desirable features of M13 vectors are as follows:**

- (1) Very large inserts can be cloned since packaging does not depend on genome size (as is the case with A. vectors).
- (2) Pure single strand copies of double-strand DNA inserts are obtained in abundance,
- (3) Since DNA inserts are accepted in either of the orientations (which is also the case for plasmid and A. vectors), some recombinant clones will produce single-strand copies of one strand of the DNA double strand, while others would produce copies of the complementary strand of DNA insert.
- (4) The phage particles in a single plaque, as a rule, will yield copies of the same single-strand. This property is very useful for a precise DNA sequencing

(using both the strands of a DNA molecule) and for the synthesis of specific radio-labelled DNA probes.

- (5) Bacterial cells infected by these vectors remain viable as in the case of plasmid vectors; this allows easy maintenance of the vector.
  - (6) They form plaques (due to slow growth of infected cells) like X phage vectors making selection of the recombinant DNAs rather easy. Finally,
  - (7) The recombinant DNA is obtained within stable bacteriophage particles.
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## **10.6 Yeast Artificial Chromosomes (YACs)**

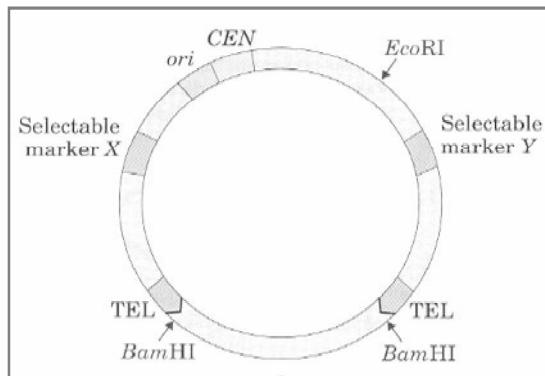
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Yeast artificial chromosomes (YACs) are plasmid shuttle vectors capable of replicating and being selected in common bacterial hosts such as *E.coli*, as well as in the budding *Saccharomyces cerevisiae* (yeast). They are of relatively small size (approximately 12 kb) and of circular form when they are amplified or manipulated in *E. coli*. These are linear vectors that behave like a yeast chromosome; hence they are called yeast artificial chromosomes (YACs). A typical YAC, e.g., pYAC3, contains the following functional elements from yeast: (1) an ARS sequence for replication, (2) CEN4 sequence for centromeric function, (3) telomeric sequences at the two ends for protection from exonuclease action, and (4) one or two selectable marker genes, viz., TRP1 and URA3, (strategy similar to other vectors); (5) SUP4, a selectable marker into which the DNA insert is integrated; and (6) the necessary sequences from *E. coli* plasmid for selection and propagation in *E. coli*.

The telomeric sequence in yeast chromosomes is a 20-70 tandem repeat of the 6 base sequence 5'CCCAA3' (its complementary sequence, 5'TTGGGG3', occurs in the other strand); there is a hairpin loop formation at the terminus, which makes the DNA duplex resistant to exonuclease action.

### **10.6.1 Features of YACs**

Large DNA (>100 kb) is ligated between two arms. Each arm ends with a yeast telomere so that the product can be stabilized in the yeast cell. Interestingly, larger YACs are more stable than shorter ones, which favor cloning of large stretches of DNA.



**Fig. 10.8 : Structure of YACs**

One arm contains an autonomous replication sequence (ARS), a centromere (CEN) and a selectable marker (*trp1*). The other arm contains a second selectable marker (*ura3*). Insertion of DNA into the cloning site inactivates a mutant expressed in the vector DNA and red yeast colonies appear.

Transformants are identified as those red colonies which grow in a yeast cell that is mutant for *trp1* and *ura3*. This ensures that the cell has received an artificial chromosome with both telomeres (because of complementation of the two mutants) and the artificial chromosome contains insert DNA (because the cell is red).

YACs are reported to suffer from many problems, including chimerism, tedious steps in YAC library construction and low yields of YAC insert DNA. The yeast genes present in different yeast vectors can become integrated into the host genome; this is called permanent transformation. It generally occurs through homologous recombination between the gene present in a vector (e.g., LEU2) and that present in the yeast chromosomes (e.g., LEU2<sup>-</sup>). Rarely, the gene may become inserted at a random chromosome site.

The homologous recombination may occur by regular crossing over or it may involve gene conversion (a non-reciprocal recombination). Vectors have been devised for high frequency stable transformation; such vectors are introduced in yeast cells in linear form and contain at their both ends sequences that are

homologous to those found at the target site (where the gene present in the vector is to be integrated) in the yeast genome. Such vectors permit integration of any specified DNA sequence at the desired site in yeast genome, i.e., they allow site-specific transformation (= integration) of genes.

### 10.6.2 Construction of YACs

After plasmid DNA purification, two distinct digestions are performed: the first with BamHI that cuts twice adjacent to the two telomeric DNA sequences flanking the HIS3 gene, which therefore is excised from the plasmid and lost. This first digestion generates a long linear fragment carrying telomeric sequences at each end. The excision of the HIS3 gene is used as negative selective marker for uncut pYAC molecules. The second digestion consists of the opening of the cloning site within the SUP4 gene. As a result of this second digestion, two linear fragments are produced as left and right arms of the future linear YAC. The selective markers are thus separated: TRP1 adjacent to ARS1 and CEN4 on the left arm and URA3 on the right arm. Large DNA fragments with ends compatible to the cloning site, obtained from the desired genome source by digestion with an appropriate restriction endonuclease, are ligated with phosphatase-treated YAC arms, to create a single yeast-transforming DNA molecule. Primary transformants can be selected for complementation of the URA3 mutation in the host, and successively for complementation of the host TRP1 mutation, thereby ensuring the presence of both chromosomal arms. Transformant colonies containing the exogenous DNA insert within the SUP4 gene are detected by their red color, due to the inactivation of the suppressor activity and the consequent accumulation of a red metabolic precursor in a host cells.

### 10.6.3 Use of YACs

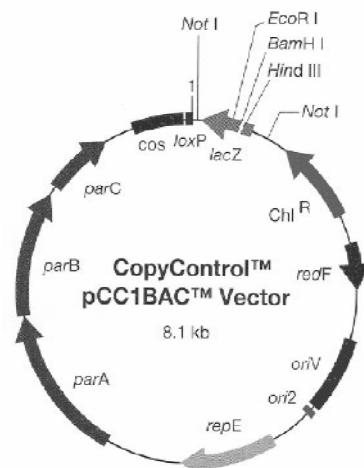
YAC vectors were initially created for the cloning of large exogenous DNA segments in *S. cerevisiae* but soon became chromosomal-like platforms for a variety of in-vivo experiments. Applications of YACs range from generating whole DNA libraries of the genomes of higher organisms to identifying essential mammalian chromosomal sequences necessary for the future construction of specialized mammalian artificial chromosomes (MACs). The availability of YAC libraries have greatly advanced the analysis of genomes previously cloned in

cosmid vectors. For example, YAC clones have been used as hybridization probes for the screening of cDNA libraries, thus simplifying the characterization of unidentified genes.

Another major application of YACs is in the study of regulation of gene expression by cis-acting, controlling DNA elements, that are present either upstream or downstream of large eukaryotic genes, after the transfer of these YACs from yeast to mammalian cells. Recent technological developments allow the transfer of YACs into mouse embryonal stem (ES) cells and the subsequent generation of transgenic mice. Investigators have begun to employ these artificial chromosomes for the *in vivo* study of multigenic loci in mammalian cells.

## 10.7 Bacterial Artificial Chromosomes (BAC)

Bacterial artificial chromosomes (BACs) are circular DNA molecules. They contain replicon that is based on the F factor comprising *oriS* and *repE* encoding an ATP-driven helicase along with *parA*, *parB*, and *parC* to facilitate accurate partitioning. The F factor is capable of carrying up to one quarter of the *E. Coli* chromosome and, thus, BACs are capable of maintaining very large DNA inserts (often up to 350 kb); however, many BAC libraries contain inserts of around 120 kb. Newer versions of BAC vectors contain sites to facilitate recovery of cloned DNA (e.g., *loxP*). A DNA fragment is cloned into BAC vectors in a similar fashion to cloning into general cloning vectors; DNA is ligated to a linearized vector and then introduced into an *E. coli* cloning strain by electroporation.

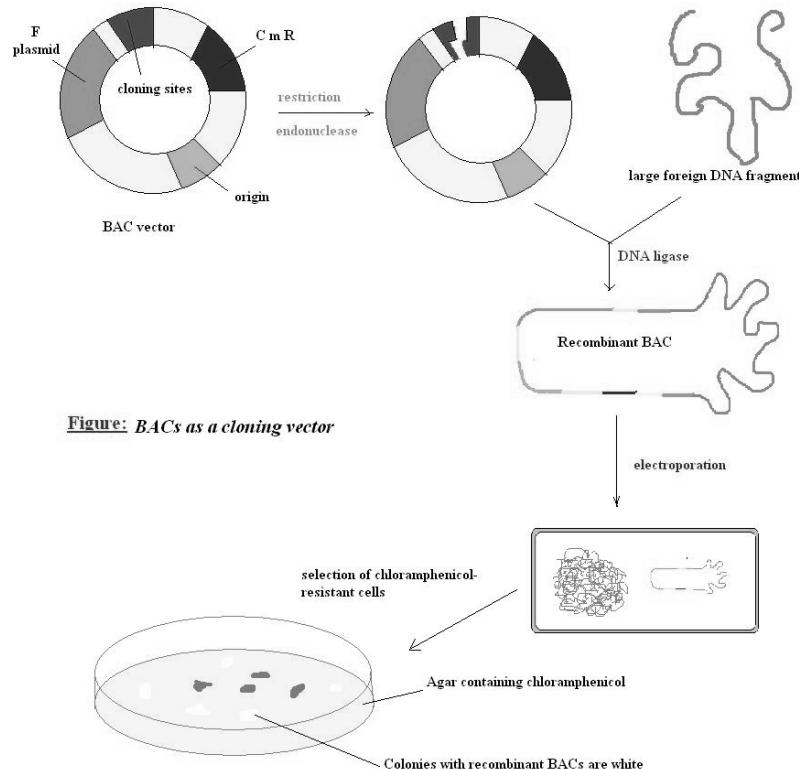


**Fig. 10.9 : Features of BAC**

BAC vectors have the origin of replication (oriS) of *E. coli* F factor, which allows a strict copy number control and stably maintains 1 to 2 copies of the vector per cell. The low copy number of BACs helps maintain the DNA inserts without any change arising from recombination between the copies of DNA inserts that is likely to happen in case of multi-copy vectors.

The low copy number also avoids any counter-selection that may arise due to an over-expression of the cloned genes. BACs were created because of the problems faced with YACs, e.g., recombination between copies of inserts and, more particularly, deletions in the DNA inserts.

The first BAC vector was pBAC108L; the other BAC vectors are pBeloBAC11, pBACe3.6, etc. Vector pBeloBAC11 is a convenient vector of 7.4 kb that allows selection of recombinant clones by lacZ $\alpha$ - complementation. This vector is maintained in *E. coli* cells at single copy per cell.



**Fig. 10.10 : Cloning of Desired Gene by BAC**

BAC vectors are stable, more user-friendly than YAC vectors, do not suffer from the problems of chimerism (variation in cloned DNA caused by recombination), and are suitable for most applications in the analysis of large genomes. These vectors are able to maintain in stable state even such genomic DNA, e.g., from genomes of Archaea and mammals, that are normally unstable in high copy number vectors.

The chief disadvantage of BAC vectors is the somewhat laborious construction of BAC libraries as in vitro manipulations, such as, restriction digestion, etc., have to be performed in agarose plugs to avoid shearing of the large DNA molecules.

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## 10.8 Summary

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This unit will attempt to summarize current understanding of the vectors. Vectors are the vehicles to transfer DNA between organisms. Vectors are capable of self replication in the host cell, so that the inserted DNA fragment will also replicate along with the vector and clones of DNA can be produced. Most cloning vectors are obtained from naturally occurring extra chromosomal elements. Commonly used vectors are plasmid, phage, cosmic, phagemid, artificial chromosomes etc. Plasmid is a circular double stranded Extra-chromosomal self replicating DNA found in bacteria. Bacteriophages are capable of cloning large pieces of DNA. Most commonly used bacteriophages are Lamda phage and M13. Cosmid is combination of plasmid vector and the cos site of Lamda phage. Plasmid part helps in selection due to the presence of marker gene. Phagemid or phasmid is hybrid of the filamentous phage M13 and plasmid. Phasmid contains the origin of replication and selectable marker from plasmid. An artificially synthesized chromosome, having a centromere, thus transmissible during cell division after introduction to a cell is called artificial chromosome e.g. YAC and BAC.

Vectors are boon to researchers who are working in the field of gene expression. The quality and yield of gene product depends upon the efficiency of expression which in turn depends upon the selection and designing of vector. Designing of a particular vector required a complete manipulation of naturally occurring vector. This unit is completely based on literature survey and summarizes cloning vector, expression vector, shuttle vector and also other vectors used in gene manipulation in plants and animals.

## 10.9 Glossary

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- **Vector** - A cloning vector is a small piece of DNA, taken from a virus, a plasmid, or the cell of a higher organism
  - **Cloning Site** - Where a vector allow a gene to be conveniently inserted into the vector or removed from it.
  - **Plasmid** - Small DNA molecule within a bacterial cell that is physically separated from a chromosomal DNA and can replicate independently.
  - **Cosmid** -A cosmid is a type of hybrid plasmid that contains a Lambda phage *cos* sequence.
  - **Transposons** - A transposable element (TE or transposon) is a DNA sequence that can change its position within the genome.
  - **BAC** -Bacterial Artificial Chromosome
  - **YAC** - Yeast Artificial Chromosome
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## 10.10 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Phasmids are hybrids formed between.
2. Which Plasmids containing phage *cos* sites?
3. Who created pBR322 plasmid?

### Section B : (Short Answer Type Questions)

1. What is cloning vectors?
2. What is cloning sites?
3. What are selectable markers?
4. What are transposons?
5. What is *cos* sequence?
6. Give full name of pBR322 and pUC plasmid.
7. How many base pairs are found in pBR322 and pUC plasmid?

### Section C : (Long Answer Type Questions)

1. What is a vector? Give properties of a vector.
2. Give the detail description of plasmid vectors.
3. Describe BAC and YAC.
4. Write discovery, properties and classification of transposons.
5. Write description and types of bacteriophage as vector.
6. Give detail description of cosmid.

### Answer Key of Section – A

1. Plasmids and bacteriophages
  2. Cosmid
  3. F. Bolivar and R. Rodriguez
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## Unit - 11

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# Recombinant Technology-III: Construction & Screening of Libraries

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### Structure of the Unit:

- 11.0 Objectives
- 11.1 Introduction
  - 11.1.1 Preparation and Principles of Gene Libraries
  - 11.1.2 Isolation of Genomic DNA
    - 11.1.2.1 Growth and Harvest of Bacterial Culture
    - 11.1.2.2 Preparation of Cell Extract
  - 11.1.3 Purification of DNA
    - 11.1.3.1 Organic Extraction for the Removal of Contaminants
    - 11.1.3.2 Using Ion-exchange Chromatography
  - 11.1.4 Storage of Nucleic Acids
- 11.2 Genomic Library
  - 11.2.1 Mechanisms for Cleaving DNA
  - 11.2.2 Construction of a Genomic Library
    - 11.2.2.1 Cloning of Genomic DNA
    - 11.2.2.2 Number of Clones Required for a Library
  - 11.2.3 Subgenomic Library
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  - 11.2.6 Applications of Genomic Library
- 11.3 cDNA Library
  - 11.3.1 Construction of a cDNA Library
    - 11.3.1.1 Isolation of mRNA
    - 11.3.1.2 cDNA Construction

- 11.3.2 Problems in cDNA Preparation
  - 11.3.3 Applications of cDNA Libraries
  - 11.3.4 Disadvantages of cDNA Libraries
  - 11.4 Genomic vs. cDNA Library
  - 11.5 Screening of DNA Libraries
    - 11.5.1 Screening by Hybridization
      - 11.5.1.1 Colony Hybridization
      - 11.5.1.2 Procedure of Colony Hybridization
    - 11.5.2 Screening by Polymerase Chain Reaction (PCR)
  - 11.6 Polymerase Chain Reaction (PCR)
    - 11.6.1 Components of PCR
    - 11.6.2 Procedure of PCR
    - 11.6.3 Types of PCR
    - 11.6.4 Applications of PCR*
    - 11.6.5 Limitations of PCR
  - 11.7 Summary
  - 11.8 Glossary
  - 11.9 Self -Learning Exercise
  - 11.10 References
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## 11.0 Objectives

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After going through this unit you will be able to understand:

- Gene/ DNA library
- Genomic library
- cDNA library
- Screening of libraries
- Polymerase Chain Reaction (PCR)

## 11.1 Introduction

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A library is a collection of DNA fragments that is stored and propagated in a population of micro-organisms through the process of molecular cloning. There are different types of DNA libraries, including cDNA libraries (formed from reverse-transcribed RNA), genomic libraries (formed from genomic DNA) and randomized mutant libraries (formed by de novo gene synthesis where alternative nucleotides or codons are incorporated). DNA library technology is a mainstay of current molecular biology, and the applications of these libraries depend on the source of the original DNA fragments. There are differences in the cloning vectors and techniques used in library preparation, but in general each DNA fragment is uniquely inserted into a cloning vector and the pool of recombinant DNA molecules is then transferred into a population of bacteria (a Bacterial Artificial Chromosome or BAC library) or yeast such that each organism contains on average one construct (vector + insert). As the population of organisms is grown in culture, the DNA molecules contained within them are copied and propagated.

If the library has been established by using fragmented cellular DNA of an organism the library is said to be a genomic library. The term genomic DNA clone or chromosomal DNA clone then refers to an individual cell carrying a cloning vector with one of the cellular DNA fragments or to a phage isolate with a specific DNA insert. Subgenomic libraries are obtained if only selected portions of the genome represented in the library, for example fragments from distinct sorted chromosomes. A characteristic of genomic libraries is the fact that the individual inserts still contain non-coding intron sequences. Such libraries are used, therefore, for determining the genomic structures of genes.

There are basically two kinds of libraries: genomic DNA and cDNA libraries. Genomic DNA libraries contain large fragments of DNA in either bacteriophages or bacterial or P1-derived artificial chromosomes (BACs and PACs). cDNA libraries are made with cloned, reverse-transcribed mRNA, and therefore lack DNA sequences corresponding to genomic regions that are not expressed, such as introns and 5' and 3' noncoding regions. cDNA libraries generally contain much smaller fragments than genomic DNA libraries, and are usually cloned into plasmid vectors.

### 11.1.1 Preparation and Principles of Gene Libraries

A suitable cloning vector (in this example a circular plasmid) is linearized by cleavage with a suitable restriction enzyme. The cellular DNA containing the gene of interest (red) is also cleaved into fragments with this enzyme. For various reasons the cellular DNA is cleaved in a way yielding overlapping fragments with a uniform length of 20 kb. Linearized vector DNA and genomic DNA fragments are then ligated *in vitro*, yielding a population of recircularized cloning vector molecules, each of which contains an insert of cellular genomic DNA. These molecules are introduced into suitable host cells. The collection of cloning vectors with inserts obtained or the collection of cells obtained called a gene library. This library is called a representative library if the sum of the genomic DNA inserts found in the cloning vectors of all cells represents the entire cellular DNA. Only a representative library, i.e., only the presence of all DNA fragments generated originally, guarantees a reasonable chance of finding the desired gene. Statistical analysis shows that for human DNA more than 600000 cell clones must be screened in order to find a particular DNA fragment containing the desired gene with a probability of 99%. In principle libraries can be made also with cellular RNA as starting material. The resulting collection of host cells containing recombinant vectors is then called a cDNA library.

There are different techniques available for screening of a library. The most important approaches involve screening by nucleic acid hybridization and screening by functional analysis. Nucleic acid hybridization requires some prior knowledge of the DNA sequence either of the gene to be cloned or of stretches of DNA in the vicinity of the gene to be cloned. Functional screening involves the use of expression vectors that allow cells containing the vector with the desired gene to express the corresponding protein. Under these circumstances, cells containing a vector with the desired gene can be identified by means of antibodies directed against the protein. Cells producing the desired gene product can also be identified in bioassays detecting protein activities, if these are available.

Many different techniques have been developed to isolate specific DNA clones from a library and if the process has been successful the specific DNA clone is said to have been cloned. Detection of an individual clone in a library can be achieved

by employing strategies of nucleic acid hybridization in which short chemically synthesized labeled oligonucleotides are used to detect complementary sequences in individual cells or phages containing an insert. The sequences of such oligonucleotides used to identify the desired gene in the library can be derived, for example, from known protein sequences according to the rules of the genetic code. Related genes can be identified by altering the hybridization conditions (for example, low stringency hybridization, i.e., hybridization under conditions that allows base mismatches) or by using so-called degenerated-oligonucleotides (mixtures of oligonucleotides that differ from each other by base substitutions at identical and/or different positions). The desired gene can be identified also by the activities of the encoded gene product. In this case, one uses a so-called expression library that have been established by cloning DNA fragments into special cloning vectors allowing the functional expression of cloned DNA fragments. Functional gene products and hence the desired clones can then be detected either by antibodies or other ligands that specifically recognize the encoded proteins or by exploiting a bioactivity of the gene product, if known.

In contrast to a genomic library which contains fragments of genomic DNA with intron and regulatory gene sequences a cDNA library contains inserts of cDNA fragments that correspond to the entire mRNAs of a cell. Such libraries therefore contain DNA fragments from which intron sequences have been removed. cDNA (or complementary DNA) is obtained by using the enzyme reverse transcriptase to copy mRNA sequences back into the corresponding DNA sequences. One of the advantages of a cDNA library is the improved frequency with which individual DNA fragments occur. This is due to the presence of multiple copies of mRNA as opposed to DNA for any given gene. In addition, the representation of inserts derived from functionally expressed mRNAs found in a cDNA library also reflects the functional activities of the cells from which the library was established. Moreover, as cDNA clones are derived from mRNA, they do not contain any intervening sequences (introns). cDNA clones can be used, therefore, directly to express the proteins encoded by them (see also: gene expression, Recombinant cytokines). Many genes encoding cytokines have been isolated originally in the form of cDNA clones.

### **11.1.2 Isolation of Genomic DNA**

Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells. The amount and purity of extracted DNA depends on the nature of the cell. The method of isolation of genomic DNA from a bacterium comprises following steps-

#### **11.1.2.1 Growth and Harvest of Bacterial Culture**

Bacterial cell culture is more convenient than any other microbe, as it requires only liquid medium (broth) containing essential nutrients at optimal concentrations, for the growth and division of bacterial cells. The bacterial cells are usually grown on a complex medium like Luria-Bertani (LB), in which the medium composition is difficult to decipher. Later, the cells are separated by centrifugation and resuspended in 1% or less of the initial culture volume.

#### **11.1.2.2 Preparation of Cell Extract**

Bacterial cell is surrounded by an additional layer called cell wall, apart from plasma membrane with some species of *E. coli* comprising multilayered cell wall. The lysis of cell wall to release the genetic material i.e. DNA can be achieved by following ways-

- Physical method by mechanical forces.
- Chemical method by metal chelating agents i.e. EDTA and surfactant i.e. SDS or enzyme (e.g. lysozyme).

#### **Lysozyme**

- Present in egg-white, salivary secretion and tears.
- Catalyzes the breakdown of cell wall i.e. the peptidoglycan layer.

#### **EDTA (Ethylene diamine tetra-acetic acid)**

- A chelating agent necessary for destabilizing the integrity of cell wall.
- Inhibits the cellular enzymes that degrade DNA.

#### **SDS (Sodium dodecyl sulphate)**

- Helps in removal of lipid molecules and denaturation of membrane proteins.

Generally, a mixture of EDTA and lysozyme is used. Cell lysis is followed by centrifugation to pellet down the cell wall fractions leaving a clear supernatant containing cell extract.

### 11.1.3 Purification of DNA

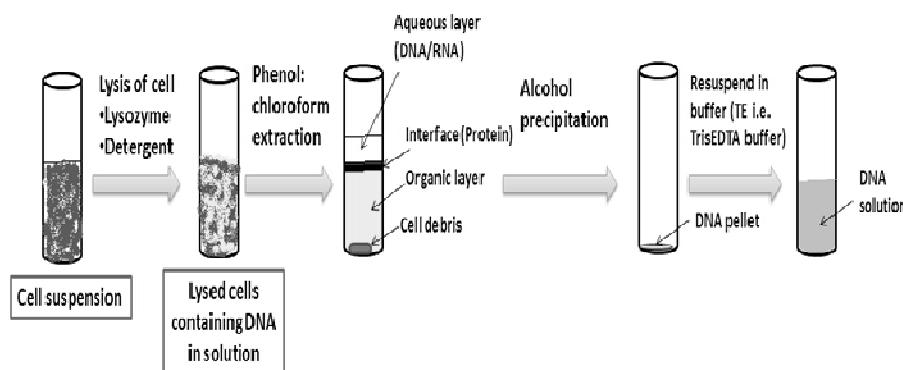
In addition to DNA, a cell extract contains significant quantities of protein and RNA which can be further purified by following methods-

#### 11.1.3.1 Organic Extraction for the Removal of Contaminants

It involves the addition of a mixture of phenol and chloroform (1:1) to the cell lysate for protein separation. The proteins aggregate as a white mass in between the aqueous phase containing DNA and RNA, and the organic layer. Treatment of lysate with pronase or protease, in addition to phenol/chloroform, ensures complete removal of proteins from the extract. The RNA can be effectively removed by using Ribonuclease, an enzyme which rapidly degrades RNA into its ribonucleotide subunits. Repeated phenol extraction is not desirable, as it damages the DNA.

#### 11.1.3.2 Using Ion-exchange Chromatography

This involves the separation of ions and polar molecules (proteins, small nucleotides and amino acids) based on their charge. DNA carrying negative charge binds to the cationic resin or matrix which can be eluted from the column by salt gradient. Gradual increase in salt concentration detaches molecules from the resin one after another.



**Fig. 11.1 : Isolation of Genomic DNA**

### 11.1.4 Storage of Nucleic Acids

The purified DNA can be stored at -20°C or -70°C under slightly basic conditions (e.g., Tris- Cl, pH 8.0) as acidic conditions result in hydrolysis of DNA. Diluted solutions of nucleic acids can be stored in aliquots and thawed once only. RNA preservation under frozen conditions is helpful.

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## 11.2 Genomic Library

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Genomic libraries are a catalogue of genes of a particular organism. They are also commonly referred to as gene banks. To create a genomic library, the complete genome of an organism is cleaved into fragments and inserted into a cloning vector. It can also refer to the collection of vector molecules. It contains all DNA sequences such as expressed genes, non-expressed genes, exons and introns, promoter and terminator regions and intervening DNA sequences.

Construction of a genomic DNA library involves isolation, purification and fragmentation of genomic DNA followed by cloning of the fragmented DNA using suitable vectors. The eukaryotic cell nuclei are purified by digestion with protease and organic (phenol-chloroform) extraction. The derived genomic DNA is too large to incorporate into a vector and needs to be broken up into desirable fragment sizes. Fragmentation of DNA can be achieved by physical method and enzymatic method. The library created contains representative copies of all DNA fragments present within the genome.

### 11.2.1 Mechanisms for Cleaving DNA

#### (a) Physical method

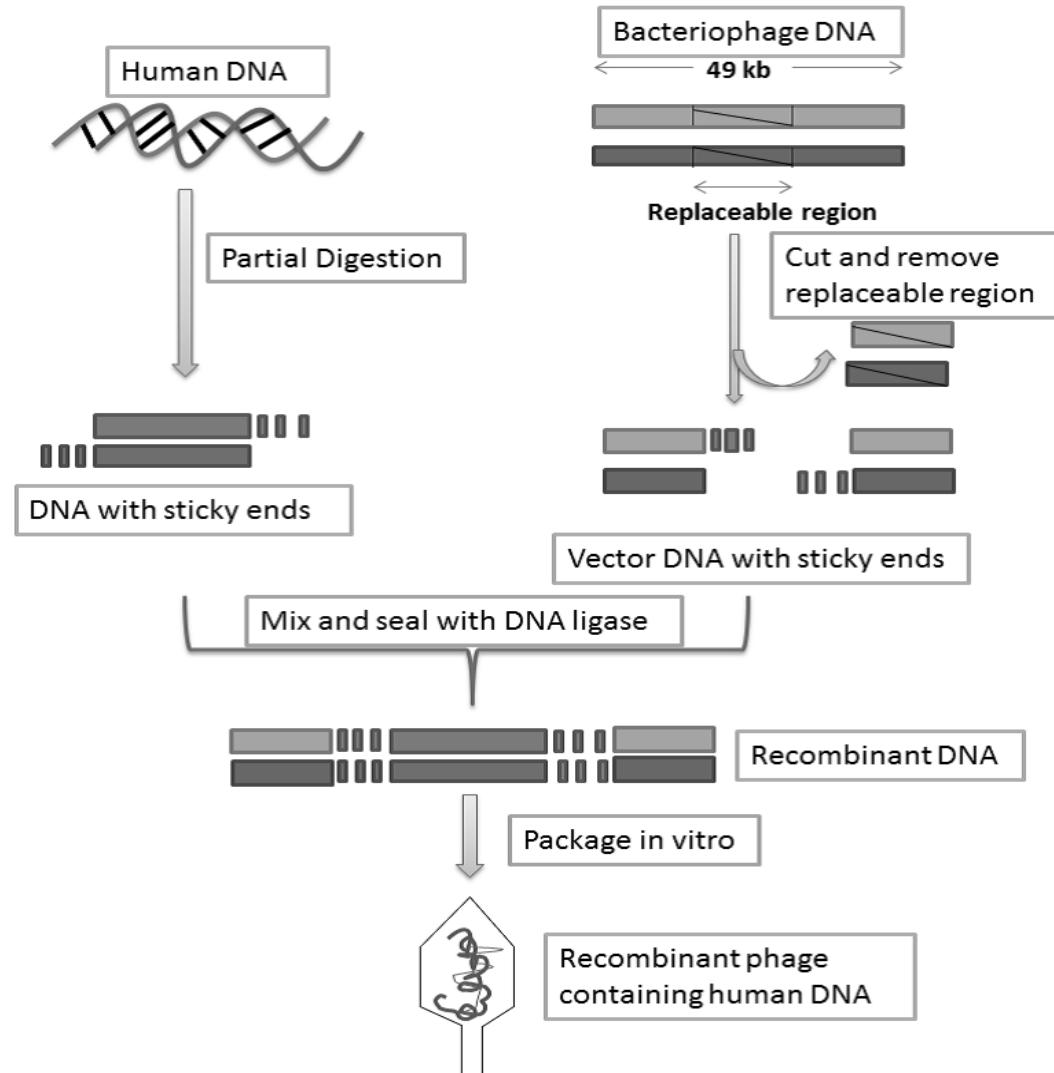
It involves mechanical shearing of genomic DNA using a narrow-gauge syringe needle or sonication to break up the DNA into suitable size fragments that can be cloned. Typically, an average DNA fragment size of about 20 kb is desirable for cloning into  $\lambda$ based vectors. DNA fragmentation is random which may result in variable sized DNA fragments. This method requires large quantities of DNA.

**(b) Enzymatic method**

- It involves use of restriction enzyme for the fragmentation of purified DNA. This method is limited by distribution probability of site prone to the action of restriction enzymes which will generate shorter DNA fragments than the desired size.
- If, a gene to be cloned contains multiple recognition sites for a particular restriction enzyme, the complete digestion will generate fragments that are generally too small to clone. As a consequence, the gene may not be represented within a library.
- To overcome this problem, partial digestion of the DNA molecule is usually carried out using known quantity of restriction enzyme to obtain fragments of ideal size.
- The fragments of desired size can be recovered by either agarose gel electrophoresis or sucrose gradient technique and ligated to suitable vectors.

**11.2.2 Construction of a Genomic Library**

First, a variety of restriction endonucleases are used to cleave at certain base pairs to create the necessary fragments of the DNA. These restriction endonucleases are chosen in such a way that the cohesive ends are compatible with a cloning vector and that the complete genome is represented by these fragments. This also means that the cloning vector is cleaved with the same restriction endonucleases and the original fragments are combined with the cloning vector using a ligase. This mixture is then inserted into a bacterial cell to produce a library. Each cell will have a different DNA molecule. Ordering of individual clones is archived by identifying overlapping sequences. This set of overlapping sequences of the catalog is defined as a cloning.



**Fig. -11.2 : Construction of Genomic Library**

### 11.2.2.1 Cloning of Genomic DNA

Various vectors are available for cloning large DNA fragments.  $\lambda$  phage, yeast artificial chromosome, bacterial artificial chromosome etc. are considered as suitable vectors for larger DNA and  $\lambda$  replacement vectors like  $\lambda DASH$  and  $EMBL3$  are preferred for construction of genomic DNA library. T4 DNA ligase is used to ligate the selected DNA sequence into the vector.

### **(1) $\lambda$ Replacement Vectors**

The  $\lambda$ EMBL series of vectors are widely used for genomic library construction. The multiple cloning sites of these vectors flanking the stuffer fragment contain opposed promoters for the T3 and T7 RNA polymerases. The restriction digestion of the recombinant vector generates short fragments of insert DNA left attached to these promoters. This generates RNA probes for the ends of the DNA insert. These vectors can be made conveniently, directly from the vector, without recourse to sub-cloning.

### **(2) High-capacity Vectors**

The high capacity cloning vectors used for the construction of genomic libraries are cosmids, bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and yeast artificial chromosomes (YACs). They are designed to handle longer DNA inserts, much larger than for  $\lambda$  replacement vectors. So they require lower number of recombinants to be screened for identification of a particular gene of interest.

#### **11.2.2 Number of Clones Required for a Library**

The number of clones to be pooled depends upon the size of the genome  $f$  and average size of the cloned DNA. Let  $(f)$  be the fraction of the genome size compared to the average individual cloned fragment size, would represent the lowest possible number of clones that the library must contain.

The minimum number of clones required can be calculated as-

$$f = \text{Genome size/ fragment size}$$

For the *E. coli* genome (4.6 Mb) with an average cloned fragment size of 5 kb,  $f$  will be 920.

#### **11.2.3 Subgenomic Library**

Subgenomic library is a library which represents only a fraction of the genome. Enhancing the fold of purification of target DNA is crucial for subgenomic DNA libraries which can be achieved by multiple, sequential digestion when information of the restriction map of the sequences of interest is known. After initial

purification of a given fragment, the purification can further be increased by redigestion with another enzyme generating a smaller (clonable) fragment relative to original DNA.

#### **11.2.4 Advantages of Genomic Libraries**

1. Identification of a clone encoding a particular gene of interest.
2. It is useful for prokaryotic organisms having relatively small genomes.
3. Genomic libraries from eukaryotic organisms are very important to study the genome sequence of a particular gene, including its regulatory sequences and its pattern of introns and exons.

#### **11.2.5 Disadvantages of Genomic Library**

1. Genome libraries from eukaryotes having very large genomes contain a lot of DNA which does not code for proteins and also contain non-coding DNA such as repetitive DNA and regulatory regions which makes them less than ideal.
2. Genomic library from a eukaryotic organism will not work if the screening method requires the expression of a gene.

#### **11.2.6 Applications of Genomic Library**

- To determine the complete genome sequence of a given organism.
- To study and generate transgenic animals through genetic engineering, serving as a source of genomic sequence.
- To study the function of regulatory sequences *in vitro*.
- To study the genetic mutations.
- Used for genome mapping, sequencing and the assembly of clone counting.

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### **11.3 cDNA Library**

Complementary DNA (cDNA) is the single-stranded or double-stranded DNA copy of a mRNA. cDNA is obtained by a number of successive enzymatic reactions *in vitro* that are known as reverse transcription .The starting material for the synthesis of cDNA can be the entire cellular or cytoplasmic RNA but also mRNA purified by affinity chromatography. The most important enzyme for the

synthesis of cDNA is RNA-dependent DNA polymerases known as reverse transcriptases. These enzymes are capable of utilizing deoxyribonucleoside triphosphates as substrate and RNA as a template for the synthesis of a DNA strand. The primary reaction product of a reverse transcription reaction is a hybrid double-stranded molecule consisting of the parent RNA strand and the newly synthesized DNA strand. Upon removal of the parent RNA strand the single-stranded DNA can be used in subsequent reactions to obtain double-stranded DNA. Several different strategies are available for efficient and high yield first strand and second strand cDNA synthesis.

A cDNA library is a combination of cloned cDNA fragments constituting some portion of the transcriptome of an organism which are inserted into a number of host cells. In eukaryotic cells, the mRNA is spliced before translation into protein. The DNA synthesized from the spliced mRNA doesn't have introns or non-coding regions of the gene. As a result, the protein under expression can be sequenced from the DNA which is the main advantage of cDNA cloning over genomic DNA cloning. A cDNA gene library contains only those sequences that represent genes actively transcribed in a given cell type. Such a library, therefore, is the ideal starting material for isolating genes that are expressed only under certain conditions or of genes that are capable to complement a genetic defect in a mutated cell line.

### **11.3.1 Construction of a cDNA Library**

cDNA is created from a mature mRNA from a eukaryotic cell with the use of an enzyme known as reverse transcriptase. In eukaryotes, a poly-(A) tail (consisting of a long sequence of adenine nucleotides) distinguishes mRNA from tRNA and rRNA and can therefore be used as a primer site for reverse transcription. This has the problem that not all transcripts, such as those for the histone, encode a poly-A tail. cDNA libraries can be constructed in different vectors, depending on the ultimate intended use. The construction of cDNA library involves following steps-

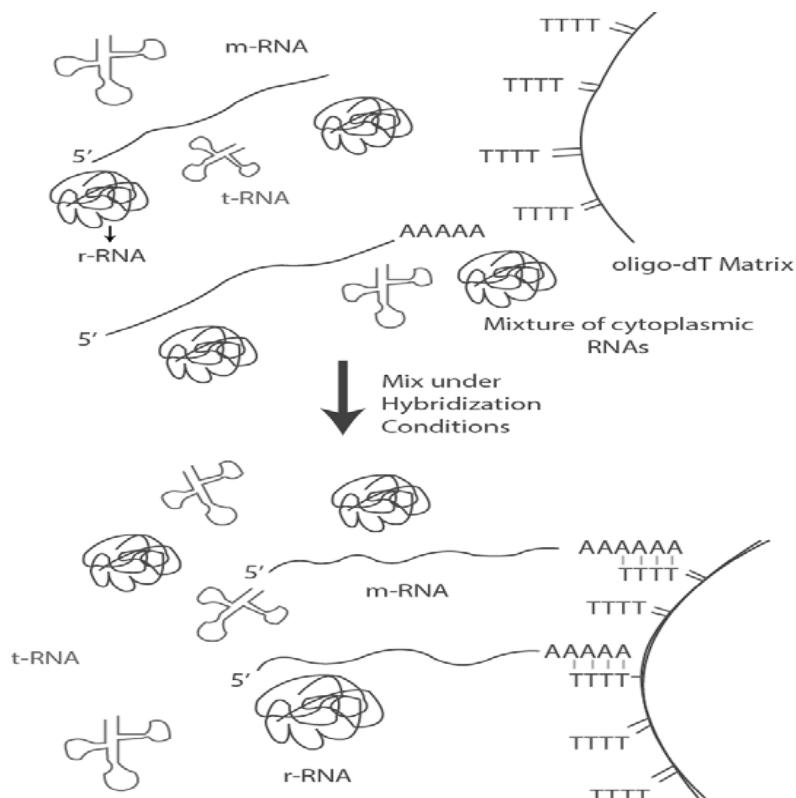
#### **11.3.1.1 Isolation of mRNA**

It involves the isolation of total mRNA from a cell type or tissue of interest. The amount of desired mRNA can be increased by following ways-

- Chromatographic purification of mRNA using oligo-dT column, which retains mRNA molecules, resulting in their enrichment.
- Spinning down mRNA by density gradient centrifugation.
- mRNA preparation from specialized cell types, e.g. developing seeds, chicken oviduct, erythrocytes,  $\beta$  cells of pancreas etc.

The 3' ends of eukaryotic mRNA consist of a string of 50 – 250 adenylate residues (poly A Tail) which makes the separation easy from the much more prevalent rRNAs and tRNAs in a cell extract using a column containing oligo-dTs tagged onto its matrix.

When a cell extract is passed through an oligo-dT column, the mRNAs bind to the column due to the complementary base-pairing between poly (A) tail and oligo-dT. Other RNAs (ribosomal RNAs and transfer RNAs) flow through as unbound fraction. The bound mRNAs can then be eluted using a low-salt buffer.



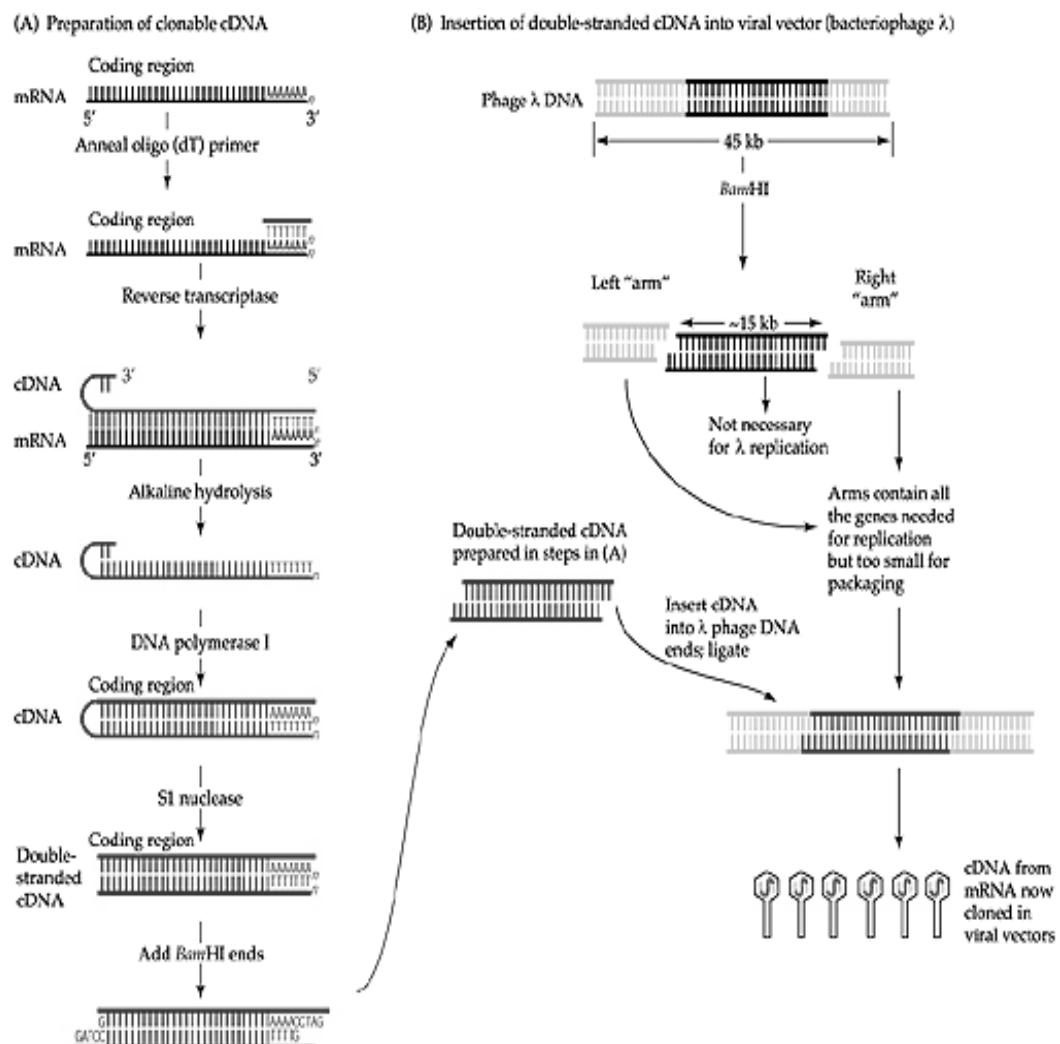
**Fig.-11.3 : Isolation of mRNA using oligo-dT Column Chromatography**

### 11.3.1.2 cDNA Construction

Once mRNA is purified, *oligo-dT* (a short sequence of deoxy-thymine nucleotides) is tagged as a complementary primer which binds to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand. Now, the mRNA is removed by using a RNase enzyme leaving a single stranded cDNA (sscDNA). This sscDNA is converted into a double stranded DNA with the help of DNA polymerase. However, for DNA polymerase to synthesize a complementary strand a free 3'-OH end is needed. This is provided by the sscDNA itself by generating a *hairpin loop* at the 3' end by coiling on it. The polymerase extends the 3'-OH end and later the loop at 3' end is opened by the scissoring action of *S<sub>1</sub> nuclease*. Restriction endonucleases and DNA ligase are then used to clone the sequences into bacterial plasmids. The whole mechanism as follows-

- mRNA being single-stranded cannot be cloned as such and is not a substrate for DNA ligase. It is first converted into DNA before insertion into a suitable vector which can be achieved using reverse transcriptase (RNA-dependent DNA polymerase or RTase) obtained from avian myeloblastosis virus (AMV).
- A short oligo (dT) primer is annealed to the Poly (A) tail on the mRNA.
- Reverse transcriptase extends the 3'-end of the primer using mRNA molecule as a template producing a cDNA: mRNA hybrid.
- The mRNA from the cDNA: mRNA hybrid can be removed by RNase H or alkaline hydrolysis to give a ss-cDNA molecule.
- No primer is required as the 3'end of this ss-cDNA serves as its own primer generating a short hairpin loop at this end. This free 3'-OH is required for the synthesis of its complementary strand.
- The single stranded (ss) cDNA is then converted into double stranded (ds) cDNA by either RTase or *E. coli* DNA polymerase.
- The ds-cDNA can be trimmed with S1 nuclease to obtain blunt-ended ds-cDNA molecule followed by addition of terminal transferase to tail the cDNA with C's and ligation into a vector.

The cloned bacteria are then selected, commonly through the use of antibiotic selection. Once selected, stocks of the bacteria are created which can later be grown and sequenced to compile the cDNA library.



**Fig.-11.4 : cDNA Construction and Formation of cDNA Library**

cDNAs are usually cloned in phage insertion vectors. Bacteriophage vectors are more suitable when a large number of recombinants are required for cloning low-abundant mRNAs as recombinant phages are produced by *in-vitro* packaging. They can easily store and handle large numbers of phage clones, as compared to the bacterial colonies carrying plasmids. Plasmid vectors are used extensively for cDNA cloning, particularly in the isolation of the desired cDNA sequence involving the screening of a relatively small number of clones.

### **11.3.2 Problems in cDNA Preparation**

- Large mRNA sequence results in inefficient synthesis of full-length cDNA. This causes problems during expression as it may not contain the entire coding sequence of the gene. This arises because of the poor processivity of RTase purified from avian myeloblastosis virus (AVM) or produced in *E.coli* from the gene of Moloney murine leukemia virus (MMLV).
- Use of S1 nuclease, the enzyme used to trim the ds cDNA, may remove some important 5' sequences.

### **11.3.3 Applications of cDNA Libraries**

- For base sequencing and gene analysis.
- For production of probes to target chromosomal genes.
- For insertion into host-vector systems for cloning and/or expression (perhaps of useful products).
- For construction of cDNA libraries (collections of random fragments of cDNA which contain all the mRNA being transcribed by the source cells at the time).

### **11.3.4 Disadvantages of cDNA Libraries**

1. cDNA libraries contain only the parts of genes found in mature mRNA. However, the sequences before and after the gene, for example, those involved in the regulation of gene expression, will not occur in a cDNA library.
2. Construction of a cDNA library cannot be used for isolating the genes expressed at low levels as there will be very little mRNA for it in any cell type and may completely be outmanoeuvred by the more abundant species.

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## **11.4 Genomic vs cDNA Library**

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A genomic library contains DNA fragments that represent the entire genome of an organism, whereas in case of cDNA library mRNA from an organism or from an organism or from specific cells of an organism are extracted and then

complementary DNA (cDNAs) are prepared from the mRNA in a multistep reaction catalysed by the enzyme reverse transcriptase.

**Table- 11.1 : Difference between Genomic and cDNA Library**

S.No.	Genomic Library	cDNA Libraries
1.	It include all possible fragments of DNA from a given cell or organism.	cDNA library carries only expressed gene sequences.
2.	It is larger	It is smaller
3.	It represents the entire genome of an organism having both coding and non coding regions.	It represents only the expressed part of the genome and contain only coding sequences called FSTs
4.	Expression of genes taken from genomic library is difficult in prokaryotic system like bacteria due to absence of splicing mechanism.	cDNA has only coding sequences therefore can be directly expressed in prokaryotic system.
5.	Vectors used genomic library include plasmid, cosmid, lambda phage, BAC and YAC in order to accommodate large fragments	Vectors used cDNA library include plasmid, phagemids, lambda phage etc to accommodate small fragments as cDNA has no introns.

## 11.5 Screening of DNA Libraries

Once a library is constructed it is screened for a particular gene of interest. Screening is based on homology between a probe and one of the clones in the library. Library screening is the process of identification of the clones carrying the gene of interest. Screening relies on a unique property of a clone in a library. The

DNA libraries consist of a collection of probably many thousand clones in the form of either plaques or colonies on a plate. Screening of libraries can be done by detecting a particular DNA sequence and gene expression. Screening large numbers of clones to identify those containing desired DNA sequences is a key step in many applications of recombinant DNA technology. One of the first large-scale screening methods introduced, hybridization, quickly became a standard technique that is still in widespread use today. Hybridization-based screening can be performed against high-density gridded macroarrays of a whole library using either a single type or mixtures of different types of radioactively labelled probes.

### **11.5.1 Screening by Hybridization**

Nucleic acid hybridization is the most commonly used method of library screening first developed by Grunstein and Hogness in 1975 to detect DNA sequences in transformed colonies using radioactive RNA probes. It relies on the fact that a single-stranded DNA molecule, used as a probe can hybridize to its complementary sequence and identify the specific sequences. This method is quick, can handle a very large number of clones and used in the identification of cDNA clones which are not full-length.

#### **11.5.1.1 Colony Hybridization**

Colony hybridization is an application of nucleic acid hybridization that is combined with conventional environmental microbiological sampling and viable planting procedures. This process utilizes a gene probes, which is radio actively marker, which attaches to complimentary base pairs from a single strand of bacteria DNA. This has proven to be a useful procedure for efficiently screening bacteria clones by RNA-DNA or DNA-DNA hybridization. This technique is accomplished by growing microbial communities from samples collecting at the research site on an agar plate, then transferring the colonies to filter paper to be lysed and denatured. After this step a labeled probe is added to bind to complementary strand of DNA and this product is assessed for any hybridization which is visualized as a black spot when looked at under x-ray.

This technique can be used to find similar microbial communities in a sample. If there is a known gene or sequence nucleic acids a researcher is looking for in a

particular samples, whether it is taken from soil, water, or the air. This can also search for particular plasmids, which is extra chromosomal DNA. This technique allows a researcher to pick a single gene from the whole genome of a bacterium of interest, while at the same time saving the researcher from having to run DNA isolation assay or a PCR assay. The con's of colony hybridization is that this assay is very time consuming, with all of the incubation this research takes days to complete. Another pitfall of this technique is that is only applies to microbes that can withstand the plating process, meaning 99% of the microbe species in the sample will not be incorporated into this experiment.

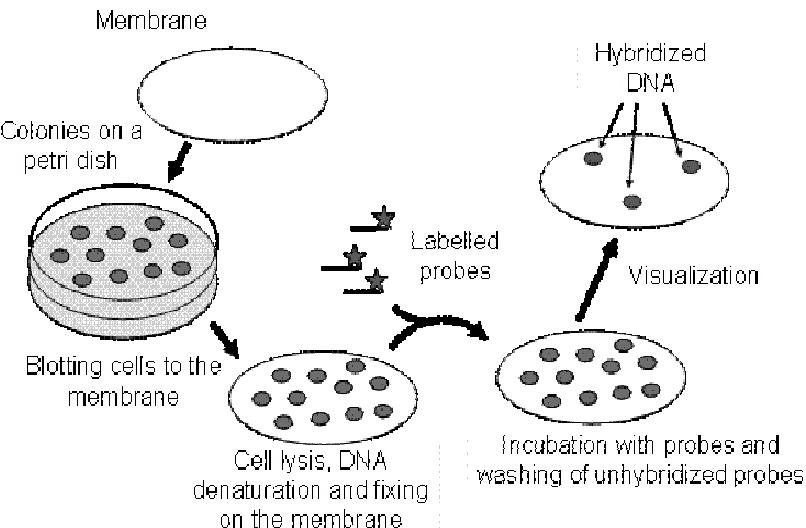
#### **11.5.1.2 Procedure of Colony Hybridization**

The overall procedures for colony hybridization are as follows-

1. Isolate and grow cultures in a suitable nutrient medium. Bacteria can be placed on selective agar for microbe colonies that are sensitive to plating; selective plates are used to facilitate growth of a particular microbe while inhibiting the growth of other microbes.
2. Transfer a sample of the colonies onto a solid matrix such as a nitrocellulose or nylon membrane. This procedure is done after the colonies have grown on the plate after incubation, which is usually done over night at 32°C however time and temperature can change depending upon the bacteria being assayed. The filter, which can be either Nylon or nitrocellulose filter, is placed on top of the bacterial colonies on the agar plate and incubated once the colonies have transferred to the filter for 24 hours at 30°C.
3. The cells on the membrane are lysed and the DNA is then denatured. After incubation the filters were placed and incubated on a series of saturated papers that lyses the cell and denatures the DNA and helps it absorb into the filter paper. The first saturated paper contained 1M sorbitol, 40 mM EDTA with a PH of 8.0, and 50 mM DTT, this was incubated for 15 min at 30° C; the second saturated paper contains 1 M sorbitol, 40 mM EDTA with a PH of 8.0, and 10% glusulase, and incubated for 3-4 h, at 37° C; the third saturated paper contained 0.5 M NaOH, and 10x SSC, and was incubated for 8 min at 37° C; the fourth and fifth saturated papers contained 0.5 M Tris-HCl (pH 7.4), 10x SSC, and were both incubated for 4 min at 37°C. The filters were then transferred to a

filtration block and, with suction applied, 200 ml of 3x SSC and 100 ml of chloroform were poured through the filter, and then the filters are baked in an oven at 80°C for 2 h.

4. A labelled gene probe is added to the matrix and hybridization takes place. The gene probe is made by isolating a piece of genetic information and the label is usually either 32P- or biotin. The hybridization occurs when the bases of the DNA match the sequence of the gene probe. When a gene probe finds its complementary DNA sequence they bind together and the specific genetic properties can be detected. This process begins with soaking the baked filter in 3x SSC for 15 minutes followed by 2 hours of incubation in the gene probe solution. Once the 2 hours incubation is completed the filter is then taken out and allowed to incubate at 65°C for 24 while the hybridizations are occurring.
5. The matrix is rinsed to remove the non-hybridized probe molecules. The wash reduces the level of background noise of the probe. The filter was washed with 3x SSC, then 2x SSC and lastly 1x SSC.
6. For radioactive probes, one uses autoradiography and the matrix is placed on x-ray film. This is when the colonies with the gene probe hybridization are visualized. If the colony has had a hybridizing event the bacteria will appear black under the X-ray autoradiograph.
7. Compare the x-ray film with the master plate to see which colonies had probe hybridization. These are the colonies that contained the specific sequence that actually hybridized with the probe. If everything is done properly it should be an easy task to compare the developed autoradiograph to the original plated bacterial colonies and to see which colony has had hybridization events.



**Fig.-11.5 : Colony Hybridization Procedure**

### 11.5.2 Screening by Polymerase Chain Reaction (PCR)

PCR screening is employed for the identification of rare DNA sequences in complex mixtures of molecular clones by increasing the abundance of a particular sequence. It is possible to identify any clone by PCR only if there is available information about its sequence to design suitable primers.

Preparation of a library for screening by PCR can be done by following ways-

- The library can be plated as plaques or colonies on agar plates and individually inoculated into the wells of the multi-well plate. However it is a labor intensive process and can lead to bias in favor of larger colonies or plaques.
- The alternative method involves diluting the library. It involves plating out a small part of the original library (the packaging mix for a phage library, transformation for a plasmid library) and calculating the titer of the library. A larger sample is diluted to give a titer of 100 colonies per mL. Dispensing 100  $\mu\text{L}$  into each well theoretically gives 10 clones in each well. These are then pooled and PCR reactions are carried out with gene-specific primers flanking a unique sequence in the target to identify the wells containing the clone of interest. This method is often used for screening commercially available libraries.

## 11.6 Polymerase Chain Reaction (PCR)

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Sometimes called "molecular photocopying (also known as People Choice Reaction)," the polymerase chain reaction (PCR) is a fast and inexpensive technique used to "amplify" - copy - small segments of DNA. Because significant amounts of a sample of DNA are necessary for molecular and genetic analyses, studies of isolated pieces of DNA are nearly impossible without PCR amplification. PCR is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies.

Though PCR occurs *in-vitro*, or outside of the body in a laboratory, it is based on the natural process of DNA replication. In its simplest form, the reaction occurs when a DNA sample and a DNA polymerase, nucleotides, primers and other reagents (man-made chemical compounds) are added to a sample tube. The reagents facilitate the reaction needed to copy the DNA code. There are three clear steps in each PCR cycle, and each cycle approximately doubles the amount of target DNA. This is an exponential reaction so more than one billion copies of the original or "target" DNA are generated in 30 to 40 PCR cycles.

### 11.6.1 Components of PCR

#### DNA template

It is the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

#### DNA polymerase

It is a type of enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is *Taq* DNA

polymerase (from *Thermis aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA. Although these enzymes are subtly different, they both have two capabilities that make them suitable for PCR. They can generate new strands of DNA using a DNA template and primers, and they are heat resistant.

### **Primers**

It is short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

### **Nucleotides (dNTPs or deoxynucleotide triphosphates)**

It is Single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands. Triphosphates of deoxynucleotides like ATP, TTP, GTP and CTP are basic components for PCR.

## **11.6.2 Procedure of PCR**

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (Fig. below). The cycling is often preceded by a single temperature step at a high temperature ( $>90^{\circ}\text{C}$ ), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. The overall mechanism of PCR is divided in three steps. These are as follows-

### **1. Denaturation-Separating the Target DNA**

During the first step of PCR, called denaturation, the tube containing the sample DNA is heated to more than  $90^{\circ}\text{C}$  ( $194^{\circ}\text{Fahrenheit}$ ), which separates the double-stranded DNA into two separate strands. The high temperature breaks the relatively weak bonds between the nucleotides that form the DNA code.

### **2. Annealing-Binding Primers to the DNA Sequence**

PCR does not copy the all of the DNA in the sample. It copies only a very specific sequence of genetic code, targeted by the PCR primers. To do this, PCR uses primers, man-made oligonucleotides (short pieces of synthetic DNA) that

bind, or anneal, only to sequences on either side of the target DNA region. Two primers are used in step two - one for each of the newly separated single DNA strands. The primers bind to the beginning of the sequence that will be copied, marking off the sequence for step three. During step two, the tube is cooled and primer binding occurs between  $40^0$  and  $60^0$ C ( $104 - 140^0$  Fahrenheit) for 20 to 40 seconds. Step two yields two separate strands of DNA, with sequences marked off by primers. The two strands are ready to be copied.

### 3. Extension-Making a Copy

In the third phase of the reaction, called extension, the temperature is increased to approximately  $72^0$ C ( $161.5^0$  Fahrenheit). Beginning at the regions marked by the primers, nucleotides in the solution are added to the annealed primers by the DNA polymerase to create a new strand of DNA complementary to each of the single template strands. After completing the extension, two identical copies of the original DNA have been made.

**Table- 11.2 : Preparation of DNA per PCR Cycle**

<b>PCR Cycles</b>	<b>Target Copies</b>
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
15	32,768
20	1,048,576
25	33,554,432
30	1,073,741,842

After making two copies of the DNA through PCR, the cycle begins again, this time using the new duplicated DNA. Each duplicate creates two new copies and after approximately 30 or 40 PCR cycles, more than one billion copies of the original DNA segment have been made. Because the PCR process is automated, it can be completed in just a few hours.

In a healthcare setting, PCR makes enough copies of target DNA from the clinical sample to allow analysis; the results of these diagnostic and monitoring tests provide clinicians and other healthcare providers with information to guide treatment.

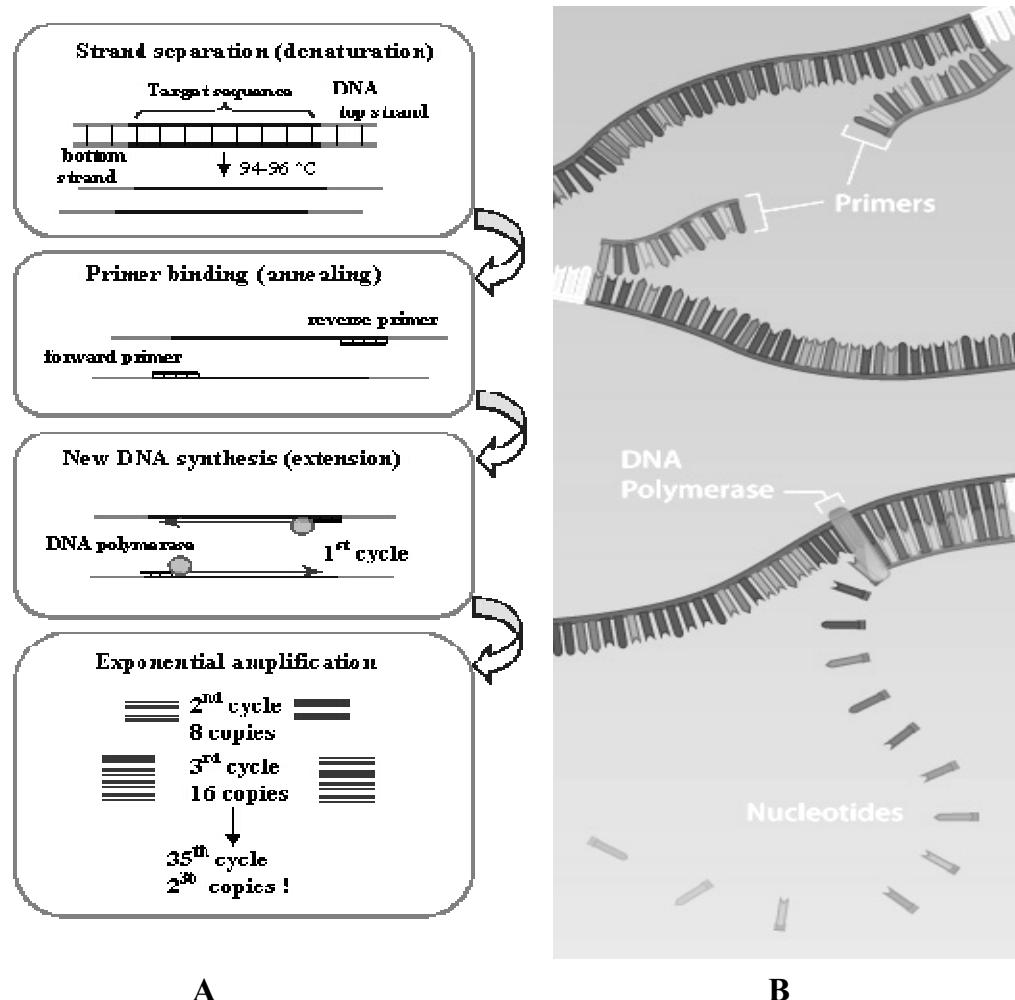


Fig.-11.6 : A & B Procedure of PCR

### 11.6.3 Types of PCR

Often only a small modification needs to be made to the standard PCR protocol to achieve a desired goal:

1. **Multiplex-PCR** uses several pairs of primers annealing to different target sequences. This permits the simultaneous analysis of multiple targets in a single sample. For example, in testing for genetic mutations, six or more amplifications might be combined. In the standard protocol for DNA Fingerprinting, the targets assayed are often amplified in groups of 3 or 4. *Multiplex Ligation-dependent Probe Amplification* (or *MLPA*) permits multiple targets to be amplified using only a single pair of primers, avoiding the resolution limitations of multiplex PCR. Multiplex PCR has also been used for analysis of microsatellites and SNPs.
2. **Variable Number of Tandem Repeats (VNTR)** PCR targets areas of the genome that exhibit length variation. The analysis of the genotypes of the sample usually involves sizing of the amplification products by gel electrophoresis. Analysis of smaller VNTR segments known as Short Tandem Repeats (or STRs) is the basis for DNA Finger printing data bases such as CODIS.
3. **Asymmetric PCR** preferentially amplifies one strand of the target DNA. It is used in some sequencing methods and hybridization probing, to generate one DNA strand as product. Thermocycling is carried out as in PCR, but with a limiting amount or leaving out one of the primers. When the limiting primer becomes depleted, replication increases arithmetically through extension of the excess primer. A modification of this process, named *L'inear-After-The-Exponential-PCR* (or *LATE-PCR*), uses a limiting primer with a higher Melting temperature ( $T_m$ ) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.
4. Some modifications are needed to perform **long PCR**. The original Klenow-based PCR process did not generate products that were larger than about 400 bp. Taq polymerase can however amplify targets of up to several thousand bp long. Since then, modified protocols with Taq enzyme have allowed targets of over 50 kb to be amplified.

5. **Nested PCR** is used to increase the specificity of DNA amplification. Two sets of primers are used in two successive reactions. In the first PCR, one pair of primers is used to generate DNA products, which may contain products amplified from non-target areas. The products from the first PCR are then used as template in a second PCR, using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity. Nested PCR is often more successful in specifically amplifying long DNA products than conventional PCR, but it requires more detailed knowledge of the sequence of the target.
6. **Quantitative PCR** is used to measure the specific amount of target DNA (or RNA) in a sample. By measuring amplification only within the phase of true exponential increase, the amount of measured product more accurately reflects the initial amount of target. Special thermal cyclers are used that monitor the amount of product during the amplification. *Quantitative Real-Time PCR* (QRT-PCR) methods use fluorescent dyes, such as Sybr Green, or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product as the amplification progresses.
7. **Hot-start PCR** is a technique performed manually by heating the reaction components to the DNA melting temperature (e.g. 95 °C) before adding the polymerase. In this way, non-specific amplification at lower temperatures is prevented. Alternatively, specialized reagents inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody, or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. 'Hot-start/cold-finish PCR' is achieved with new hybrid polymerases that are inactive at ambient temperature and are only activated at elevated temperatures.
8. In **Touchdown PCR**, the annealing temperature is gradually decreased in later cycles. The annealing temperature in the early cycles is usually 3-5 °C above the standard  $T_m$  of the primers used, while in the later cycles it is a similar amount below the  $T_m$ . The initial higher annealing temperature leads to greater specificity for primer binding, while the lower temperatures permit more efficient amplification at the end of the reaction.

9. **Assembly PCR** (also known as Polymerase Cycling Assembly or *PCA*) is the synthesis of long DNA structures by performing PCR on a pool of long oligonucleotides with short overlapping segments, to assemble two or more pieces of DNA into one piece. It involves an initial PCR with primers that have an overlap and a second PCR using the products as the template that generates the final full-length product. This technique may substitute for ligation-based assembly.
10. In **Colony PCR**, bacterial colonies are screened directly by PCR, for example, the screen for correct DNA vector constructs. Colonies are sampled with a sterile pipette tip and a small quantity of cells transferred into a PCR mix. To release the DNA from the cells, the PCR is either started with an extended time at 95 °C (when standard polymerase is used), or with a shortened denaturation step at 100 °C and special chimeric DNA polymerase.
11. The **Digital PCR** simultaneously amplifies thousands of samples, each in a separate droplet within an emulsion.
12. **Suicide PCR** is typically used in paleogenetics or other studies where avoiding false positives and ensuring the specificity of the amplified fragment is the highest priority. It was originally described in a study to verify the presence of the microbe *Yersinia pestis* in dental samples obtained from 14th Century graves of people supposedly killed by plague during the medieval Black Death epidemic. The method prescribes the use of any primer combination only once in a PCR (hence the term "suicide"), which should never have been used in any positive control PCR reaction, and the primers should always target a genomic region never amplified before in the lab using this or any other set of primers. This ensures that no contaminating DNA from previous PCR reactions is present in the lab, which could otherwise generate false positives.

#### 11.6.4 Applications of PCR

##### 1. Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA,

representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid, phage, or cosmid (depending on size) or the genetic material of another organism. Bacterial colonies (*E.coli*) can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

Some PCR 'fingerprints' methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing. This technique may also be used to determine evolutionary relationships among organisms when certain molecular clocks are used.

## **2. Amplification and Quantification of DNA**

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ADNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar and the body of English king Richard III.

## **3. PCR in Diagnosis of Diseases**

PCR permits early diagnosis of malignant diseases such as leukaemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000 fold higher than that of other methods.

PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses. PCR also permits identification of non-cultivable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

### **11.6.5 Limitations of PCR**

DNA polymerase is prone to error, which in turn causes mutations in the PCR fragments, which are made. Additionally, the specificity of the PCR fragments can be mutated to the template DNA, due to nonspecific binding of primers. Furthermore information on the sequence is necessary prior, in order to generate the primers. The PCR reaction starts to generate copies of the target sequence exponentially. Only during the exponential phase of the PCR reaction is it possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Because of inhibitors of the polymerase reaction found in the sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs, making the end point quantification of PCR products unreliable. This is the attribute of PCR that makes Real-Time Quantitative RT-PCR so necessary.

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## **11.7 Summary**

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Library is a collection of DNA fragments that is stored and propagated in a population of micro-organisms through the process of molecular cloning. There are different types of DNA libraries, including cDNA libraries, genomic libraries and randomized mutant libraries. If the library has been established by using fragmented cellular DNA of an organism the library is said to be a genomic library. The term genomic DNA clone or chromosomal DNA clone then refers to an individual cell carrying a cloning vector with one of the cellular DNA fragments or to a phage isolate with a specific DNA insert. Subgenomic libraries are obtained if only selected portions of the genome, for example fragments from distinct sorted

chromosomes, are represented in the library. A characteristic of genomic libraries is the fact that the individual inserts still contain non-coding intron sequences. Such libraries are used, therefore, for determining the genomic structures of genes.

Once a library is constructed it is screened for a particular gene of interest. Screening is based on homology between a probe and one of the clones in the library. Library screening is the process of identification of the clones carrying the gene of interest. Screening relies on a unique property of a clone in a library. The DNA libraries consist of a collection of probably many thousand clones in the form of either plaques or colonies on a plate. Screening of libraries can be done by detecting a particular DNA sequence and gene expression. Screening large numbers of clones to identify those containing desired DNA sequences is a key step in many applications of recombinant DNA technology. One of the first large-scale screening methods introduced, hybridization, quickly became a standard technique that is still in widespread use today. Hybridization-based screening can be performed against high-density gridded macroarrays of a whole library using either a single type or mixtures of different types of radioactively labelled probes.

Colony hybridization is an application of nucleic acid hybridization that is combined with conventional environmental microbiological sampling and viable planting procedures. This process utilizes a gene probes, which is radio actively marker, which attaches to complimentary base pairs from a single strand of bacteria DNA. This has proven to be a useful procedure for efficiently screening bacteria clones by RNA-DNA or DNA-DNA hybridization. PCR screening is employed for the identification of rare DNA sequences in complex mixtures of molecular clones by increasing the abundance of a particular sequence. It is possible to identify any clone by PCR only if there is available information about its sequence to design suitable primers.

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## 11.8 Glossary

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- **DNA Library** : A library is a collection of DNA fragments that is stored and propagated in a population of micro-organisms through the process of molecular cloning.

- **Genomic DNA** : Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA remaining unchanged (helical ribbon).
  - **Subgenomic library** : Subgenomic library is a library which represents only a fraction of the genome.
  - **cDNA library** : A cDNA library is a combination of cloned cDNA fragments constituting some portion of the transcriptome of an organism which are inserted into a number of host cells.
  - **Colony hybridization** : Colony hybridization is an application of nucleic acid hybridization that is combined with conventional environmental microbiological sampling and viable planting procedures.
  - **Library** : Library screening is the process of identification of the clones carrying the gene of interest.
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## 11.9 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Write name of two libraries.
2. Define genomic library.
3. Elaborate term PCR.

### Section B : (Short Answer Type Questions)

1. Write the methods for DNA purification.
2. Describe procedure of cell extract preparation.
3. What is subgenomic library?
4. Write advantages and disadvantages of genomic library.
5. Write difference between genomic and cDNA library.
6. What is colony hybridization? Write procedure for colony hybridization.

### Section C : (Long Answer Type Questions)

1. Give detail description about cDNA library construction.
2. Describe components and procedure of PCR.
3. Write applications of PCR.

**Answer Key of Section – A**

1. Genomic library and cDNA library.
  2. Genomic libraries are a catalogue of genes of a particular organism. They are also commonly referred to as gene banks.
  3. Polymerase Chain Reaction.
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**11.10 References**

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## Unit - 12

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# Recombinant Technology-IV: Molecular Markers

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### **Structure of the Unit:**

- 12.0 Objectives
- 12.1 Introduction
- 12.2 Molecular Markers
  - 12.2.1 Genetic Markers
  - 12.2.2 Morphological Markers
  - 12.2.3 Cytological Markers
  - 12.2.4 Biochemical (Protein) Markers
  - 12.2.5 DNA-based Molecular Markers
- 12.3 Properties of Molecular Markers
- 12.4 Types of DNA Markers
  - 12.4.1 RFLP Markers
  - 12.4.2 RAPD Markers
  - 12.4.3 AFLP Markers
  - 12.4.4 SSR Markers
  - 12.4.5 SNP Markers
  - 12.4.6 Transposable Elements
  - 12.4.7 Alu-repeats
  - 12.4.8 Repeat Complementary Primers
- 12.5 Marker Assisted Selection (MAS)
- 12.6 Applications of Molecular Markers
- 12.7 Molecular Markers in Crop Improvement Program
- 12.8 Glossary

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- 12.9 Summary
  - 12.10 Self Learning Exercise
  - 12.11 References
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## 12.0 Objectives

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After going through this unit you will be able to understand about:

- Molecular Markers, Genetic Markers
  - Types of DNA Markers: RFLP Markers, RAPD Markers, AFLP Markers, SSR Markers, SNP Markers, Transposable Elements, Alu-repeats, Repeat Complementary Primers, Marker Assisted Selection (MAS)
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## 12.1 Introduction

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A molecular marker is a molecule contained within a sample taken from an organism (biological markers) or other matter. It can be used to reveal certain characteristics about the respective source. With the advent of molecular markers, a new generation of markers has been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. They are no longer looked upon as simple DNA fingerprinting markers in variability studies or as mere forensic tools. Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis. The discovery of PCR (polymerase chain reaction) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker-based gene tags, map-based cloning of agronomic ally important genes, variability studies, phylogenetic analysis, system mapping, marker-assisted selection of desirable genotype etc. Thus giving new dimensions to concerted efforts of breeding and marker-aided selection that can reduce the time span of developing new and better varieties and it will make the dream of super varieties come true. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively.

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## 12.2 Molecular Markers

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### 12.2.1 Genetic Markers

Genetic markers are the biological features that are determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another, and thus they can be used as experimental probes or tags to keep track of an individual, a tissue, a cell, a nucleus, a chromosome or a gene. Genetic markers used in genetics and plant breeding can be classified into two categories: classical markers and DNA markers. Classical markers include morphological markers, cytological markers and biochemical markers. DNA markers have developed into many systems based on different polymorphism-detecting techniques or methods (southern blotting – nuclear acid hybridization, PCR- polymerase chain reaction, and DNA sequencing) such as RFLP, AFLP, RAPD, SSR, SNP, etc.

### 12.2.2 Morphological Markers

Use of markers as an assisting tool to select the plants with desired traits had started in breeding long time ago. During the early history of plant breeding, the markers used mainly included visible traits, such as leaf shape, flower color, pubescence color, pod color, seed color, seed shape, hilum color, awn type and length, fruit shape, rind(exocarp) color and stripe, flesh color, stem length, etc. These morphological markers generally represent genetic polymorphisms which are easily identified and manipulated. Therefore, they are usually used in construction of linkage maps by classical two- and/or three point tests. Some of these markers are linked with other agronomic traits and thus can be used as indirect selection criteria in practical breeding.

### 12.2.3 Cytological Markers

In cytology, the structural features of chromosomes can be shown by chromosome karyotype and bands. The banding patterns, displayed in color, width, order and position, reveal the difference in distributions of euchromatin and heterochromatin. For instance, Q bands are produced by quinacrine hydrochloride, G bands are produced by Giemsa stain, and R bands are the reversed G bands. These chromosome landmarks are used not only for characterization of normal

chromosomes and detection of chromosome mutation, but also widely used in physical mapping and linkage group identification. The physical maps based on morphological and cytological markers lay a foundation for genetic linkage mapping with the aid of molecular techniques. However, direct use of cytological markers has been very limited in genetic mapping and plant breeding.

#### **12.2.4 Biochemical (Protein Markers)**

Protein markers may also be categorized into molecular markers though the latter are more referred to DNA markers. Isozymes are alternative forms or structural variants of an enzyme that have different molecular weights and electrophoretic mobility but have the same catalytic activity or function. Isozymes reflect the products of different alleles rather than different genes because the difference in electrophoretic mobility is caused by point mutation as a result of amino acid substitution. Therefore, Isozymes markers used to map other genes. They are also used in seed purity test and occasionally in plant breeding. There are only a small number of isozymes in most crop species and some of them can be identified only with a specific strain. Therefore, the use of enzyme markers is limited.

#### **12.2.5 DNA-based Molecular Markers**

Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is expensive and laborious. A wide variety of techniques have, therefore, been developed in the past few years for visualizing DNA sequence polymorphism.

DNA markers are defined as a fragment of DNA revealing mutations or variations, which can be used to detect polymorphism between different genotypes or alleles of a gene for a particular sequence of DNA in a population or gene pool. Such fragments are associated with a certain location within the genome and may be detected by means of certain molecular technology. Simply DNA marker is a small region of DNA sequence showing polymorphism (base deletion, insertion and substitution) between different individuals. There are two basic methods to detect the polymorphism: Southern blotting, a nuclear acid hybridization technique

developed by Southern 1975, and PCR, a polymerase chain reaction technique by Mullis, 1990. Using PCR and/or molecular hybridization followed by electrophoresis (e.g. PAGE- polyacrylamide gel electrophoresis, AGE – agarose gel electrophoresis, CE-capillary electrophoresis), the variation in DNA samples or polymorphism for a specific region of DNA sequence can be identified based on the product features, such as band size and mobility. In addition to Sothern blotting and PCR, more detection systems have been also developed. For instance, several new array chip techniques use DNA hybridization combined with labelled nucleotides, and new sequencing techniques detect polymorphism by sequencing.

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### 12.3 Properties of Molecular Markers

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DNA markers are also called molecular markers in many cases and play a major role in molecular breeding. Depending on application and species involved; ideal DNA markers for efficient use in marker-assisted breeding should meet the following criteria:

- High level of polymorphism.
- Even distribution across the whole genome (not clustered in certain regions).
- Co-dominance in expression (so that heterozygotes can be distinguished from homozygotes).
- Clear distinct allelic features (so that the different alleles can be easily identified).
- Single copy and no pleiotropic effect.
- Low cost to use (or cost-efficient marker development and genotyping).
- Easy assay or detection and automation.
- High availability (un-restricted use) and suitability to be duplicated or multiplexed (so that the data can be accumulated and shared between laboratories).
- Genome-specific in nature (especially with polyploids).
- No detrimental effect on phenotype.

Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR-based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermo-stable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography. PCR is a versatile technique invented during the mid-1980s. Ever since thermostable DNA polymerase was introduced in 1988, the use of PCR in research and clinical laboratories has increased tremendously. The primer sequences are chosen to allow base-specific binding to the template in reverse orientation. PCR is extremely sensitive and operates at a very high speed. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology.

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## 12.4 Types of DNA Markers

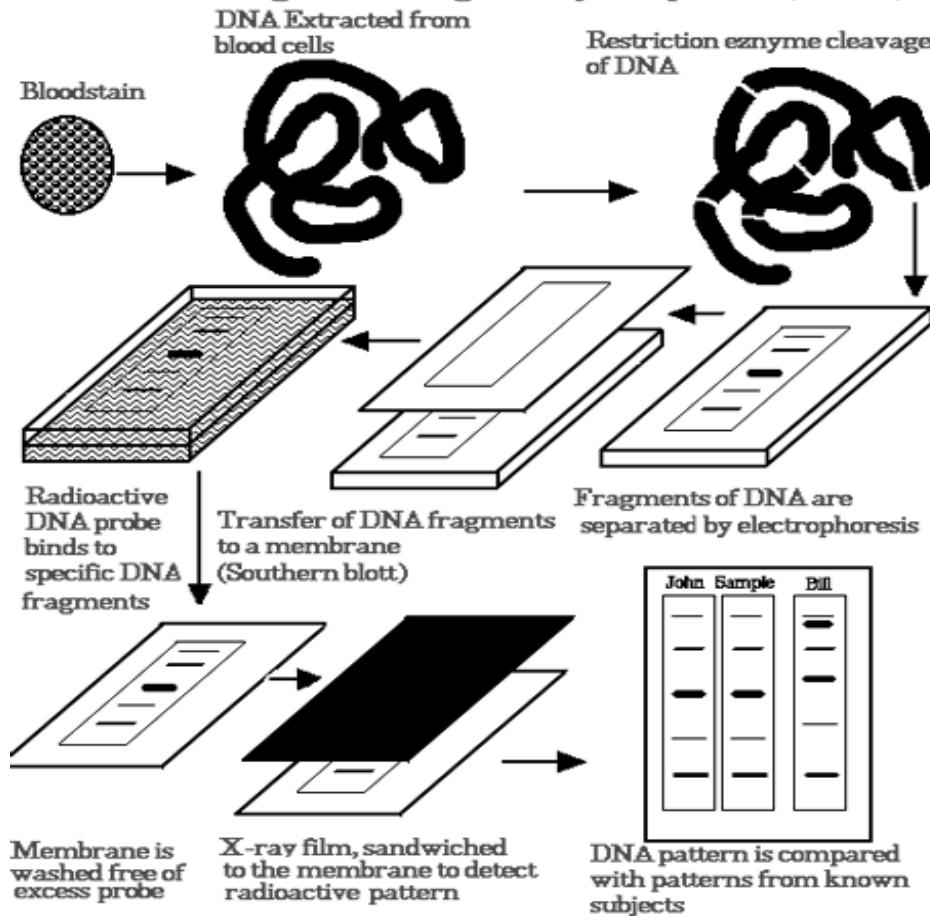
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On the basis of functions and structure of markers the DNA markers can be divided in following types:

### 12.4.1 RFLP Markers

RFLP (Restriction Fragment Length polymorphism) markers are the first generation of DNA markers and one of the important tools for plant genome mapping. They are a type of Southern-Bolting-based markers. In living organisms, mutation events (deletion and insertion) may occur at restriction sites or between adjacent restriction sites in the genome. Gain or loss of restriction sites resulting from base pair changes and insertions or deletions at restriction sites within the restriction fragments may cause differences in size of restriction fragments. These variations may cause alternation or elimination of the recognition sites for restriction enzymes. As a consequence, when homologous chromosomes are subjected to restriction enzyme digestion, different restriction products are produced and can be detected by electrophoresis and DNA probing techniques.

### Restriction Fragment Length Polymorphism (RFLP)



**Fig:- 12.1 : Process of RFLP**

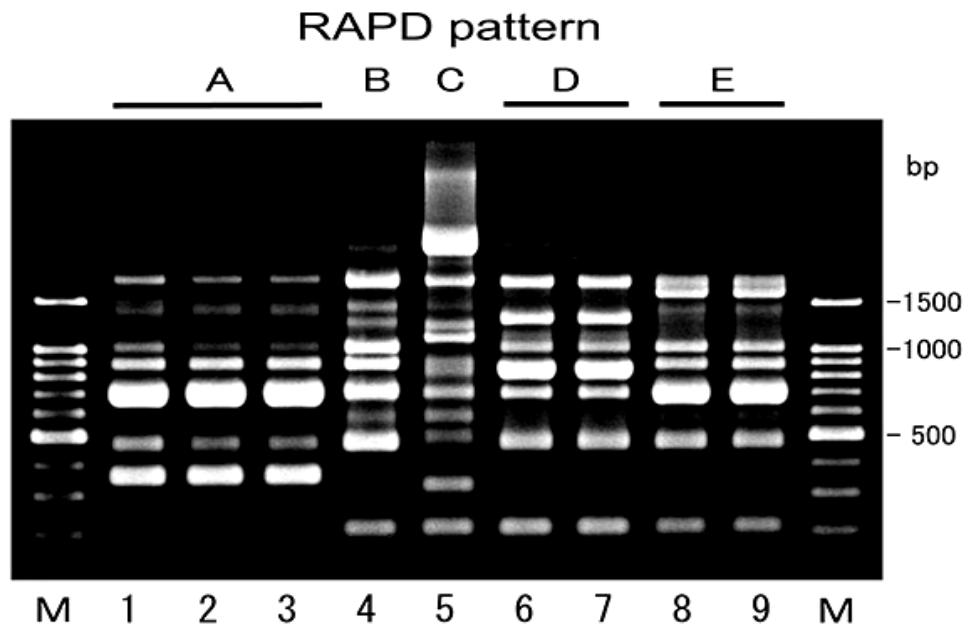
RFLP markers are powerful tools for comparative mapping. Most RFLP markers are co-dominant and locus-specific. RFLP genotyping is highly reproducible, and the methodology is simple and no special equipment is required. By using an improved RFLP technique, i.e., cleaved amplified polymorphism sequence (CAPS), also known as PCRRFLP, high-throughput markers can be developed from RFLP probe sequences. Very few CAPS are developed from probe sequences, which are complex to interpret. Most CAPS are developed from SNPs found in other sequences followed by PCR and detection of restriction sites. CAPS technique consists of digesting a PCR-amplified fragment and detecting the polymorphism by the presence/absence of restriction sites.

Another advantage of RFLP is that the sequence used as a probe need not be known. All that a researcher needs is genomic clones that can be used to detect the

polymorphism. Very few RFLPs have been sequenced to determine what sequence variation is responsible for the polymorphism. However, it may be problematic to interpret complex RFLP allelic systems in the absence of sequence information. RFLP analysis requires large amounts of high-quality DNA, has low genotyping throughput, and is very difficult to automate. Radioactive autorography involving in genotyping and physical maintenance of RFLP probes limit its use and share between laboratories. RFLP markers were predominantly used in 1980s and 1990s, but since last decade fewer direct uses of RFLP markers in genetic research and plant breeding have been reported. Most plant breeders would think that RFLP is too laborious and demands too much pure DNA to be important for plant breeding. It was and is, however, central for various types of scientific studies.

#### **12.4.2 RAPD Markers**

RAPD (Random Amplified Polymorphic DNA) is a PCR-based marker system. In this system, the total genomic DNA of an individual is amplified by PCR using a single, short (usually about ten nucleotides/bases) and random primer. The primer which binds to many different loci is used to amplify random sequences from a complex DNA template that is complementary to it (maybe including limited number of mismatches). Amplification can take place during the PCR, if two hybridization sites are similar to one another (at least 3000 bp) and in opposite directions. The amplified fragments generated by PCR depend on the length and size of both the primer and the target genome. The PCR products (up to 3 kb) are separated by agarose gel electrophoresis and imaged by ethidium bromide (EB) staining. Polymorphisms resulted from mutations or rearrangements either at or between the primer-binding sites are visible in the electrophoresis as the presence or absence of a particular RAPD band.



**Fig.- 12.2 : Pattern of RAPD**

RAPD predominantly provides dominant markers. This system yields high levels of polymorphism and is simple and easy to be conducted. First, neither DNA probes nor sequence information is required for the design of specific primers. Second, the procedure does not involve blotting or hybridization steps, and thus it is a quick, simple and efficient technique.

#### 12.4.3 AFLP Markers

AFLPs (Amplified Fragment Length Polymorphism) are PCR-based markers, simply RFLPs visualized by selective PCR amplification of DNA restriction fragments. Technically, AFLP is based on the selective PCR amplification of restriction fragments from a total double-digest of genomic DNA under high stringency conditions, i.e., the combination of polymorphism at restriction sites and hybridization of arbitrary primers. Because of this AFLP is also called selective restriction fragment amplification (SRFA). An AFLP primer (17-21 nucleotides in length) consists of a synthetic adaptor sequence, the restriction endonuclease recognition sequence and an arbitrary, non-degenerate ‘selective’ sequence (1-3 nucleotides). The primers used in this technique are capable of annealing perfectly to their target sequences (the adapter and restriction sites) as well as a small number of nucleotides adjacent to the restriction sites.

# AFLP PCR

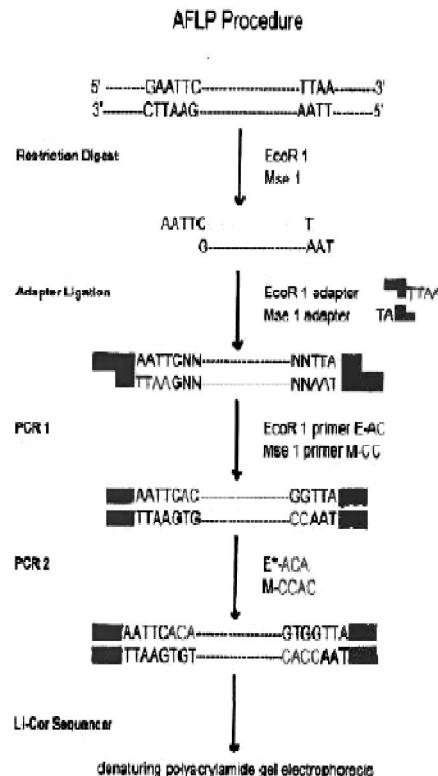
**AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci**

- Genomic DNA is digested with one or more restriction enzymes, tetracutter (*Mse*I) and a hexacutter (*Eco*RI).

- Ligation of linkers to all restriction fragments.

- Pre-selective PCR is performed using primers which match the linkers and restriction site specific sequences.

- Electrophoretic separation and amplicons on a gel matrix, followed by visualisation of the band pattern.



**Fig.- 12.3 : Schematic Presentation of AFLP**

The first step in AFLP involves restriction digestion of genomic DNA (about 500 ng) with two restriction enzymes, a rare cutter (6-bp recognition site, *Eco*RI, *Pst*I or *Hind*III) and a frequent cutter (4-bp recognition site, *Mse*I or *Taq*I). The adaptors are then ligated to both ends of the fragments to provide known sequences for PCR amplification. The double-stranded oligonucleotide adaptors are designed in such a way that the initial restriction site is not restored after ligation. Therefore, only the fragments which have been cut by the frequent cutter and rare cutter will be amplified. This property of AFLP makes it very reliable, robust and immune to small variations in PCR amplification parameters (e.g., thermal cycles, template concentration), and it also can produce a high marker density. The AFLP products can be separated in high-resolution electrophoresis systems. The fragments in gel-based or capillary DNA sequencers can be detected by dye-labelling primers radioactively or fluorescently. The number of bands produced can be manipulated by the number of selective nucleotides and the nucleotide motifs used.

#### 12.4.4 SSR Markers

SSRs (Simple Sequence Repeat), also called microsatellites, short tandem repeat (STRs) or sequence-tagged microsatellite sites (STMS) are PCR-based markers. They are randomly tandem repeats of short nucleotide motifs (2-6 bp/nucleotides long). Di-, tri- and tetra-nucleotide repeats, e.g. (GT) $n$ , (AAT) $n$  and (GATA) $n$ , are widely distributed throughout the genomes of plants and animals. The copy number of these repeats varies among individuals and is a source of polymorphism in plants. Because the DNA sequences flanking microsatellite regions are usually conserved, primers specific for these regions are designed for use in the PCR reaction.

One of the most important attributes of microsatellite loci is their high level of allelic variation, thus making them valuable genetic markers. The unique sequences bordering the SSR motifs provide templates for specific primers to amplify the SSR alleles via PCR. SSR loci are individually amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence. The PCR-amplified products can be separated in high-resolution electrophoresis systems (e.g. AGE and PAGE) and the bands can be visually recorded by fluorescent labeling or silver-staining.

#### 12.4.5 SNP Markers

An SNP (Single Nucleotide Polymorphism) is a single nucleotide base difference between two DNA sequences or individuals. SNPs can be categorized according to nucleotide substitutions either as transitions(C/T or G/A) or transversions (C/G, A/T, C/A or T/G). In practice, single base variants in cDNA (mRNA) are considered to be SNPs as are single base insertions and deletions in the genome. SNPs provide the ultimate/simplest form of molecular markers as a single nucleotide base is the smallest unit of inheritance, and thus they can provide maximum markers. SNPs occur very commonly in animals and plants. Typically, SNP frequencies are in a range of one SNP every 100-300 bp in plants. SNPs may present within coding sequences of genes, non-coding regions of genes or in the intergenic regions between genes at different frequencies in different chromosome regions.

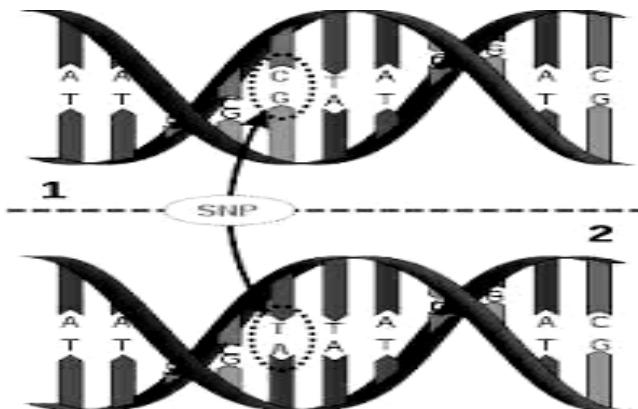
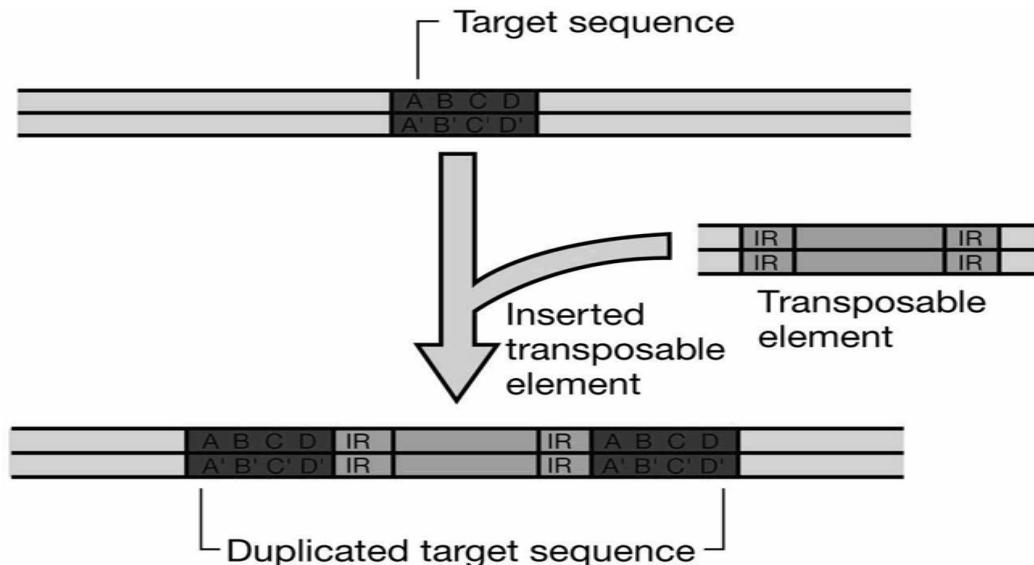


Fig.- 12.4 : SNP Markers

#### 12.4.6 Transposable Elements

A transposable element (TE or transposon) is a DNA sequence that can change its position within the genome, sometimes creating or reversing mutations and altering the cell's genome size. Transposition often results in duplication of the TE. TEs make up a large fraction of the C-value of eukaryotic cells. They are generally considered non-coding DNA, although it has been shown that TEs are important in genome function and evolution. In *Oxytricha*, which has a unique genetic system, they play a critical role in development. They are also very useful to researchers as a means to alter DNA inside a living organism.

A large number of transposable repeat elements have been studied in plants; however, only a few have been exploited as molecular markers. In evolutionary terms, they have contributed to genetic differences between species and individuals by playing a role in retrotransposition events promoting unequal crossing over. Retrotransposon-mediated fingerprinting has been shown to be an efficient fingerprinting method for detection of genetic differences between different species.



**Fig.- 12.5 : Transposable Elements**

#### 12.4.7 Alu-repeats

This strategy was developed to fingerprint genotypes using semi specific primers, complementary to repetitive DNA elements called ‘Alu-repeats’, in human genome analysis. Alu repeats are a class of randomly repeated interspersed DNA, preferentially used for Alu PCR as they reveal considerable levels of polymorphism<sup>35</sup>. These representatives of short and long interspersed nuclear elements are known as SINES. Alu elements are approximately 300 bp in size and have been suggested to be originated from special RNA species that have been reintegrated at a rate of approximately one integration event per 10000 years. These repeats have been studied largely in humans, while their function in plants remains largely unexplored.

#### 12.4.8 Repeat Complementary Primers

As an alternative to the interspersed repeats, primers complementary to other repetitive sequence elements were also successfully used for generation of polymorphisms, e.g. introns/exons splice junctions<sup>36</sup>, tRNA genes<sup>37</sup>, 5sRNA genes<sup>38</sup> and Zn-finger protein genes<sup>39</sup>. Primers complementary to specific exons, resulting in the amplification of the intervening introns have been studied by Lessa *et al.* One of the strengths of these new strategies is that they are more amenable to automation than the conventional hybridization-based techniques.

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## 12.5 Marker Assisted Selection (MAS)

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To improve plant varieties, since the late 19<sup>th</sup> century plant breeders relied on phenotypic selection, achieving breeding progress through the assessment of external and internal traits such as plant habits, disease resistances, yield, or quality traits. New, improved varieties were developed by solely selecting plants with desirable phenotypes. Plant breeding techniques became very sophisticated over the years but time demanding too. Developing a new, improved plant variety by means of phenotypic selection can easily exceed 10 years.

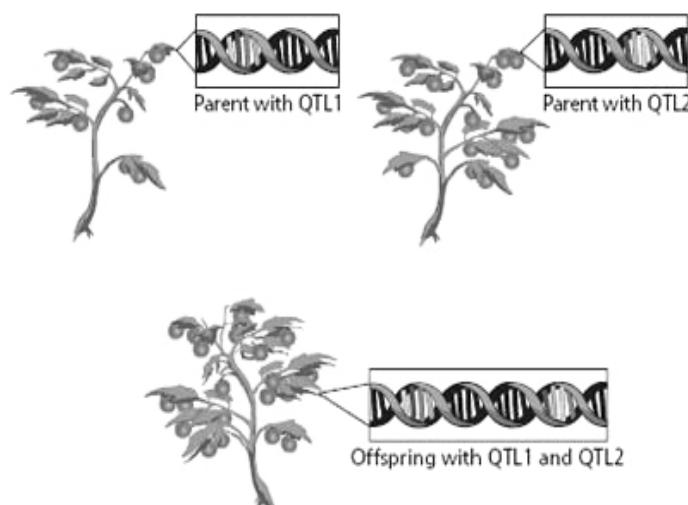
Sometimes the term “Smart Breeding”, an acronym for “Selection with Markers and Advanced Reproductive Technologies”, is used to describe marker supported breeding strategies. The idea of MAS or smart breeding was taken up with great enthusiasm, and several breeders expressed the hope to “skip several breeding cycles and condense timelines” and to finally having found a tool, “to control all allelic variation for all genes of agronomic importance. According to Xuand Crouch (2008) the greatest benefits of MAS are the possibilities to “achieve the same breeding progress in a much shorter time than through conventional breeding”, “pyramid combinations of genes that could not be readily combined through other means” and to “assemble target traits more precisely, with less unintentional losses”.

MAS which is sometimes referred to as genomics is a form of biotechnology which uses genetic finger-printing techniques to assist plant breeders in matching molecular profile to the physical properties of the variety. It is the identification of DNA sequences located near genes that can be tracked to breed for traits that are difficult to observe. MAS refer to the use of DNA markers that are tight-linked to target loci as a substitute for or to assist phenotype screening. By determining the allele of a DNA marker, plants that possess particular genes or quantitative trait loci (QTL) may be identified based on their genotype rather than their phenotype. Collard and Mackill (2006) reported the fundamental advantages of MAS compared to conventional phenotypic selection which are:-

- Simpler compared to phenotypic breeding

- Selection may be carried out at breeding stage and single plants may be selected with high reliability.

In this technique, linkages are sought between DNA markers and agronomic important traits such as resistance to pathogens, insects and nematodes, tolerance to biotic stresses, quality parameters and quantitative traits. MAS is in contrast to genetic engineering which involves the artificial insertion of such individuals genes from one organism into the genetic material of another (typically, but not exclusively from other unrelated Species).



**Fig.- 12.6 : Selection Based on Markers**

Marker-assisted selection (MAS) refers to such a breeding procedure in which DNA marker detection and selection are integrated into a traditional breeding program. Taking a single cross as an example, the general procedure can be described as follow:

- Select parents and make the cross, at least one (or both) possesses the DNA marker allele(s) for the desired trait of interest.
- Plant F<sub>1</sub> population and detect the presence of the marker alleles to eliminate false hybrids.
- Plant segregating F<sub>2</sub> population, screen individuals for the marker(s), and harvest the individuals carrying the desired marker allele(s).

- Plant F<sub>2</sub>:3 plant rows, and screen individual plants with the marker(s). A bulk of F<sub>3</sub> individuals within a plant row may be used for the marker screening for further confirmation in case needed if the preceding F<sub>2</sub> plant is homozygous for the markers. Select and harvest the individuals with required marker alleles and other desirable traits.
- In the subsequent generations (F<sub>4</sub> and F<sub>5</sub>), conduct marker screening and make selection similarly as for F<sub>2</sub>:3s, but more attention is given to superior individuals within homozygous lines/rows of markers.
- In F<sub>5</sub>:6 or F<sub>4</sub>:5 generations, bulk the best lines according to the phenotypic evaluation of target trait and the performance of other traits, in addition to marker data.
- Plant yield trials and comprehensively evaluate the selected lines for yield, quality, resistance and other characters of interest.

## **12.6 Applications of Molecular Markers**

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it.

The two major applications of DNA markers in plant sciences are detailed chromosome mapping and selection and introgression of both simple and quantitative traits. In addition, DNA markers are also used for germplasm evaluation, genetic diagnostics, phylogenetic analysis, study of genome organization and screening of transformants. In this review chromosome mapping will not be covered; the focus is on the selection purposes of DNA markers. Also, not all crops can be covered in this review, thus the focus is restricted to marker application in the main agricultural crops grown in temperate zones, mainly wheat

(*Triticum aestivum*), barley (*Hordeum vulgare*), potato (*Solanum tuberosum*), maize (*Zea mays*), fruits and vegetables, particularly tomato (*Solanum lycopersicum*), complemented with some examples from rice (*Oryza sativa*) and soybean (*Glycine max*).

## 12.7 Molecular Markers in Crop Improvement Program

Agriculture is one of the most important occupations in India with almost 70% of the population being dependent on it. A noteworthy research in conventional breeding for several years has made this country self-sufficient in many respects. However, the ever-increasing population has alarmed food security in India and attempts have been initiated to integrate modern biotechnology tools in conventional breeding to improve the most important crops such as rice, wheat and legumes.

Extensive research using DNA markers is in progress in many institutions all over India. Markers tagged and mapped with specific genes have been identified and some such examples include resistance genes for blast<sup>137</sup> and gall midge<sup>138, 139</sup> using RFLP- and PCR-based approaches in rice. Similarly, in wheat, leaf rust resistance gene LR 28, and pre-harvest sprout tolerance gene<sup>140</sup> have been tagged. QTLs such as protein content in wheat<sup>141</sup> and heterosis in rice<sup>142</sup> have also been identified. While efforts for tagging genes providing resistance to BPH, WBPH, sheath rot and drought are going on, many attempts are also being made towards pyramiding different resistance genes for a specific disease or pest attack like blast, bacterial blight, gall midge, BPH, WBPH, etc. in rice in order to increase the field life of the crop.

Germplasm analysis to study genetic diversity is another important area in which a lot of efforts have been put in. Fingerprinting of crops like rice<sup>124, 125, 129</sup>, wheat<sup>108, 143</sup>, chickpea<sup>130</sup>, pigeonpea<sup>144</sup>, pearl millet<sup>117, 145</sup> etc. is being carried out extensively. This information has potential in strategic planning of future breeding towards crop sustainability in India. Apart from use of molecular markers in crop plants, efforts are also underway in other horticultural plants. Early identification of sex in dioecious papaya using molecular marker is one such example<sup>135</sup>.

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## 12.8 Summary

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With the advent of molecular markers in the late 1970s marker-assisted selection (MAS), a breeding approach based on the genotype of plants rather than assessing the phenotype only, became possible. From the beginning, the expectations about the potentials of MAS have been huge. Nevertheless, there is little systematic knowledge about the degree to which MAS is being used in modern plant breeding programs and for what purposes.

The two major applications of DNA markers in plant science today are detailed chromosome mapping and selection and introgression of both simple and quantitative traits. In addition, DNA markers are also used for germplasm evaluation, genetic diagnostics, phylogenetic analysis, study of genome organization and screening of transformants. In this review chromosome mapping is not covered; the focus is on the use of molecular markers for selection purposes. While marker application has become an important tool in some breeding areas e.g. hybrid breeding, it is much less applied in breeding self-pollinating cereals as e.g. wheat or barley. The reason for this is that the decision for or against the application of molecular markers is solely based on economic reasons.

Besides their usefulness in breeding, markers are also potent tools for the identification and study of biodiversity. Therefore, the questions arise, if and how marker technology is or could be useful in the conservation and use of biodiversity within the current legal framework. These questions are discussed in the last part of this study.

Another aspect of marker application is the valorization of plant genetic resources. The value of genetic resources being stored in gene banks could possibly rise through the application of markers as markers can help to reveal the presence of traits in PGR and may permit for their efficient use in the improvement of crop varieties. However, some respondents pointed out that until now an increase in variability in agricultural crops through the utilization of MAS cannot be observed.

## 12.9 Glossary

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- **Molecular marker :** A molecular marker is a molecule contained within a sample taken from an organism (biological markers) or other matter.
  - **Genetic marker :** Genetic markers are the biological features that are determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another.
  - **RFLP :** Restriction Fragment Length Polymorphism.
  - **RAPD :** Random Amplified Polymorphic DNA.
  - **AFLPs :-**Amplified Fragment Length Polymorphism.
  - **SSRs :** Simple Sequence Repeat.
  - **SNP :** Single Nucleotide Polymorphism.
  - **MAS :** Marker Assisted Selection.
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## 12.9 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Which marker is a powerful tool for comparative mapping?
2. Which marker is PCR based marker?
3. Mention alternative name of AFLP marker?

### Section B : (Short Answer Type Questions)

1. What are Morphological Markers?
2. Write about SSR markers.
3. What is transposable element?
4. Give applications of markers.
5. Describe the role of molecular markers in crop improvement program.

### Section C : (Long Answer Type Questions)

1. Give properties of molecular markers.
2. Describe RFLP markers.

3. Write types of DNA markers and give detail description about RAPD and AFLP markers.

**Answer Key of Section – A**

1. RFLP
  2. RAPD
  3. SRFA
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## Unit - 13

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# Genetic Engineering of Plants

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### Structure of the Unit:

- 13.0 Objectives
- 13.1 Introduction
- 13.2 Aim of Genetic Engineering in Plants
- 13.3 Strategies for Development of Transgenic
  - 13.3.1 Direct Gene transfer Method (Vector less)
    - 13.3.1.1 Chemical methods
    - 13.3.1.2 Physical methods
  - 13.3.2 Indirect Gene Transfer Method (Vector Mediated)
    - 13.3.2.1 Important genes on the Ti plasmid
    - 13.3.2.2 DNA transfer into the plant genome
- 13.4 Transposon Tagging
  - 13.4.1 Obtaining the Sequence of the Transposon "Tag"
  - 13.4.2 Transposon and Transposase Engineering
  - 13.4.3 Transposon Tagging Strategies
    - 13.4.3.1 Random Mutagenesis
    - 13.4.3.2 Directed Mutagenesis
  - 13.4.4 Cloning tagged genes: from insertion to sequence
  - 13.4.5 Confirmation
- 13.5 Chloroplast Transformation
  - 13.5.1 Transformation methods for chloroplast transformation
  - 13.5.2 Vector design for chloroplast transformation
  - 13.5.3 Advantages of chloroplast transformation
  - 13.5.4 Limitations of chloroplast transformation
- 13.6 Risk and Controversies
  - 13.6.1 Concerns about human health
  - 13.6.2 Concerns about damage to the environment

- 13.6.3 Concerns about damage to current farming practices
  - 13.6.4 Controversial studies
  - 13.6.5 Indian controversies
  - 13.7 Summary
  - 13.8 Glossary
  - 13.9 Self-Learning Exercise
  - 13.10 References
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## 13.0 Objectives

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After going through this unit you will be able to understand:

- The various aim of genetic engineering
  - Various methods for direct DNA transfer
  - Biology of *Agrobacterium*; and their use in genetic engineering
  - Chloroplast transformation and their application
  - Risk and controversies on genetic engineering of plant including impact of genetic engineering on health, environment and economy
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### 13.1 Introduction

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Traditional plant breeding has been going on for hundreds of years and is still commonly used today, but it has limitations such as the new traits that can be added to those that already exist in that species or plant must be mate to each other by sexual method. The advanced technology genetic engineering overcomes such type of limitation of plant breeding because it does not require crossing between plants. In this technique target DNA is isolated from particular organism and subsequently transfers to desire organism and produces transgenic organism. These transgenic are produces by various DNA delivery methods such as direct or indirect DNA transfer method. Direct DNA transfer method involve various physical and chemical method in which there is no requirement of any mediator while indirect DNA transfer method require mediator such as *Agro bacterium*: a natural engineer. By using all this technique various transgenic plant and their food product which known as genetically modified food has been produced. Currently a controversies on the use of these genetically modified is going on in all over world

but simultaneously many countries including developed as well as developing are producing various transgenic food.

### **13.2 Aim of Genetic Engineering in Plants**

The improvement of plants production with the use of genetics has been occurring for years. Traditionally, crop improvement was achieved by selecting the best appeared plants/seeds and saving them to plant for the next year's crop. Once the science of genetics became better understood, plant breeders used what they knew about the genes of a plant to select for specific desirable traits. This type of genetic modification, known as traditional plant breeding, modifies the genetic composition of plants by making crosses and selecting new superior genotype combinations. Traditional plant breeding has been going on for hundreds of years and is still commonly used today. Plant breeding is an important tool, but it has limitations such as breeding can only be done between those plants that can sexually mate with each other. This limits the new traits that can be added to those that already exist in that species. Another disadvantage is that when plants are mated, (crossed), many traits are transferred along with the trait of interest including traits with undesirable effects on yield potential.

Genetic engineering is not bound by such type of limitations of traditional plant breeding. Genetic engineering physically removes the DNA from one organism and transfers the target gene(s) for one or a few traits, into another organism. The crossing is not necessary in genetic engineering; therefore the 'sexual' barrier between species is overcome and traits from any living organism can be transferred into a plant. This method is also more specific in that a single trait can be added to a plant.

The main aims of genetic engineering of plants as follow:

1. Genetic engineering can boost the growth and yield of a variety food crops-- pulses and cereals-- and alleviate the problems of food shortage and mitigate the suffering of the starving millions across the world.
2. To increase the growth rates of agricultural plants and their resistance potential to different diseases caused by various pathogens and parasites. This is beneficial for human as it can greatly increase the production of food sources with the usage of fewer resources.

3. To produce the modified crops which are able to grow without usage of chemicals, such as fertilizers and pesticides, and therefore decrease the severity and frequency of the damages produced by these chemical pollution.
4. One of the best-known applications of genetic engineering is the creation and use of genetically modified crops, which are used to produce genetically modified food and materials with diverse uses.
5. One goal, and the first to be realized commercially, is to provide protection from environmental threats, such as cold, or pathogens, such as insects or viruses, and/or resistance to herbicides. There are also fungal and virus resistant crops developed or in development.
6. They have been developed to make the insect and weed management of crops easier and can indirectly increase crop yield.
7. Another goal of genetic engineering is to modify the quality of produce plants, for instance, increasing the nutritional value or providing more industrially useful qualities or quantities. The Amflora potato, for example, produces a more industrially useful blend of starches. Soybeans and canola have been genetically modified to produce more healthy oils.
8. Another goal consists of driving the GMO to produce materials that it does not normally make. One example is "pharming", which uses crops as bioreactors to produce vaccines, drug intermediates, or drug themselves; the useful product is purified from the harvest and then used in the standard pharmaceutical production process.
9. Another goal in generating GMOs, is to directly improve yield by accelerating growth, or making the organism hardier (for plants, by improving salt, cold or drought tolerance).
10. Genetic engineering may help make crops resistant to herbicides used to kill the unwanted plants and weeds which obstruct their full growth. Though some herbicides are selective and kill only the specifically targeted unwanted plants, there are others which are non-selective and besides killing the useless and obstructive weeds, kill any plants they come in contact thus killing the plants which are sought to be protected.
11. In addition genetic engineering also aimed to make crops which are resistance against Fungal and bacterial infections also resistant to viruses.

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### 13.3 Strategies for Development of Transgenic

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During the last decades, a tremendous progress has been made in the development of transgenic plants using the various techniques of genetic engineering. The plants, in which a functional foreign gene has been incorporated by any biotechnological methods that generally are not present in the plant, are called transgenic plants. Transgenic plants have many beneficial traits like insect resistance, herbicide tolerance, delayed fruit ripening, improved oil quality, weed control etc. Some of the commercially grown transgenic plants in developed countries are: "Roundup Ready" soybean, 'Freedom II' squash, 'High-lauric' rapeseed (canola), 'Flavr Savr' and 'Endless Summer' tomatoes.

To achieve genetic transformation in plants, we need the construction of a vector (genetic vehicle) which transports the genes of interest, flanked by the necessary controlling sequences i.e. promoter and terminator, and deliver the genes into the host plant. The two kinds of gene transfer methods in plants are: direct gene transfer method (vector less) and indirect gene transfer method (Vector mediated)

#### 13.3.1 Direct gene transfer method (Vector less)

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The methods used for direct gene transfer in plants are:

##### 13.3.1.1 Chemical methods

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compounds are introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell.

An ideal chemical used for DNA transfer should have the ability to-

1. Protect DNA against nuclease degradation.
2. Transport DNA to the target cells.
3. Facilitate transport of DNA across the plasma membrane.
4. Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

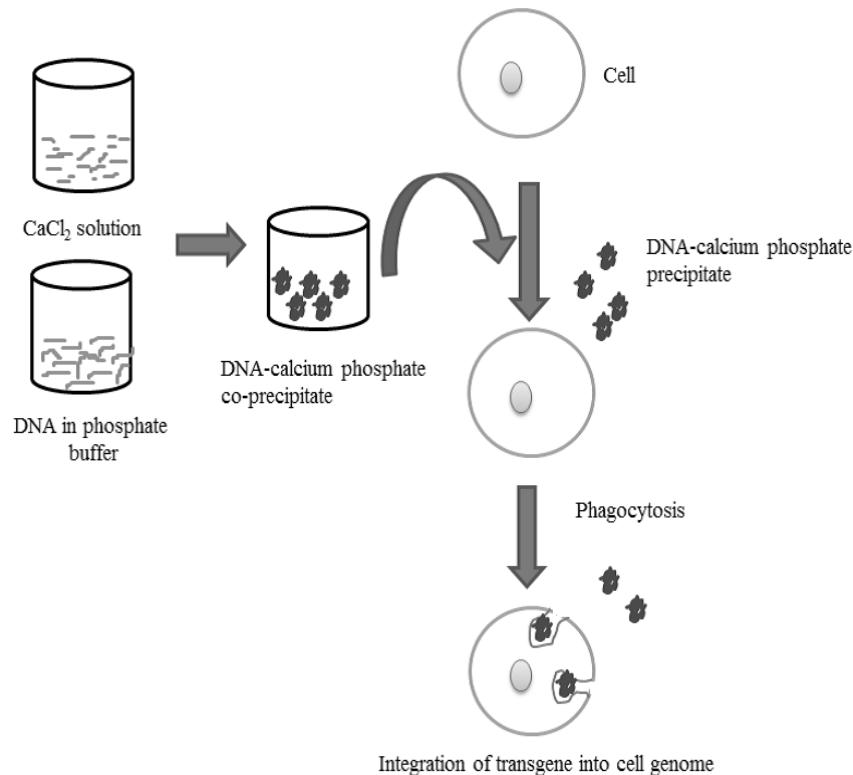
1. Calcium phosphate
2. DEAE dextran
3. Cationic Lipid
4. Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

### **1. Calcium phosphate**

One of the cheapest methods uses calcium phosphate, originally discovered by F. L. Graham and A. J. van der Eb in 1973. A HEPES-buffered saline solution (HeBS) containing phosphate ion is combined with a calcium chloride solution containing the DNA to be transfected. When the two are combined, a fine precipitate of the positively charged calcium and the negatively charged phosphate will form, binding the DNA to be transfected on its surface. The suspension of the precipitate is then added to the cells to be transfected (usually a cell culture grown in a monolayer). By a process not entirely understood, the cells take up some of the precipitate, and with it, the DNA. This process has been a preferred method of identifying many oncogenes.

This method is mainly used in the production of recombinant viral vectors.

It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.



**Fig. 13.1: Transfer of DNA by using Calcium phosphate**

### Advantages

1. Simple and inexpensive
2. Applicability to generate stably transfected cell lines
3. Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
4. Can be used for stable or transient transfection

### Disadvantages

1. Toxic especially to primary cells
2. Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
3. Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
4. Limited by the composition and size of the precipitate

### **2. DEAE-Dextran (Diethylaminoethyl Dextran) mediated DNA transfer**

This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.

Diethylaminoethyl dextran (DEAE-dextran) is a soluble poly-cationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell. In this method, the negatively charged DNA and positively charged DEAE-dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE-dextran in mixture results in net positive charge in the DEAE-dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. The method can be improved by osmotic shock using DMSO or glycerol.

Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

### **Advantages**

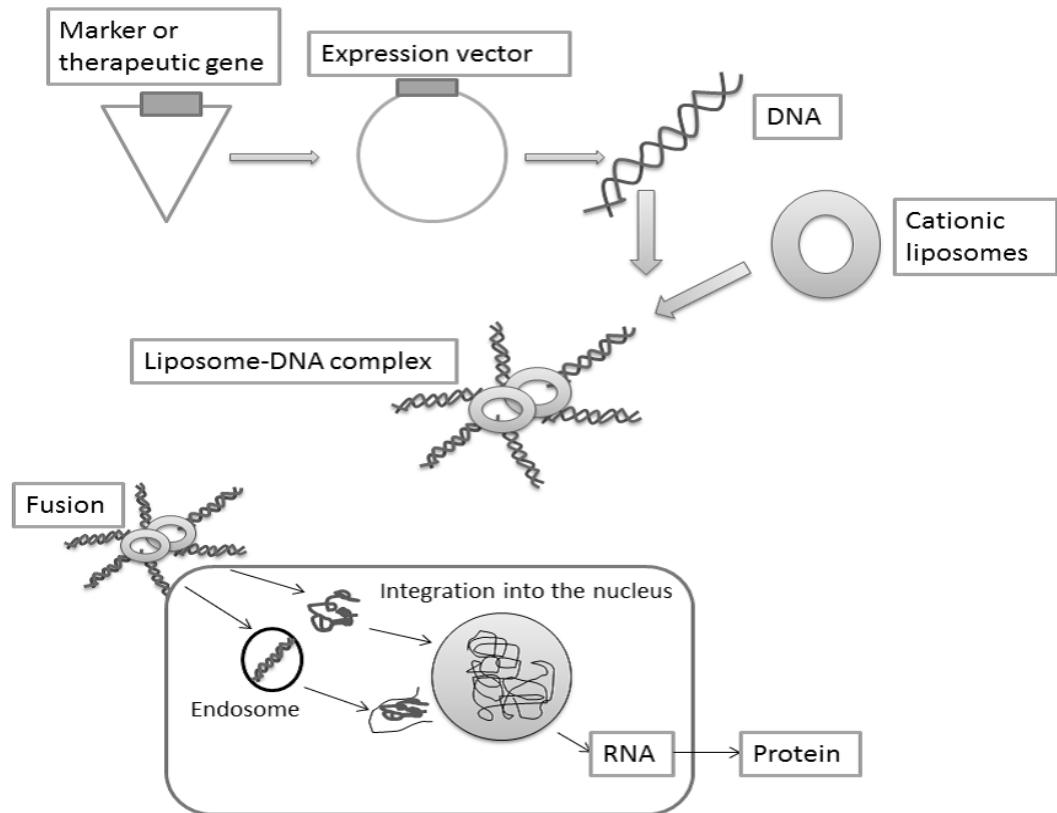
1. Simple and inexpensive
2. More sensitive
3. Can be applied to a wide range of cell types
4. Can be used for transient transfection.

### **Disadvantages**

1. Toxic to cells at high concentrations
2. Transfection efficiency varies with cell type
3. Can only be used for transient transfection but not for stable transfection
4. Typically produces less than 10% delivery in primary cells

### **3. Lipofection**

Lipofection is a method of transformation, first described in 1965 as a model of cellular membranes using liposomes which are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of 0.1 to 10 micrometer or 20-25 nanometers respectively. They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA-liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.



**Fig. 13.2 : Lipofection method for DNA delivery**

### Advantages

1. It is cost effective method
2. Efficient delivery of nucleic acids to cells in a culture dish.
3. Delivery of the nucleic acids with minimal toxicity.
4. Protection of nucleic acids from degradation.
5. Measurable changes due to transfected nucleic acids in sequential processes.
6. Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

### Disadvantages

1. It is not applicable to all cell types.
2. It fails for the transfection of some cell lines with lipids.

#### 13.3.1.2 Physical Method

It has been discussed earlier that due to amphipathic nature of the phospholipid bilayer of the plasma membrane, polar molecules such as DNA and protein are

unable to freely pass through the membrane. Various physical or mechanical methods are employed to overcome this and aid in gene transfer as listed below-

1. Electroporation
2. Microinjection
3. Particle Bombardment
4. Sonoporation
5. Laser induced
6. Bead transfection

### **1. Electroporation**

This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells. It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells. It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus. The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

#### **Advantages**

1. It is highly versatile and effective for nearly all cell types and species.
2. It is highly efficient method as majority of cells take in the target DNA molecule.
3. It can be performed at a small scale and only a small amount of DNA is required as compared to other methods.

#### **Disadvantages**

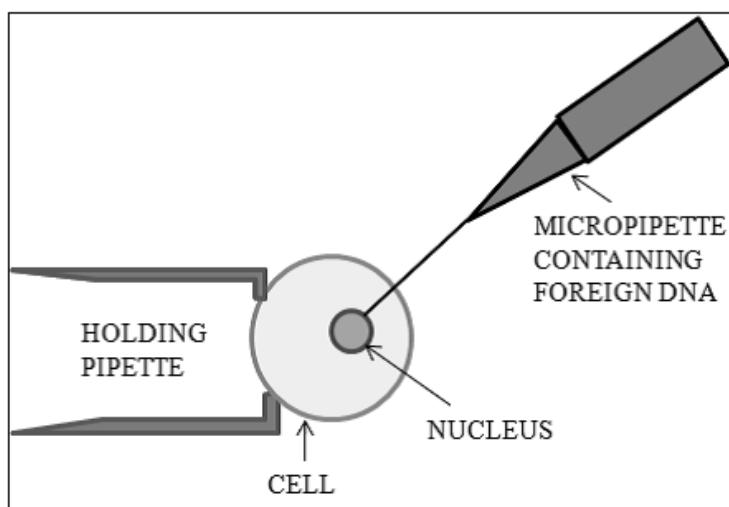
1. Cell damage is one of the limitations of this method caused by irregular intensity pulses resulting in too large pores which fail to close after membrane discharge.
2. Another limitation is the non-specific transport which may result in an ion imbalance causing improper cell function and cell death.

## 2. Microinjection

DNA microinjection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century. It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette (0.5 - 1.0 micrometer diameter). The introduced DNA may lead to the over or under expression of certain genes. It is used to identify the characteristic function of dominant genes. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs, and the cells of early embryo.

### Advantages

1. No requirement of a marker gene.
2. Introduction of the target gene directly into a single cell.
3. Easy identification of transformed cells upon injection of dye along with the DNA.
4. No requirement of selection of the transformed cells using antibiotic resistance or herbicide resistance markers.
5. It can be used for creating transgenic organisms, particularly mammals.



**Fig. 13.3: Microinjection method for DNA transfer**

## 3. Particle bombardment

Prof Sanford and colleagues at Cornell University (USA) developed the original bombardment concept in 1987 and coined the term “biolistic” (short for “biological

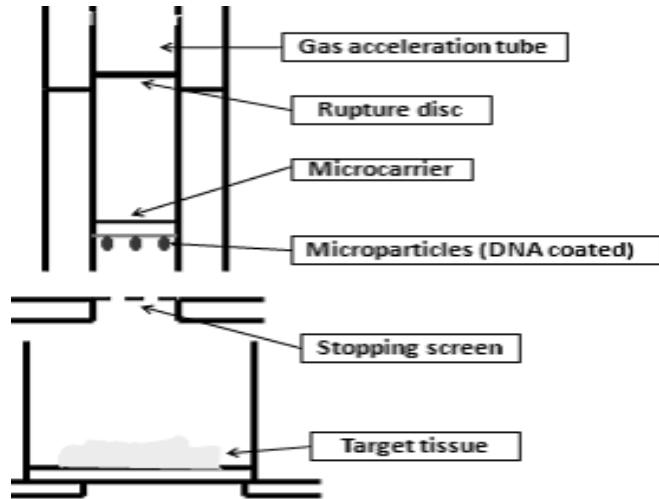
ballistics") for both the process and the device. It also termed as particle bombardment, particle gun, micro projectile bombardment and particle acceleration. It employs high-velocity micro projectiles to deliver substances into cells and tissues. In this method, the foreign DNA containing the genes to be transferred is coated onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded onto the target tissue or cells using a particle gun (also called as gene gun/shot gun/microprojectile gun).

### **Apparatus**

The biolistic gun employs the principle of conservation of momentum and uses the passage of helium gas through the cylinder with arrange of velocities required for optimal transformation of various cell types. It consists of a bombardment chamber which is connected to an outlet for vacuum creation. The bombardment chamber consists of a plastic rupture disk below which macro carrier is loaded with micro carriers. These micro carriers consist of gold or tungsten micro pellets coated with DNA for transformation.

### **Working system of particle bombardment gun**

The apparatus is placed in Laminar flow while working to maintain sterile conditions. The target cells/tissue is placed in the apparatus and a stopping screen is placed between the target cells and micro carrier assembly. The passage of high pressure helium ruptures the plastic rupture disk propelling the macro carrier and micro carriers. The stopping screen prevents the passage of macro projectiles but allows the DNA coated micro pellets to pass through it thereby, delivering DNA into the target cells.



**Fig. 13.4: Biostatic gun method**

### Advantages

1. Simple and convenient method involving coating DNA or RNA on to gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.
2. No need to obtain protoplast as the intact cell wall can be penetrated.
3. Manipulation of genome of sub-cellular organelles can be done.
4. Eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.
5. This device offers to place DNA or RNA exactly where it is needed into any organism.

### Disadvantages

1. The transformation efficiency may be lower than *Agrobacterium*- mediated transformation.
2. Specialized equipment is needed. Moreover the device and consumables are costly.
3. Associated cell damage can occur.
4. The target tissue should have regeneration capacity.
5. Random integration is also a concern.
6. Chances of multiple copy insertions could cause gene silencing.

#### **4. Sonoporation**

Sonoporation involves the use of ultrasound for temporary permeabilization of the cell membrane allowing the uptake of DNA, drugs or other therapeutic compounds from the extracellular environment. This method leaves the compound trapped inside the cell after ultrasound exposure. It employs the acoustic cavitation of micro bubbles for enhancing the delivery of large molecules like DNA. The micro bubbles form complex with DNA followed by injection and ultrasound treatment to deliver DNA into the target cells. Unlike other methods of transfection, sonoporation combines the capability to enhance gene and drug transfer.

#### **Advantages**

1. Simple and highly efficient gene transfer method.
2. No significant damage is cause to the target tissue.

#### **Disadvantages**

1. Not suitable for tissues with open or cavitated structures.
2. High exposure to low-frequency (<MHz) ultrasounds result in complete cellular death (rupture of the cell). Thus cellular viability must be taken into consideration while employing this technique.

#### **5. Laser induced transfection**

It involves the use of a brief pulse of focused laser beam. In this method, DNA is mixed with the cells present in the culture and then a fine focus of laser beam is passed on the cell surface that forms a small pore sufficient for DNA uptake into the cells. The pore thus formed is transitory and repairs soon.

#### **6. Bead transfection**

Bead transfection combines the principle of physically producing breaks in the cellular membrane using beads. In this method, the adherent cells are incubated for a brief period with glass beads in a solution containing the DNA. The efficiency of this rapid technique depends on Concentration of DNA in a solution, Timing of the addition of DNA and Size and condition of the beads and the buffers utilized.

### 13.3.2 Indirect Gene Transfer Method (Vector mediated)

*Agrobacterium* is considered as the natural genetic engineer. The genus *Agrobacterium* has been divided in many species on the basis of symptoms of disease and range of host plant such as *A. radiobacter* is a “non-virulent” species, *A. tumefaciens* causes crown gall disease, *A. rubi* causes cane gall disease, *A. rhizogenes* causes hairy root disease and *A. Vitis* causes galls on grape and a few other plant species. The *Agrobacterium* can transform a remarkably broad group of organisms including dicots, monocots, gymnosperms as well as fungi, such as member of ascomycetes, basidiomycetes and yeasts.

The soil bacterium *Agrobacterium tumefaciens* is a rod shaped gram negative bacteria that causes tumorous growth termed as crown gall disease in dicot plants. The involvement of bacteria in this disease was established by Smith and Townsend in 1907. It possesses the unique ability to transfer some of its genetic material to the chromosome of a dicotyledonous plant cell. Bacterial DNA which is transferred to the plant induces the formation of a tumor, and also contains information that directs the plant cell to produce and secrete compounds that *Agrobacterium* can utilize as a source of carbon and nitrogen. In nature, the tumor often appears at the soil/air junction, also termed ‘crown’. It is thought that *Agrobacterium* gain access to susceptible plant cells by wounding.

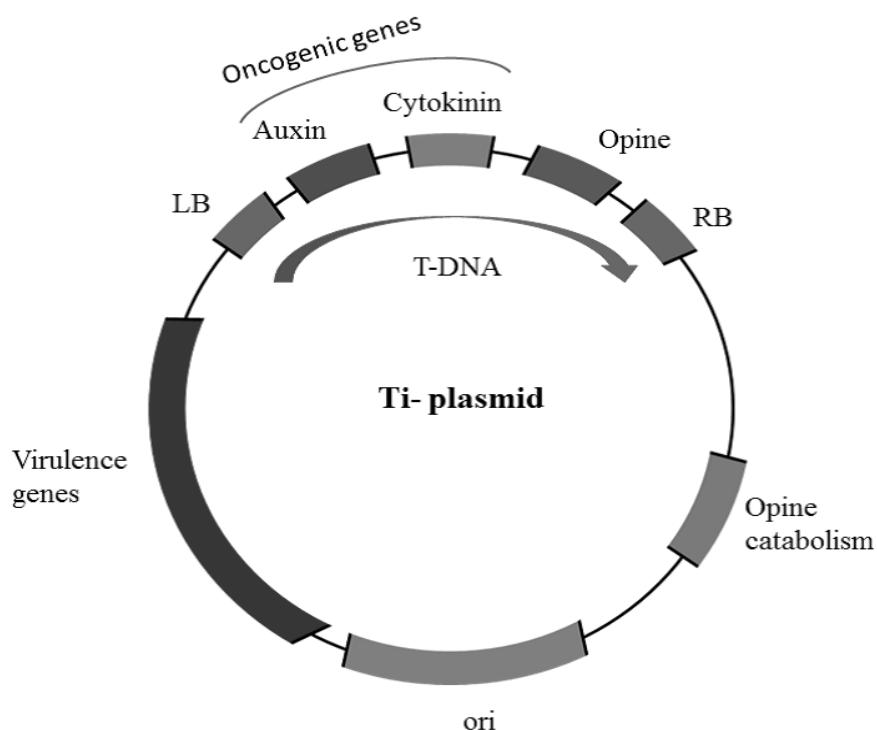
*Agrobacterium* contains a transfer DNA (T-DNA) located in its tumor-inducing (Ti) plasmid that is transferred into the nucleus of an infected plant cell. The T-DNA gets incorporated into the plant genome and is subsequently transcribed. The T-DNA integrated into the plant genome carries not only oncogenic genes but also opine synthesizing genes.

#### 13.2.2.1 Important genes on the Ti (Tumor-inducing) plasmid

The virulent strains of *A. tumefaciens* possess large plasmids (140–235 kbp) known as tumor-inducing (Ti) plasmid. The Ti plasmid encodes for proteins that function in the DNA transfer process in addition to containing the actual DNA that is transferred to the plant. The Ti plasmid contain following elements like T-DNA, vir region, origin of replication, region enabling conjugative transfer and o-cat region (required for catabolism of opines).

**T-DNA:** It is a small, specific segment of the plasmid, about 24kb in size and it is flanked by two imperfect 25bp repeat motifs at either end, called the *left border*

(LB) and *right border* (RB). Importantly, the LB and RB, together with small surrounding elements, are the only two DNA sequence elements that define which piece of DNA is transferable and which is not! Therefore, any piece of DNA inserted between the two borders can be transferred from *Agrobacterium* to the plant.



**Fig. 13.5: Ti Plasmid and their gene**

#### Genes on T-DNA

The T-DNA contains two groups of genes, as follows:

- **Oncogenes** for synthesis of auxins and cytokinins (phytohormones) and the over-production of phytohormones lead to proliferation of callus or tumor formation.
- **Opine synthesizing genes** for the synthesis of opines (a product from amino acids and sugars secreted by the crown gall infected cells and utilized by *A. tumefaciens* as carbon and nitrogen sources). Thus opines act as source of nutrient for bacterial growth, e.g. Octopine, Nopaline.

**Table 13.1: Various gene on T-DNA**

<b>Gene</b>	<b>Product</b>	<b>Function</b>
<b><i>ocs</i></b>	Octopine synthase	Opine synthesis
<b><i>nos</i></b>	Nopaline synthase	Opine synthesis
<b><i>frs</i></b>	Fructopine synthase	Opine synthesis
<b><i>mas</i></b>	Mannopine synthase	Opine synthesis
<b><i>ags</i></b>	Agropine synthase	Opine synthesis
<b><i>trns1 (iaaH, auxA)</i></b>	Tryptophan-2-mono-oxygenase	Auxin synthesis
<b><i>trns2 (iaaM, auxB)</i></b>	Indoleacetamide hydrolase	Auxin synthesis
<b><i>trnr (ipt, cyt)</i></b>	Isopentyltransferase	Cytokinin synthesis

### The ***vir***-genes and their function

The physical transfer of the T-DNA from *Agrobacterium* into the plant cell and its subsequent integration into the host plant nuclear genome are complex processes that are still not entirely understood at the molecular and cellular level. Virulence genes aid in the transfer of T-DNA into the host plant cell. Ti plasmid contains 35 ***vir*** genes arranged in 8 operons (***vir A, B, C, D, E, F, G and H***). The function of various product of ***vir*** gene as follow:

***vir A***: Signaling molecules (phenolic compounds, e.g. acetosyringone) produced by the plant are perceived by *Agrobacterium* through ***vir A***

- Also induces the expression of other ***vir*** genes (via ***vir G***).

***vir C, D***: Involved in generating and processing of the single stranded T-DNA copy

***vir D1, D2***- T strand synthesis

- ***D1***- cause relax in the DNA (topoisomerase)
- ***D2*** - multiple functions, such as:
  1. Cut the T DNA
  2. Act as Pilot at the 5' end of T DNA.
  3. Covalently bound to the 5' end of T-strand.

4. Assist T-strand transfer out of the bacteria and into the nucleus (D2-gus-localizes to the nucleus).

***vir C1, C2:*** Enhance T-DNA strand production when D1, D2 are limiting.

- Recognition of the right border sequence.

***vir D2 and E2:*** Assist in the nuclear uptake

- ***virE2*** is a single stranded binding protein
- T-strand is fully coated with E2
- ***virE2***- transgenic plants that produce E2 complement ***virE2*** mutants
- Transportation is polar, the 5' end enter first

***virB:*** Allows passage through the bacterial membrane

- VirB forms a channel or pore.
- Contains 11 open reading frames, many secreted or membrane proteins.

### 13.3.2.2 DNA transfer into the plant genome

The schematic representation of *Agrobacterium*-mediated transformation method is shown in Fig.. T-DNA transfer and integration into the plant genome involves following steps:

#### Recognition and *vir* gene induction

*Agrobacterium* perceives signaling molecules (phenolic compounds and sugars) released by the wounded plant cells. These signaling molecules recognised by the bacterial VirA/VirG2-component signal transduction system.

The process begins when a plant is wounded. A wounding event, which often occurs at the base of the stem of the plant, causes the plant to release compounds that act as chemotactic attractants for *Agrobacterium*. When *Agrobacterium* reaches the plant, it attaches to a receptor on the surface of the plant cell and begins to synthesize cellulose, resulting in a strong connection between the two cells. Subsequently, the phenolic compounds (acetosyringone, Catechol, Pyrogallic acid, Syringyl alcohol, Chalcone derivatives etc.) secreted by the plant are recognized by a protein kinase on the surface of the bacteria. These compounds also serve as inducers of the *vir* genes. In this case, the kinase (VirA) auto-phosphorylates in response to the phenolics and then transfers the phosphate to another protein

(VirG) within the bacterium. In its phosphorylated state, VirG is able to induce transcription of genes on the Ti (tumor inducing) plasmid.

### **Formation of T-DNA complex**

The Ti plasmid encodes for proteins that function in the DNA transfer process in addition to containing the actual DNA that is transferred to the plant. Vir D1/D2 border-specific endonucleases recognize the left and right borders of T-DNA. Vir D2 induces single stranded nicks in Ti plasmid causing the release of an approximately 20 kb piece of single-stranded T-DNA. Vir D2 then attaches to the 5'-end of the displaced ss-T DNA forming an immature T-complex.

The T-DNA remains single-stranded and is coated with VirE2 proteins that serve to both protect the DNA and target it to the plant cell nucleus by virtue of nuclear localization signals (NLS) found on it.

### **Transfer of T-DNA and integration into the plant cell**

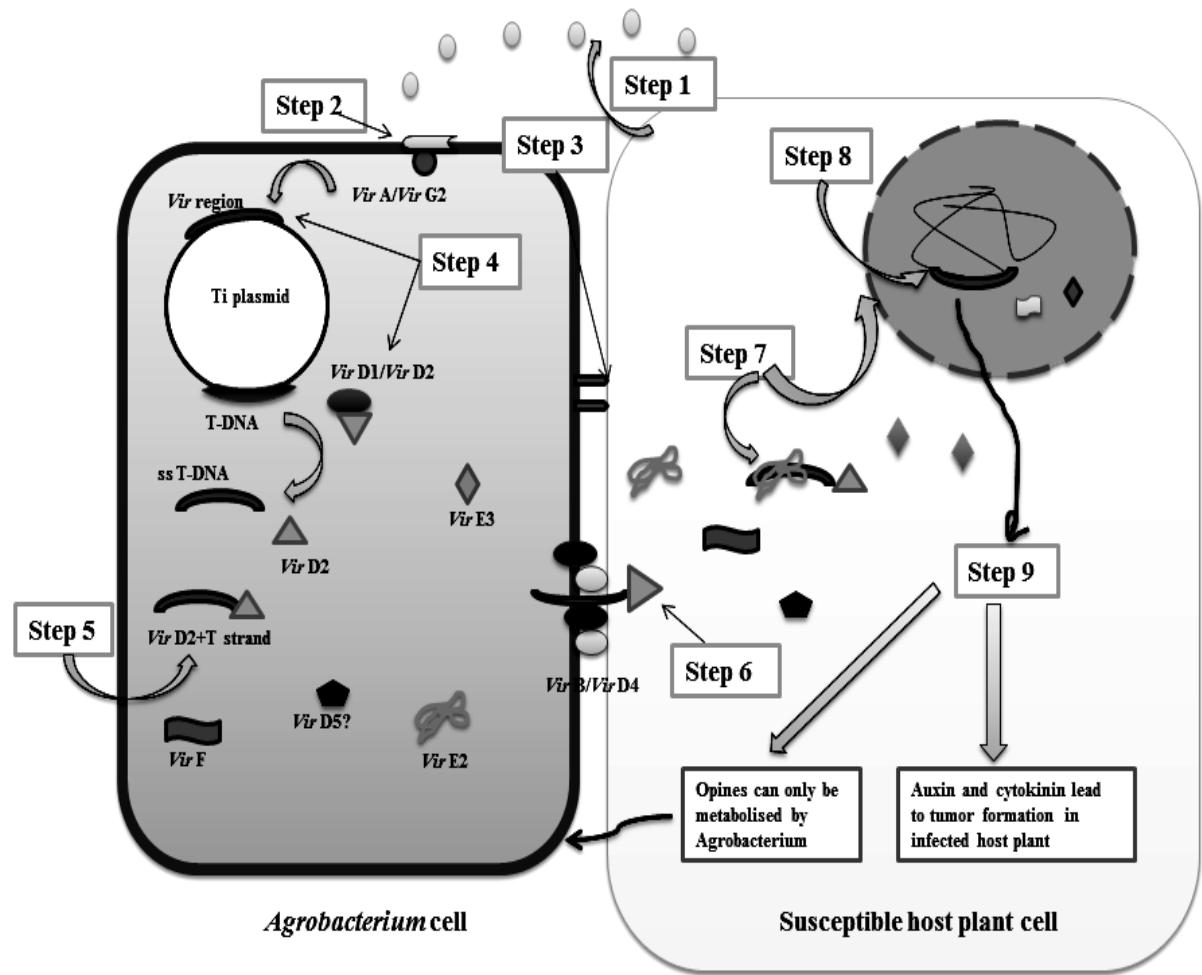
The transfer of T-DNA to the plant cell is mediated by *Vir B* and *Vir D4* that form a conjugative pilus (T-pilus). VirD4 serves as a “linker” that helps in the interaction of the processed T-DNA/VirD2 complex with the VirB- encoded pilus. Other vir genes products (*Vir E2*, *Vir E3*, *VirF*, *Vir D5*) also pass through this T-pilus to aid in the assembly of T-DNA/vir protein complex in the plant cytoplasm forming a mature T-complex.

Most VirB proteins help in the formation of the membrane channel or act as ATPases to provide energy for assembly and export processes of channel. VirB proteins (including VirB2, VirB5 and VirB7) help in the formation of the T-pilus. VirB2 is the major pilin protein that undergoes processing and cyclization.

Vir D2 and Vir E2 protect the ss-T strand from nucleases inside the plant cytoplasm by attaching to the 5'end. Both VirD2 and VirE2 proteins have nuclear localization signals (NLS) which serves as pilot proteins to guide the mature T-complex to the plant nucleus.

The efficiency of transfer is enhanced by VirC2 proteins, which recognize and bind to the overdrive enhancer element.

Some additional proteins like importins, VIP1 and VirF may interact with the T-strand, either directly or indirectly, to form larger T-complexes in the plant cell. Vir F directs the proteins coating T-complex (VIP1 and Vir E2) for destruction in proteasome.

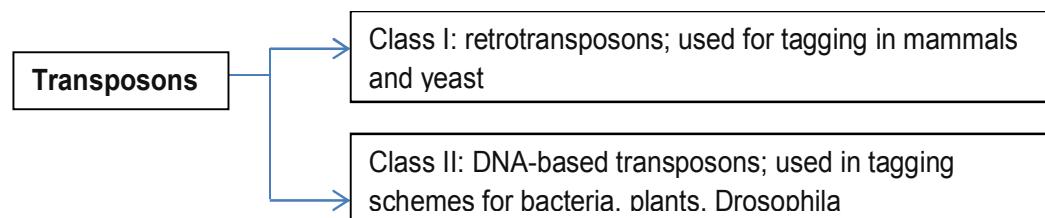


**Fig. 13.6: *Agrobacterium* mediated transformation: From crown gall tumors to genetic transformation** (Adapted from Pacurar DI, Christensen HT, Pacurar ML, Pamfil D, Botez C, Bellini C. 2011, Physiological and Molecular Plant Pathology, 76: 76-81)

### 13.4 Transposon Tagging

Transposons are mobile genetic elements that move (transpose) from one region of the genome to another. Transposons are used as tools for gene cloning because insertion of a transposon into a gene disrupts its function and produce a visible mutant phenotype. When the DNA sequence of the transposon is known, it is possible to clone the disrupted gene by using the transposable element as a "tag" to identify the segment of DNA harboring the element. Transposon tagging involves

inducing transposition, screening for mutations caused by transposon insertion, identifying the element causing the mutation, and cloning the tagged gene.



Class II elements have inverted repeats at their ends and produce the products needed for their own excision and insertion, termed transposase.

### 13.4.1 Obtaining the Sequence of the Transposon "Tag"

To use a transposon as a molecular "tag" for gene cloning, it is necessary to use a transposon with a known DNA sequence. Many transposons have already been well characterized. New transposons can be "trapped" by first mobilizing transposition and then identifying transposon insertions into known genes (generally by looking for unstable alleles of the gene). Then the mutant allele is sequenced, thus determining the sequence of the inserted transposon.

### 13.4.2 Transposon and Transposase Engineering

It is useful to have transposons designed so that they carry selectable markers and they carry part of a plasmid that can be selected for in *E. coli* to facilitate cloning of flanking DNA by plasmid rescue. A marker linked to the transposase source facilitates removal of the transposase later by segregation from the tagged gene. It is also sometimes possible to increase transposition frequencies by deleting or altering part of the transposase gene, by using a powerful constitutive promoter to drive transposase, or by altering transposon size.

### 13.4.3 Transposon Tagging Strategies

#### 13.4.3.1 Random Mutagenesis

In random mutagenesis, transposons are mobilized to create a library of individuals with different transposon insertions. Then the library of insertions is screened for mutant phenotypes of interest. In a two-element system, transposons are mobilized by putting stable transposase into the background of the nonautonomous transposons, either by genetic crosses or, in the case of the *Drosophila P* element, by microinjecting the transposase into an embryo containing nonautonomous

elements (9). In other organisms, growth at low temperature mobilizes transposition. Dominant visible mutations are seen in the M1 generation, and recessive visible mutations are seen segregating in the M2 progeny of M1 individuals. Individuals containing a transposed element are often termed "transposants".

#### **13.4.3.2 Directed Mutagenesis**

There are two types of directed tagging schemes. In both cases, the target gene has been identified previously by its mutant phenotype. In the first type of directed mutagenetic scheme, insertion into a specific gene is selected for by crossing an individual homozygous for the previously identified recessive mutation with a wild-type individual carrying an autonomous transposon (alternatively, an individual carrying both a stable transposase source and the mutation of interest is crossed with a wild-type individual carrying a nonautonomous transposon). The next generation, the M1, is screened for individuals exhibiting the mutant phenotype. These individuals carry both the original mutation and a new transposon-induced allele of the same gene.

The second type of directed mutagenetic experiment is useful in systems where the transposon is known to move preferentially to linked sites on the chromosome. A mapped transposable element that is known to be linked to the gene of interest is mobilized. Insertions into the gene of interest are identified in the M1 by the strategy outlined previously, or the linked transposon is mobilized in a wild-type background, and the M2 is screened for mutations. In either case, if there is a strong preference for transposition to linked sites, the frequency of mutations induced in the specific gene should increase relative to a random tagging procedure.

#### **13.4.4 Cloning tagged genes: from insertion to sequence**

The first step in cloning a tagged gene is to identify a transposon linked to the mutation. This is done by Southern blotting DNA from progeny obtained from the putatively tagged mutant. A transposon-specific probe probes the blot to identify a band present in the homozygous mutant progeny and absent in wild-type progeny that do not segregate for the mutant phenotype. If many transposons segregate in the background, finding a transposon linked to the mutant phenotype is difficult. A large number of progeny need to be examined, or the mutant must back-crossed to

a strain lacking transposons to segregate background transposons. Once a linked transposon is identified, there are a number of different ways to clone the gene in which a transposon has inserted. Three commonly used methods are described here.

1. **Plasmid rescue.** If the transposon used for tagging contains part of a selectable plasmid (eg, pBR322) at one end of the transposon, plasmid rescue is used to isolate a fragment of flanking host DNA. Genomic DNA from the tagged individual is digested with a restriction enzyme that releases the selectable plasmid from the transposon but does not cut within the plasmid itself. This creates a linear piece of DNA containing the plasmid sequence and a small fragment of flanking host DNA. The products of the digestion are religated at a high dilution to ensure intramolecular ligation products. The ligation products are transformed into *E. coli*, and then the cloned flanking DNA is isolated.
2. **Inverse polymerase chain reaction (IPCR).** IPCR is another method for isolating host sequences flanking a transposon insertion. As for plasmid rescue, genomic DNA from a tagged individual is isolated and digested with a restriction enzyme that releases the end of the transposon and a piece of adjoining host DNA. Ligation is used to circularize the linear host DNA-transposon fragment. PCR using two transposon-specific oligonucleotide primers, each reading outward from the ends of the transposon sequence into the flanking DNA, are used to amplify the flanking host DNA. Then the amplified product is cloned.
3. **Library screening.** A third option is to make a library using genomic DNA from the tagged mutant that has been digested with an enzyme that does not cut within the inserted transposon. The recombinant vector containing the transposon also contains some flanking host DNA. A transposon-specific probe is used to screen the library and identify clones containing the transposon and the flanking DNA.

#### 13.4.5 Confirmation

Once the flanking host DNA is cloned, it should be used to probe Southern blots of DNA extracted from homozygous mutant and wild-type plants to look for a band difference, indicating transposon insertion into the complementary sequences in

the mutant. This is done to prove that the cloned DNA actually represents the host DNA flanking the transposon and is not an artifact of cloning. Then the cloned flanking sequences are sequenced directly or used as probes to obtain full-length sequences for further analysis. If the transposon has not inserted into the coding sequence of the disrupted gene itself (or in the case of an enhancer trap, if the transposon is not in a gene), it is necessary to use the flanking sequences for initiating chromosome walk to identify the gene of interest.

The most convincing way to prove that a mutant phenotype results from transposon disruption of the cloned gene is to rescue the mutant phenotype by transforming the cloned gene back into the mutant. It is also possible to isolate and analyze different alleles of the gene from independently derived mutants. If all of the mutant alleles harbor mutations in the cloned gene, it is good evidence that the correct gene has been identified. Phenotypic revertants that show a loss of the transposon from the cloned sequence, when they are examined by sequencing or Southern blotting techniques, also confirm the identity of the tagged gene. In the case of enhancer-trapped genes, where there is no mutant phenotype associated with the insertion, expression analysis of the cloned gene should closely mimic that of the enhancer-trap reporter gene.

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### 13.5 Chloroplast Transformation

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Chloroplast is a specialized organelles present in photosynthetic plant cells (Algae to higher plants). Their principal function is to capture energy from light for the fixation of atmospheric CO<sub>2</sub> and convert it into sugars. In addition it also harbour many other important biosynthetic pathways. They possess their own genome with a variable size up to several hundred kilobases; each chloroplast can contain up to 100 copies of its own genome. The number of chloroplast varies between 1 and more than 100 in higher plants. Chloroplast transformation offers an important tool to investigate in term of many aspects of plant physiology and the regulation of gene expression. It has also got much attention for applications in biotechnology due to several advantages as compared with transformation of the nuclear genome. It is also important that in the most of flowering plants including crops, the chloroplast genome is inherited to progeny through the maternal parent, while transmission of chloroplast through pollen is very rare. Thus, plastid transformation provides a strong level of biological containment. Another

advantage is that the integration of a transgene in the plastid genome proceeds by homologous recombination and is therefore precise and predictable. Hence, variable position effects on gene expression or the inadvertent inactivation of a host gene by integration of the transgene are avoided. Furthermore, plastid genes are not subject to gene silencing or RNA interference. Recent reviews have focused on the numerous applications of plastid transformation for the production of pharmaceuticals or biofuels, and on the development of transformation protocols in a rapidly increasing number of plant and algal species. Plastid transformation can involve delivery of DNA into chloroplasts or non-green plastids. Once stable transformation has been achieved, all plastid types within the plant will contain the same transgenic plastome. Thus, in flowering plants containing a variety of plastid developmental forms, the term plastid transformation is more accurate than chloroplast transformation.

First time chloroplast transformation was achieved in the alga *Chlamydomonas reinhardtii*. In addition, the *aadA* marker and methods for removal of marker were first demonstrated. In higher plants, Tobacco gained significant attention for chloroplast transformation due to its easy to culture and regeneration. Tobacco protoplasts were co-cultivated with *Agrobacterium* but the resulted transgenic lines showed the unstable integration of foreign DNA into the chloroplast genome. The interested genes were introduced in isolated intact chloroplasts and then into protoplasts resulting in transgenic plants. Gene gun, a transformation device, was developed by John Sanford to enable the transformation of plant chloroplasts without using isolated plastids.

### **13.5.1 Transformation methods for chloroplast transformation**

First successful chloroplast transformation was performed in *Chlamydomonas reinhardtii* by particle Biolistic/Particle bombardment method. Subsequently the stable plastid transformation has also been established in higher plants, *Nicotiana tabacum*, *Arabidopsis*, rape, *Lesquerella*, rice, potato, lettuce, soybean, cotton, carrot and tomato. However, plastid transformation is routinely performed only in tobacco because of higher efficiency of transformation in tobacco than in other plants. Simple operation and high transformation efficiency makes it a favorable way for plastid or chloroplast transformation.

PEG-mediated and *Agrobacterium*-mediated transformation method was also employed in the early days. In this method a protoplasts (plant cell without cell wall) treated with PEG which allows permeabilization of the plasma membrane and facilitates uptake of DNA. Subsequently, with a mechanism largely uncharacterized, the plasmid DNA passes the plastid membranes and reaches the stroma where it integrates into the plastome as during biotic transformation.

The plastid transformation can also be done by using vector. Initial transformation vectors carried a plastid 16S rRNA (*rrn16*) gene with point mutations that confer spectinomycin and streptomycin resistance. The recessive *rrn16* marker genes were, 100-fold less efficient than the currently used *aadA* gene. The *aadA* gene encodes aminoglycoside 3'-adenylyltransferase, an enzyme that inactivates spectinomycin and streptomycin by adenylation.

### **13.5.2 Vector design for chloroplast transformation**

#### **Selectable marker genes**

Due to the presence of multi-copy plastid DNA, selectable marker genes are very important to achieve uniform transformation of all genome copies. The non-transformed plastids are gradually left out during an enrichment process using a selective medium. Initially plastid 16S rRNA gene (*rrn16*) was used as a selection marker in process of chloroplast transformation. The transgenic lines were selected by spectinomycin resistance but efficiency was very low. A range of selectable markers for chloroplast transformation have been developed based on various features like dominance, cell-autonomy or portability.

- Dominant markers confer high transformation efficiency due to the expression at early stages although present in a minority of the plastomes. e. g. *aadA* (aminoglycoside 3'-adenylyltransferase) gene confers resistance to streptomycin and spectinomycin by inactivation of antibiotics.
- Recessive markers confer lower transformation efficiency. They confer resistance only when enough transformed plastome copies are produced by random segregation resulting in a selectable phenotype. e. g. point mutation in the ribosomal RNA, *rrnS* and *rrnL* genes, confer antibiotic resistance by relieving the sensitivity of individual ribosome.

- Plastid- or cell-autonomous markers confer their phenotype only to the organelle or the cell in which they reside. Some markers may integrate at a specific locus of the plastid genome e.g. *rrnS* or *rrnL* genes.
- Autonomous and portable markers can be inserted virtually in any locus of the plastome e.g. *aadA* gene.

Some markers have a property to confer a phenotype strong enough for direct selection of transformants.



**Fig. 13.7: Selectable marker is under the control of expression signals such as promoter, a 3' UTR and a 5' UTR.**

### Insertion sites

Plastid expression vectors possess left and right flanking sequences each with 1–2 kb in size from the host plastid genome, which facilitates foreign gene insertion into plastid DNA via homologous recombination. The insertion site in the plastid genome is determined by the choice of plastid DNA segment flanking the marker gene and the gene of interest. The foreign DNA is inserted in intergenic regions of the plastid genome.

Various insertion sites such as *trnV-3'rps12*, *trnI-trnA* and *trnfM-trnG* are frequently used in this technique. The *trnV-3'rps12* and *trnI-trnA* sites are located in the 25 kb inverted repeat region of plastid DNA and insertion of gene into these sites results in the rapid copying into two copies in the inverted repeat region. The insertion site *trnfM-trnG* is located in the large single copy region of the plastid DNA. The insertion of gene between *trnfM* and *trnG* should contain only one copy per plastid DNA. *pSBL- CTV2* was the first vector developed in the

Daniell laboratory for expression of several proteins. This vector inserts the foreign gene in *trnI-trnA* intergenic region.

### **Regulatory sequences**

The level of gene expression in plastids is predominately regulated by regulatory sequences such as promoter as well as 5' UTR elements. Strong promoter is required for high mRNA level, for high-level of protein accumulation e.g. rRNA operon (*rrn*) promoter (*Prrn*). Most commonly used promoter is *CaMV* 35S from cauliflower mosaic virus which drives high level of transgene expression in dicots.

In plastid expression vectors, a suitable 5' untranslated region (5'-UTRs) containing a ribosomal binding site (RBS) is an important element.

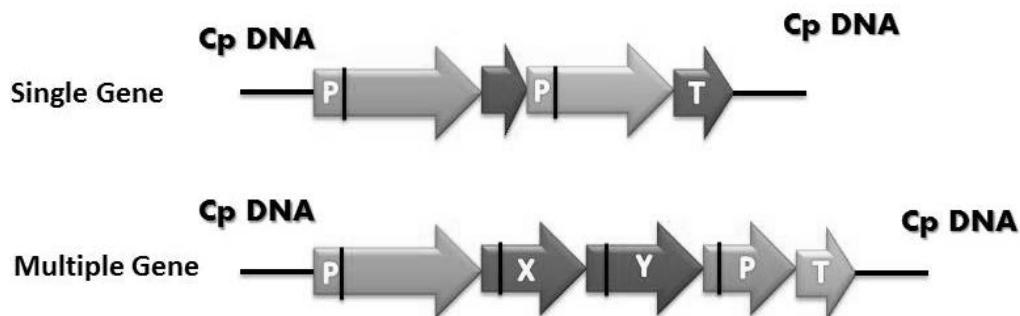
Stability of the transgenic mRNA is ensured by the 5' UTR and 3' UTR sequences flanking the transgene. Protein accumulation from the transgene depends on the 5'-UTR inserted upstream of the open reading frame encoding the genes of interest.

#### **13.5.3 Advantages of chloroplast transformation**

Chloroplast transformation offer several advantages in compare to nuclear transformation which are as follows-

- **Risk of transgene escape:** Chloroplast genome is maternally inherited and there is rare occurrence of pollen transmission. It provides a strong level of biological containment and thus reduces the escape of transgene from one cell to other.
- **Expression level:** It exhibits higher level expression of transgene and thus higher level of protein production due to the presence of multiple copies of chloroplast transgenes per cell and in addition it remains unaffected by phenomenon such as pre or post-transcriptional silencing.
- **Homologous recombination:** Chloroplast transformation involves homologous recombination and is therefore precise and predictable. It minimizes the insertion of unwanted DNA that accompanies in nuclear genome transformation. This also avoids the deletions and rearrangements of transgene DNA, and host genome DNA at the site of insertion.
- **Gene silencing/ RNA interference:** Gene silencing or RNA interference does not occur in genetically engineered chloroplasts.

- **Position effect:** Absence of position effect due to lack of a compact chromatin structure and efficient transgene integration by homologous recombination. Avoids inadvertent inactivation of host gene by transgene integration
- **Disulphide bond formation:** Ability to form disulfide bonds and folding human proteins results in high-level production of biopharmaceuticals in plants.
- **Multiple gene expression:** Multiple transgene expression is possible due to polycistronic mRNA transcription.



**Fig. 13.8: Multigene expression**

(Adapted from Daniell H, Khan MS, Allison L. 2002. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends in Plant Science 7 (2): 84-91.)

- **Expression of edible vaccine:** High level of expression and engineering foreign genes without the use of antibiotic resistant genes makes this compartment ideal for the development of edible vaccines.
- **Codon usage:** Chloroplast is originated from cyanobacteria through endosymbiosis. It shows significant similarities with the bacterial genome. Thus, any bacterial genome can be inserted in chloroplast genome.
- **Expression of toxic proteins:** Foreign proteins observed to be toxic in the cytosol are non-toxic when accumulated within transgenic chloroplasts as they are compartmentalized inside chloroplast.

#### 13.5.4 Limitations of Chloroplast Transformation

1. Transformation frequencies are much lower than those for nuclear genes.
2. Prolonged selection procedures under high selection pressure are required for the recovery of transformants.

3. The methods of transgene transfer into chloroplasts are limited, and they are either expensive or require regeneration from protoplasts.
  4. These transformation systems are far more successful with tobacco than with other plant species.
  5. Products of transgenes ordinarily accumulate in green parts only.
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### **13.6 Risk and Controversies**

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The introduction of transgenic crops and foods into the existing food production system has generated a number of questions about possible negative consequences. The dispute involves consumers, farmers, biotechnology companies, governmental regulators, non-governmental organizations, and scientists. The key areas of controversy related to genetically modified food (GMO food), whether such food should be labeled, the role of government regulators, the objectivity of scientific research and publication, the effect of genetically modified crops on health and the environment, the effect on pesticide resistance, the impact of such crops for farmers, and the role of the crops in feeding the world population. People with concerns about this technology have reacted in many ways, from participating in letter-writing campaigns to demonstrating in the streets to vandalizing institutions where transgenic research is being conducted. What are the main concerns? What scientific support is there for these concerns?

The issues surrounding objections to transgenic crops can be broadly grouped into concerns about

- **Damage to human health**  
Allergenicity, horizontal transfer and antibiotic resistance, eating foreign DNA, cauliflower mosaic virus promoter, changed nutrient levels
- **Damage to the natural environment**  
Monarch butterfly, crop-to-weed gene flow, antibiotic resistance, leakage of GM proteins into soil, reductions in pesticide spraying: are they real?
- **Disruption of current practices of farming and food production in developed countries**  
crop-to-crop gene flow
- **Disruption of traditional practices and economies in less developed countries**

### 13.6.1 Concerns about human health

#### Allergenicity

The possibility that we might see an increase in the number of allergic reactions to food as a result of genetic engineering has a powerful emotional appeal because many of us experienced this problem before the advent of transgenic crops, or know of someone who did. However, there is no evidence so far that genetically engineered foods are more likely to cause allergic reactions than are conventional foods. Tests of several dozen transgenic foods for allergenicity have uncovered only a soybean that was never marketed and the now-famous StarLink corn. Although the preliminary finding is that StarLink corn is probably not allergenic, the scientific debate continues. Every year some people discover that they have developed an allergy to a common food such as wheat or eggs, and some people may develop allergies to transgenic foods in the future, but there is no evidence that transgenic foods pose more of a risk than conventional foods do.

#### Horizontal transfer and antibiotic resistance

The use of antibiotic resistance markers in the development of transgenic crops has raised concerns about whether transgenic foods will play a part in our loss of ability to treat illnesses with antibiotic drugs. At several stages of the laboratory process, developers of transgenic crops use DNA that codes for resistance to certain antibiotics, and this DNA becomes a permanent feature of the final product although it serves no purpose beyond the laboratory stage. Will transgenic foods contribute to the existing problems with antibiotic resistance?

One concern is that the enzyme product of the DNA might be produced at low levels in transgenic plant cells. While high processing temperatures would inactivate the enzyme in processed foods, ingestion of fresh or raw transgenic foods could result in the stomach containing a small amount of an enzyme that inactivates an orally administered dose of the antibiotic. This issue was raised during the approval processes for Calgene's FlavrSavr tomato and Ciba-Geigy's *Bt* corn 176. In both cases, tests showed that orally administered antibiotics would remain effective. While the risks from antibiotic resistance genes in transgenic plants appear to be low, steps are being taken to reduce the risk and to phase out their use.

### **Eating foreign DNA**

When scientists make a transgenic plant, they insert pieces of DNA that did not originally occur in that plant. Often these pieces of DNA come from entirely different species, such as viruses and bacteria. Is there any danger from eating this "foreign" DNA? We eat DNA every time we eat a meal. DNA is the blueprint for life and all living things contain DNA in many of their cells. What happens to this DNA? Most of it is broken down into more basic molecules when we digest a meal. A small amount is not broken down and is either absorbed into the blood stream or excreted in the feces. So far there is no evidence that DNA from transgenic crops is more dangerous to us than DNA from the conventional crops, animals, and their attendant micro-organisms that we have been eating all our lives.

### **Changed nutrient levels**

How do genetically engineered foods compare with conventional foods in nutritional quality? This is an important issue, and one for which there will probably be much research in the future, as crops that are engineered specifically for improved nutritional quality are marketed. However, there have been only a few studies to date comparing the nutritional quality of genetically modified foods to their unmodified counterparts.

The central question for GE crops that are currently available is whether plant breeders have accidentally changed the nutritional components that we associate with conventional cultivars of a crop. Because isoflavones are thought to play a role in preventing heart disease, breast cancer, and osteoporosis, the isoflavone content of Roundup Ready soybeans has been investigated by several researchers. The studies completed so far do not resolve the issue of whether RoundupReady soybeans have isoflavone levels comparable to conventional varieties, but the differences found in experiments appear to be small or moderate in comparison with natural variation in isoflavone levels. Additional evidence may clarify the arguments for and against Roundup applications as a risk factor in soybean cultivation.

Industry studies submitted in support of applications for permission to sell transgenic crops indicate that the nutritional components that are commonly tested are similar in transgenic foods and conventional foods.

### **13.6.2 Concerns about damage to the environment**

#### **Biodiversity**

Crop genetic diversity might decrease due to the development of superior GM strains that crowd others out of the market. Indirect effects might affect other organisms. To the extent that agrochemicals impact biodiversity, modifications that increase their use, either because successful strains require them or because the accompanying development of resistance will require increased amounts of chemicals to offset increased resistance in target organisms.

Studies comparing the genetic diversity of cotton found that in the US diversity has either increased or stayed the same, while in India it has declined. This difference was attributed to the larger number of modified varieties in the US compared to India. A review of the effects of *Bt* crops on soil ecosystems found that in general they "appear to have no consistent, significant, and long-term effects on the microbiota and their activities in soil". The diversity and number of weed populations has been shown to decrease in farm-scale trials in the United Kingdom and in Denmark when comparing herbicide-resistant crops to their conventional counterparts. The UK trial suggested that the diversity of birds could be adversely affected by the decrease in weed seeds available for foraging.

A 2005 study designed to "simulate the impact of a direct overspray on a wetland" with four different agrochemicals (carbaryl (Sevin), malathion, 2,4-dichlorophenoxyacetic acid, and glyphosate in a Roundup formulation) by creating artificial ecosystems in tanks and then applying "each chemical at the manufacturer's maximum recommended application rates" found that "species richness was reduced by 15% with Sevin, 30% with malathion, and 22% with Roundup, whereas 2,4-D had no effect". The study has been used by environmental groups to argue that use of agrochemicals causes unintended harm to the environment and to biodiversity.

#### **Secondary pests**

Several studies documented surges in secondary pests within a few years of adoption of *Bt* cotton. In China, the main problem has been with mirids, which have in some cases "completely eroded all benefits from *Bt* cotton cultivation". A 2009 study in China concluded that the increase in secondary pests depended on local temperature and rainfall conditions and occurred in half the villages studied.

The increase in insecticide use for the control of these secondary insects was far smaller than the reduction in total insecticide use due to *Bt* cotton adoption. The finding was consistent with a hypothesis that more pesticide sprayings are needed over time to control emerging secondary pests, such as aphids, spider mites, and lygus bugs. Similar problems have been reported in India, with mealy bugs and aphids.

### **Crop-to-weed gene flow**

Hybridization of crops with nearby weeds may enable weeds to acquire traits we wish they didn't have, such as resistance to herbicides. Research results indicate that crop traits may escape from cultivation and persist for many years in wild populations. Genes that provide a competitive edge, such as resistance to viral disease, could benefit weed populations around a crop field.

Many cultivated crops have sexually compatible wild relatives with which they hybridize under favorable circumstances. The likelihood that transgenes will spread can be different for each crop in each area of the world.

For example, there are no wild relatives of corn in the United States or in Europe for transgenic corn to pollinate, but such wild relatives exist in Mexico. Soybeans and wheat are self-pollinating crops, so the risk of transgenic pollen moving to nearby weeds is small. However, that small risk must be balanced against the fact that there are wild relatives of wheat in the United States. There are no wild relatives of soybean in the United States, but such wild relatives exist in China. Thus each crop must be evaluated individually for the risk of gene flow in the area where it will be grown.

### **Antibiotic resistance**

There is also concern that transgenic plants growing in the field will transfer their antibiotic resistance genes to soil micro-organisms, thus causing a general increase in the level of antibiotic resistance in the environment. However, many soil organisms have naturally occurring resistance as a defense against other organisms that generate antibiotics, so genes contributed occasionally by transgenic plants are unlikely to cause a change in the existing level of antibiotic resistance in the environment.

### **Leakage of GM proteins into soil**

Many plants leak chemical compounds into the soil through their roots. There are concerns that transgenic plants may leak different compounds than conventional plants do, as an unintended consequence of their changed DNA.

Speculation that this may be happening leads to concern about whether the communities of micro-organisms living near transgenic plants may be affected. The interaction between plants and soil micro-organisms is very complex, with the micro-organisms that live around plant roots also leaking chemical compounds into the soil. Much more research must be done before we understand the relationships that occur between micro-organisms and conventional crops. Attempts to discover whether transgenic plants are changing the soil environment, and whether they are changing it in good ways or bad ways, are hindered by our lack of basic scientific knowledge.

### **Reductions in pesticide spraying**

One of the most appealing arguments in favor of transgenic plants is the potential for reducing the damage we do to our environment with conventional methods of farming. Pest-resistant crops such as *Bt* corn and *Bt* cotton have been promoted as a means to reduce the spraying of pesticides, while herbicide-tolerant crops such as Roundup Ready soybeans are said to reduce the application of herbicides. Large reductions in chemical spraying have been claimed to result from the introduction of these transgenic varieties. Are the claims true? *Bt* cotton is the only crop for which claims of reduced spraying are clear. Analysts paint a mixed picture on the results of planting Roundup Ready soybeans. *Bt* corn and herbicide-tolerant cotton and corn have not resulted in clear reductions in the spraying of chemicals.

#### **13.6.3 Concerns about damage to current farming practices**

##### **Crop-to-crop gene flow**

Hybridization of transgenic crops with nearby conventional crops raises concerns about separation distances to ensure purity of crops and about who must pay if unwanted genes move into a neighbor's crop. As "Identity Preservation" and segregation of GM from non-GM crops become factors in marketing products, it will be important to ensure that hybridization is not occurring in the field.

Many factors influence the potential for gene flow from crop to crop. Some crops are highly outcrossing, with pollen carried to other fields by wind and by insects.

Other species are highly self-pollinating, with little potential for pollen transfer to neighboring plants. Because of the differences among crops species, every case must be evaluated individually for potential to contribute to gene flow from transgenic to conventional crops.

If GM pollen pollinates plants in a neighboring field, then the issue of genetic trespass may arise. What level of GM presence, if any, should be allowed in products that are sold as organic or conventional? Should GM farmers and companies bear responsibility for preventing gene flow, or should conventional and organic farmers pay to protect their products from gene flow? Should GM versions of outcrossing plants be banned as too risky, while GM versions of self-pollinating plants are permitted? These issues have already prompted several lawsuits and they will continue to be a factor in the development and use of transgenic plants for years to come.

#### **13.6.4 Controversial studies**

##### **Pusztai affair**

Arpad Pusztai published the first peer-reviewed paper to find negative effects from GM food consumption in 1999. Pusztai fed rats potatoes transformed with the *Galanthus nivalis* agglutinin (GNA) gene from the *Galanthus* (snowdrop) plant, allowing the tuber to synthesise the GNA lectin protein. While some companies were considering growing GM crops expressing lectin, GNA was an unlikely candidate. Lectin is toxic, especially to gut epithelia. Pusztai reported significant differences in the thickness of the gut epithelium, but no differences in growth or immune system function. On June 22, 1998, an interview on Granada Television's current affairs programme *World in Action*, Pusztai said that rats fed on the potatoes had stunted growth and a repressed immune system. A media frenzy resulted. Pusztai was suspended from the Rowett Institute. Misconduct procedures were used to seize his data and ban him from speaking publicly.

##### ***Bt* corn**

A 2011 study was the first to evaluate the correlation between maternal and fetal exposure to *Bt* toxin produced in GM maize and to determine exposure levels of the pesticides and their metabolites. It reported the presence of pesticides associated with the modified foods in women and in pregnant women's foetuses. The paper and related media reports were criticized for overstating the

results. Food Standards Australia New Zealand (FSANZ) posted a direct response, saying that the suitability of the ELISA method for detecting the Cry1Ab protein was not validated and that no evidence showed that GM food was the protein's source. The organization also suggested that even had the protein been detected its source was more likely conventional or organic food.

### **13.6.5 Indian controversies**

India is an agrarian country with around 60% of its people depending directly or indirectly upon agriculture. From 1995 to 2013, a total of 296,438 farmers have killed themselves in India, or an average of 16,469 suicides per year. During the same period, about 9.5 million people died per year in India from other causes including malnutrition, diseases and suicides that were non-farming related, or about 171 million deaths from 1995 to 2013. Activists and scholars have offered a number of conflicting reasons for farmer suicides, such as monsoon failure, high *deBt* burdens, genetically modified crops, government policies, public mental health, personal issues and family problems. There is also accusation of states fudging the data on farmer suicides. In India, GM cotton yields in Maharashtra, Karnataka, and Tamil Nadu resulted in an average 42% increase in yield in 2002, the first year of commercial planting. A severe drought in Andhra Pradesh that year prevented any increase in yield, because the GM strain was not drought tolerant. Drought-tolerant variants were later developed. Driven by substantially reduced losses to insect predation, by 2011 88% of Indian cotton was modified. Though disputed, the economic and environmental benefits of GM cotton to farmers have been documented. A study from 2002 through 2008 on the economic impacts of *Bt* cotton in India, showed that *Bt* cotton increased yields, profits and living standards of smallholder farmers. However, recently cotton bollworm has been developing resistance to *Bt* cotton. Consequently, in 2012 Maharashtra banned *Bt* cotton and ordered an independent socioeconomic study of its use. Indian regulators cleared the *Bt* brinjal, a genetically modified eggplant, for commercialisation in October 2009. After opposition by some scientists, farmers and environmental groups, a moratorium was imposed on its release in February 2010 "for as long as it is needed to establish public trust and confidence".

As of 1 January 2013, all foods containing GMOs must be labelled. The Legal Metrology (Packaged Commodities) Rules, 2011 states that "every package

containing the genetically modified food shall bear at the top of its principal display panel the letters 'GM'" The rules apply to 19 products including biscuits, breads, cereals and pulses, and a few others. The law faced criticism from consumer rights activists as well as from the packaged-food industry; both sides had major concerns that no logistical framework or regulations had been established to guide the law's implementation and enforcement. On March 21, 2014, the Indian government revalidated 10 GM-based food crops and allowed field trials of GM food crops, including wheat, rice and maize.

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### 13.7 Summary

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The plants, in which a functional foreign gene has been incorporated by any biotechnological methods that generally are not present in the plant, are called transgenic plants. The main aims of genetic engineering of plants are boost the growth and yield of a variety food crops; provide protection to plants from environmental threats, such as cold, or pathogens, such as insects or viruses, and/or resistance to herbicides and also increase the nutritional value of the food etc. For making transgenic there are two kinds of gene transfer methods in plants are: direct gene transfer or vector less and Vector mediated method. Direct gene transfer consists of various chemical and physical methods while vector mediated require a biological agent such as *Agrobacterium*. Transposons tagging and chloroplast transformation are also important method for the construction of transgenic plants. The introduction of transgenic crops and foods into the existing food production system has generated a number of questions about possible negative consequences. The dispute involves consumers, farmers, biotechnology companies, governmental regulators, non-governmental organizations, and scientists.

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### 13.8 Glossary

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- **Electroporation:** It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.
- **Microinjection:** It involves delivery of foreign DNA into a living cell through a fine glass micropipette.
- **Ti- plasmid:** The virulent strains of *A. tumefaciens* possess large plasmids (140–235 kbp) known as tumor-inducing (Ti) plasmid.

- **T-DNA:** Transfer DNA is a small, specific segment of the Ti plasmid, about 24kb in size.
- **Transposons:** They are mobile genetic elements that move (transpose) from one region of the genome to another.
- **Plastid transformation:** It involves the delivery of DNA into chloroplasts or non-green plastids.

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### 13.9 Self-Learning Exercises

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#### Section A : (Very Short Answer Type Questions)

1. Who discover the particle bombardment method?
2. Which bacteria known as natural engineer?
3. What is size of the T DNA?
4. Name the product of gene *aadA*.
5. Define the Transposons.

#### Section B : (Short Answer Type Questions)

1. Define electroporation.
2. What are the limitations of chloroplast transformation?
3. Give the name of any two GMO crops.
4. Define the Ti plasmid.
5. Define the vir gene.

#### Section C : (Long Answer Type Questions)

1. Write a note on the aim of genetic engineering.
2. Write a detail account on direct DNA transfer method.
3. Describe the role of Agrobacterium in development of transgenic plants.
4. Give a detail account on chloroplast transformation.

#### Answer Key of Section-A

1. Prof Sanford and colleagues
2. *Agrobacterium tumefaciens*
3. 24kb
4. aminoglycoside 3' adenylyltransferase

5. Transposons are mobile genetic elements that move (transpose) from one region of the genome to another.
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## Unit - 14

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### Transgenic Plants and Crop Improvement

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#### Structure of the Unit:

- 14.0 Objectives
- 14.1 Introduction
- 14.2 Transgenic Plants in Crop Improvement
  - 14.2.1 Genetically Modified plants and their goal
  - 14.2.2 Variety of transgenic crops and their growing area
- 14.3 Insect and Pest Resistant Plants
  - 14.3.1 The mechanism of *Bt* toxicity
  - 14.3.2 Current Status of *Bt* Technology
  - 14.3.3 Safety Aspects of *Bt* Technology
  - 14.3.4 Advantages of *Bt* Crops
- 14.4 Herbicide Resistance Plants
  - 14.4.1 Glyphosate-tolerant crops
  - 14.4.2 Glufosinate-tolerant crops
  - 14.4.3 Advantages of Herbicide Tolerant Crops
  - 14.4.4 Safety Aspects of Herbicide Tolerance Technology
- 14.5 Abiotic Stress Tolerance Plants
  - 14.5.1 Heat tolerance plants
  - 14.5.2 Drought resistance plants
- 14.6 Quality Improvement
  - 14.6.1 Nutritional Improvement
  - 14.6.2 Golden Rice-with Pro-Vitamin A
  - 14.6.3 Improvement of Aroma
  - 14.6.4 Seedless Vegetables
- 14.7 Summary
- 14.8 Glossary
- 14.9 Self-Learning Exercise

14.10 References

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**14.0 Objective**

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After going through this unit you will be able to understand -

- Role of genetic engineering in crop improvement
  - Insect resistance plant and their risk
  - Herbicide resistance plant and their resistance mechanism
  - Heat and drought resistance plants and resistance mechanism
  - Role of transgenic plant in quality improvement of plant
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**14.1 Introduction**

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Transgenic plants are often referred to as genetically engineered (GE) plants, or genetically modified (GM) plants which include, *inter alia*, pest resistant cotton, maize, canola (mainly *Bt* or *Bacillus thuringiensis*), herbicide glyphosate resistant soybean, cotton and viral disease resistant potatoes, papaya and squash. In addition to the ability to produce crops with novel traits, genetic engineering also offers the promise of making plant breeding more efficient or for reducing the time required to make new varieties. Research using transgenic plants is an extremely valuable and powerful tool to help scientists learn about how plants function. The knowledge gained from this kind of research can be applied in many areas of plant science, not just in the creation of new crop varieties with novel traits. Most of the genetically engineered insect-resistant crop varieties produced so far uses specific genes taken from *Bacillus thuringiensis*. Currently, *Bt* corn and *Bt* cotton varieties are being grown in the U.S. and other countries. Genetically engineered herbicide tolerant varieties of major crops have been developed for use with glyphosate (Roundup®) or glufosinate (Liberty®) herbicides, and some cotton varieties grown in the U.S. have genetically engineered tolerance to bromoxynil or sulfonylurea herbicides. Monsanto's Roundup Ready® soybean varieties are the most widely grown type of genetically engineered plant. The plant biotechnology is not only used for improvements of crop plants for agronomic traits and but also significant efforts are also made to improve the nutritional content and organoleptic qualities such as taste and aroma in fruits and vegetables.

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## 14.2 Transgenic Plants in Crop Improvement

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### 14.2.1 Genetically Modified Plants and their goal

Higher productivity holds the key in the fight against rural poverty. Biotechnology promises to boost productivity and thus raise rural incomes, much in the same way that the green revolution did in large parts of Asia during the 1960s to 1980s. Productivity gains encompass essentially all factors of agricultural production. This may mean higher crop and livestock yields, lower pesticide and fertilizer applications, less demanding production techniques, higher product quality, better storage and easier processing, or enhanced methods to monitor the health of plants and animals. One type of technology, however, has given rise to a host of concerns and questions, transgenic plants. Transgenic plants are often referred to as genetically engineered (GE) plants, or genetically modified (GM) plants, although the latter term is not preferred by some who point out that all crop plants have been genetically modified from their original wild state by domestication, selection and controlled breeding over long periods of time. The U.S. Food and Drug Administration uses the term bioengineered to describe transgenic crops. GMOs are those organisms that have been modified by the application of recombinant DNA technology or genetic engineering, a technique used for altering a living organism's genetic material. With the rapid advances in biotechnology, a number of genetically modified (GM) crops or transgenic crops carrying novel traits have been developed and released for commercial agriculture production. These include, *inter alia*, pest resistant cotton, maize, canola (mainly *Bt* or *Bacillus thuringiensis*), herbicide glyphosate resistant soybean, cotton and viral disease resistant potatoes, papaya and squash. In addition, various transgenic crops are under development and not yet commercially released with traits for biofortification, phytoremediation and production of pharmaceuticals, such as rice with high level of carotenoid for production of Vitamin A (e.g. golden rice) and bananas with vaccines. Commercial cultivation of transgenic crops started in the early 1990s. Herbicide tolerance and insect resistance are the main GM traits that are currently under commercial cultivation, and the main crops are: soybean, maize, canola and cotton.

Very simply, the primary benefit derived from the ability to use genes from other organisms is to increase the amount of genetic variability available for breeders to use beyond that accessible by conventional breeding methods.

The goal is to allow plant breeders to produce more useful and productive crop varieties by exploiting genes from a wide range of living sources, not just those that can be found within the crop species itself. Progress in traditional plant breeding is limited by the genetic diversity within each crop species, the diversity sometimes available from closely related species, or occasionally useful diversity created within the crop itself by inducing mutations. Often, genes for traits that could be of benefit are not found in a particular crop species, so the ability to make plants with new, desirable traits borrowed from other species represents a major technological advance over conventional breeding methods.

The methods used to make transgenic plants also allow scientists to change crop traits by altering the crop's own genetic code, changing the function of the products coded for by genes or changing the way genes are expressed (switched on or off). This strategy has been used, for example, to modify the way tomato fruits ripen to preserve quality.

In addition to the ability to produce crops with novel traits, genetic engineering also offers the promise of making plant breeding more efficient or for reducing the time required to make new varieties. The ability to insert just one or a few specific genes into varieties without also introducing many other genes that might negatively affect the quality of crop varieties is often cited as a major benefit of using technology to produce transgenic plants. Given the enormous investment of time and money spent identifying useful genes, applying cloning and transformation techniques, and testing and evaluating newly transformed plants, all before conventional crossing to incorporate the genetically engineered traits into adapted crop varieties, some would argue that we are far from realizing this potential benefit.

Research using transgenic plants is an extremely valuable and powerful tool to help scientists learn about how plants function. The knowledge gained from this kind of research can be applied in many areas of plant science, not just in the creation of new crop varieties with novel traits.

The most common genetically engineered (GE) crops now being grown are transgenic varieties of soybean, canola, cotton, and corn. Varieties of each of these crops have been engineered to have either herbicide tolerance or insect resistance (or in a few cases, both).

#### **14.2.2 Variety of transgenic crops and their growing area**

All of the genetically engineered insect-resistant crop varieties produced so far use specific genes taken from *Bacillus thuringiensis*, a common soil bacterium, to produce proteins that are toxic to certain groups of insects that feed on them. Currently, only *Bt* corn and *Bt* cotton varieties are being grown in the U.S., but *Bt* potatoes were on the market for several years until being discontinued in 2001.

Several different genetic modifications have been used to engineer tolerance to herbicides, the most widely adopted GE trait overall. Genetically engineered herbicide tolerant varieties of each of the four major crops listed above have been developed for use with glyphosate (Roundup®) or glufosinate (Liberty®) herbicides, and some cotton varieties grown in the U.S. have genetically engineered tolerance to bromoxynil or sulfonylurea herbicides. Monsanto's Roundup Ready soybean varieties are the most widely grown type of genetically engineered plant.

GM crops are now commercially planted on about 100 million hectares in some 22 developed and developing countries. Argentina, Brazil, China and India are the largest developing-country producers of transgenic crops. The choice of GM crops varies among the developing countries, with insect resistant cotton being the most important commercially produced transgenic crop in Asian and African countries, while herbicide-resistant soybean followed by insect-resistant corn is predominant in the Latin American continent. In most cases these GM technologies are proprietary, developed by the private sector and released for commercial production through licensing agreements. Cultivation and commercial production of GM crops are capital intensive owing to high costs of seed and technology. Nevertheless, their cultivation has generally increased, mainly because of the benefits accrued from lower labour and production costs, reduction in use of chemical inputs and improved economic gain. The United States of America, Argentina and Canada are the major producers and exporters of GM crops and products. The four main global GM crops are among the major commodities traded on world markets. The increasing cultivation of GM crops has raised a wide range of concerns with respect to food safety, environmental effects and socio-economic issues. In current, about 50 different kinds of genetically engineered plants (each developed from a unique "transformation event") have been approved for

commercial production in the U.S. These include 12 different crops modified to have six general kinds of traits:

**Table 14.1: Variety of Transgenic Plants and their Traits**

S.No.	Transgenic trait	Crops
1.	Insect resistance	Corn, Cotton, Potato, Tomato
2.	Herbicide tolerance	Corn, Soybean, Cotton, Canola, Sugar beet, Rice, Flax
3.	Virus resistance	Papaya, Squash, Potato
4.	Altered oil composition	Canola, Soybean
5.	Delayed fruit ripening	Tomato
6.	Male sterility and restorer system (used to facilitate plant breeding)	Chicory, Corn

In 1999, the area planted to transgenic varieties was approximately half of the U.S. soybean crop and about 25% of the U.S. corn crop. The estimated worldwide area planted to transgenic varieties in 2000 increased 11% over the 1999 area (James, 2000). Most of the transgenic crop varieties currently grown by farmers are either herbicide tolerant or insect pest-resistant. In addition to the crops listed below, minor acreages were planted to transgenic potato, squash, and papaya.

**Table 14.2: Transgenic Crop Production Area by Country**

(Source: James, 2000)

S.No.	Country	Area planted in 2000 (millions of acres)	Crops grown
1.	USA	74.8	soybean, corn, cotton, canola
2.	Argentina	24.7	soybean, corn, cotton
3.	Canada	7.4	soybean, corn, canola
4.	China	1.2	Cotton
5.	South Africa	0.5	corn, cotton
6.	Australia	0.4	Cotton
7.	Mexico	minor	Cotton

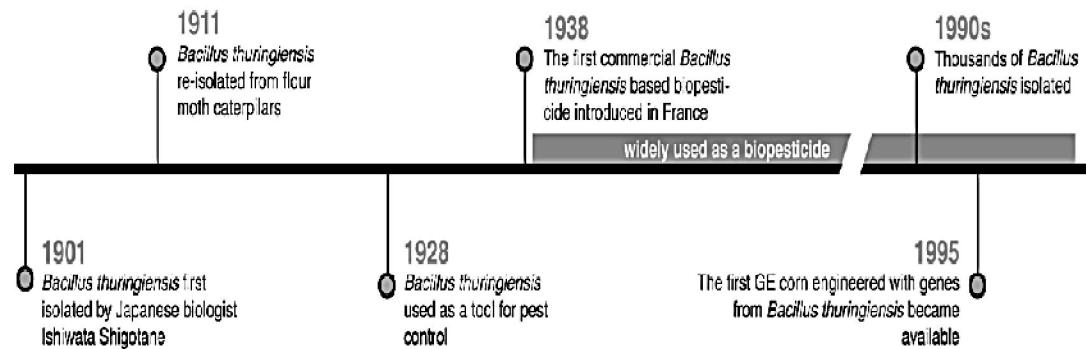
8.	Bulgaria	Minor	Corn
9.	Romania	Minor	soybean, potato
10.	Spain	Minor	Corn
11.	Germany	Minor	Corn
12.	France	Minor	Corn
13.	Uruguay	Minor	Soybean

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### 14.3 Insect and Pest Resistant Plants

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*Bacillus thuringiensis (Bt)* is a very common bacterium found in a variety of distinct environments, from soil, to dessert, to tundra. It was first isolated in 1901 by Japanese biologist Ishiwata Shigetane as he studied the causes of a disease afflicting silkworms. Then in 1911, the German scientist Ernst Berliner re-isolated *Bt* from flour moth caterpillars that had been collected from Thuringia, Germany (hence the species name). Soon Berliner determined that the *Bt* bacterium produces toxin which was specifically toxic to certain larva including the cotton bollworm and the Asian and European corn borers, all of which are common plant pests whose infestations produce devastating effects on important crops. The toxin, known as *Bt* toxin, is produced in an inactive, crystalline form but when it ingested by the larva of the target insect, the *Bt* protein is activated in the gut's alkaline condition and punctures the mid-gut leaving the insect unable to eat. The gene which encodes the *Bt* toxin known as cry gene. However, it wasn't until 1928 that anyone attempted to harness *Bt* as a tool for pest control. ***Bt*** preparations are commonly used in organic agriculture to control insects, as *Bt* toxin occurs naturally and is completely safe for humans. It is because of its ability to produce the insecticidal protein that much research is being done to exploit the organism's agronomic value. To date, there are more than 200 types of ***Bt*** proteins identified with varying degrees of toxicity to some insects. The different variations have different target insect specificity. For example, the toxins classified under Cry1a group, target Lepidoptera (butterflies), while toxins in the Cry3 group are effective against beetles.



**Fig. 14.1: Developmental History of Insect Resistant Plants**

*Bacillus thuringiensis* has been used to control pests for almost a century, with its first agricultural application dating back to 1928 and first commercialization a decade later.

**Table 14.3: List of Cry Gene Group and Target Group of Pest**

S.No.	Cry gene designation	Toxic to these insect orders
1.	CryIA(a), CryIA(b), CryIA(c)	Lepidoptera
2.	Cry1B, Cry1C, Cry1D	Lepidoptera
3.	CryII	Lepidoptera, Diptera
4.	CryIII	Coleoptera
5.	CryIV	Diptera
6.	CryV	Lepidoptera, Coleoptera

### Earlier *Bt* Technology

*Bt* is easily cultured by fermentation. Thus, over the last 40 years, *Bt* has been used as an insecticide by farmers worldwide. Organic farming in particular has benefited from *Bt* insecticide, as it is one of the very few pesticides permitted by organic standards. The insecticide is applied either as a spray, or as ground applications. It comes in both granules and liquefied form.

The efficiency of both applications is quite limited, as target organisms often do not come in contact with the insecticide as they are found on the underside of

leaves or have already penetrated into the plant. Scientists are working to overcome this problem through the use of modern biotechnology.

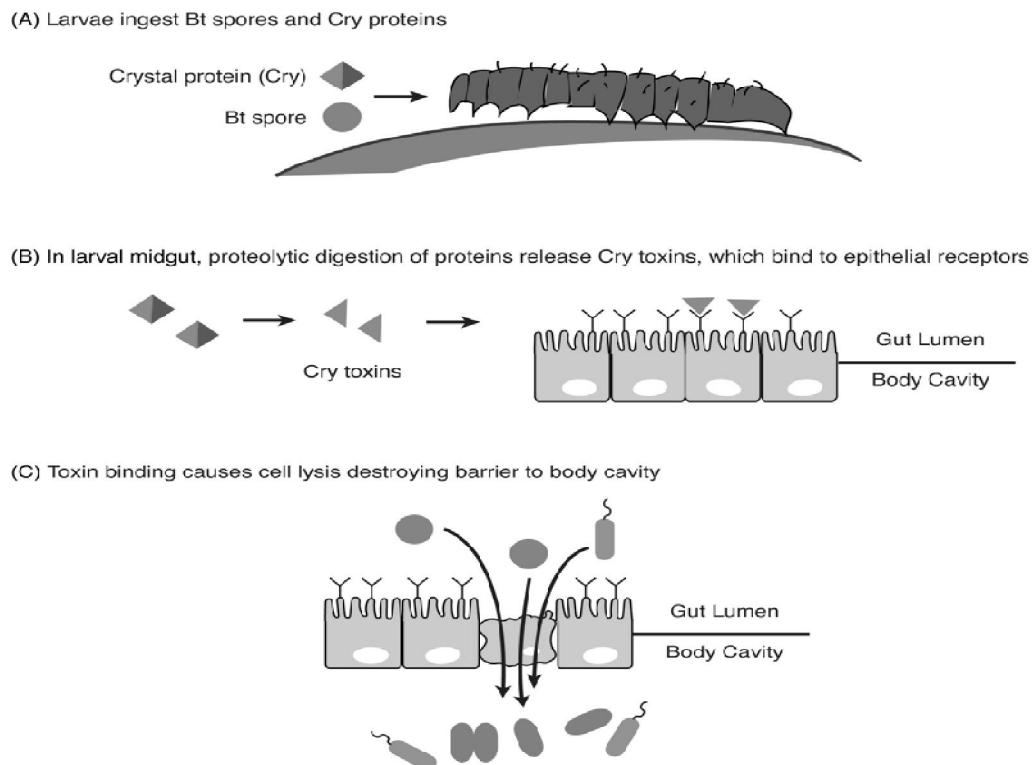
### **Modern *Bt* Technology**

Scientists have taken the *Bt* gene responsible for the production of the insecticidal protein from the bacterium and incorporated it into the genome of plants. . If plants produce *Bt* toxin on their own, they can defend themselves against specific types of insects. This means farmers no longer have to use chemical insecticides to control certain insect problems. Thus, these plants have a built-in mechanism of protection against targeted pests. The protein produced by the plants does not get washed away, nor is it destroyed by sunlight. The plant is thus protected from the bollworm or the corn borer round the clock regardless of the situation. Researchers have used genetic engineering to take the bacterial genes needed to produce *Bt* toxins and introduce them into plants

Critics claim that in some cases the use of insect resistant crops can harm beneficial insects and other non-target organisms. Extensive ecological impact assessments have been addressing these issues. In the field, no significant adverse effects on non-target wildlife nor long term effects of higher *Bt* concentrations in soil have yet been observed.

#### **14.3.1 The mechanism of *Bt* toxicity**

After *Bt* was first discovered, the mechanism of its toxicity still remained a mystery for many years. But in the 1950s, scientists discovered that the crystalline proteins that formed in *Bt* spores, previously observed by Berliner, were responsible for *Bt* toxicity. These crystal proteins, called Cry proteins, exhibit such a high degree of target specificity because of their mode of action within insect larvae.



**Fig. 14.2 :The production of *Bt* toxins is coupled to the organism's sporulation, and the multi-stage toxic mechanism by which *Bt* kills insects directly benefits the proliferation of the bacteria.**

(Source: <http://sitn.hms.harvard.edu/flash/2015/insecticidal-plants/>)

When the *Cry* protein reaches the gut, it is partially degraded, releasing a smaller and potentially toxic part of the protein. But this toxin will only be active if it finds the right matching protein receptor sticking off the cells lining the gut of a larval insect. This is the most important aspect of the *Cry* toxin mechanism. Much in the same way that a certain key will only open a certain lock, the *Cry* toxin can only exert its toxic effect on a particular cell receptor. Consequently, the toxin tends to only impact insects within a particular taxonomic order.

Once the toxin is bound, the process is fairly straightforward. The toxin recruits other *Cry* toxins to the same cell and together they form a hole in cell's membrane that ultimately causes the cell to burst. The cumulative effect of this happening to many cells is the irreversible destruction to the midgut membrane, compromising the barrier between the body cavity and gut. Without this barrier, *Bt* spores and

other native gut bacteria can infiltrate and grow within the nutrient-rich body of the insect.

What makes *Bt* such a great candidate for pesticide and GM applications is that while these *Cry* toxins are highly effective against insects, they have been shown to be safe for consumption by mammals. Tests by the EPA have demonstrated that *Cry* proteins, like any other benign dietary protein, are very unstable in the acidic stomach environment. Furthermore, an oral toxicity test, which involves giving mice exceptionally high doses of purified toxic *Bt* proteins, showed no significant health impacts. In their 2001 reassessment of several *Bt Cry* proteins, the EPA concluded from these findings that “there is reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the *Cry1AB* and *Cry1F* proteins and the genetic material necessary for their production.” Similar conclusions were drawn about the *Cry1Ac* protein of *Bt* cotton. Other mouse studies on have shown that even high doses of truncated *Cry* proteins, such that only the toxic region is conserved, have no deleterious effects . A paper in Annual Review of Entomology from 2002 also makes the strong point that, in addition to no demonstrated toxicity of *Bt* toxins, their use provides important health benefits to livestock and humans by preventing certain insect-caused crop diseases that produce toxic and carcinogenic compounds.

#### **14.3.2 Current Status of *Bt* Technology**

At the end of 2013, an estimated 28.8 million hectares of land were planted with crops containing the *Bt* gene. The following table shows the countries that have commercialized *Bt* crops and its products, from 1996 to 2013.

*Bt* insect-resistant crops currently on the market include

1. Corn: primarily for control of European corn borer, but also corn earworm and Southwestern corn borer. A list of approved *Bt* hybrids is available through the National Corn Growers Association web site
2. Cotton: for control of tobacco budworm and cotton bollworm
3. Potato: for control of Colorado potato beetle. *Bt* potato has been discontinued as a commercial product.

**Table 14.4: List of *Bt* Insect-Resistant Crops with Respective Country**

S.No.	<i>Bt</i> crop	Country
1.	Cotton	Argentina, Australia, Brazil, Burkina Faso, Canada, China, Colombia, Costa Rica, European Union (EU), India, Japan, Mexico, Myanmar, New Zealand, Pakistan, Paraguay, Philippines, Singapore, South Africa, South Korea, USA
2.	Eggplant	Bangladesh
3.	Maize	Argentina, Australia, Brazil, Canada, Chile, China, Colombia, Egypt, El Salvador, EU, Honduras, Indonesia, Japan, Malaysia, Mexico, New Zealand, Panama, Paraguay, Philippines, Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, USA,
4.	Poplar	China
5.	Potato	Australia, Canada, Japan, Mexico, New Zealand, Philippines, Russian Federation, South Korea, USA
6.	Rice	China, Iran
7.	Soybean	Argentina, Australia, Brazil, Canada, China, Colombia, EU, Japan, Mexico, New Zealand, Paraguay, South Korea, Taiwan, Thailand, USA, Uruguay
8.	Tomato	Canada, Chile, USA

(Source: ISAAA's GM Approval Database.)

### 14.3.3 Safety Aspects of *Bt* Technology

#### 1. Effects on Human Health

So how safe is the *Bt* protein to non-target organisms? The specificity of *Bt* for its target insects is one of the characteristics that make it an ideal method of biological pest control. In fact, different strains of *Bt* have specific toxicity to certain target insects. The specificity rests on the fact that the toxicity of the *Bt* protein is receptor-mediated. This means that for an insect to be affected by the *Bt* protein, it must have specific receptor sites in its gut where the proteins can bind. Fortunately, humans and majority of beneficial insects do not have these receptors. Before *Bt*

crops are placed on the market, they must pass very stringent regulatory tests, including those for toxicity and allergenicity.

The U.S. Environmental Protection Agency (US-EPA) has already administered toxicology assessments, and *Bt* proteins have already been tested even at relatively higher dosages. According to the Extension Toxicology Network (Extoxnet), a pesticide information project of several universities in the US, “no complaints were made after 18 humans ate one gram of commercial *Bt* preparation daily for five days, on alternate days. Humans who ate one gram per day for three consecutive days were not poisoned or infected.

## **2. Effects on the Environment: Soil ecosystems and groundwater**

The *Bt* protein is moderately persistent in soil and is classified as immobile, as it does not move, or leach, with groundwater. It does not particularly persist in acidic soil conditions and, when exposed to sunlight, is rapidly destroyed due to UV radiation.

Independent experts have conducted studies to investigate the impact of *Bt* crops on soil organisms and other insect species that are considered beneficial in agriculture. No adverse effects have been found on non-target soil organisms, even when these organisms were exposed to quantities of *Bt* far higher than what would actually occur under natural crop-growing conditions.

## **3. Effects on Animals and Insects**

On tests conducted on dogs, guinea pigs, rats, fish, frogs, salamanders, and even birds, the *Bt* protein was found not to have any harmful effects. It is also noteworthy that no toxic effects were found on beneficial or predator insects, such as honeybees and lady beetles.

In 1999, it was reported that pollen from *Bt* corn had a negative impact on Monarch butterfly larvae. This report raised concerns and questions about the risks of *Bt* crops on non-target organisms. Recent studies, however, show that *Bt* corn poses “negligible” threat to Monarch butterflies in the field. A collaborative research effort by scientists in the US and in Canada has produced information to develop a formal risk assessment of the impact of *Bt* corn on Monarch butterfly populations. They concluded that in most commercial hybrids, *Bt* expression in pollen is low, and laboratory and field studies show no acute toxic effects at any pollen density that would be encountered in the field.

#### **14.3.4 Advantages of *Bt* Crops**

##### **1. Improved pest management**

Insect-protected *Bt* crops provide the farmer with season-long protection against several damaging insect pests, and reduce or eliminate the need for insecticide sprays. This eliminates the yield loss that results from less than optimal pest control by applied farm insecticides, and it allows the farmer more time for other farm management duties.

##### **2. Reduction in insecticide use**

A study by the US Department of Agriculture reported that 8.2 million pounds of pesticide active ingredients were eliminated by farmers who planted *Bt* crops in 1998. Significant reductions have also been reported in China and Argentina, where the use of *Bt* cotton resulted in a 60-70% reduction in pesticide use.)

##### **3. Greater net return**

Lower input costs often contribute to a higher net return compared to conventional crops. *Bt* cotton farmers in the US earned an incremental \$99 million as a result of decreased pesticide costs and/or increased yields. Similarly, *Bt* cotton farmers in Argentina reported that *Bt* cotton generated an average incremental benefit of \$65.05/ha

##### **4. Improved conditions for non-target organisms**

Since *Bt* crops are able to defend themselves against pests, the use of chemical insecticides is significantly reduced, thereby encouraging the proliferation of beneficial organisms. These beneficial organisms can help control other secondary pests, which can often become a problem when predator and parasite populations are reduced by conventional broad-spectrum insecticides.

##### **5. Less mycotoxin in corn**

Aside from being effective against insect pests, *Bt* crops have lower incidences of opportunistic microbial pathogens, such as the fungus *Fusarium*. This fungus produces mycotoxins that can be deadly to livestock and also cause cancer in humans.

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## 14.4 Herbicide Resistance Plants

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Weed control is one of the farmer's biggest challenges in crop production, because poorly controlled weeds drastically reduce crop yield and quality. Many herbicides on the market control only certain types of weeds, and are approved for use only on certain crops at specific growth stages. Residues of some herbicides remain in the soil for a year or more, so that farmers must pay close attention to the herbicide history of a field when planning what to plant there. Development of transgenic plants resistance to certain biodegradable herbicides was the first major achievement from genetic engineering in plants. In fact, this activity generated the basic tool and technique for gene transfer in plants, and several genes that confer herbicide resistance serve as useful selectable marker. Herbicide tolerant crops resolve many of those problems because they include transgenes providing tolerance to the herbicides Roundup® (chemical name: glyphosate) or Liberty® (glufosinate). These herbicides are broad-spectrum, meaning that they kill nearly all kinds of plants except those that have the tolerance gene. Thus, a farmer can apply a single herbicide to his fields of herbicide tolerant crops, and he can use Roundup and Liberty effectively at most crop growth stages as needed. Another important benefit is that this class of herbicides breaks down quickly in the soil, eliminating residue carry-over problems and reducing environmental impact.

Herbicide resistant crops also facilitate low or no tillage cultural practices, which many consider to be more sustainable. Another advantage is that farmers can manage weeds without turning to some of the more environmentally suspect types of herbicides.

Critics claim that in some cases, the use of herbicide resistant crops can lead to an increase in herbicide use, promote the development of herbicide resistant weeds, and damage biodiversity on the farm. Extensive ecological impact assessments have been addressing these issues. Among the field trials conducted on herbicide resistant crops, studies in the United Kingdom have shown that different herbicides and different herbicide application practices can affect the amount of wild plants on the farm. In comparison with conventional cropping systems, weed and animal populations were negatively affected by herbicide tolerant sugar beet and rapeseed, but biodiversity was increased with the use of herbicide tolerant maize.

**Table 14.5: Herbicide Resistance Gene and their Sources**

S.No.	Herbicide	Resistance Gene	Gene Source
1.	Glufosinate, phosphinothricin, bialaphos	bar, PAT (phosphinothricin acetyl transferase)	Streptomyces sp. Alcaligenes sp.
2.	Glyphosate	aroA, EPSPS (3-enoyl pyruvyl shikimate 5- phosphatesynthase) gene	Agrobacterium sp.
3.	Bromoxynil	BXN (Bromoxynil nitrilase)	Klebsiella pneumoniae
4.	Sulfonamides	DHPS (dihydropteroate synthase), sul	Broad host range plasmid
5.	Sulfonylurea	ALS (acetolactate synthase)	Nicotiana tabacum

From 1996 to 2013, herbicide-tolerant crops consistently occupied the largest planting area of biotech crops. In 2013 alone, herbicide tolerant crops occupied 99.4 million hectares or 57% of the 175.2 million hectares of biotech crops planted globally. The most common are the glyphosate and glufosinate tolerant varieties. The following table shows countries that have approved major HT crops for food, feed and/or cultivation.

#### 14.4.1 Glyphosate-tolerant crops

Glyphosate herbicide kills plants by blocking the EPSPS enzyme, an enzyme involved in the biosynthesis of aromatic amino acids, vitamins and many secondary plant metabolites. There are several ways by which crops can be modified to be glyphosate-tolerant. One strategy is to incorporate a soil bacterium gene that produces a glyphosate-tolerant form of EPSPS. Another way is to incorporate a different soil bacterium gene that produces a glyphosate degrading enzyme.

1. *P. hybrida* EPSPS has been overexpressed in other crops as well, but the increased level of glyphosate tolerance was not enough to protect these transgenics from the herbicide under field conditions.
2. Mutants of *S. typhimuriurh* and *E. coli* showed tolerance to glyphosate due to a mutant form of EPSPS that is tolerant to glyphosate. Several different mutant forms of *E. coli* and petunia EPSPS were expressed in transgenic tobacco, soybean, canola and tomato. These transgenics showed increased tolerance to glyphosate, but the level of tolerance was inadequate for commercial use. *Bacterium Agrobacterium* strain CP4 has a glyphosate tolerant EPSPS enzyme.

This EPSPS gene was transferred into soybean. The transgenic soybean showed no visible injury due to up to 1.68 kg acid equivalent per hectare glyphosate. Subsequently, this gene has been expressed in cotton and several other crops to obtain glyphosate tolerant lines. In plants, the enzyme is localized in chloroplasts, by using a signal peptide sequence specific for chloroplasts.

3. Glyphosate is extensively and rapidly metabolised by glyphosate oxidoreductase (GOX), which breakdown glyphosate into glyoxylate and aminomethyl phosphonic acid. The gene encoding GOX has been isolated from *Achromobacter* sp. strain LBAA and expressed in transgenic plants.

Expression of gox and localization of GOX into chloroplasts provided excellent vegetative and reproductive tolerance to glyphosate. The gene gox has been expressed in plants in combination with *Agrobacterium* sp. CP4 EPSPS gene; several such transgenic varieties have been approved for commercial cultivation, e.g., Roundup Ready maize variety developed by Monsanto, U.S.A.

**Table 14.6: Organism resistance to the glyphosate and their mechanism**

<b>Organism</b>	<b>Source of glyphosate tolerant strain/EPSPS enzyme</b>	<b>Mechanism of glyphosate tolerance</b>
Tobacco ( <i>N. tabacum</i> )	Cell lines selected for resistance to glyphosate	Overproduction of EPSPS due to gene amplification
Carrot ( <i>D. carota</i> )	Cell lines selected for glyphosate resistance	Overproduction of EPSPS due to gene amplification
<i>Salmonella typhimurium</i>	Mutant strain	Tolerance of EPSPS to glyphosate (Pro at position 101 replaced by Ser)
<i>E. coli</i>	Mutant strain	Tolerance of EPSPS to glyphosate (Ala at position 96 replaced by Gly)
<i>Agrobacterium</i> sp.	Natural strain CP4 (tolerant to glyphosate)	High tolerance of EPSPS to glyphosate, and tight binding to PEP
<i>E. coli</i>	Site-directed mutagenesis	Tolerance of EPSPS to glyphosate due to amino acid replacement
<i>Arabidopsis thaliana</i>	Site-directed mutagenesis	Tolerance of EPSPS to glyphosate due to amino acid replacement
Petunia	Site-directed mutagenesis	Tolerance of EPSPS to glyphosate due to amino acid replacement
<i>Achromobacter</i> sp. strain LBA44	Natural isolate	Inactivation of glyphosate by glyphosate oxidoreductase enzyme.

(Source: [http://cdn.yourarticlerepository.com/wp-content/uploads/2014/04/clip\\_image00464.jpg](http://cdn.yourarticlerepository.com/wp-content/uploads/2014/04/clip_image00464.jpg))

#### **14.4.2 Glufosinate-tolerant crops**

Glufosinate herbicides contain the active ingredient phosphinothrinicin, which kills plants by blocking the enzyme responsible for nitrogen metabolism and for detoxifying ammonia, a by-product of plant metabolism. Crops modified to tolerate glufosinate contain a bacterial gene that produces an enzyme that detoxifies phosphinothrinicin and prevents it from doing damage.

Other methods by which crops are genetically modified to survive exposure to herbicides including: 1) producing a new protein that detoxifies the herbicide; 2) modifying the herbicide's target protein so that it will not be affected by the herbicide; or 3) producing physical or physiological barriers preventing the entry of the herbicide into the plant. The first two approaches are the most common ways scientists develop herbicide tolerant crops.

#### **14.4.3 Advantages of Herbicide Tolerant Crops**

- Excellent weed control and hence higher crop yields
- Flexibility – possible to control weeds later in the plant's growth;
- Reduced numbers of sprays in a season;

- Reduced fuel use (because of less spraying);
- Reduced soil compaction (because of less need to go on the land to spray);
- Use of low toxicity compounds which do not remain active in the soil; and
- The ability to use no-till or conservation-till systems, with consequent benefits to soil structure and organisms (Felsot, 2000).

#### **14.4.4 Safety Aspects of Herbicide Tolerance Technology**

##### **1. Toxicity and Allergenicity**

Government regulatory agencies in several countries have ruled that crops possessing herbicide-tolerant conferring proteins do not pose any other environmental and health risks as compared to their non-GM counterparts.

Introduced proteins are assessed for potential toxic and allergenic activity in accordance with guidelines developed by relevant international organizations. They are from sources with no history of allergenicity or toxicity; they do not resemble known toxins or allergens; and they have functions, which are well understood.

##### **2. Effects on the Plants**

The expression of these proteins does not damage the plant's growth nor result in poorer agronomic performance compared to parental crops. Except for expression of an additional enzyme for herbicide tolerance or the alteration of an already existing enzyme, no other metabolic changes occur in the plant.

##### **3. Persistence or invasiveness of crops**

A major environmental concern associated with herbicide-tolerant crops is their potential to create new weeds through outcrossing with wild relatives or simply by persisting in the wild themselves. This potential, however, is assessed prior to introduction and is also monitored after the crop is planted. The current scientific evidence indicates that, in the absence of herbicide applications, GM herbicide-tolerant crops are no more likely to be invasive in agricultural fields or in natural habitats than their non-GM counterparts (Dale et al., 2002).

The herbicide-tolerant crops currently in the market show little evidence of enhanced persistence or invasiveness.

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## 14.5 Abiotic Stress Tolerance Plants

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The negative impact of the non-living factors on living organisms in specific conditions define to abiotic stress. And in case of plants those plants express resistant to such factors called abiotic stress resistant plants. The examples of abiotic stresses are extreme temperatures, high winds; drought, flood etc. are most common stressors. Adverse environmental factors, of which water scarcity represents the most severe constraint to agriculture, account for about 70 percent of potential yield loses worldwide.

### 14.5.1 Heat tolerance plants

The most important kinds of cultivated plants like corn, soybean, tomato, etc generates only 50% of genetically possible yields per year. One main change in plant created by heat stress is accumulation of low molecular compounds (glycine betaine, sugar, amino acids) which is mainly used in cells vital functions. Researchers transferred genes for coline oxidase in *Arabidopsis thaliana* from *Arthrobacter globiformis*. The enzyme is involved in the production of glycine betaine which caused way of over production of glycine betanin in heat tolerant transgenic plants.

### 14.5.2 Drought resistance plants

Plants respond to their changing environment in a complex, integrated way that allows them to react to the specific set of conditions and constraints present at a given time. Therefore, the genetic control of tolerance to abiotic stresses is not only very complex, but is also highly influenced by other environmental factors and by the developmental stage of the plant.

The physiological responses of plants to a deficit of water include leaf wilting, a reduction in leaf area, leaf abscission, and the stimulation of root growth by directing nutrients to the underground parts of the plants. Plants are more susceptible to drought during flowering and seed development (the reproductive stages), as plant's resources are deviated to support root growth. In addition, abscisic acid (ABA), a plant stress hormone, induces the closure of leaf stomata (microscopic pores involved in gas exchange), thereby reducing water loss through transpiration, and decreasing the rate of photosynthesis. These responses improve the water-use efficiency of the plant on the short term. Stress like drought created high osmotic changes in response to which proline is formed in the tissues of the

plants. The gene of the pyrroline-5-carboxylsynthetase that is used for the proline synthesis was transferred from a bean (*Vigna aconitifolia*) into rice (*Oryza sativa*). The transgenic plants showed a better growth under salt and drought stress conditions than the respective control plants.

A second group of genes activated by drought is comprised by regulatory proteins that further regulate the transduction of the stress signal and modulate gene expression. At least four independent stress-responsive genetic regulatory pathways are known to exist in plants, forming a highly complex and redundant gene network<sup>8,9</sup>. Two of the pathways are dependent on the hormone ABA, and two are ABA-independent. These pathways are also implicated in the perception and response to additional stress factors, including cold, high temperature and salinity.

### **Genetic Engineering Drought Tolerant Plants**

Although not a crop plant, *Arabidopsis* has played a vital role in the elucidation of the basic processes underlying stress tolerance, and the knowledge obtained has been transferred to a certain degree to important food plants<sup>10</sup>. Many of the genes known to be involved in stress tolerance have been isolated initially in *Arabidopsis*. The introduction of several stress-inducible genes into plants by genetic engineering has resulted in increased tolerance of transgenics to drought, cold and salinity stresses<sup>8,9</sup>. Some examples are reviewed in the following section.

#### **1. Genetic manipulation of the stress response to abscisic acid (ABA)**

ABA levels in the plant greatly increase in response to water stress, resulting in the closure of stomata thereby reducing the level of water loss through transpiration from leaves and activate stress response genes. The reaction is reversible: once water becomes available again, the level of ABA drops, and stomata re-opens. Increasing the plant's sensitivity to ABA has therefore been a very important target for improving drought tolerance.

*ERA1*, a gene identified in *Arabidopsis*, encodes the β-subunit of a farnesyl-transferase, and is involved in ABA signaling. Plants lacking *ERA1* activity have increased tolerance to drought, however are also severely compromised in yield. In order to have a conditional, reversible down-regulation of ABA, a group of Canadian researchers used a drought-inducible promoter to drive the antisense expression of *ERA1*, in both *Arabidopsis* and canola plants<sup>11</sup>. Transgenic plants

performed significantly better under water stress, with consistently higher yields over conventional varieties. Importantly, there was no difference in performance between transgenic and controls in conditions of sufficient water, demonstrating that the technology has no yield-drag<sup>11</sup>. Multi-location trials have confirmed yield increases due to enhanced protection to drought to be 15-25 percent compared to non-transgenic controls.

Performance Plants Inc, a Canadian plant biotechnology company, is developing the technology for commercialization, under the name Yield Protection Technology™ (YPT™). YPT™ is also being developed for maize, soybean, cotton, ornamentals and turf grass to be available to farmers in early 2011.

## **2. ABA-independent gene regulation to drought stress**

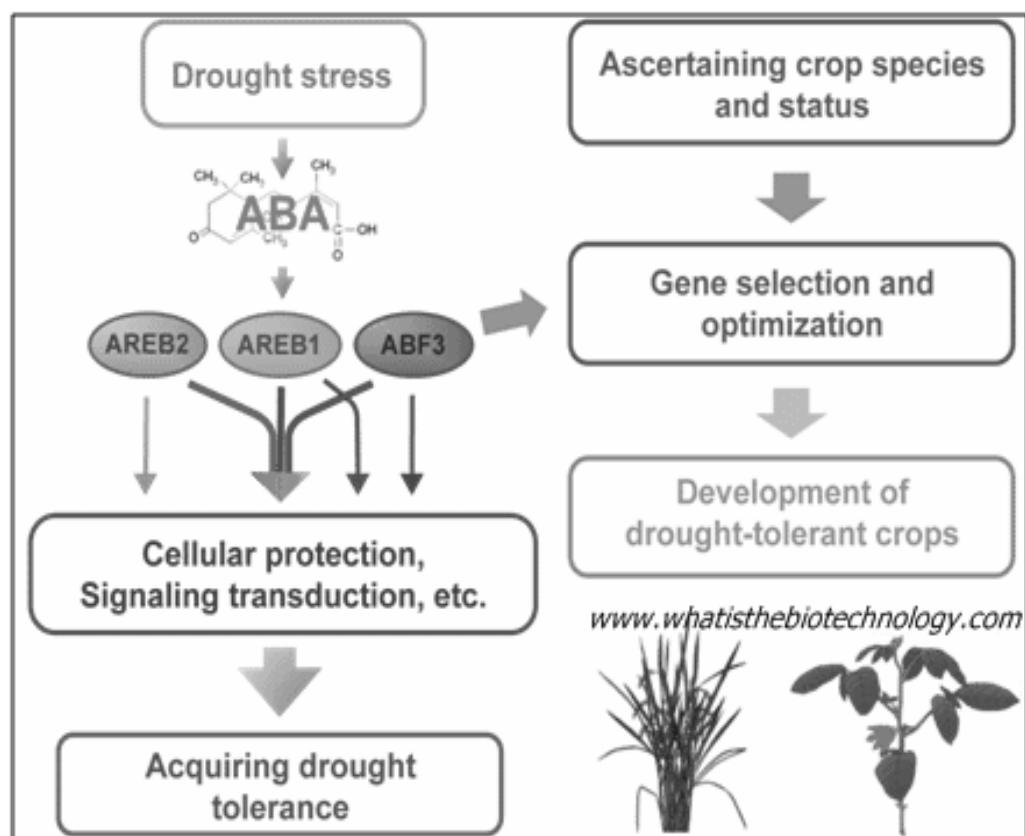
The transcription factors *DREB1* and *DREB2*, are important in the ABA-independent drought tolerant pathways, that induce the expression of stress response genes. Over-expression of the native form of *DREB1*, and of a constitutively active form of *DREB2*, increases the tolerance of transgenic *Arabidopsis* plants to drought, high salinity and cold. Although these genes were initially identified in *Arabidopsis* plants, their presence and role in stress tolerance have been reported in many other important crops, such as rice, tomato, barley, canola, maize, soybean, rye, wheat and maize, indicating that this is a conserved, universal stress defense mechanism in plants<sup>9</sup>. This functional conservation makes the *DREB* genes important targets for crop improvement for drought tolerance through genetic engineering.

## **Conclusion**

Although significant progress has been made in elucidating the genetic mechanisms underlying drought tolerance, considerable challenges remain. In field conditions, crops are subjected to variable levels of multiple stresses, thus one area of studies that deserves much more attention is the response of plants to a combination of stresses. There, plant's response to multiple stresses cannot be inferred from the response to individual stress. It is thus essential to test newly developed varieties to multiple stresses, and to carry out extensive field studies in a large range of conditions that assess tolerance as absolute yield increases.

Another major challenge is the increasing difficulty and expense in obtaining approvals for field trials of GM plants. As a number of measures are in place to

ensure the safe and responsible design of field tests, excessive precaution should not become a barrier to making sure we use all the tools available to us for a more sustainable agriculture.



**Fig. 14.3: Abiotic stress resistance in plants**

(Source: <http://www.whatisthebiotechnology.com>)

Arabidopsis has showed a vital role in the elucidation of the basic processes underlying stress tolerance, and the knowledge gained has been transferred to a certain degree to important grain plants. Many of the genes known to be involved in stress tolerance have been isolated initially in Arabidopsis. The introduction of several stress-inducible genes into plants by genetic engineering has resulted to increased tolerance of transgenics to drought, cold and salinity stresses.

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## 14.6 Quality Improvement

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The goal of plant biotechnology is not confined to improvements of crop plants for agronomic traits and significant efforts are also being made to improve the nutritional content and organoleptic qualities such as taste and aroma in fruits and vegetables.

### 14.6.1 Nutritional Improvement

Plants produce several compounds like storage proteins, vitamin, flavonoids, carotenoids etc., all are performing vital role for plants and also have nutritional importance for human beings. However, some of the vegetables are deficient in essential amino acids such as methionine and lysine but although these are good sources of minerals, proteins, macro-micronutrients, vitamins, antioxidants, phytosterols and dietary fibre. The amino acid content can be modified or enhanced by expression of synthetic protein, over expression of homologous or heterogeneous proteins, modifying the amino acid sequence of the protein or through metabolic engineering.

Potato is an important food crop; the nutritive value of potato protein is diminished due to deficiency in essential amino acids lysine, tyrosine and the sulphur containing amino acids methionine and cysteine. To improve the nutritive value of potato an Amaranthus seed albumin gene *AmA1* has been expressed in transgenic potato tubers. This protein is non-allergenic and rich in all essential amino acids corresponding with WHO standards for human diet requirements. Similarly, a 292 bp artificial gene (*asp-1*) encoding a storage protein composed of essential amino acids was transferred in sweet potato. One of the transgenic lines showed a fourfold increase in protein as compared to that of storage roots of control plants.

Carotenoids, such as B-carotene and lycopene, give the fruit its characteristic colour. Carotenoids are good antioxidants and also are precursors of vitamin A. These are synthesized through the isoprenoid biosynthetic pathway. Provitamin content of tomato was increased by transferring a bacterial gene encoding for the phytoene – desaturase enzyme that converts phytoene to lycopene into transgenic tomato.

These transgenic plants produced three-fold more B-carotene content than that of control plants. Similarly, a six-fold increase in carotenoid content and two to three

fold increase in tocopherol content was achieved in transgenic potato plants by antisense technology.

Another group of metabolites exploited for its antioxidant property are the flavonoids. These are a diverse group of polyphenolic secondary metabolites, which impart colour to the fruits. Flavonoids are present only in tomato peel. A transgenic approach has been used to increase the flavonoid content by over-expression of either the enzymes involved in flavonoids biosynthesis or transcription factors that regulate the genes of this pathway.

Transgenic tomato plants expressing petunia CHI-A gene encoding chalcone isomerase showed significant increase in flavonoids content. Similarly, a 10-fold increase in flavonoid content has been achieved by ectopic expression of the maize transcription factors LC and CI in transgenic tomato.

#### **14.6.2 Golden Rice-with Pro-Vitamin A**

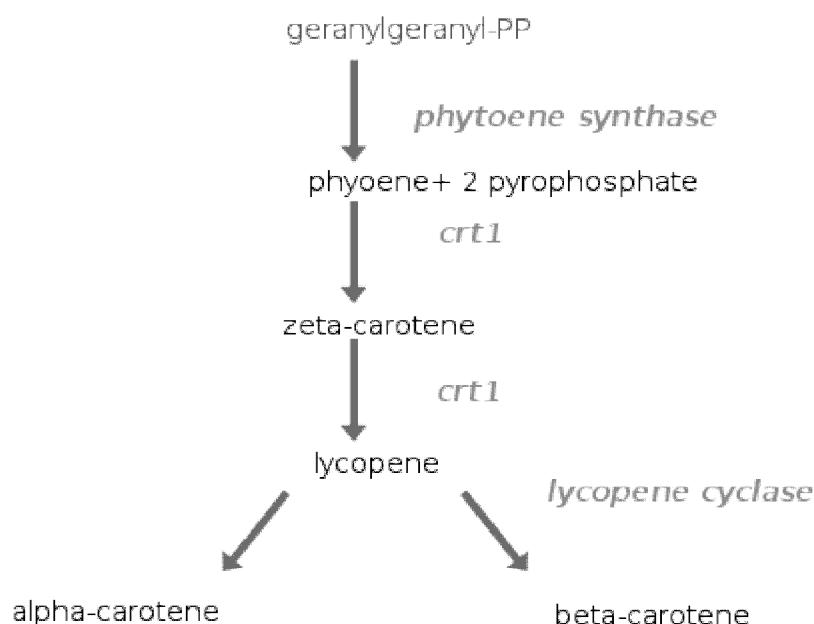
According to the World Health Organization (WHO), vitamin A deficiency (VAD) is the leading causes of preventable blindness in children. For children, a lack of vitamin A causes severe visual impairments and blindness and significantly increases the risk of severe illness and even death from common infections such as diarrhea and measles. The genes from daffodil and one from the bacterium *Erwinia uredovora* were inserted in the rice genome. These three genes produce the enzymes necessary to convert GGDP to pro vitamin- A. The inserted genes are controlled by specific promoters such that the enzymes and the provitamin-A are only produced in the rice endosperm. Provitamin-A is not produced by traditional rice varieties. However, geranylgeranyl diphosphate (GGDP), a compound naturally present in immature rice endosperm, with the help of several enzymes not normally found in rice can be used to produce provitamin-A.

Through the work of two European scientists, Dr. Ingo Potrykus of the Swiss Federal Institute of Technology in Zurich and Dr. Peter Beyer of the University of Freiburg in Germany, rice plants were developed containing two daffodil genes and one bacterial gene that carry out the four steps required for the production of beta-carotene in rice endosperm. Golden rice is the result of an effort to develop rice varieties that produce pro-vitamin-A (beta- carotene) as a means of alleviating vitamin A (retinol) deficiencies in the diets of poor and disadvantaged people in developing countries. Because traditional rice varieties do not produce vitamin-A,

transgenic technologies were required. Golden rice was created by transforming rice with only two beta-carotene biosynthesis genes:

- *psy* (phytoene synthase) from daffodil (*Narcissus pseudonarcissus*)
- *crtI* (carotene desaturase) from the soil bacterium *Erwinia uredovora*

The *psy* and *crtI* genes were transformed into the rice nuclear genome and placed under the control of an endosperm-specific promoter, so they are only expressed in the endosperm. The exogenous *lcy* gene has a transit peptide sequence attached so it is targeted to the plastid, where geranylgeranyl diphosphate formation occurs. The bacterial *crtI* gene was an important inclusion to complete the pathway, since it can catalyze multiple steps in the synthesis of carotenoids up to lycopene, while these steps require more than one enzyme in plants. The end product of the engineered pathway is lycopene, but if the plant accumulated lycopene, the rice would be red. Recent analysis has shown the plant's endogenous enzymes process the lycopene to beta-carotene in the endosperm, giving the rice the distinctive yellow color for which it is named. The original golden rice was called SGR1, and under greenhouse conditions it produced 1.6 µg/g of carotenoids.



**Fig. 14.4: Gene for Golden Rice and their function**

When golden rice is ingested, the human body splits the pro-vitamin-A to make vitamin A. Detailed information can only be obtained once the golden rice trait is transferred to local varieties and produced in quantities sufficient to support necessary field experiments. According to Swiss scientist Potrykus, “The intent of golden rice is to supplement to diet with vitamin A, not provide 100% of the Recommended Daily Allowance (RDA)”.

#### **14.6.3 Improvement of Aroma**

The aroma of fruits, vegetables and flowers are mixtures of volatile metabolites such as alcohols, phenols, ethers, aldehydes, ketones etc. Some of the short-chain aldehydes and alcohols are derived from lipid components by the action of lipases, hydro-peroxide lipases and alcohol dehydrogenases. When yeast  $\Delta$ -9 desaturase gene was transferred in tomato plants, changes in certain flavour compounds such as cw-3-hexenol, 1-hexanol, hexanal and cis-3-hexenal was recorded.

Linalool, an acyclic monoterpene alcohol, markedly influences the flavour of tomatoes. Linalool imparts a sweet, floral alcoholic note to fresh tomatoes. Hence linalool levels were altered by engineering the S-linalool synthase (LIS) gene from Clarkia breweri in tomato plants. The expression of S-linalool synthase enzyme, which catalyses the formation of linalool, resulted in elevated levels of linalool in the transgenic fruit.

#### **14.6.4 Seedless Vegetables**

The seedless nature of parthenocarpic (development of fruit without fertilization) fruits increases consumer acceptance, makes processing of vegetables easier, and also improves the quality of vegetables, e.g., brinjal (where seeds are associated with bitter substances).

Parthenocarpy has been shown to be regulated by auxins. Hence, efforts have been made to increase the auxin production or the sensitivity of ovary to auxins, towards inducing parthenocarpy. Expression of *iaaM* gene driven by the ovule specific promoter *defH9* has been shown to confer parthenocarpy to transgenic tomato and eggplant.

In another approach, the *Agrobacterium rhizogenes* derived gene *rolB* has been used for the induction of parthenocarpy in tomato. Transgenic tomato plants transformed with the *rolB* under the control of ovary and young fruit specific promoter TPRP-F1 developed parthenocarpic fruits.

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## 14.7 Summary

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With the rapid advances in biotechnology, a number of genetically modified (GM) crops or transgenic crops carrying novel traits have been developed and released for commercial agriculture production. Commercial cultivation of transgenic crops started in the early 1990s. Herbicide tolerance and insect resistance are the main GM traits that are currently under commercial cultivation, and the main crops are: soybean, maize, canola and cotton. The most common genetically engineered (GE) crops now being grown are transgenic varieties of soybean, canola, cotton, and corn. Varieties of each of these crops have been engineered to have either herbicide tolerance or insect resistance. The introduction of several stress-inducible genes into plants by genetic engineering has resulted to increased tolerance of transgenics to drought, cold and salinity stresses. In addition the genetic engineering also contributed in enhancement of nutritional level of crops such as Golden rice is the result of an effort to develop rice varieties that produce pro-vitamin-A (beta-carotene) as a means of alleviating vitamin A (retinol) deficiencies in the diets of poor and disadvantaged people in developing countries.

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## 14.8 Glossary

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- ***Bacillus thuringiensis***: It is a very common bacterium found in a variety of distinct environments, from soil, to dessert, to tundra.
  - **cry gene**: It encodes crystal protein which is toxic for pest
  - **Golden rice**: It is transgenic rice which was created by transforming rice with two beta-carotene biosynthesis genes
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## 14.9 Self-Learning Exercises

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### Section A : (Very Short Answer Type Questions)

1. Name the bacteria which used in production of insect resistant plant.
2. Herbicide resistant gene bar encode \_\_\_\_\_.
3. Name the any two insect-resistant crops.
4. Name the gene involved in production of golden rice.

### **Section B : (Short Answer Type Questions)**

1. Write the mechanism of *Bt* toxicity.
2. Write short note on cry protein.
3. Write short notes on heat tolerance plants.

### **Section C : (Long Answer Type Questions)**

1. Give a detail account on insect resistant plant.
2. Write an explanatory note on herbicide resistant plants.
3. Discuss the importance and risk of transgenic plants.
4. Short notes on following
  - I. Golden rice
  - II. Seed less fruit
  - III. Drought resistant plants

### **Answer Key of Section-A**

1. *Bacillus thuringiensis*
2. phosphinothricin acetyl transferase
3. *Bt* Cotton and *Bt* corn
4. psy (phytoene synthase) and *crtI* (carotene desaturase)

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## Unit - 15

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# Microbial Genetic Manipulation

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## 15.0 Objectives

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After going through this unit you will be able to understand:

- Process of transformation in bacteria and their type
  - Selectable marker gene and Selection of transformed bacterial cell
  - Role of genetic engineering in development of industrial microbe as well as nitrogen fixing microbe
  - Fermentation technology
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### 15.1 Introduction

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In molecular biology, **transformation** used to describe the insertion of new genetic material into nonbacterial cells, including animal and plant cells. Transformation can occur in two ways: natural transformation and artificial transformation. In cloning protocols, artificial transformation is used to introduce recombinant DNA into host bacteria (*E. coli*). Once the bacteria are transformed, those cells containing the plasmid are selected for and maintained using selective pressure from the selectable marker. Artificial transformation by using genetic engineering offers unlimited opportunities for creating new combinations of genes which leads to the development or improvement of strains and can be used by several industries. In addition transfer of *nif* gene among nitrogen fixing microbe leads the development in biological nitrogen fixation. Several microorganisms are used in fermentation technology, techniques for large-scale production of microbial products. It must both provide an optimum environment for the microbial synthesis of the desired product and be economically feasible on a large scale. They can be divided into surface (emersion) and submersion techniques. The latter may be run in batch, fed batch, continuous reactors

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### 15.2 Bacterial Transformation

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In molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s). In 1928, the English scientist Frederick Griffiths was studying the bacterium *Streptococcus pneumoniae*. This organism causes pneumonia, which in 1928 was the leading cause of death in the Western countries. He was working with two strains of *S.*

*pneumoniae*: among these one was virulent and caused disease (a pathogenic strain) while another one was non virulent did not cause disease. The pathogenic form of the organism produced an external polysaccharide coating that caused colonies of this strain growing on agar medium to appear smooth while non-pathogenic strain did not produce the coating, and its colonies appeared rough. Griffiths' experiments involved injecting mice with the *S. pneumonia* strains. When he used the smooth strain, the mice became ill and died. When he used the rough strain, they stayed healthy. In one series of experiments, Griffiths mixed heat-killed smooth cells (which had no effect when injected into mice) with living rough cells (which also had no effect when injected into mice) and injected the combination into mice. To his surprise, the mice became ill and died, as if they had been injected with living smooth cells. When Griffiths isolated *S. pneumonia* from the dead mice, he found that they produced smooth colonies. Griffiths concluded that the living rough cells had been transformed into smooth cells as the result of being mixed with the dead smooth cells. It was sixteen years before another group of investigators (Avery, McCarty, and MacLeod) showed that the "transforming principle," the substance from the heat-killed smooth strain that caused the transformation, was DNA. Transformation occurs naturally in some species of bacteria, but it can also be affected by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence, which might occur as a time-limited response to environmental conditions such as starvation and cell density.

"Transformation" may also be used to describe the insertion of new genetic material into nonbacterial cells, including animal and plant cells; however, because "transformation" has a special meaning in relation to animal cells, indicating progression to a cancerous state, the term should be avoided for animal cells when describing introduction of exogenous genetic material. Introduction of foreign DNA into eukaryotic cells is often called "transfection". Transformation can occur in two ways: natural transformation and artificial transformation.

### 15.2.1 Natural transformation

Natural transformation describes the uptake and incorporation of naked DNA from the cell's natural environment. It is a bacterial adaptation for DNA transfer that depends on the expression of numerous bacterial genes whose products appear to

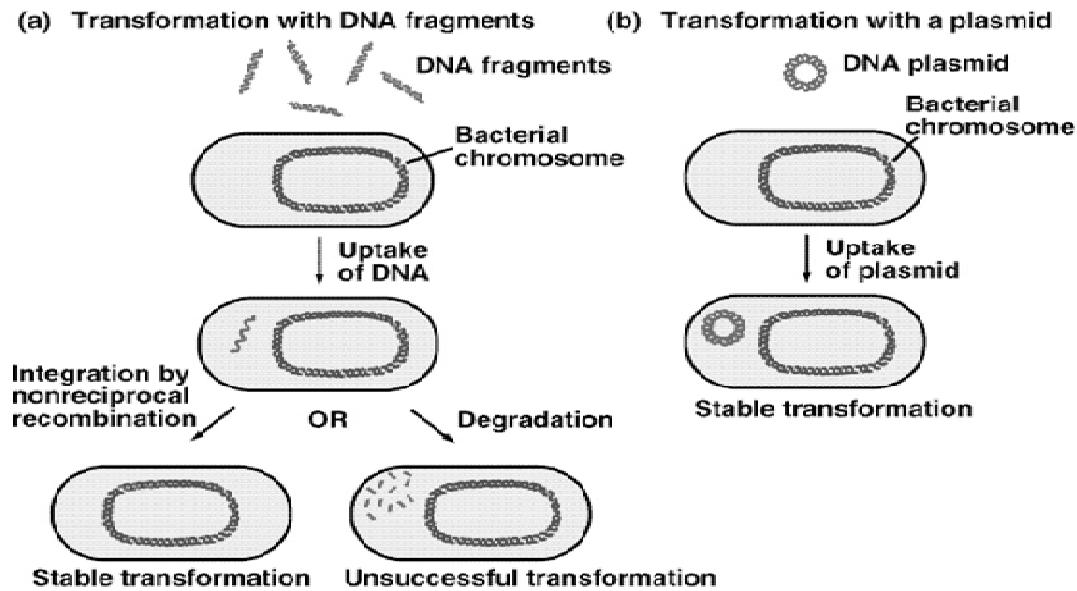
be responsible for this process. Some bacteria undergo transformation naturally. *Streptococcus pneumonia* is one of these, as are *Neisseria gonorrhoea* (the causative agent of gonorrhea) and *Haemophilus influenza* (the principle cause of meningitis in children). Each of these organisms has surface proteins that bind to DNA in the environment and transport it into the cell. Once inside the cell, the base sequence of the new DNA is compared to the bacterium's DNA. If enough similarity in sequence exists, the new DNA can be substituted for the homologous region of the bacterium's DNA. This is known as recombination. If the new DNA is not similar to the bacterium's DNA, it is not incorporated into the genome and is broken down by intracellular enzymes. In general, transformation is a complex, energy-requiring developmental process. In order for a bacterium to bind, take up and recombine exogenous DNA into its chromosome, it must become competent, that is, enter a special physiological state. Competence development in *Bacillus subtilis* requires expression of about 40 genes. The DNA integrated into the host chromosome is usually (but with rare exceptions) derived from another bacterium of the same species, and is thus homologous to the resident chromosome.

In *B. subtilis* the length of the transferred DNA is greater than 1271 kb (more than 1 million bases). The length transferred is likely double stranded DNA and is often more than a third of the total chromosome length of 4215 kb. It appears that about 7-9% of the recipient cells take up an entire chromosome. The capacity for natural transformation appears to occur in a number of prokaryotes, and thus far 67 prokaryotic species (in seven different phyla) are known to undergo this process.

### 15.2.2 Artificial transformation

Still, it is rare for most bacteria to take up DNA naturally from the environment. But by subjecting bacteria to certain artificial conditions, we can enable many of them to take up DNA. Artificial transformation encompasses a wide array of methods for inducing uptake of exogenous DNA. In cloning protocols, artificial transformation is used to introduce recombinant DNA into host bacteria (*E. coli*). The most common method of artificial transformation of bacteria involves use of divalent cations (e.g., calcium chloride) to increase the permeability of the bacterium's membrane, making them chemically competent, thereby increasing the likelihood of DNA acquisition. Another artificial method of transformation is electroporation, in which cells are shocked with an electric current, to create holes

in the bacterial membrane. With a newly-compromised cell membrane, the transforming DNA is free to pass into the cytosol of the bacterium. Regardless of which method of transformation is used, outgrowth of bacteria following transformation allows repair of the bacterial surface and selection of recombinant cells if the newly acquired DNA conveys antibiotic resistance to the transformed cells. Once DNA is taken into a cell, the use of that DNA by the cell to make RNA and proteins is referred to as expression. In nature, the expression of the newly acquired DNA depends upon its being integrated into the DNA of the host cell. As discussed above, the process of integration is known as recombination, and it requires that the new DNA be very similar in sequence to the host genome. However, researchers usually want to introduce into a cell DNA that is quite different from the existing genome. Such DNA would not be recombined into the genome and would be lost. To avoid this problem, scientists transform host cells with plasmid DNA. A plasmid is a small, circular piece of double-stranded DNA that has an origin of replication. An origin of replication is a sequence of bases at which DNA replication begins. Because they contain origins of replication, plasmids are copied by the host cell's DNA replication enzymes, and each daughter cell receives copies of the plasmid upon cell division. Therefore, plasmids do not need to be recombined into the genome to be maintained and expressed. Additionally, since plasmids do not have to have DNA that is similar to the host cell's DNA, DNA from other organisms can be maintained as a plasmid. Fortunately, it is relatively easy to introduce new DNA sequences into plasmids. Plasmids naturally occur in bacteria and yeast, and they are widely used as vehicles for introducing foreign DNA into these organisms. Thus far, no analogs of plasmids are known for higher plants and animals, which is one reason why genetic engineering is so much more difficult in higher organisms.



**Fig. 15.1 : Transformation of Bacteria**

### 15.2.3 Selection of transformed bacteria

Once the bacteria are transformed, those cells containing the plasmid are selected for and maintained using selective pressure from the selectable marker. Various antibiotic resistance gene acts as selectable marker. The antibiotics used in transformation are very similar (or the same) as antibiotics used to treat bacterial infections in humans. In medical situations, the term antibiotic resistance has a very negative connotation since it indicates an infection that cannot be successfully treated with antibiotics. However, antibiotic resistance has a far more positive meaning in biotechnology, since it is the end result of a successful transformation experiment. In a typical transformation, billions of bacteria are treated and exposed to plasmid DNA. Only a fraction (usually less than 1 in 1000) will acquire the plasmid. Antibiotic resistance genes provide a means of finding the bacteria which acquired the plasmid DNA in the midst of all of those bacteria which did not if the plasmid used to transform the DNA contains a gene for resistance to an antibiotic, then after transformation, bacteria that acquired the plasmid (transformants) can be distinguished from those that did not by plating the bacteria on a medium containing the antibiotic. Only the bacteria that acquired the plasmid will overcome the killing effect of the antibiotic and grow to form colonies on the plate.

So the only colonies on an antibiotic plate after a transformation are the bacteria that acquired the plasmid. This procedure accomplishes our two goals of giving an advantage to cells that have a plasmid so the plasmid is retained and of having a marker so we know our cells contain new DNA. Resistance to an antibiotic is known as a selectable marker; that is, we can select for cells that contain it. There are other marker genes as well. One class of marker genes are color marker genes, which change the color of a bacterial colony.

#### 15.2.4 Marker genes

Several of the plasmids contain an additional marker gene that causes the transformed cells to be colored. The plasmids and their marker genes are listed in Table 1.

**Table 1 : Plasmids and their Selectable Marker**

Plasmid	Selectable Marker	Colour Marker	Phenotype of transformants
pAMP	beta-lactamase	None	ampicillin-resistant colonies
pVIB	beta-lactamase	lux genes	ampicillin-resistant, glow-in-the-dark colonies
pGREEN	beta-lactamase	mutant GFP fusion gene	ampicillin-resistant, yellow-green colonies
pBLU	beta-lactamase	beta-galactosidase	ampicillin-resistant, blue colonies in the presence of X-gal

#### Selectable marker gene: Beta-lactamase

Ampicillin is a member of the penicillin family of antibiotics. Ampicillin and the other penicillins prevent the formation of the bacterial cell walls which leads the killing of the bacteria. Ampicillin and the other penicillin antibiotics contain a chemical group called a beta-lactam ring. The ampicillin-resistance gene encodes beta-lactamase, an enzyme that destroys the activity of ampicillin by breaking down the beta-lactam ring. When a bacterium is transformed with a plasmid

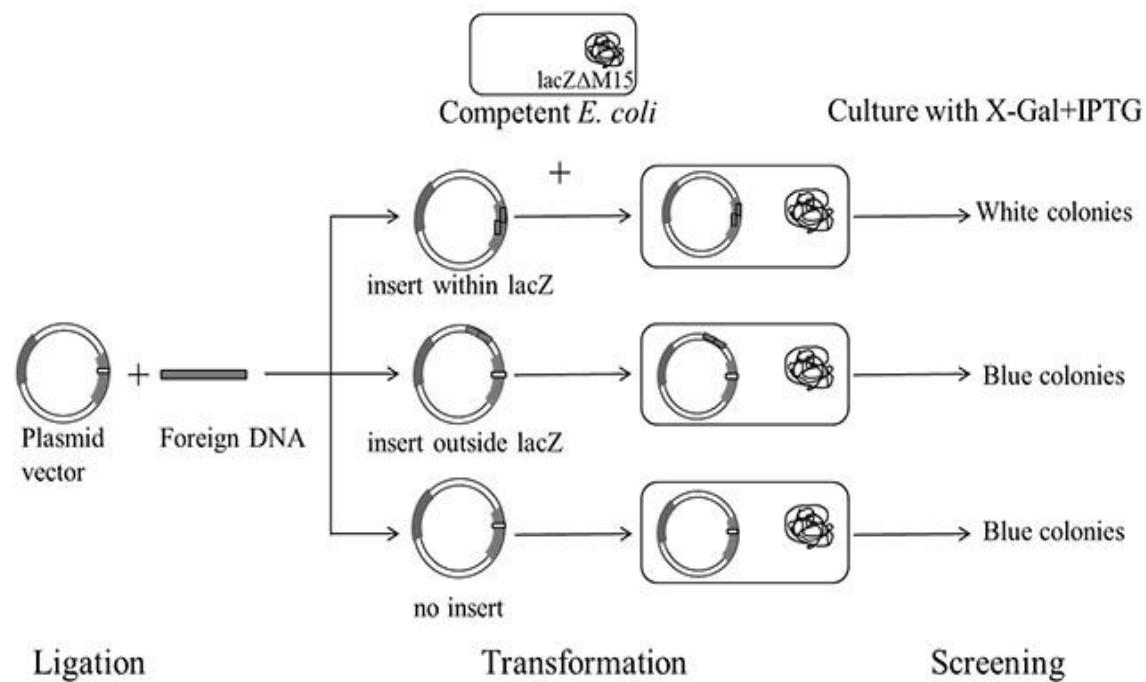
containing the beta-lactamase gene, it expresses the gene and synthesizes the beta-lactamase protein. The beta-lactamase protein is secreted from the bacterium and destroys the ampicillin in the surrounding medium by the mechanism described above. As the ampicillin is broken down, the transformed bacterium regains its ability to form its cell wall and is able to replicate to form a colony. The colony continues to secrete beta-lactamase and forms a relatively ampicillin-free zone around it. After prolonged incubation, small satellite colonies of non-transformed bacteria that are still sensitive to ampicillin grow in these relatively ampicillin-free zones. The plasmid pAMP contain the beta-lactamase gene. The presence of the beta-lactamase gene in the bacteria after they are transformed with pAMP allows the bacteria to grow in ampicillin-containing media. The beta-lactamase gene is called a selectable marker because in the presence of ampicillin, it allows you to select for cells that have been successfully transformed with the pAMP plasmid. Bacteria cells that have not been transformed and do not contain the plasmid and its beta-lactamase gene will not be able to grow in the presence of ampicillin.

### **Blue-White Screening**

Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of  $\beta$ -galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose. The presence of lactose in the surrounding environment triggers the lacZ operon in *E. coli*. The operon activity results in the production of  $\beta$ -galactosidase enzyme that metabolizes the lactose. Most plasmid vectors carry a short segment of lacZ gene that contains coding information for the first 146 amino acids of  $\beta$ -galactosidase. The host *E. coli* strains used are competent cells containing lacZ $\Delta$ M15 deletion mutation. When the plasmid vector is taken up by such cells, due to  $\alpha$ -complementation process, a functional  $\beta$ -galactosidase enzyme is produced. The plasmid vectors used in cloning are manipulated in such a way that this  $\alpha$ -complementation process serves as a marker for recombination. A multiple cloning site (MCS) is present within the lacZ sequence in the plasmid vector. This sequence can be nicked by restriction enzymes to insert the foreign DNA. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the  $\alpha$ -complementation does not occur, therefore, a functional  $\beta$ -galactosidase enzyme is

not produced. If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the lacZ gene in the plasmid vector complements the lacZ deletion mutation in the host E. coli producing a functional enzyme.

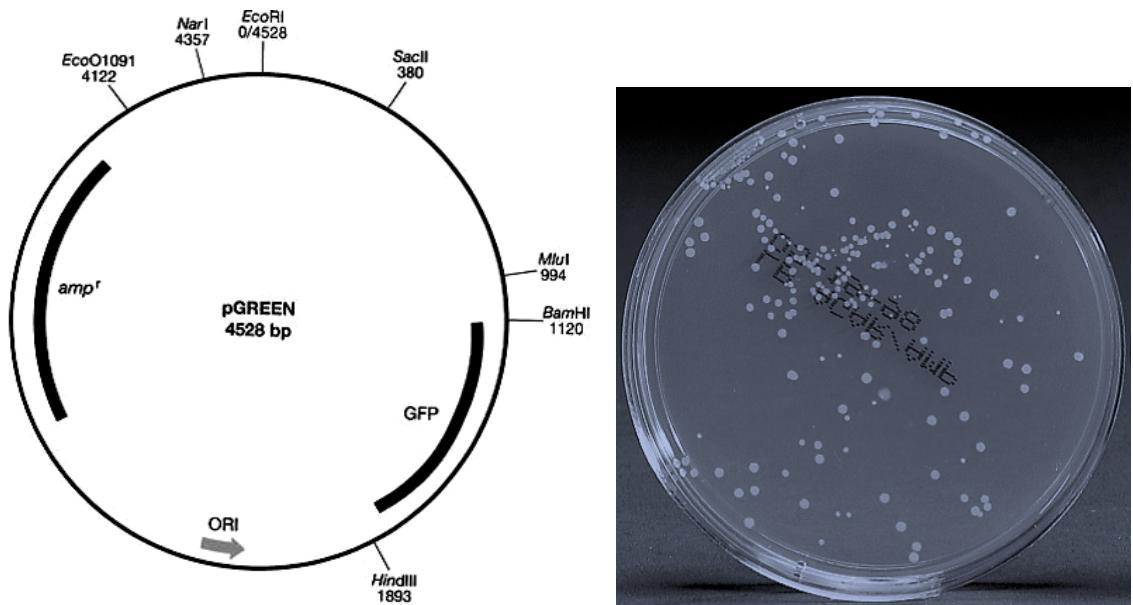
For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If  $\beta$ -galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analog of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for  $\beta$ -galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.



**Fig. - 15.2 : Selection of transformed by Blue-White Screening Method**

**Color marker gene: Mutant GFP fusion gene**

GFP stands for Green Fluorescent Protein, and the GFP gene is from a bioluminescent jellyfish, *Aequorea victoria*. These jellyfish emit a green glow from the edges of their bell like structures. This glow is easily seen in the coastal waters inhabited by the jellyfish. GFP glows by itself; it is auto-fluorescent in the presence of ultraviolet light. Because of this self-glowing feature, GFP has become widely used in research as a reporter molecule. A reporter molecule is one protein (such as the gene for GFP) linked to the protein that you are actually interested in studying. Then you follow what your protein is doing by locating it with the reporter molecule. For instance, if you wanted to know whether gene X was involved in the formation of blood vessels, you could link (or fuse) gene X to the GFP gene. Then, instead of making protein X, the cells would make a protein that was X plus GFP. The type of protein that results from linking the sequences for two different genes together is known as a fusion protein. If the blood vessels began glowing with GFP, it would be a clue that protein X was usually present and a sign that X might indeed be involved in blood vessel formation. The pGREEN plasmid contains a GFP gene and a gene for ampicillin resistance. It has a mutant version of GFP that turns bacteria yellow-green, even in normal light. If you expose the colonies to a UV light, they also fluoresce. This plasmid contains an ampicillin-resistance gene in addition to the GFP gene. Ampicillin is an antibiotic and works by preventing *E.coli* from constructing cell walls, thereby killing the bacteria. When the ampicillin resistance gene is present it directs the production of an enzyme that blocks the action of the chemical and the bacteria are able to survive. Bacteria without the plasmid and hence the resistance gene are unable to grow on a plate containing ampicillin in the medium and only the transformants will survive.



**Fig. 15.3 : Plasmid pGREEN and selection of transformant**

### 15.3 Genetic Improvement of Industrial Microbes

Industrial microbiology is a field which deals with the development of technologies to control and manipulate the growth and activities of selected microbe to create desirable products and economic gain. In addition to bacteria and yeasts; animal and plant cell cultures are now used to produce sophisticated products such as monoclonal antibodies, immunomodulating compounds and complex plant metabolites.

Traditional fermentations were originally performed (and still are in many cases) by a mixture of wild microorganisms emanating from the raw materials or the local environment, e.g., some food and alcoholic beverage fermentations. The first attempts to improve the microorganisms occurred little more than 120 years ago, when they were first isolated from these processes as pure cultures from which the most useful strains were then selected. Those fermentation processes developed in the first 80 years of the 20th century have mostly used pure monocultures. The targeted microorganisms were often isolated from the natural environment by the random screening of a large number of isolates. Alternatively, suitable microorganisms were acquired from culture collections. Most of these microorganisms, irrespective of their origins, were subsequently modified by

conventional strain improvement strategies, using mutagenesis or breeding programmes, to improve their properties for industrial use. Several processes developed in the past 20 years have involved recombinant microorganisms and genetic engineering technologies have increasingly been used to improve established industrial strains.

Industrial Products are produced by the wild strains isolated from nature. They are selected on the basis of particular product produced by strain. E.g. *Bacillus* is isolated from soil sample for the production of amylase. (Amylase can act on starch to give glucose). These naturally isolated organisms usually produce commercially important products in very low concentrations and therefore it is essential to increase the productivity of the selected organism. For increase in yield of the desired product, medium and growth conditions should be optimized. Medium and growth optimization is having limited effect on increase in the product due to organism's maximum ability to synthesize the product which is controlled by its genome. Thus if one want to increase the product then one should modify the genome. Once the desired genome is created for the increased desired product, then cultural requirements of the modified organisms should be tested. Once genome is modified again it is further modified for improvement. Thus the process of strain improvement require continuous changing of genes of given organism with changes in the medium components. Their ability to readily undergo genetic manipulation has also opened up almost limitless further possibilities for new products and services from the fermentation industries.

### **15.3.1 Industrial strains and strain improvement**

Irrespective of the origins of an individual microorganism, it should ideally exhibit:

1. Genetic stability.
2. Efficient production of the target product, whose route of biosynthesis should preferably be well characterized.
3. Limited or no need for vitamins and additional growth factors.
4. Utilization of a wide range of low-cost and readily available carbon sources.
5. Amenability to genetic manipulation.
6. Safety, non-pathogenicity and should not produce toxic agents, unless this is the target product.

7. Ready harvesting from the fermentation.
8. Ready breakage, if the target product is intracellular.

In biotechnological processes the aim is primarily to improve/optimize the particular characteristics sought in an organism. Advances have been achieved in this area by using screening and selection techniques to obtain better organisms. In a selection system, all rare or novel strains grow while the rest do not. In a screening system, all strains grow but certain strains or cultures are chosen because they show the desired qualities required by the industry in question.

### **15.3.2 Genetic manipulation of microorganisms**

Genetic manipulations are used to produce microorganisms with new and desirable characteristics. Genetic modification can be achieved by: Mutation and recombination.

#### **15.3.2.1 Mutation**

Mutation can be spontaneous or induced. Spontaneous mutation can lead to the change in the genetic make-up of the organisms but chances are there that culture may have problem of yield degeneration because variants are usually inferior producers. Variants or mutants can be obtained giving better yield by inducing mutation to the wild strain. Once a promising culture is found, a variety of techniques can be used for culture improvement, including chemical mutagenesis and ultraviolet light. Mutation can creates so many mutants of which some are superior producers and some are inferior producers of the product. It is not easy to select them on the basis of one or two criteria but superior mutant is selected using more than one criterion for the formation of product. However, such methods normally lead only to the loss of undesirable traits or increased production due to loss of control functions. It has rarely led to the appearance of a new function or property. Thus, an organism with a desired feature will be selected from the natural environment, propagated and subjected to a mutational programme, then screened to select the best progeny. As an example, the first cultures of *Penicillium notatum*, which could be grown only under static conditions, yielded low concentrations of penicillin. In 1943 a strain of *P. chrysogenum* was isolated – strain NRRL 1951 – which was further improved through mutation (using X-ray treatment, UV and mustard gas the yield was increased from 120 IU to 2 580 IU). Today most

penicillin is produced with *P. chrysogenum* grown in aerobic stirred fermenters, which gives 55-fold higher penicillin yields than the original static cultures.

### **Selection of Mutants giving better primary metabolites:**

Any metabolite is produced by particular microorganisms via certain pathway. When concentration of particular metabolite increases then organisms have regulatory mechanism to control over the production of those metabolites. If by mutation such control is blocked then improved product can be produced. **Example:** *Corynebacterium glutamicum* produces glutamic acid; this product can be increased by mutation. Kinoshita isolated mutant of *C. glutamicum* which is deficient in the production of biotin as well as in the synthesis of enzyme  $\alpha$ -ketoglutarate dehydrogenase. Biotin deficient strain do not produce proper membrane and thus deficient in selective permeability. While defect in the production of enzyme  $\alpha$ -ketoglutarate dehydrogenase will not allow the formation of succinic acid from the  $\alpha$ -ketoglutaric acid, and  $\alpha$ -ketoglutarate is diverted to glutamic acid synthesis.

Organisms used for the commercial production of primary metabolites are rarely modified at only one genetic site, sometime it is necessary to alter several control sites to produce desire product in high quantity.

### **Selection of mutants producing improved levels of secondary metabolites:**

The design of producers for the isolation of mutants overproducing secondary metabolites is more difficult due to the fact that far less information is available on the control of production and, also, that the end products of secondary metabolism are not required for the growth.

Screening is used and achieves considerable success in selecting mutant for the production of secondary metabolites. Several workers have obtained improved secondary metabolite producing strains by isolating auxotrophic mutants. In many cases there is no correlation between the compound and the secondary metabolites produced. Possible explanation for this may be that they are double mutants and their auxotrophy was not directly related to the improved productivity.

Organisms may be exploited by using Nitrosoguanidine (NTG) as a mutagen. NTG causes clusters of mutations around the replication fork of the bacterial chromosome. Thus if one of the mutations were selectable it may be possible to

isolate a strain containing the selectable mutation which is close by, for this one should require the accurate knowledge about the positions of the genes important in secondary metabolites.

The technique of selecting mutants resistant to inhibitory analogues has found some application in the selection of secondary metabolites overproducers.

For example Elander et al. (1971) isolated tryptophan analogue resistant mutant of *Pseudomonas aureofaciens* which overproduced antibiotic pyrrolnitrin. Tryptophan is precursor for this pyrrolnitrin and resistant mutant can produce more of this limiting precursor.

#### **15.3.2.2 Recombination**

Recombination is process of creating new combination of genes in given organisms. Recombination process is not that much successful as the use of induced mutant and selection, this is due to success of mutation programme.

Now recombination technique is used for the strain improvement after the different techniques available which help us to use this technique more conveniently. Recombination process can be carried out naturally or artificially. Recombination that is occurring naturally is applicable to few organisms, due to limitation of genetic exchange between these organisms. While artificial recombination can occur in any cell and almost any gene can be inserted in any cell.

##### **Natural recombination included:**

**Parasexual cycle in some fungi:** For this process genetically unlike nuclei must be present in one of the fungi. After the fusion of genetic material of two different organisms, heterokaryon is produced. These heterokaryons contain genetic information of two different organisms or recombinant gene.

**Conjugation:** In this process the genetic information of one bacterium is transferred to other via cell to cell contact. The chromosome of the ‘donor’ cell is mobilized by the integration of a normally extrachromosomal DNA particle into the recipient. This technique is used in the preparation of strain producing particular compound in excess, or producing compound which is not previously present in recipient organism. Conjugation is also demonstrated in *Streptomyces* which have enormous industrial significance.

In recent years industrial genetics has come to depend increasingly on two new ways of manipulating DNA – protoplast and cell fusion and recombinant DNA technology (genetic engineering).

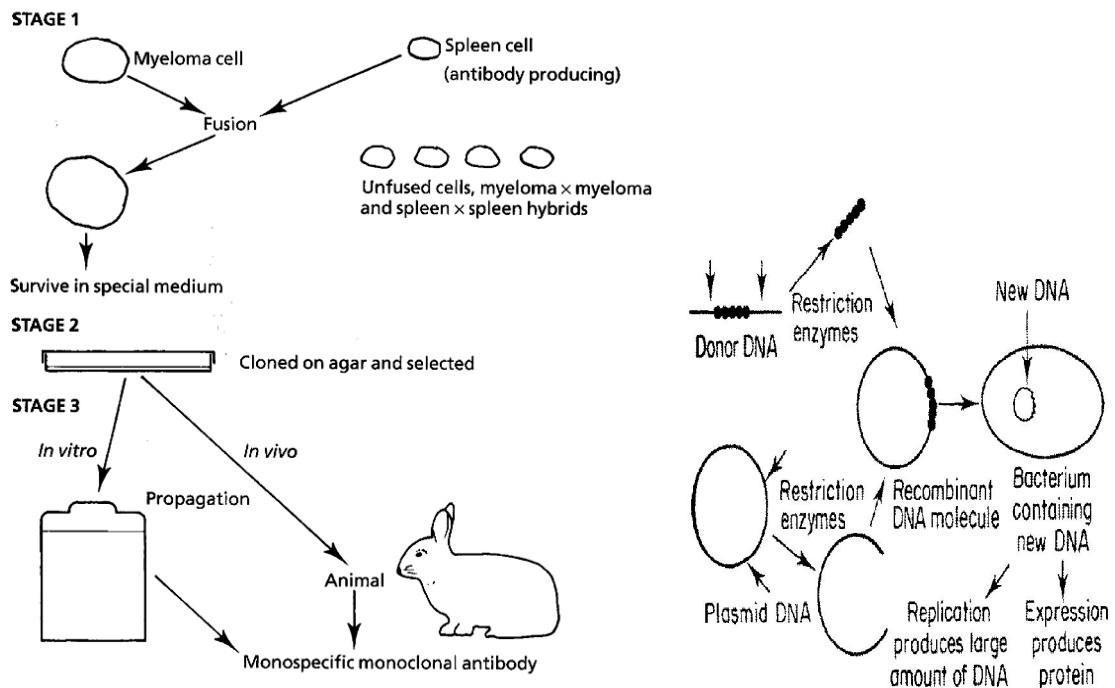
#### **Natural recombination by Protoplast Fusion:**

Protoplasts are the cells devoid of cell wall. Cell fusion, followed by nuclear fusion will occur between protoplasts that would not otherwise fuse and resulting in fused protoplast may generate cell wall and grow into mutant cell. If fusion occurs to form hybrids, desired recombinants are identified by means of selective plating techniques. After regeneration of the cell wall, the new protoplasm fusion product can be used in further studies. A major advantage of the protoplast fusion technique is that protoplasts of different microbial species can be fused, even if they are not closely linked taxonomically. For example, protoplasts of *Penicillium roquefortii* have been fused with *P. chrysogenum*. Even yeast protoplasts and erythrocytes can be fused. One of the most exciting and commercially rewarding areas of biotechnology involves a form of mammalian cell fusion to the formation of monoclonal antibodies. In 1975, pure monoclonal antibodies were produced from the fusion product of (hybridoma) of  $\beta$ -lymphocytes and myeloma tumour cells. The monoclonal antibody technique changes antibody secreting cells (with a limited life span) to cells that are capable of continuous growth (immortalisation) while maintaining their specific antibody secreting potential (Fig. 3.1). Monoclonal antibodies are now widely applied in many diagnostic techniques which require a high degree of specificity. Specific monoclonal antibodies have been combined into diagnostic kits in health care, in plant and animal agriculture and food manufacture.

#### **15.3.2.3 Genetic Engineering**

Recombinant DNA technology or genetic engineering offers unlimited opportunities for creating new combinations of genes. In this technique Host's chromosomal or extrachromosomal DNA is cut and the desired small DNA is inserted into it, this lead to the recombination of the Host DNA. The desired DNA can be isolated from plants, animals or microorganisms (the donors), and fragmented into groups of one or more genes. Such fragments can then be couples to another piece of DNA (the vector) and then passed into the host or recipient cell, becoming part of the genetic complement of the new host. The host cell can then

be propagated in mass to form novel genetic properties and chemical abilities that were unattainable by conventional ways of breeding or mutation. Short lengths of chemically synthesized DNA sequences can be inserted into recipient microorganisms by the process of site-directed mutagenesis. This can create small genetic alterations leading to a change of one or several amino acids in a target protein. Such minor amino acid changes have been found to lead, in many cases, to unexpected changes in protein characteristics and have resulted in new products such as more environmentally resistant enzymes and enzymes that can catalyze desired reactions. These approaches are part of the field of protein engineering. Enzymes and bioactive peptides with markedly different characteristics (stability, kinetics and activities) can be created. The molecular basis for the functioning of these modified products also can be better understood. One of the most interesting areas is the design of enzyme-active sites to promote the modification of "unnatural substrates". This approach may lead to improved transformation of recalcitrant materials, or even the degradation of materials that have previously not been amenable to biological processing. The majority of the recombinant DNA prepared by this technique is used for the improvement of organisms producing primary metabolites. The efficiency of the organisms used in the single cell protein process, *Methylophilus methylotrophus*, has been improved by the incorporation of a plasmid containing the glutamate dehydrogenase from *E. coli*. This technique is also available for bacteria, yeast and fungi. But it is limited to improvement of organism for primary metabolites, due to lack of information regarding basic genetics of secondary metabolites production.



**Fig 15.4 : Application of Genetic Engineering**

## 15.4 Nitrogen Fixing Microbe and their Development

Biological nitrogen fixation is carried out by a special class of prokaryotes. Most of the nitrogen fixing prokaryotes found in soil is free living in nature. They fix the nitrogen without the direct interaction with other organisms. The examples in this category include *Azotobacter*, *Bacillus*, *Clostridium* and *Klebsiella*. They contribute to a very less amount of global nitrogen fixation due to scarcity of appropriate carbon sources. Associative types of microorganisms remain in close association with the rhizosphere region of members of family Poaceae (Rice, wheat, corn, oats, barley etc.). These bacteria fix an appreciable amount of nitrogen. Associative nitrogen fixation can supply 20–25% of total nitrogen requirements in rice and maize. Symbiosis is a naturally occurring phenomenon where the microbes and higher plants manually and beneficially contribute to the process of nitrogen fixation. Such an association requires that bacteria provide the host plant with fixed nitrogen for the growth of plant in exchange of nutrients and carbohydrates that are utilized by them to fulfill the energy requirement of the

process of nitrogen fixation. Most commonly, the symbiotic associations occur between the plants of family Leguminosae and bacteria of genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*. These bacteria are collectively referred as rhizobia. Another common type of symbiosis occurs between water fern *Azolla* with a cyanobacterium *Anabaena azollae*. Anabaena colonizes cavities of *Azolla* fronds where the cyanobacteria fix sufficient amount of nitrogen in heterocysts, the specialized cells meant for nitrogen fixation. The amount of nitrogen fixation by cyanobacteria is comparatively more in symbiotic state than on free living condition. Another example is the symbiosis between several woody plant species such as alder trees (*Alnus* sp.) with the soil bacteria of genus *Frankia*, an actinomycete. Nitrogen fixation is an “old” topic in scientific terms since it is over a century ago that scientists experimentally proved that some “unique” species of plants with the help of microbes that were later found bearing nitrogenase, are capable of utilizing atmospheric nitrogen. The thought of engineering major crops to adapt such a capability, either by association/symbiosis with microbes or by introducing nitrogenase directly into the plant, was proposed several decades ago but only limited experimental approaches were carried out due to the complexity of the nature of the biological nitrogen fixation process.

#### **15.4.1 Organization of Nitrogen fixing genes**

Nitrogen fixation is carried out by three groups of genes. These are; Nod gene (responsible for nodule formation), Nif gene (responsible for nitrogen fixation) and Hup gene (responsible for nitrogen uptake). All these three types of genes are present in a group on a single chromosome. This makes their copying and transfer mechanism simple for genetic engineering purposes.

Though the mechanism of nodule formation is complex nod gene is responsible for nodule formation as well as host recognition and specificity. However, a few genes located on plasmids can produce nodules. Plasmid of *R. leguminosarum* is less than 10 kb even then it has property to recognize host and nodule formation.

Nitrogen fixation has been thoroughly covered during the last years, since genetic engineering fosters the hope for techniques improving the nitrogen supply of plants. The production of synthetic nitrogen fertilizer is expensive and extraordinarily costly in terms of energy. Bacteria, too, are not able to produce ammonia at low energy costs, since the triple bond of nitrogen belongs to the

strongest covalent bonds occurring in biologically important molecules. The conversion of 1 mole nitrogen to 2 mole ammonia requires 25 mole ATP, i.e. the fixation of 1 gram nitrogen costs 10 g glucose under favorable conditions. *Azotobacter*'s reaction is especially pricey: it needs 100 g glucose for the fixation of 1 g nitrogen.

The genetic basis of nitrogen fixation is largely known. The preferred test object was and still is *Klebsiella pneumoniae*, an enterobacterium belonging to the kinship of *Escherichia coli* and the salmonellas. In nitrogen fixation, the nitrogenase complex takes up a key position. The encoding and the regulation of this protein are controlled by a certain DNA region, the *nif*-region, that contains 16 (or 17) genes in the case of *Klebsiella*. The *nif*-genes belong to seven different operons (transcription units). Except for one gene that is located on the complementary strand, all of them are located on the same (the 'encoding') strand. In *Azobacter*, the genes are scattered over the whole genome. The *nif*-region of *Klebsiella* has been isolated, cloned, and expressed in *Escherichia coli*. Nevertheless, the transformation of green plants poses several principal problems:

1. The genes have to be coupled to a eukaryotic promoter in order to be expressed.
2. Oxygen-free compartments or zones are required, since nitrogenase is extremely sensitive towards oxygen.
3. The electron transport chain of the plant cell has to be in tune with that of the nitrogenase, and finally.
4. have sufficient amounts of ATP to be provided.

The problem is thus not the transformation of the plant cells or the integration of the foreign genes into the plant genome, but to obtain control of both their expression and the activity of their gene products. No practicable solutions exist as yet. It is rather tried to increase the efficiency of soil bacteria or to optimize the conditions of the plants' rhizosphere.

### **Nod gene**

Most of the biological nitrogen fixing bacteria contains a large plasmid called mega-plasmid. In several functions it is similar to Ti plasmid and contains genes responsible for auxin and cytokinin production. Excess production of these plant growth regulators helps in nodule formation. According to Rosenberg (1981)

several special genes are present along with nod genes. Such plasmids are absent in non-symbiotic bacteria.

A nod gene is a group of genes containing Nod A, B, C, D genes having 8.5 kb length. These genes form polypeptides of different lengths (196, 197, 402, 211 amino acid). Nod genes of different rhizobium species have almost 70% homologies which are called common Nod genes.

### **Nif genes**

This gene is responsible for nitrogen fixation and present in the genome of symbiotic and non-symbiotic nitrogen fixing bacteria. In symbiotic bacteria *Rhizobium*, it is present near nod genes on the mega plasmid, while in non-symbiotic cyanobacteria it is present on the main DNA. Initially Nif gene has been transferred in *E. coli*.

In higher plants, chloroplast is a cell organelle which might have been originated from prokaryotes, therefore attempt are made to transfer Nif gene into chloroplast. Easy availability of ATP and NADPH<sub>2</sub> in chloroplast also makes them ideal recipient for this gene transfer.

Most of the cereal plants are monocots and any such effort to transfer such Nif gene will revolutionize the yield, economics and environmental pollution. However, there are many difficulties in transferring, integration and expression of a prokaryotic gene into a monocot.

### **Hup gene**

Gene responsible for nitrogen uptake is Hup gene. In symbiotic bacteria this gene recycles the hydrogen produced during nitrogen fixation as shown in the Fig. 11.4. Hydrogen produced at different steps is assimilated in the reduction of nitrogen. In most of the legumes 30-50% energy (in the form of ATP) is spent on hydrogen liberation. This results in loss in capacity of nitrogen fixation. If this hydrogen can be recycled by nitrogenase enzyme we can save a lot of energy, and this can be carried out by improved Hup gene.

*Klebsiella pneumoniae* strain M5 a1 (Enterobacteriaceae) is a free living bacteria which has been studied extensively for genetics of nitrogen fixation. This bacterial genome is quite similar to that of *E. coli* and *Salmonella typhimurium*. Therefore most of the techniques of genetic engineering can be applied to *Klebsiella*.

### **15.4.2 Transgenes with Nif Genes**

It is important to develop transgenic plants containing Nif genes to solve the problem of nitrogen fertilizer supplement to crop plants. This will have beneficial effects of economics and environment also. For this purpose, Ti based plasmid and Cauliflower mosaic virus based promoter (CAM promoter) was used to transfer Nif genes in to non-legume plants. To test the efficacy of this system, phaseolin gene from legume (pulses) has been transferred to sunflower where it was expressed and produced phaseolin.

Protoplasts isolation from root nodules and preparations of rhizobia were used to develop hybrids by protoplasts fusion and organelles uptake. Major contributions were made by groups headed by Prof. E.C. Cocking, Prof. M.R. Davey, Dr. I. Portykus (inventor of Golden rice) and Prof. I.K.Vasil. However, true nitrogen fixing hybrids are yet to be obtained by this method.

## **15.5 Fermentation Technology**

### **15.5.1 Fermentation and Fermented Product**

The term ‘fermentation’ is derived from the Latin verb, *fevere*, to boil. Fermentation technology is one of the oldest food technologies that have been used for several thousand years as an effective and low cost means for preserving foods and beverages. Food fermentation is of prime importance in the developing countries where the limitation of resources encourages the use of locally available fermented food products for additional nutrition. These fermented products are more common among people belonging to rural areas, without much awareness about the microflora involved in their production. In order to be viable in any specific industrial context, bioprocessing must possess advantages over competing methods of production such as chemical technology. In practice, many bioprocessing techniques will be used industrially because they are the only practical way in which a specific product can be made (e.g. vaccines, antibiotics). Biochemical engineering covers the design of vessels and apparatus suitable for performing such biochemical reactions or transformations. The very beginnings of fermentation technology, or as it is now better recognized, bioprocess technology, were derived in part from the use of microorganisms for the production of foods such as cheeses, yoghurts, sauerkraut, fermented pickles and sausages, soya sauce and other Oriental products, and beverages such as beers, wines and derived spirits.

In many cases, the present-day production processes for such products are still remarkably similar. These forms of bioprocessing were long viewed as arts or crafts, but are now increasingly subjected to the full array of modern science and technology. Paralleling these useful product formations was the identification of the roles microorganisms could play in removing obnoxious and unhealthful wastes, which has resulted in the worldwide service industries involved in water purification, effluent treatment and solid waste management.

**Table 15.2 : Fermentation Products in various Industrial branches**

Branch	Products
Chemical Organic (bulk)	Ethanol, acetone, butanol, organic acids (citric, itaconic)
Organic (fine)	Enzyme, perfumeries, polymers (polysaccharides)
Inorganic	Metal beneficiation, bioaccumulation and leaching (Cu, U)
Pharmaceutical	Antibiotics, diagnostic agents (enzymes, monoclonal antibodies), enzyme inhibitors, steroids, vaccines
Energy	Ethanol (gasohol), methane (biogas), biomass
Food	Dairy products (cheeses, yogurts, fish and meat products), Beverages (alcoholic, tea and coffee), baker's yeast, Food additives (antioxidants, colours, flavours, stabilizers) Mushroom products, novel foods (soy sauce, tempeh, miso), Amino acids, vitamins, starch products, glucose and high fructose syrups, functional modifications of proteins, pectins
Agriculture	Animal feedstuffs (SCP), veterinary vaccines, microbial pesticides, ensilage and composting processes, plant cell and tissue culture (vegetative propagation, genetic improvement)

Although the traditional forms of bioprocess technology relate to food and beverages, new products are increasingly being derived from microbial fermentations, namely:

1. To produce essential primary metabolites such as acetic and lactic acids, glycerol, amino acids, vitamins, etc.

2. To produce secondary metabolites (metabolites that do not have an obvious role in the metabolism of the producer organism) such as penicillin, cephalosporin, streptomycin, etc.
3. To produce many forms of industrially useful enzymes, e.g. exocellular enzymes and intracellular enzymes, etc.

The product formation stages in bioprocess technology are very similar no matter what organism is selected, what medium are used and what product is formed. All biotechnological processes are performed within containment systems or bioreactors. Large numbers of cells are involved in these processes and the bioreactor ensures their close involvement with correct medium and conditions for growth and product formation. Examples of the diverse product categories produced industrially in bioreactors are given in Table 15.3.

**Table 15.3 : Examples of Products in Different Categories Produced in Industrial Bioreactors**

Category	Example
Cell mass	Baker's yeast, single cell protein
Cell components	Intracellular proteins
Biosynthetic products	Antibiotics, vitamins, amino and organic acids High fructose corn syrup, 6- aminopenicillanic acid Activated sludge, anaerobic digestion
Catabolic products Bioconversion	High fructose corn syrup, 6- aminopenicillanic acid
Waste treatment	Activated sludge, anaerobic digestion

### **15.5.2 Principles of Microbial Growth**

The growth of organisms depends on the availability and transport of necessary nutrients to the cell and subsequent uptake and on environmental parameters such as temperature, pH and aeration. The quantity of biomass or specific cellular component in a bioreactor can be determined gravimetrically (by dry weight, DNA or protein) or numerically (by number of cells). Doubling time means the period of time required for the doubling in the weight of biomass. Average doubling times increase with increasing cell size and complexity, e.g.: bacteria 0.25-1h; yeast 1-

2h; mould fungi 2-6,5h; plant cells 20-70h; and animal cells 15-48 h. In normal practice an organism seldom has ideal conditions for unlimited growth – growth often depends on a limiting factor, e.g. an essential nutrient. As the concentration of this factor drops so will the growth potential of the organism decrease. In biotechnological processes there are three main ways of growing microorganisms in the bioreactor, namely batch, semi-continuous or continuous.

In a **batch culture** the microorganisms are inoculated into a fixed volume of medium and as growth takes place nutrients are consumed and products of growth (biomass, metabolites) accumulate. The nutrient environment within the bioreactor is continuously changing and thus, in turn, enforcing changes to cell metabolism. Cell multiplication ceases because of exhaustion or limitation of nutrient(s) and accumulation of toxic excreted products. The initial lag phase is a time of no apparent growth, when the cells are adapted to the environmental conditions. Then there is a transient acceleration phase when the inoculum begins to grow and an exponential phase then quickly follows. In the exponential phase microbial growth proceeds at the maximum possible rate for that organism with excess of nutrients and absence of growth inhibitors. After the nutrient conditions change growth rate decreases, entering the deceleration phase and then the stationary phase is followed, when overall growth does not depend on nutrient concentration. The final phase is the death phase when growth rate has ceased. Most biotechnological processes are stopped before this stage because of decreasing metabolism and cell lysis. In industrial usage, batch cultivation has been operated to optimize organism or biomass production and then to allow the organism to perform specific biochemical transformations such as end-product formation (e.g. amino acids, enzymes) or decomposition of substances (sewage treatment, etc). Many important products such as antibiotics are formed during the stationary phase of the growth cycle in batch cultivation.

In contrast to batch conditions the practice of **continuous cultivation** gives near-balanced growth with little fluctuation of nutrients, metabolites, cell numbers or biomass. This practice depends on fresh medium entering a batch system at the exponential phase of growth with a corresponding withdrawal of medium and cells. Continuous methods of cultivation permit organisms to grow under unchanging conditions in which growth occurs at a constant rate and in a constant

environment. Factors such as pH and the concentrations of nutrients and metabolic products can be held near constant. In industrial practice continuously operated systems are of limited use and include only single cell protein and ethanol and some forms of waste-water treatment processes. The full range of cultivation methods for microorganisms is shown in Table 15.4.

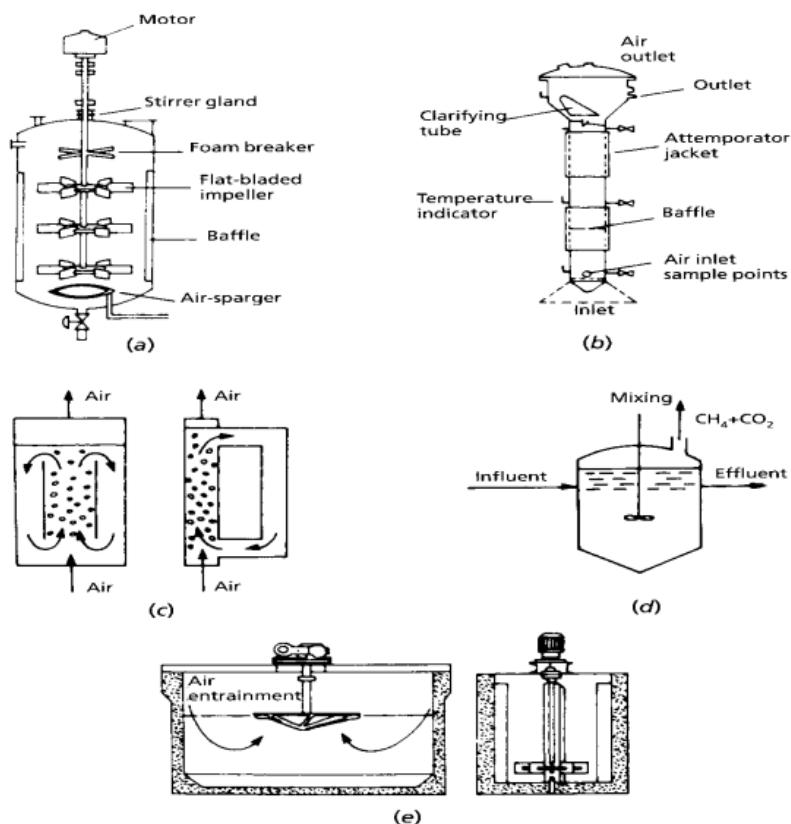
**Table 15.4 : Characteristics of Cultivation Methods**

Type of culture	Operation characteristics	Application
Solid	Simple, cheap, selection of colonies from single cell possible; process control limited	Maintenance of strains, genetic studies; production of enzymes; composting
Film	Various types of bioreactors	Waste-water treatment, monolayer culture (animal cells); bacterial leaching; vinegar production
Submerged homogeneous distribution of cells; batch	'Spontaneous' reaction, various types of reactors, agitation by stirred, air, liquid process control for physical parameters, less for chemical and biological parameters	Standard type of cultivation antibiotics, solvents, acids, etc.
Fed-batch	Simple method for control of regulatory effects, e.g. glucose repression	Production of baker's yeast
Continuous one stage homogeneous	Proper control of reaction; excellent for kinetic and regulatory studies; high cost for experiment; problems of aseptic operation; the need for highly trained operators	Few cases of application in industrial scale; production of single cell protein; wastewater treatment

### 15.5.3 The Bioreactor (Fermenter)

Bioreactors, which are used in biotechnological processes range from simple stirred or non-stirred open containers to complex, aseptic systems involving

varying levels of advanced computer inputs. Bioreactors are of two distinct types (Fig. 5). In the first type they are non-aseptic systems where it is impossible to operate with entirely pure cultures; in the second type they are aseptic systems allow producing antibiotics, vitamins, etc. In all forms of fermentation the main aim is to ensure that all parts of the system are subject to the same conditions. Within the bioreactor the microorganisms are suspended in the aqueous nutrient medium containing the necessary substrates for growth of the organism and required product formation. All nutrients, including oxygen, must be diffused into each cell, and waste products, such as heat, CO<sub>2</sub> and waste metabolites must be removed.



(a) Continuous stirred tank bioreactor (b) Tower bioreactor (c) Loop (recycle) bioreactor  
 (d) Anaerobic digester or bioreactor (e) Activated sludge bioreactor

**Fig 15.5 : Various forms of Bioreactor**

The concentration of the nutrients must be held within a definite range, since low values will limit the rate of organism metabolism whereas excessive concentrations can be toxic. Fermentation reactions are multiphase, involving a gas phase

(containing N<sub>2</sub>, O<sub>2</sub> and CO<sub>2</sub>), one or more liquid phases (aqueous medium and liquid substrate) and solid phase (the microorganisms and possibly solid substrates). All phases must be kept in close contact to achieve rapid mass and heat transfer. To achieve optimization of the bioreactor system it must be necessary adhered to the following guidelines:

1. The bioreactor should be designed to exclude entrance of contaminating organisms
2. The culture volume should remain constant, i.e. no leakage or evaporation
3. The dissolved oxygen level must be maintained above critical levels of aeration and culture agitation for aerobic organisms
4. Environmental parameters such as temperature, pH, etc. must be controlled; and the culture volume must be well mixed.
5. The standard of materials used in the construction of sophisticated fermenters is also important.

### **Standards of materials used in fermenter design**

All materials contacting with the solutions or organism culture in the bioreactor must be corrosion resistant.

- The materials must be non-toxic so that do not inhibit culture growth.
- The materials of bioreactor must withstand repeated sterilization with high pressure steam.
- The bioreactor stirrer system, entry ports and end plates must not be deformed or broken under mechanical stress.
- Transparent materials should be used wherever possible to visual inspection of the medium and culture

#### **15.5.4 Media Design for Fermentation Processes**

Water is at the centre of all biotechnological processes and in most cases is the dominant component of the media in which microorganisms grow. The quality of water is highly relevant because it affects microbial growth and the production of specific bioproducts. The basic nutritional requirements of microorganisms are an energy or carbon source, an available nitrogen source, inorganic elements and, for some cell types, specific growth factors. In most biotechnological processes carbon and nitrogen sources are more often derived from relatively complex mixtures of

cheap natural products or by-products (Table 15.5). Availability and type of nutrient can exert strong physiological control over fermentation. Raw material input to fermentation will be dependent on the cost of the material.

Sterilization practices for biotechnological media must achieve maximum kill of contaminating microorganisms, with minimum temperature damage to medium components. The media preparation is one of the important parts of the overall bioprocess leading to high efficiency of growth and concomitant rich product formation.

**Table 15.5 : Sources of Carbohydrate and Nitrogen for Industrial Media**

Sources of carbohydrate	Form	Sources of nitrogen (% nitrogen by weight)
Glucose	Glucose monohydrate, Hydrolyzed starch	Barley (1,5-2,0), beet molasses (1,5-2.0), corn-steep liquor (4,5),
Starch	Pure lactose, whey powder	groundnut meal (8,0), oat flour (1,5-2,0), rye flour (1,5-2,0), soya bean meal (8,0), whey powder (4,5)
Lactose	Barley, groundnut meal, oat flour, soya bean meal, rye flour	
Sucrose	Beet molasses, cane molasses, crude and pure sugar	

### 15.5.5 Solid Substrate Fermentation

There are many biotechnological processes that involve the growth of microorganisms on solid substrates in the absence or near absence of free water (Table 4.6). The most regularly used solid substrates are cereal grains, legume seeds, wheat bran, lignocellulose materials such as straws, sawdust, and a wide range of plant and animal materials. Most of these compounds are polymeric molecules, insoluble or sparingly soluble in water, but are mostly cheap, easily obtainable and represent a concentrated source of nutrients for microbial growth.

**Table 15.6 : Some examples of Solid Substrate Fermentations**

Example	Substrate	Microorganism(s) involved
Mushroom	Straw, manure	<i>Agaricus bisporus</i>
Sauerkraut	Cabbage	Lactic acid bacteria
Soy sauce	Soya beans and wheat	<i>Aspergillus oryzae</i>

Tempeh	Soya beans	<i>Rhizopus oligosporus</i>
Ontjom	Peanut press cake	<i>Neurospora sitophila</i>
Cheeses	Milk curd	<i>Penicillium roquefortii</i>
Leaching of metals	Low grade ores	<i>Thiobacillus sp.</i>
Organic acids	Cane sugar, molasses	<i>Aspergillus niger</i>
Enzymes	Wheat bran, etc	<i>Aspergillus niger</i>
Composting	Mixed organic materials	Fungi, bacteria, actinomycetes
Sewage treatment	Components of sewage	Bacteria, fungi and protozoa

Many of these fermentations have great antiquity and in many instances there are records dating back hundreds of years. In the Orient there is a wide array of food fermentations including soy sauce and tempeh as well as many large industrial enzyme processes. In the West, the fermentation processes have centered on the production of silage, mushroom cultivation, cheese and sauerkraut production, and the composting of plant and animal wastes. The microbial components of solid substrate fermentations can occur as single pure cultures, mixed identifiable cultures or totally mixed indigenous microorganisms. Bioreactors designs for solid substrate fermentations are simpler than for liquid cultivations. They are classified into fermentations (a) without agitation, (b) with occasional agitation, and (c) with continuous agitation.

## 15.6 Summary

Transformation discovered by English scientist Frederick Griffiths in 1928, which is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s). The genetic engineering cause artificial transformation and offers unlimited opportunities for creating new combinations of genes which leads to the development or improvement of strains and can be used by several industries. Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria (transformed bacteria). In addition genetic manipulations are used to produce microorganisms with new and desirable characteristics. Genetic modification can be achieved by: Mutation and recombination and genetic engineering, which leads to development of new strains.

In addition fermentation technology is a part of biotechnological process, which is performed within containment systems or bioreactors. Large numbers of cells are involved in these processes and the bioreactor ensures their close involvement with correct medium and conditions for growth and product formation.

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## 15.7 Glossary

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- **Transformation:** It is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s)
  - **Transfection:** Introduction of foreign DNA into eukaryotic cells is often called "transfection".
  - **Ampicillin:** It is a member of the penicillin family of antibiotics. Ampicillin and the other penicillins prevent the formation of the bacterial cell walls which leads the killing of the bacteria.
  - **GFP:** It stands for Green Fluorescent Protein, and the GFP gene is from a bioluminescent jellyfish, *Aequorea victoria*.
  - **$\beta$ -galactosidase:** It is an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.
  - **Recombination:** It is a process of creating new combination of genes in given organisms.
  - **Conjugation:** In this process the genetic information of one bacterium is transferred to other via cell to cell contact.
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## 15.8 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Name the any two selectable markers.
2. What is conjugation?
3. Name the biological source of GFP.
4. Who discovered the transformation?

### Section B : (Short Answer Type Questions)

1. What is transformation?

- 2 Define the vector.
3. Describe the blue white screening.
4. What is bioreactor?

### **Section C : (Long Answer Type Questions)**

1. Give a detail account bacterial transformation.
2. Describe the role of genetic engineering in strain improvement.
3. Describe the fermentation technology in detail.

### **Answer Key of Section-A**

1. beta-galactosidase and beta-lactamase
2. Conjugation: In this process the genetic information of one bacterium is transferred to other via cell to cell contact.
3. *Aequorea victoria*
4. Frederick Griffiths

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## **15.9 References**

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## Unit - 16

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# Genomics

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### Structure of the Unit:

- 16.0 Objectives
  - 16.1 Introduction
  - 16.2 Genome
  - 16.3 Genomics
  - 16.4 Plant Genomics
  - 16.5 Genomics Research
  - 16.6 Practical Applications of Genomics
  - 16.7 Summary
  - 16.8 Glossary
  - 16.9 Self-Learning Exercise
  - 16.10 References
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## 16.0 Objectives

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After studying this unit you will understand about –

- Genome, Genomics, Plant Genomics,
  - Genomic Research and its Practical Applications.
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## 16.1 Introduction

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Genomics is a branch of Genetics that concerns the sequencing and analysis of an organism's genome. The genome is the entire DNA content that is present within one cell of an organism. It determines complete DNA sequences and perform genetic mapping to help understand disease. The term genomics was first coined in 1986 by Tom Roderick, a geneticist at the Jackson Laboratory in Maine, during a meeting about the mapping of the human genome.

Some biological entities have additional genetic material besides they reside in their chromosomes. "Genome" stores information in auxiliary material, which is carried in plasmids. It describes all of the genes and information on non-coding DNA that have the potential to be present. In plants, protozoans and animals, "genome" carries the typical association of only information on chromosomal DNA. Although these organisms contain chloroplasts or mitochondria that have their own DNA, the genetic information contained by DNA within these organelles is not considered part of the genome. However, mitochondria have their own genome and referred as the "mitochondrial genome". The DNA found within the chloroplast may be referred to as the "plastome".

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## 16.2 Genome

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Genome is the genetic material of an organism or it is an organism's complete set of DNA, including all of its genes. It contains all necessary information needed to build and maintain that organism. In humans, a copy of the entire genome more than 3 billion DNA base pairs is contained in all cells that have a nucleus. It is encoded either in DNA or, for RNA viruses, in RNA. The genome includes both the genes and the non-coding sequences of the DNA/RNA. Some organisms have multiple copies of chromosomes: diploid, triploid, tetraploid and so on. In classical genetics, in a sexually reproducing organism the gamete has half the number of chromosomes of the somatic cell and the genome is a full set of chromosomes in a diploid cell. The halving of the genetic material in gametes is accomplished by the segregation of homologous chromosomes during meiosis. In haploid organisms, including cells of bacteria, archaea, and in organelles including mitochondria and chloroplasts, or viruses, that similarly contain genes, the single or set of circular or linear chains of DNA (or RNA for some viruses), likewise constitute the genome. The term genome can be applied specifically to mean what is stored on a complete set of nuclear DNA (i.e., the "nuclear genome") but can also be applied to what is stored within organelles that contain their own DNA, as with the "mitochondrial genome" or the "chloroplast genome". Additionally, the genome can comprise non-chromosomal genetic elements such as viruses, plasmids, and transposable elements.

## Genome compositions

Genome composition constitutes haploid genome, which includes genome size, proportions of non-repetitive DNA and repetitive DNA. Comparison of genome compositions between genomes helps in understand the evolutionary history of a given genome. In prokaryotes, most of the genome (85–90%) is non-repetitive DNA, which means coding DNA mainly forms it, while non-coding regions only take a small part. Whereas, eukaryotes have the feature of exon-intron organization of protein coding genes; the variation of repetitive DNA content in eukaryotes is also extremely high.

### Genome size

Genome size is the total number of DNA base pairs in one copy of a haploid genome. The genome size is positively correlated with the morphological complexity among prokaryotes and lower eukaryotes; however, in higher eukaryotes, this correlation is no longer effective. This phenomenon also indicates the mighty influence coming from repetitive DNA act on the genomes.

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## 16.3 Genomics

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Genomics involves the study of intragenomic processes such as epistasis, heterosis and pleiotropy as well as the interactions between loci and alleles within the genome. The fields of molecular biology and genetics are mainly concerned with the study of the role and function of single genes.

Genomics is a concept that was first developed by Fred Sanger who first sequenced the complete genome of a virus and of a mitochondrion. He initiated the practice of sequencing and genome mapping as well as developing bioinformatics and data storage. The knowledge about genes that has so far been gathered has led to the emergence of functional genomics, a field concerned with trying to understand the pattern of gene expression, especially across different environmental conditions.

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## 16.4 Plant Genomics

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Plant genomics is a growing and constantly evolving field of study, which includes methods and protocols, expert researchers explore the current issues and methodologies of this expanding field, specifically addressing areas of gene

discovery and the functional analysis of genes with a focus on the primary tools and sub-disciplines of genetic mapping, mRNA, protein and metabolite profiling.

The molecular analysis of plants often focused on the single gene level. Genomic approaches are permeating every aspect of plant biology, and since they rely on DNA-coded information, they expand molecular analyses from a single to a multispecies level. Plant genomics is reversing the previous paradigm of identifying genes behind biological functions and instead focuses on finding biological functions behind genes. It also reduces the gap between phenotype and genotype and helps to comprehend not only the isolated effect of a gene, but also the way its genetic context and the genetic networks it interacts with can modulate its activity. Plant genomes are described in terms of genome size, gene content, extent of repetitive sequences and polyploidy/duplication events. Although plants are also possess mitochondrial and chloroplast genomes, their nuclear genome is the largest and most complex. Plant genomes contain various repetitive sequences and retrovirus-like retrotransposons containing long terminal repeats and other retro elements, such as long interspersed nuclear elements and short-interspersed nuclear elements. Retro element insertions contribute to the large difference in size between collinear genome segments in different plant species.

Almost many economically important plant species, such as corn, wheat, potato, and oat are either ancient or more recent polyploidy, comprising more than one, and in cases such as wheat, three different homologous genomes within a single species. Duplicated segments also account for a significant fraction of the rice genome. About 60% of the *Arabidopsis* genome is present in 24 duplicated segments, each more than 100 kilobases (kb) in size. Ancestral polyploidy contributes to create genetic variation through gene duplication and gene silencing. Genome duplication and subsequent divergence is an important generator of protein diversity in plants.

### **Model plant species**

Model organisms (*Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*) provide genetic and molecular insights into the biology of more complex species. Since the genomes of most plant species are either too large or too complex to be fully analyzed, the plant scientific community has adopted model organisms. They share features such as being diploid and

appropriate for genetic analysis, being amenable to genetic transformation, having a (relatively) small genome and a short growth cycle, having commonly available tools and resources, and being the focus of research by a large scientific community. Although the advent of tissue culture techniques fostered the use of tobacco and petunia, the species now used as model organisms for mono- and dicotyledonous plants are rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*) respectively.

Arabidopsis is a small Cruciferae plant without agricultural use sets seed in only 6 weeks from planting, has a small genome of 120 Mega bases (Mb) and only five chromosomes. There are extensive tools available for its genomic analysis, whole genome sequence, Expressed Sequence Tags (ESTs) collections, characterized mutants and large populations mutagenized with insertion elements (transposons or the T-DNA of *Agrobacterium*). Arabidopsis can be genetically transformed on a large scale with *Agrobacterium tumefaciens* and biolistics. Other tools available for this model plant are saturated genetic and physical maps.

Unlike Arabidopsis, rice is one of the world's most important cereals. More than 500 million tons of rice is produced each year, and it is the staple food for more than half of the World's population. There are two main rice subspecies. Japonica is mostly grown in Japan, while indica is grown in China and other Asia-Pacific regions. Rice also has very saturated genetic maps, physical maps, whole genome sequences, as well as EST collections pooled from different tissues and developmental stages. It has 12 chromosomes, a genome size of 420 Mb, and like Arabidopsis, it can be transformed through biolistics and *A. tumefaciens*. Although, efficient transposon-tagging systems for gene knockouts and gene detection have not yet become available for saturation mutagenesis in rice, however some recent successes have been reported.

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## 16.5 Genomic Research

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Genomics is a branch of Science which includes studying genome at molecular, chromosomal, biochemical and phenotypic levels. The advent of these technologies resulted in a rapid amplification in the scope and speed of completion of projects. First of all in 1972, Walter Fieres and workers of University of Ghent, Belgium determine the sequence of gene. In 1977, first DNA based genome of

Bacteriophage OX174 (5368 bp) was sequenced. The first complete genome sequence of a eukaryotic organelle, the human mitochondrion (16,568 bp, about 16.6 kb kilobase), was reported in 1981, and the first chloroplast genomes followed in 1986. In 1992, the first eukaryotic chromosome, chromosome III of brewer's yeast *Saccharomyces cerevisiae* (315 kb) was sequenced. The first free-living organism to be sequenced was that of *Haemophilus influenzae* (1.8 Mb: megabase) in 1995. The following year a consortium of researchers from laboratories across North America, Europe, and Japan announced the completion of the first complete genome sequence of a eukaryote, *S. cerevisiae* (12.1 Mb), and since then genomes have continued being sequenced at an exponentially growing pace. As of October 2011, the complete sequences are available for: 2,719 viruses, 1,115 archaea and bacteria, and 36 eukaryotes, of which about half are fungi.

The number of genome projects has increased as technological improvements continue to lower the cost of sequencing. (A) Exponential growth of genome sequence databases since 1995. (B) The cost in US Dollars (USD) to sequence one million bases. (C) The cost in USD to sequence a 3,000 Mb (human-sized) genome on a log-transformed scale.

### **Functional genomics**

Functional genomics is a field of molecular biology that attempts to make use of the vast wealth of data produced by genomic projects (such as genome sequencing projects) to describe gene functions and interactions. Functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein–protein interactions, as opposed to the static aspects of the genomic information such as DNA sequence or structures. Moreover it helps in functioning of DNA at gene level, RNA transcripts, and protein products. Besides, it involved in high-throughput methods rather than a more traditional “gene-by-gene” approach.

### **Structural genomics**

Structural genomics involves taking a large number of approaches to structure determination, including experimental methods using genomic sequences or modeling-based approaches based on sequence or structural homology to a protein of known structure or based on chemical and physical principles for a protein with no homology to any known structure. It seeks to describe the 3-dimensional

structure of every protein encoded by a given genome. This genome-based approach allows for a high-throughput method of structure determination by a combination of experimental and modeling approaches.

### **Epigenomics**

Epigenomics is the study of the complete set of epigenetic modifications on the genetic material of a cell, known as the epigenome. Epigenetic modifications are reversible modifications on a cell's DNA or histones that affect gene expression without altering the DNA sequence. Two of the most characterized epigenetic modifications are DNA methylation and histone modification. Epigenetic modifications play an important role in gene expression and regulation, and are involved in numerous cellular processes such as in differentiation/development and tumorigenesis. The study of epigenetics on a global level has been made possible only recently through the adaptation of genomic high-throughput assays.

### **Metagenomics**

Metagenomics is the study of metagenomes, genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. While traditional microbiology and microbial genome sequencing rely upon cultivated clonal cultures, early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of diversity in a natural sample.

### **Genome analysis**

Genome analysis projects involve three components: the sequencing of DNA, the assembly of that sequence to create a representation of the original chromosome, and the annotation and analysis of that representation.

#### **1. DNA Sequencing**

DNA sequencing was done in sequencing centers, centralized facilities which contain research laboratories with the costly instrumentation and technical support necessary. As sequencing technology continues to improve, however, a new generation of effective fast turnaround bench top sequencers has come within reach of the average academic laboratory.

## 2. Shotgun sequencing

Shotgun sequencing is a sequencing method designed for analysis of DNA sequences longer than 1000 base pairs, up to and including entire chromosomes. It is named by analogy with the rapidly expanding, quasi-random firing pattern of a shotgun. Since the chain termination method of DNA sequencing can only be used for fairly short strands (100 to 1000 basepairs), longer DNA sequences must be broken into random small segments which are then sequenced to obtain reads. Multiple overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. Shotgun sequencing is a random sampling process, requiring over-sampling to ensure a given nucleotide is represented in the reconstructed sequence; the average number of reads by which a genome is over-sampled is referred to as coverage.

## 3. High-throughput sequencing

The high demand for low-cost sequencing has driven the development of high-throughput sequencing technologies that parallelize the sequencing process, producing thousands or millions of sequences at once. High-throughput sequencing technologies are intended to lower the cost of DNA sequencing beyond what is possible with standard dye-terminator methods. In ultra-high-throughput sequencing as many as 500,000 sequencing-by-synthesis operations may be run in parallel.

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## 16.6 Practical Applications of Genomics

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Genomics play important roles in many fields, including medicine, biotechnology, anthropology and other social sciences. Genome sequence data provide tools for the development of practical uses for genetic information. DNA is an invaluable tool in forensics because - aside from identical twins - every individual has a uniquely different DNA sequence. Repeated DNA sequences in the human genome are sufficiently variable among individuals that they can be used in human identity testing. The FBI uses a set of thirteen short tandem repeat (STR) DNA sequences for the Combined DNA Index System (CODIS) database, which contains the DNA fingerprint or profile of convicted criminals. Investigators of a crime scene can use this information in an attempt to match the DNA profile of an unknown sample to

a convicted criminal. DNA fingerprinting can also identify victims of crime or catastrophes, as well as many family relationships, such as paternity.

### **(i) Genomic medicine**

The basis of many diseases is the alteration of one or more genes. Testing for such diseases requires the examination of DNA from an individual for some change that is known to be associated with the disease. Genomics involves study of many diseases, Sickle cell anemia, cystic fibrosis; Huntington's disease caused by abnormalities in DNA sequence that code for specific proteins. Sometimes the change is easy to detect, such as a large addition or deletion of DNA, or even a whole chromosome. Many changes are very small, whereas, other changes can affect the regulation of a gene and result in too much or too little of the gene product. In most cases if a person inherits only one mutant copy of a gene from a parent, then the normal copy is dominant and the person does not have the disease; however, that person is a carrier and can pass the disease on to offspring. Next-generation genomic technologies allow clinicians and biomedical researchers to drastically increase the amount of genomic data collected on large study populations. When combined with new informatics approaches that integrate many kinds of data with genomic data in disease research, allowing researchers to better understand the genetic bases of drug response and disease.

### **(ii) Synthetic biology and bioengineering**

The growth of genomic knowledge has enabled increasingly sophisticated applications of synthetic biology. In 2010 researchers at the J. Craig Venter Institute announced the creation of a partially synthetic species of bacterium, *Mycoplasma laboratorium*, derived from the genome of *Mycoplasma genitalium*.

### **(iii) Gene therapy**

Gene therapy is used for treatment or cures a disease by providing a normal copy of the individual's mutated gene. The first step in gene therapy is the introduction of the new gene into the cells of the individual. This must be done using a vector (a gene carrier molecule), which can be engineered in a test tube to contain the gene of interest. Viruses are the most common vectors because they are naturally able to invade the human host cells. These viral vectors are modified so that they can no longer cause a viral disease.

#### (iv) Synthetic biology and bioengineering

Genomics involves the study of intragenomic processes such as epistasis, heterosis and pleiotropy as well as the interactions between loci and alleles within the genome.

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### 16.7 Summary

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Genomics is the study of entire genome, usually starting with whole genome sequencing. It employs exciting new methods to investigate molecular plant breeding technology and gene functional analysis via transformation, mutation, protein function, and gene expression. Composed in the highly successful Methods in Plant Genomics methods and protocols are essential guide for all plant scientists for further studies in the area of genomics research. The fields of molecular biology and genetics are mainly concerned with the study of the role and function of single genes. By contrast, genomics does not involve single gene research unless the purpose is to understand a single gene's effects in context of the entire genome. Genome sequence data provide tools for the development of practical uses for genetic information. DNA is an invaluable tool in forensics because - aside from identical twins - every individual has a uniquely different DNA sequence. Repeated DNA sequences in the human genome are sufficiently variable among individuals that they can be used in human identity testing.

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### 16.8 Glossary

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- **Genome** : is the genetic material of an organism or it is an organism's complete set of DNA, including all of its genes.
- **Genomics** : involves the study of intragenomic processes such as epistasis, heterosis and pleiotropy as well as the interactions between loci and alleles within the genome. Or Genomics is a branch of Science which includes studying genome at molecular, chromosomal, biochemical and phenotypic levels.
- **Epigenomics** : is the study of the complete set of epigenetic modifications on the genetic material of a cell, known as the epigenome.
- **Gene therapy** : is used for treatment or cures a disease by providing a normal copy of the individual's mutated gene.

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## 16.9 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Genome is a \_\_\_\_\_.
2. Epigenomics is the study of \_\_\_\_\_.
3. Name two model plant species of Genomic research?
4. Genome project involves \_\_\_\_\_.
5. Genomics is the study of \_\_\_\_\_.

### Section B : (Short Answer Type Questions)

1. Describe the Functional genomics.
2. Explain Gene therapy.

### Section C : (Long Answer Type Questions)

1. Write an essay on Genomics.
2. Describe the advantages of genomic research areas?

### Answer key of Section – A

1. Genetic material of organism.
2. Study of the complete set of epigenetic modifications on the genetic material of a cell.
3. Arabidopsis sps , Rice
4. DNA sequencing, representation and annotation of chromosomes.
5. Branch of Genetics involve in sequencing and analysis of an organisms genome.

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## 16.9 References

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- P K Gupta - Genetic
- Gene IV,IX
- Alberts - Molecular biology of cell

## Unit - 17

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# Proteomics

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### Structure of the Unit:

- 17.0 Objectives
- 17.1 Introduction
- 17.2 Proteomics
- 17.3 Branches of Proteomics
- 17.4 Plant Proteomics
- 17.5 Proteomic Research
- 17.6 Practical Applications of Proteomic
- 17.7 Summary
- 17.8 Glossary
- 17.9 Self-Learning Exercise
- 17.10 References

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## 17.0 Objectives

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After studying this unit you will understand about –

- Proteomics, Plant Proteomics,
- Proteomic Research and its Practical Applications.

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## 17.1 Introduction

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Proteomics is the large-scale study of proteins, particularly their structures and functions. Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. The proteome is the complete set of proteins produced by the genome at any one time. The proteome is much more complex than either the genome or the transcriptome. This is because each protein can be chemically modified in different ways after synthesis. This

varies with time and distinct requirements, or stresses, that a cell or organism undergoes.

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## 17.2 Proteomics

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The term proteomics was first coined in 1997 to make an analogy with genomics, the study of the genome. The word proteome is a portmanteau of protein and genome, and was coined by Marc Wilkins in 1994. Proteomics is an interdisciplinary domain formed on the basis of the research and development of the Human Genome Project; it is also emerging scientific research and exploration of proteomes from the overall level of intracellular protein composition, structure, and its own unique activity patterns. It is an important component of functional genomics. The proteome is also very dynamic. Most of our cells contain the same genome regardless of the cell type, developmental stage or environmental conditions. The proteome, however, varies considerably in these differing circumstances due to different patterns of gene expression and different patterns of protein modification.

Proteomics defined as the study of the proteome: the collection of all the proteins expressed from the genome in all its forms, polymorphisms and post-translational modifications (PTMs). Broad-based proteomics is a strategy wherein disease or physiological models are analyzed with the most well-suited proteomics technologies to reveal changes or differences in protein make-up between the experimental condition and controls. This approach allows for the optimal discovery of the changes which define the model system. Screening for the maximum number of protein changes limits experimental bias by not excluding groups of proteins, therefore making it imperative to use the most appropriate technology for the biological system and hypothesis being addressed.

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## 17.3 Branches of Proteomics

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Proteomics covers a number of different aspects of protein function, including the following:

### (i) Structural proteomics

This is the large-scale analysis of protein structures. Protein structure comparisons can help to identify the functions of newly discovered genes. Structural analysis

can also show where drugs bind to proteins and where proteins interact with each other. This is achieved using technologies such as X-ray crystallography and NMR spectroscopy.

### **(ii) Expression proteomics**

The large-scale analysis of protein expression is known as Expression proteomics. This can help to identify the main proteins found in a particular sample and proteins differentially expressed in related samples, such as diseased vs. healthy tissue. A protein found only in a diseased sample may represent a useful drug target or diagnostic marker. Proteins with similar expression profiles may also be functionally related. Technologies such as 2D-PAGE and mass spectrometry are used here.

### **(iii) Interaction proteomics**

The large-scale analysis of protein interactions .The characterization of protein-protein interactions helps to determine protein functions and can also show how proteins assemble in larger complexes. Technologies such as affinity purification, mass spectrometry and the yeast two-hybrid system are particularly useful.

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## **17.4 Plant Proteomics**

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Proteomics has greatly advanced our understanding of proteome composition, modulation and modification in model plant systems, and crop research. Proteomics approaches have also been used to characterize proteome modulation during plant development processes as well as to profile proteomes of plant organs. In order to understand and modelize development processes, it is particularly important to access complete proteome profile (i.e. high proteome coverage) and in some cases to perform proteome profiling of specific cell types. Both aspects are still limited in plant proteomics, even for model plant species. A number of descriptive studies have characterized proteomes and phosphoproteomes of cell dedifferentiation phloem, plant organs including leaves, stems, internodes, roots, pollen and seeds.

Although significant advances in the comprehensive profiling, functional analysis, and regulation of proteins has occurred in model organisms such as yeast (*Saccharomyces cerevisiae*) and in humans, proteomics research in plants has not

advanced at the same pace. The availability of the complete Arabidopsis (*Arabidopsis thaliana*) genome, which is small compared to that of other plants, along with an increasingly comprehensive catalog of protein-coding information from large-scale cDNA sequencing and transcript mapping experiments, set it apart as a complex but accessible model organism to study plant proteomics.

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## 17.5 Proteomic Research

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Proteomics has recently come into the act as a promising force to transform biology and medicine. It is becoming increasingly apparent that changes in mRNA expression correlate poorly with protein expression changes. Proteins change enormously in patterns of expressions across developmental and physiological responses. Proteins also face changes on the act of environmental perturbations. Proteins are the actual effectors driving cell behavior. The field of proteomics strives to characterize protein structure and function, protein-protein, protein-nucleic acid, protein-lipid, and enzyme-substrate interactions, protein processing and folding, protein activation, cellular and sub-cellular localization, protein turnover and synthesis rates, and even promoter usage. Integrating proteomic data with information such as gene, mRNA and metabolic profiles helps in better understanding of how the system works.

### **Protein modification**

Almost all proteins are modified from their pure translated amino-acid sequence, so-called post-translational modification. Specialized methods have been developed to study phosphorylation (phosphoproteomics) and glycosylation (glycoproteomics).

### **Cellular proteomics**

Cellular proteomics is a new branch of proteomics that goal is to map the location of proteins and protein-protein interactions in whole cells during key cell events. Centers are around the use of techniques such as X-ray Tomography and optical fluorescence microscopy.

## Experimental bioinformatics

A branch of bioinformatics, as it is applied in proteomics, coined by Mathias Mann. It involves the mutual design of experimental and bioinformatics methods to create (extract) new types of information from proteomics experiments.

Proteomics has steadily gained momentum over the past decade with the evolution of several approaches. Few of these are new and others build on traditional methods. Mass spectrometry-based methods and micro arrays are the most common technologies for large-scale study of proteins. There are two mass spectrometry-based methods currently used for protein profiling. The more established and widespread method uses high resolution, two-dimensional electrophoresis to separate proteins from different samples in parallel, followed by selection and staining of differentially expressed proteins to be identified by mass spectrometry. Despite the advances in 2DE and its maturity, it has its limits as well. The central concern is the inability to resolve all the proteins within a sample, given their dramatic range in expression level and differing properties.

The second quantitative approach uses stable isotope tags to differentially label proteins from two different complex mixtures. Here, the proteins within a complex mixture are labeled first isotopically, and then digested to yield labeled peptides. The labeled mixtures are then combined, the peptides separated by multidimensional liquid chromatography and analyzed by tandem mass spectrometry. Isotope coded affinity tag (ICAT) reagents are the widely used isotope tags. In this method, the cysteine residues of proteins get covalently attached to the ICAT reagent, thereby reducing the complexity of the mixtures omitting the non-cysteine residues.

Quantitative proteomics using stable isotopic tagging is an increasingly useful tool in modern development. Firstly, chemical reactions have been used to introduce tags into specific sites or proteins for the purpose of probing specific protein functionalities. The isolation of phosphorylated peptides has been achieved using isotopic labeling and selective chemistries to capture the fraction of protein among the complex mixture. Secondly, the ICAT technology was used to differentiate between partially purified or purified macromolecular complexes such as large RNA polymerase II pre-initiation complex and the proteins complexed with yeast transcription factor. Thirdly, ICAT labeling was recently combined with chromatin

isolation to identify and quantify chromatin-associated proteins. Finally ICAT reagents are useful for proteomic profiling of cellular organelles and specific cellular fractions.

Another quantitative approach is the Accurate Mass and Time (AMT) tag approach developed by Richard D. Smith and coworkers at Pacific Northwest National Laboratory. In this approach, increased throughput and sensitivity is achieved by avoiding the need for tandem mass spectrometry, and making use of precisely determined separation time information and highly accurate mass determinations for peptide and protein identifications.

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## 17.6 Applications of Proteomics

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One major development to come from the study of human genes and proteins has been the identification of potential new drugs for the treatment of disease. This relies on genome and proteome information to identify proteins associated with a disease, which computer software can then use as targets for new drugs. For example, if a certain protein is implicated in a disease, its 3D structure provides the information to design drugs to interfere with the action of the protein. A molecule that fits the active site of an enzyme, but cannot be released by the enzyme, inactivates the enzyme. This is the basis of new drug-discovery tools, which aim to find new drugs to inactivate proteins involved in disease. As genetic differences among individuals are found, researchers expect to use these techniques to develop personalized drugs that are more effective for the individual.

Proteomics is also used to reveal complex plant-insect interactions that help identify candidate genes involved in the defensive response of plants to herbivory.

Proteome analysis of *Arabidopsis* peroxisomes has been established as the major unbiased approach for identifying new peroxisomal proteins on a large scale. There are many approaches to characterizing the human proteome, which is estimated to contain between 20,000 and 25,000 non-redundant proteins. The number of unique protein species will likely increase by between 50,000 and 500,000 due to RNA splicing and proteolysis events, and when post-translational modification are also considered, the total number of unique human proteins is estimated to range in the low millions.

**(i) Proteomics as protein biomarkers**

Biomarkers of drug efficacy and toxicity are becoming a key need in the drug development process. Mass spectral-based proteomic technologies are ideally suited for the discovery of protein biomarkers in the absence of any prior knowledge of quantitative changes in protein levels. The success of any biomarker discovery effort will depend upon the quality of samples analyzed, the ability to generate quantitative information on relative protein levels and the ability to readily interpret the data generated.

**(ii) Proteomics in Tumor Metastasis**

Tumor metastasis is the dominant cause of death in cancer patients. However, the molecular and cellular mechanisms underlying tumor metastasis are still elusive. The identification of protein molecules with their expressions correlated to the metastatic process would help to understand the metastatic mechanisms and thus facilitate the development of strategies for the therapeutic interventions and clinical management of cancer. Proteomics is a systematic research approach aiming to provide the global characterization of protein expression and function under given conditions. Proteomic technology has been widely used in biomarker discovery and pathogenetic studies including tumor metastasis. The combination of proteomics with other experimental approaches in biochemistry, cell biology, molecular genetics and chemistry, together with the development of new technologies and improvements in existing methodologies will continue to extend its application in studying cancer metastasis.

**(iii) Proteomics in renal disease diagnosis**

Proteomics is widely envisioned as playing a significant role in the translation of genomics to clinically useful applications, especially in the areas of diagnostics and prognostics. In the diagnosis and treatment of kidney disease, a major priority is the identification of disease-associated biomarkers. Proteomics, with its high-throughput and unbiased approach to the analysis of variations in protein expression patterns (actual phenotypic expression of genetic variation), promises to be the most suitable platform for biomarker discovery. Combining such classic analytical techniques as two-dimensional gel electrophoresis with more sophisticated techniques, such as MS, has enabled considerable progress to be

made in cataloguing and quantifying proteins present in urine and various kidney tissue compartments in both normal and diseased physiological states.

#### **(iv) Proteomics in Neurology**

Proteomics have been widely adopted in most areas of biology and medicine. In neurology and neuroscience, many applications of proteomics have involved neurotoxicology and neurometabolism, as well as in the determination of specific proteomic aspects of individual brain areas and body fluids in neurodegeneration. Investigation of brain protein groups in neurodegeneration, such as enzymes, cytoskeleton proteins, chaperones, synaptosomal proteins and antioxidant proteins, is in progress as phenotype related proteomics. The concomitant detection of several hundred proteins on a gel provides sufficiently comprehensive data to determine a path physiological protein network and its peripheral representatives. The rapid spread of proteomics technology, which principally consists of two-dimensional gel electrophoresis (2-DE) with in-gel protein digestion of protein spots and identification by mass spectrometry, has provided an explosive amount of results.

#### **(v) Proteomics to fetal and maternal medicine**

The recent elucidation of the human genome sequence has provided a wealth of useful information but does not provide information on diseases caused by changes at the protein level. Proteomics includes the characterization and functional analysis of all proteins that are expressed by the genome at a certain moment, under certain conditions. Since expression levels of many proteins strongly depend on complex, but well-balanced regulatory systems, the proteome, unlike the genome, is highly dynamic. This variation depends on the biological function of a cell, but also on signals from its environment. In (bio) medical research it has become increasingly apparent that cellular processes, in particular in disease, are determined by multiple proteins. Hence it is important not to focus on one single gene product (one protein), but to study the complete set of gene products (the proteome). In this way the multi-factorial relations underlying certain diseases may be unraveled potentially identifying therapeutically targets. For many diseases characterization of the functional proteome is crucial for elucidating alterations in protein expression and modifications. When proteins undergo non-genetically determined alterations such as alternative splicing, or post-translational

modifications, e.g. phosphorylation or glycosylation, it may affect their function. Although abnormalities in splicing or post-translational modifications can cause a disease process, they can also be a consequence. An example is that patients with diabetes have high blood glucose which glycosylates hundreds or even thousands of proteins, including HbA1c which is used to monitor diabetic control.

#### **(vi) Proteomics in Urological Cancer Research**

Proteomic analysis allows the comparison of the proteins present in a diseased tissue sample with the proteins present in a normal tissue sample. Any proteins, which have been altered either quantitatively or qualitatively between the normal and diseased sample are likely to be associated with the disease process. These proteins can be identified and may be useful as diagnostic markers for the early detection of the disease or prognostic markers to predict the outcome of the disease or they may be used as drug targets for the development of new therapeutic agents.

#### **(vii) Proteomics in Autoantibody profiling**

Proteomics technologies enable profiling of autoantibody responses using biological fluids derived from patients with autoimmune disease. They provide a powerful tool to characterize auto reactive B-cell responses in diseases including rheumatoid arthritis, multiple sclerosis, autoimmune diabetes, and systemic lupus erythematosus. Autoantibody profiling may serve purposes including classification of individual patients and subsets of patients based on their 'autoantibody fingerprint', examination of epitope spreading and antibody isotype usage, discovery and characterization of candidate auto antigens, and tailoring antigen-specific therapy. In the coming decades, proteomics technologies will broaden our understanding of the underlying mechanisms of and will further our ability to diagnose, prognosticate and treat autoimmune disease.

#### **(viii) Proteomics in Cardiovascular research.**

The development of proteomics is a timely one for cardiovascular research. Analyses at the organ, sub cellular, and molecular levels have revealed dynamic, complex, and subtle intracellular processes associated with heart and vascular disease. The power and flexibility of proteomic analyses, which facilitate protein separation, identification, and characterization, should hasten our understanding of these processes at the protein level. Properly applied, proteomics provides

researchers with cellular protein “inventories” at specific moments in time, making it ideal for documenting protein modification due to a particular disease, condition, or treatment. This is accomplished through the establishment of species- and tissue-specific protein databases, providing a foundation for subsequent proteomic studies.

#### **(ix) Proteomics to diabetes research.**

Proteomics is the investigation of all the proteins and their various modifications making up a system, be that a cell, tissue or organism. The techniques involved in proteomics allow the global screening of complex samples of proteins and provide qualitative and quantitative evidence of altered protein expression. This lends itself to the investigation of the molecular mechanisms underpinning disease processes and the effects of treatment.

#### **(x) Proteomics in Nutrition Research**

Proteomics holds great promise for discoveries in nutrition research, including profiles and characteristics of dietary and body proteins; digestion, absorption, and metabolism of nutrients; functions of nutrients and other dietary factors in growth, reproduction, and health; biomarkers of the nutritional status and disease; and individualized requirements of nutrients. The proteome analysis is expected to play an important role in solving major nutrition-associated problems in humans and animals, such as obesity, diabetes, cardiovascular disease, cancer, aging, and intrauterine fetal retardation.

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## **17.7 Summary**

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Proteomics, the study of the proteome, is important because proteins represent the actual functional molecules in the cell. When mutations occur in the DNA, it is the proteins that are ultimately affected. Drugs, when they have beneficial effects, do so by interacting with proteins. The human genome contains the complete set of genes required to build a functional human being. However, the genome is only a source of information. In order to function, it must be expressed. The transcription of genes is the first stage of gene expression and is followed by the translation of messenger RNA to produce proteins.

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## 17.8 Glossary

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- **Gel electrophoresis** : One- and two-dimensional gel electrophoresis is used to identify the relative mass of a protein and its isoelectric point.
- **X-ray crystallography and nuclear magnetic resonance** : they are used to characterize the three-dimensional structure of peptides and proteins. However, low-resolution techniques such as circular dichroism, Fourier transform infrared spectroscopy and small angle x-ray scattering can be used to study the secondary structure of proteins.
- **Tandem mass spectrometry**: is combined with reverse phase chromatography or 2-D electrophoresis is used to identify (by de novo peptide sequencing) and quantify all the levels of proteins found in cells.
- **Mass spectrometry (no-tandem)** : often MALDI-TOF, is used to identify proteins by peptide mass fingerprinting. Less commonly this approach is used with chromatography and/or high resolution mass spectrometry. This technique is becoming less used and the scientific world no longer accepts absolute identification of a protein based solely on peptide mass fingerprint data.
- Affinity chromatography, yeast two hybrid techniques, fluorescence resonance energy transfer (FRET), and Surface Plasmon Resonance (SPR) are used to identify protein-protein and protein-DNA binding reactions.
- X-ray Tomography used to determine the location of labeled proteins or protein complexes in an intact cell. Frequently correlated with images of cells from light based microscopes.

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## 17.9 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. A proteome is a \_\_\_\_\_.
2. Expression proteomics involves analysis of \_\_\_\_\_.
3. Name two applications of proteomics in medicine
4. The term Proteome was coined by \_\_\_\_\_.

5. Full name of FRET \_\_\_\_\_.

**Section B : (Short Answer Type Questions)**

1. What do you mean by Bioinformatics?
2. Explain Structural proteomics?

**Section C : (Long Answer Type Questions)**

1. Define proteomics and discuss its various applications.
2. Describe the advantages of proteomic research?

**Answer key of Section – A**

1. A complete set of protein.
2. Expression and differential expression of proteins.
3. Diabetes and Cancer.
4. Marc Wilkins.
5. Fluorescenes resonance energy transfer.

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## **17.10 References**

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- P K Gupta - Genetic
- Alberts - Molecular biology of cell
- Srivastava S.- Proteins and Proteomics

## Unit - 18

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# Bioactive Compounds

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### Structure of the Unit:

- 18.0 Objectives
  - 18.1 Introduction
  - 18.2 Secondary Metabolite
  - 18.3 Alkaloids
  - 18.4 Antioxidants
  - 18.5 Flavonoid
  - 18.6 Protein
  - 18.7 Terpenoids
  - 18.8 Phenols
  - 18.9 Glycosides
  - 18.10 Summary
  - 18.11 Glossary
  - 18.12 Self-Learning Exercise
  - 18.13 References
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## 18.0 Objectives

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After studying this units you will be understand about –

- various types of Secondary Metabolite, their uses and applications,
  - Alkaloids, Antioxidants, Flavonoid, Protein, Terpenoids, Phenols and Glycosides.
- 

## 18.1 Introduction

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Bioactive compounds have an effect on a living organism, tissue or cell and have an influence on health. They are found in both plant and animal products or can be

synthetically produced. Some examples of bioactive compounds are flavonoids, caffeine, carotenoids, carnitine, choline, coenzyme Q, creatine, dithiolthiones, phytosterols, phytoestrogens, glucosinolates, polyphenols, anthocyanins prebiotics, and taurine. Plant bioactive compounds are carotenoids and polyphenols (fruits and vegetables), or phytosterols (from oils) as well as in animals fatty acids, found in milk and fish.

Metabolites are compounds synthesized by plants for both essential functions, such as growth and development (primary metabolites), and specific functions, such as pollinator attraction or defense against herbivory (secondary metabolites). Besides, metabolites are organic compounds synthesized by organisms using enzyme-mediated chemical reactions called metabolic pathways. Primary metabolism in a plant comprises all metabolic pathways that are essential to the plant's survival. They are directly involved in the growth and development of a plant whereas secondary metabolites are compounds produced in other metabolic pathways that, although important, are not essential to the functioning of the plant. However, secondary plant metabolites are useful in the long term, often for defense purposes, and give plants characteristics such as colour. Besides, secondary plant metabolites are also used in signaling and regulation of primary metabolic pathways.

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## 18.2 Secondary Metabolites

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Plant secondary metabolites are helpful in growth and development of plants but are not required for the plant to survive. Secondary metabolism facilitates the primary metabolism in plants. This primary metabolism consists of chemical reactions that allow the plant to live. In order for the plants to stay healthy, secondary metabolism plays a pinnacle role in keeping all the of plants' systems working properly. A common role of secondary metabolites in plants is defense mechanisms. They are used to fight off herbivores, pests, and pathogens. Besides, secondary metabolites are used in anti-feeding activity, toxicity or acting as precursors to physical defense systems.

Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups:

1. Flavonoids and allied phenolic and polyphenolic compounds,
2. Terpenoids and

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3. Nitrogen-containing alkaloids and sulphur-containing compounds.

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### **18.3 Alkaloids**

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Alkaloids are a large group of nitrogen-containing compounds occur in certain families of flowering plants. More than 3,000 different types of alkaloids have been identified in a total of more than 4,000 plant species. Many plant families are particularly rich in alkaloids; all plants of the poppy family (Papaveraceae) are thought to contain them, for example. The Ranunculaceae (buttercups), Apocynaceae (*Catranthus roseus*), Solanaceae (nightshades), and Amaryllidaceae (amaryllis) are other prominent alkaloid-containing families. A few alkaloids have been found in animal species, such as the New World beaver (*Castor canadensis*) and poison-dart frogs (*Phyllobates*). Ergot and a few other fungi also produce them. Alkaloids accumulate in plant organs such as leaves or fruits and are ingested by animals that consume those plant parts. Many alkaloids are extremely toxic, especially to mammals, and act as potent nerve poisons, enzyme inhibitors, or membrane transport inhibitors. In addition to being toxic; many alkaloids are also bitter or otherwise bad-tasting. Therefore, the presence of alkaloids and other toxic secondary metabolites can serve as a deterrent to animals, which learn to avoid eating such plants. Many potentially toxic plant-derived alkaloids have medicinal properties as long as they are administered in carefully regulated doses. Alkaloids with important medicinal uses include morphine and codeine from the opium poppy and cocaine from the coca plant. These alkaloids act on the nervous system and are used as painkillers. Atropine from the deadly nightshade plant also acts on the nervous system, anesthesia and ophthalmology.

#### **Classification of Alkaloids**

Alkaloids are naturally occurring organic nitrogen-containing bases. Alkaloids are characterized by a great structural diversity and there is no unique classification of alkaloids. Alkaloids are often classified on the basis of their chemical structure. For example, those alkaloids that contain a ring system called indole are known as indole alkaloids. On this basis of chemical structure, the principal classes of alkaloids are the pyrrolidines, pyridines, tropanes, pyrrolizidines, isoquinolines, indoles, quinolines, and the terpenoids and steroids. Alternatively, alkaloids can be

classified according to the biological system in which they occur. For example, the opium alkaloids occur in the opium poppy (*Papaver somniferum*).

Hegnauer (1963) conveniently classified alkaloids into six important groups, corresponding to the six amino-acids legitimately considered as the starting points for their biosynthesis, such as: anthranilic acid, histidine, lysine, ornithine phenylalanine and tryptophan. Price (1963) further took a leading clue from the earlier observation and considered in details the alkaloids present in one of the families, (Rutaceae) and logically placed them in the following nine chemical-structural categories, namely: acridines, amides, amines, benzylisoquinolines, canthinones, imidazoles, indolquinazolines, furoquinolines, and quinazolines. Moreover, recent classifications are based on similarity of the carbon skeleton (e.g., indole-, isoquinoline-, and pyridine-like) or biogenetic precursor (ornithine, lysine, tyrosine, tryptophan, etc.).

Alkaloids are also divided into the following major groups:

1. "True alkaloids", which contain nitrogen in the heterocycle and originate from amino acids. Examples are atropine, nicotine, and morphine. This group also includes some alkaloids that besides nitrogen heterocycle contain terpene (e.g., evonine) or peptide fragments (e.g. ergotamine). This group also includes piperidine alkaloids coniine and coniceine although they do not originate from amino acids.
2. "Protoalkaloids", which contain nitrogen and also originate from amino acids. Examples include mescaline, adrenaline and ephedrine.
3. Polyamine alkaloids – derivatives of putrescine, spermidine, and spermine.
4. Peptide and cyclopeptide alkaloids.
5. Pseudalkaloids – alkaloid-like compounds that do not originate from amino acids. This group includes, terpene-like and steroid-like alkaloids, as well as purine-like alkaloids such as caffeine, theobromine, theacrine and theophylline.

### **The Biological role**

Alkaloids are multifunctional in nature and have diverse and important physiological effects on humans and other animals. Well-known alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine. The function of alkaloids

in plants is not yet understood. They are known to be simply waste products of plants' metabolic processes and serves specific biological functions. In some plants, the concentration of alkaloids increases prior to seed formation and then drops off when the seed is ripe. Alkaloids may also protect some plants from destruction by certain insect species. Although it was assumed that the alkaloids are the final products of nitrogen metabolism in plants, as urea in mammals however, later shown that alkaloid concentration varies over time.

### **(i) Biological significance**

1. Protect plants against fungi, insects and animals.
2. Excretory product of plants.
3. Some alkaloids are reservoir of N and help in protein synthesis.
4. Behaves as growth regulator hormones.
5. Helpful in neutralize the harmful effect of acids in plants.
6. Also protect plants from plant diseases.
7. Colchicine is used in multiplication of chromosomes.
8. They inhibit enzyme activity and seed germination. Some remove bad effect of tannins.

### **(ii) Medicinal significance**

The medicinal properties of alkaloids are very diverse. Vincristine and vinblastine from the Periwinkle plant is inhibitors of cell division and is used to treat cancers of the blood and lymphatic systems. Quinine from the bark of the cinchona tree is toxic to the Plasmodium parasite, which causes malaria, and has long been used in tropical and subtropical regions of the world. Other alkaloids are used as stimulants, including caffeine, present in coffee, tea, and cola plants, and nicotine, which is present in tobacco. Nicotine preparations are, paradoxically, also used as an aid in smoking cessation. Nicotine is also a very potent insecticide. For many years ground-up tobacco leaves were used for insect control, but this practice was superseded by the use of special formulations of nicotine. Recently the use of nicotine as an insecticide has been discouraged because of its toxicity to humans.

Morphine is a powerful narcotic used for the relief of pain, though it's addictive properties limit its usefulness. Codeine, the methyl ether derivative of morphine found in the opium poppy, is an excellent analgesic that is relatively non addictive.

Certain alkaloids act as cardiac or respiratory stimulants. Quinidine, which is obtained from plants of the genus *Cinchona*, is used to treat arrhythmias, or irregular rhythms of the heartbeat. Many alkaloids affect respiration, but in a complicated manner such that severe respiratory depression may follow stimulation. The drug lobeline (*Lobelia inflata*) is safer in this respect and is therefore clinically useful. Ergonovine (*Claviceps purpurea*) and ephedrine (*Ephedra* species) act as blood-vessel constrictors. Ergonovine is used to reduce uterine hemorrhage after childbirth, and ephedrine is used to relieve the discomfort of common colds, sinusitis, hay fever, and bronchial asthma.

### **(iii) Use in agriculture**

In addition to the development of a wide range of relatively low-toxic synthetic pesticides, some alkaloids, such as salts of nicotine and anabasine, were used as insecticides. Their use was limited by their high toxicity to humans. At the same time Azadirachtin isolated from neem tree is also used as biopesticides.

### **(iv) Use as psychoactive drugs**

Alkaloids and their extracts, and later pure alkaloids, have been used as psychoactive substances. Cocaine, caffeine, and cathinone are stimulants of the central nervous system. Mescaline and many of indole alkaloids (such as psilocybin, dimethyl tryptamine and ibogaine) have hallucinogenic effect. Morphine and codeine are strong narcotic pain killers. However there are alkaloids that do not have strong psychoactive effect themselves, but are precursors for semi-synthetic psychoactive drugs. Example, ephedrine and pseudoephedrine are used to produce methcathinone and methamphetamine. At the same time, Thebaine is used in the synthesis of many painkillers such as Oxychodone.

## **18.4 Antioxidants**

Antioxidant inhibits the oxidation of other molecules. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized

themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols.

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxides. However, insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress damage the cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation.

The reactive oxygen species produced in cells include hydrogen peroxide ( $H_2O_2$ ), hypochlorous acid ( $HClO$ ), and free radicals such as the hydroxyl radical ( $\cdot OH$ ) and the superoxide anion ( $O_2^-$ ). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms, while damage to proteins causes enzyme inhibition, denaturation and protein degradation.

### **Classification of Antioxidant**

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (lipophilic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. These compounds may be synthesized in the body or obtained from the diet. The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors. Some common antioxidants are -Uric acid, Ascorbic acid (vitamin C), Glutathione, Melatonin and Tocopherols and tocotrienols (vitamin E).

Uric acid is by far the highest concentration antioxidant in human blood. Uric acid (UA) is an antioxidant oxypurine produced from xanthine by the enzyme xanthine oxidase, and is an intermediate product of purine metabolism. Ascorbic acid or "vitamin C" is a monosaccharide oxidation-reduction (redox) catalyst found in both animals and plants. As one of the enzymes needed to make ascorbic acid has been lost by mutation during primate evolution, humans must obtain it from the diet; it is therefore a vitamin.

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. Melatonin is a powerful antioxidant. Melatonin easily crosses cell membranes and the blood-brain barrier. Unlike other antioxidants, melatonin does not undergo redox cycling, which is the ability of a molecule to undergo repeated reduction and oxidation. Redox cycling may allow other antioxidants (such as vitamin C) to act as pro-oxidants and promote free radical formation. Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties. Of these, -tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolizing this form.

### **Biological roles**

Oxidative stress seems to play a significant role in many human diseases, including cancers. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials of antioxidant supplements including beta-carotene, vitamin A, and vitamin E singly or in different combinations suggest that supplementation has no effect on mortality or possibly increases it. Randomized clinical trials of antioxidants including beta carotene, vitamin E, vitamin C and selenium have shown no effect on cancer risk or increased cancer risk associated with supplementation. Supplementation with

selenium or vitamin E does not reduce the risk of cardiovascular disease. Antioxidants also have many industrial uses, such as preservatives in food and cosmetics and to prevent the degradation of rubber and gasoline.

Besides, oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease, the pathologies caused by diabetes, rheumatoid arthritis, and neurodegeneration in motor neuron diseases. In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease and from general tissue damage; one case in which this link is particularly well-understood is the role of oxidative stress in cardiovascular disease. Here, low density lipoprotein (LDL) oxidation appears to trigger the process of atherosclerosis, which results in atherosclerosis, and finally cardiovascular disease.

Oxidative damage in DNA can cause cancer. Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase etc. protect DNA from oxidative stress. It has been proposed that polymorphisms in these enzymes are associated with DNA damage and subsequently the individual's risk of cancer susceptibility.

### **(i) Food**

Fruits and vegetables are good sources of nutrients and photochemical which suggests that antioxidant compounds might lower risk against several diseases. Fruits and vegetables are good sources of antioxidant vitamins. Antioxidant vitamins are found in vegetables, fruits, eggs, legumes and nuts. Vitamins A, C or E can be destroyed by long-term storage or prolonged cooking. The effects of cooking and food processing are complex, as these processes can also increase the bioavailability of antioxidants, such as some carotenoids in vegetables. Processed food contains fewer antioxidant vitamins than fresh and uncooked foods, as preparation exposes food to heat and oxygen. Antioxidants are used as food additives to help guard against food deterioration. Exposure to oxygen and sunlight are the two main factors in the oxidation of food, so food is preserved by keeping in the dark and sealing it in containers or even coating it in wax, as with cucumbers. However, as oxygen is also important for plant respiration, storing plant materials in anaerobic conditions produces unpleasant flavours and unappealing colours. Consequently, packaging of fresh fruits and vegetables

contains a ~8% oxygen atmosphere. Antioxidants are an especially important class of preservatives as, unlike bacterial or fungal spoilage, oxidation reactions still occur relatively rapidly in frozen or refrigerated food. These preservatives include natural antioxidants such as ascorbic acid (AA, E300) and tocopherols (E306), as well as synthetic antioxidants such as propyl gallate (PG, E310), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA, E320) and butylated hydroxytoluene (BHT, E321)

### **(ii) Pharmaceuticals**

Based on the favourable effect observed for methylprednisolone in patients with head injury, a number of experimental therapeutics have been designed with the goal of providing neuroprotection or other therapeutic effects via an antioxidant mechanism. Tirilazad mesylate is an anti-oxidant steroid derivative that was designed to inhibit the lipid peroxidation observed and believed to play a key role in neuronal death in stroke and head injury. It demonstrated activity in preclinical models of head injury and stroke. Clinical trials demonstrated no effect on mortality or other outcomes in subarachnoid haemorrhage, and worsened results in ischemic stroke.

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## **18.5 Flavonoid**

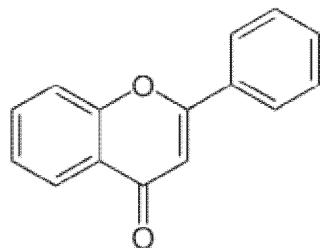
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Flavonoids are polyphenolic molecules containing 15 carbon atoms and are soluble in water. They consist of two benzene rings connected by a short three carbon chain. One of the carbons in this chain is connected to a carbon in one of the benzene rings, either through an oxygen bridge or directly, which gives a third middle ring. The flavonoids can be divided into six major subtypes, which include chalcones, flavones, isoflavonoids, flavanones, anthoxanthins and anthocyanins. Many of these molecules, particularly the anthoxanthins give rise to the yellow colour of some petals, while anthocyanins are often responsible for the red colour of buds and the purple-red colour of autumn leaves. Flavonoids word is derived from the Latin word *flavus* meaning yellow, their colour in nature) are a class of plant secondary metabolites. Flavonoids were referred to as Vitamin P because of the effect they had on the permeability of vascular capillaries .Flavonoids are a group of plant metabolites thought to provide health benefits through cell signaling

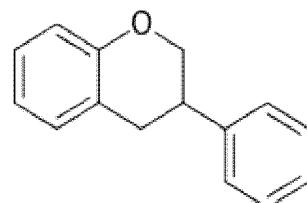
pathways and antioxidant effects. These molecules are found in a variety of fruits and vegetables.

### Classification of Flavonoids

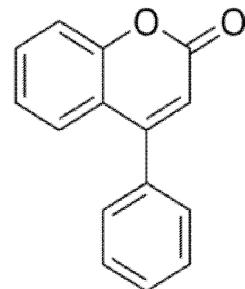
They can be subdivided into different subgroups depending on the carbon of the C ring on which B ring is attached, and the degree of unsaturation and oxidation of the C ring. Flavonoids in which B ring is linked in position 3 of the ring C are called isoflavones; those in which B ring is linked in position 4, neoflavanoids, while those in which the B ring is linked in position 2 can be further subdivided into several subgroups on the basis of the structural features of the C ring. This subgroup is flavones, flavonols, flavanones,flavanonols, flavanols or, catechins and anthocyanins. Finally, flavonoids with open C ring are called chalcones.



Flavone (2-phenyl-1, 4-benzopyrone)



Isoflavan structure



Neoflavanoids structure

### Flavones

They have a double bond between positions 2 and 3 and a ketone in position 4 of the C ring. Most flavones of vegetables and fruits has a hydroxyl group in position 5 of the A ring, while the hydroxylation in other positions, for the most part in position 7 of the A ring or 3' and 4' of the B ring may vary according to the

taxonomic classification of the particular vegetable or fruit. Some flavones, such as nobiletin and tangeretin, are polymethoxylated.

### **Flavonols**

Compared to flavones, they have a hydroxyl group in position 3 of the C ring, which may also be glycosylated. Like flavones, flavonols are very diverse in methylation and hydroxylation patterns as well, and, considering the different glycosylation patterns, they are perhaps the most common and largest subgroup of flavonoids in fruits and vegetables. For example, quercetin is present in many plant foods.

### **Flavanones**

Flavanones also called dihydroflavones, have the C ring saturated; therefore, unlike flavones, the double bond between positions 2 and 3 is saturated and this is the only structural difference between the two subgroups of flavonoids. The flavanones can be multi-hydroxylated, and several hydroxyl groups can be glycosylated and/or methylated.

### **Isoflavones**

As anticipated, isoflavones are a subgroup of flavonoids in which the B ring is attached to position 3 of the C ring. They have structural similarities to estrogens, such as estradiol, and for this reason they are also called phytoestrogens.

### **Neoflavonoids**

They have the B ring attached to position 4 of the C ring.

### **Flavanols or flavan-3-ols or catechins**

Flavanols are also referred to flavan-3-ols as the hydroxyl group is almost always bound to position 3 of C ring; they are called **catechins** as well. Unlike many flavonoids, there is no double bond between positions 2 and 3. Another distinctive feature, e.g. compared to flavanonols, with which they share a hydroxyl group in position 3, is the lack of a carbonyl group, that is, a keto group, in position 4. This particular chemical structure allows flavanols to have two chiral centres in the molecule, on positions 2 and 3, then four possible diastereoisomers. Epicatechin is the isomer with the *cis* configuration and catechin is the one with the *trans* configuration. Each of these configurations has two stereoisomers, namely, (+)-epicatechin and (-)-epicatechin, (+)-catechin and (-)-catechin. (+)-Catechin and

(-) -epicatechin are the two isomers most often present in edible plants.

### **Anthocyanidins**

chemically, anthocyanidins are flavylium cations and are generally present as chloride salts. They are the only group of flavonoids that gives plants colours (all other flavonoids are colourless). Anthocyanins are glycosides of anthocyanidins. Sugar units are bound mostly to position 3 of the C ring and they are often conjugated with phenolic acids, such as ferulic acid. The colour of the anthocyanins depends on the pH and also by methylation or acylation at the hydroxyl groups on the A and B rings.

### **Chalcones**

Chalcones and dihydrochalcones are flavonoids with open structure; they are classified as flavonoids because they have similar synthetic pathways.

### **Biological roles**

Flavonoids are widely distributed in plants, fulfilling many functions. Flavonoids are the most important plant pigments for flower coloration, producing yellow or red/blue pigmentation in petals designed to attract pollinator animals. In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation. They may also act as chemical messengers, physiological regulators, and cell cycle inhibitors. Flavonoids secreted by the root of their host plant help Rhizobia in the infection stage of their symbiotic relationship with legumes like peas, beans, clover, and soy. Rhizobia living in soil are able to sense the flavonoids and this triggers the secretion of Nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses such as ion fluxes and the formation of a root nodule. In addition, some flavonoids have inhibitory activity against organisms that cause plant diseases, e.g. *Fusarium oxysporum*. Flavonoids are important antioxidants, and promote several health effects. Aside from antioxidant activity, these molecules provide the following beneficial effects: Anti-viral, Anti-cancer, Anti-inflammatory, and Anti-allergic.

Quercetin can help to alleviate eczema, sinusitis, asthma, and hay fever. Some studies have shown that flavonoid intake is inversely related to heart disease, with these molecules inhibiting the oxidation of low-density lipoproteins and therefore reducing the risk of atherosclerosis developing. Flavonoids are also abundant in

red wine, which some have theorized is the reason why the incidence of heart disease may be lower among the French compared with other Europeans, despite a higher consumption of foods rich in cholesterol . Many studies have also shown that one to two glasses of wine a day can help protect against heart disease. Some types of tea are also rich in flavonoids and their consumption is thought to lower levels of triglycerides and cholesterol in the blood. Soy flavonoids or isoflavones also lower cholesterol, as well as protecting against osteoporosis and alleviating the symptoms of menopause.

### **(i) Food sources**

Almost all fruits, vegetables and herbs contain a certain amount of flavonoids. They can also be found in other food sources including dry beans, grains, red wine and green and black teas. The general rule is that the more colorful a food item is, the richer it will be in flavonoids. Oranges however are an exception to the rule because the flavonoids contained in this fruit are mainly found in the white and pulp interior of the skin. Flavonoids such as the catechins are "the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants". Flavonols, the original bioflavonoids such as quercetin, are also found ubiquitously, but in lesser quantities. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Foods with a high flavonoid content include parsley, onions, blueberries and other berries, black tea, green tea and oolong tea, bananas, all citrus fruits, *Ginkgo biloba*, red wine, sea-buckthorns, and dark chocolate . The citrus flavonoids include hesperidin (a glycoside of the flavanone hesperetin), quercetin, rutin (two glycosides of the flavonol quercetin), and the flavone tangeritin.

### **(ii) Medical research**

Flavonoids have been shown to have a wide range of biological and pharmacological activities in *in-vitro* studies. Examples include anti-allergic, anti-inflammatory, antioxidant, anti-microbial (antibacterial, antifungal, and antiviral), anti-cancer, and anti-diarrheal activities. Flavonoids have also been shown to inhibit topoisomerase enzymes and to induce DNA mutations in the mixed-lineage

leukemia (MLL) gene in *in-vitro* studies. Some common diseases are Inflammation, Cancer, Cardiovascular diseases and Antibacterial.

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## 18.6 Proteins

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Proteins are large biological molecules, or macromolecules, consisting of one or chains of amino acid residues. Proteins perform a vast array of functions within living organisms, including catalyzing metabolic reactions, replicating DNA, responding to stimuli, and transporting molecules from one location to another. Proteins differ from one another primarily in their sequence of amino acids, usually results in folding of the protein into a specific three-dimensional structure that determines its activity.

A linear chain of amino acid residues is called a polypeptide. A protein contains at least one long polypeptide. Short polypeptides, containing less than about 20-30 residues, are rarely considered to be proteins and are commonly called peptides, or sometimes oligopeptides. The individual amino acid residues are bonded together by peptide bonds and adjacent amino acid residues. The sequence of amino acid residues in a protein is defined by the sequence of a gene, which is encoded in the genetic code. In general, the genetic code specifies 20 standard amino acids; however, in certain organisms the genetic code can include selenocysteine and—in certain archaea—pyrrolysine.

### Classification of Protein

Proteins are not entirely rigid molecules; they may shift between several related structures while they perform their functions. In the context of functional rearrangements, tertiary or quaternary structures are usually referred to as "conformations", and transitions between them are called conformational changes. Such changes are often induced by the binding of a substrate molecule to an enzyme's active site, or the physical region of the protein that participates in chemical catalysis. In solution proteins also undergo variation in structure through thermal vibration and the collision with other molecules.

Proteins can be informally divided into three main classes, which correlate with typical tertiary structures: globular proteins, fibrous proteins, and membrane proteins. Almost all globular proteins are soluble and many are enzymes. Fibrous proteins are often structural, such as collagen, the major component of connective

tissue, or keratin, the protein component of hair and nails. Membrane proteins often serve as receptors or provide channels for polar or charged molecules to pass through the cell membrane.

### **Biological roles**

Proteins have many functions. They serve as enzymatic catalysts, are used as transport molecules and storage; they are used in movement; they are needed for mechanical support (skin and bone contain collagen-a fibrous protein); they mediate cell responses; antibody proteins are needed for immune protection; control of growth and cell differentiation uses proteins.

#### **(i) Cellular functions**

Proteins are the chief actors within the cell, carrying out the duties specified by the information encoded in genes.

The chief characteristic of proteins that also allows their diverse set of functions is their ability to bind other molecules specifically and tightly. The region of the protein responsible for binding another molecule is known as the binding site and is often a depression or "pocket" on the molecular surface. This binding ability is mediated by the tertiary structure of the protein, which defines the binding site pocket, and by the chemical properties of the surrounding amino acids' side chains.

Proteins can bind to other proteins as well as to small-molecule substrates. When proteins bind specifically to other copies of the same molecule, they can oligomerize to form fibrils; this process occurs often in structural proteins that consist of globular monomers that self-associate to form rigid fibers. Protein–protein interactions also regulate enzymatic activity, control progression through the cell cycle, and allow the assembly of large protein complexes that carry out many closely related reactions with a common biological function. Proteins can also bind to, or even be integrated into, cell membranes. The ability of binding partners to induce conformational changes in proteins allows the construction of enormously complex signaling networks.

#### **(ii) Structural proteins**

Structural proteins confer stiffness and rigidity to otherwise-fluid biological components. Most structural proteins are fibrous proteins; for example, collagen and elastin are critical components of connective tissue such as cartilage, and

keratin is found in hard or filamentous structures such as hair, nails, feathers, hooves, and some animal shells. Some globular proteins can also play structural functions, for example, actin and tubulin are globular and soluble as monomers, but polymerize to form long, stiff fibers that make up the cytoskeleton, which allows the cell to maintain its shape and size.

Other proteins that serve structural functions are motor proteins such as myosin, kinesin, and dynein, which are capable of generating mechanical forces. These proteins are crucial for cellular motility of single celled organisms and the sperm of many multicellular organisms which reproduce sexually. They also generate the forces exerted by contracting muscles and play essential roles in intracellular transport.

### (iii) Proteomics

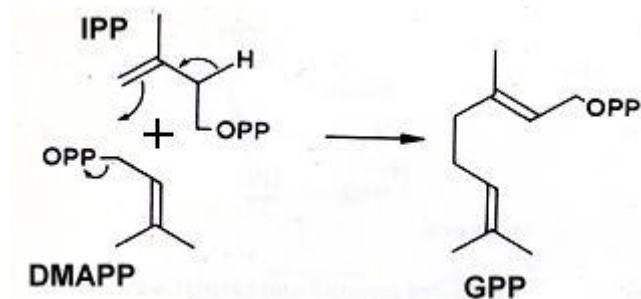
The total complement of proteins present at a time in a cell or cell type is known as its proteome, and the study of such large-scale data sets defines the field of proteomics, named by analogy to the related field of genomics. Proteomics include 2D electrophoresis which allows the separation of a large number of proteins, mass spectrometry, which allows rapid high-throughput identification of proteins and sequencing of peptides, protein microarrays, which allow the detection of the relative levels of a large number of proteins present in a cell, and two-hybrid screening, which allows the systematic exploration of protein–protein interactions.

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## 18.7 Terpenoids

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Terpenoids is a subclass of the prenyl lipids (terpenes, prenylquinones, and sterols), represents the oldest group of small molecular products synthesized by plants and is most widespread group of natural products. Terpenoids are described as modified terpenes, where methyl groups are moved or removed, or oxygen atoms added. It is also known as "terpenes" more broadly, to include the terpenoids. Terpenoids are derived from acetyl coenzyme A or from intermediates in glycolysis. Terpenoids are extraordinarily diverse but they all originate through the condensation of the universal phosphorylated derivative of hemiterpene, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) giving geranyl pyrophosphate (GPP).



In higher plants, IPP is derived from the classic mevalonic acid pathway in the cytosol but from the methylerythritol phosphate pathway in plastids. It is generally accepted that the cytosolic pool of IPP serves as a precursor of sesquiterpenes, triterpenes, sterols and polyterpenes whereas the plastid pool of IPP provides the precursors of mono-, di- and tetraterpenes. Mono-, sesqui-, di-, and sesterterpenes contain the isoprene units linked in a head to tail fashion. The triterpenes and carotenoids (tetraterpenes) contain two C15 and C20 units and linked in head to head. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones are also found.

### **Classification of the Terpenoids**

They are classified by the number of five-carbon isoprenoid units they contain. Monoterpene (containing two C5- units) are exemplified by the aromatic oils (such as menthol) contained in the leaves of members of the mint family. The pyrethrins, which are monoterpene esters from the flowers of chrysanthemum and related species, are used commercially as insecticides. They affect the nervous systems of insects while being biodegradable and nontoxic to mammals, including humans. Diterpenes are formed from four C5-units. Paclitaxel (commonly known by the brand name Taxol), a diterpene found in bark of the Pacific yew tree, is a potent inhibitor of cell division in animals. Triterpenoids (formed from six C5-units) comprise the plant steroids, some of which act as plant hormones.

These also can protect plants from insect attack though their mode of action is quite different from that of the pyrethrins. Tetraterpenoids (eight C5- units) include important pigments such as beta-carotene, which is a precursor of vitamin A, and lycopene, which gives tomatoes their red colour. Rather than functioning in plant defense, the coloured pigments that accumulate in ripening fruits can serve as attractants to animals, which actually aid the plant in seed dispersal. The

polyterpenes are polymers that may contain several thousand isoprenoid units. Rubber, a polyterpene in the latex of rubber trees that probably aids in wound healing in the plant, is also very important for the manufacture of tires and other products.

Classification of the terpenoids is based upon the number of isoprene (or isopentane) units incorporated in the basic molecular skeleton:

Hemiterpenoids, 1 isoprene unit (5 carbons)

Monoterpenoids, 2 isoprene units (10C)

Sesquiterpenoids, 3 isoprene units (15C)

Diterpenoids, 4 isoprene units (20C) (e.g. ginkgolides)

Sesterterpenoids, 5 isoprene units (25C)

Triterpenoids, 6 isoprene units (30C) (e.g. sterols)

Tetraterpenoids, 8 isoprene units (40C) (e.g. carotenoids)

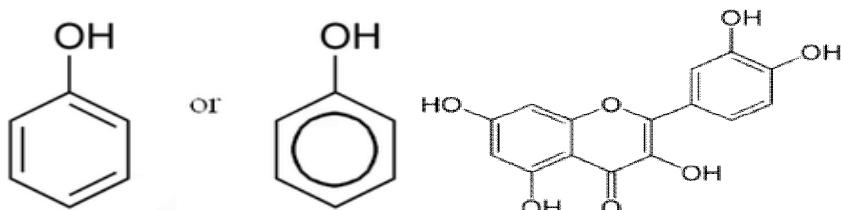
Polyterpenoid with a larger number of isoprene units

### **Biological roles**

They are universally present in small amounts in living organisms, where they play numerous vital roles in plant physiology as well as important functions in all cellular membranes. Plant antioxidants are composed of a broad variety of different substances like ascorbic acid and tocopherols, polyphenolic compounds, or terpenoids. They perform several important functions in plants and humans. Monoterpene and diterpenes, which are the main components of essential oils, act as allelopathic agents, attractants in plant-plant or plant-pathogen/herbivore interactions or repellants. For humans, carotenoids play an important role for health, carotenoids with provitamin A activity are important for vision; other carotenoids influence the human immune function and gap-junctional communication (GJC). Additionally, their antioxidative capacity is believed to be responsible for the health promoting properties of fruits and vegetables. Three main ways of action of carotenoids have been detected i.e., quenching of singlet oxygen, hydrogen transfer, or electron transfer.

## 18.8 Phenols

Phenols, also called phenolics, are a class of chemical compounds consisting of a hydroxyl group ( $-\text{OH}$ ) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol, which is also called carbolic acid  $\text{C}_6\text{H}_5\text{OH}$ . Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule.



Phenol - the simplest of the phenols

Quercetin, a typical flavonoid

Phenolic compounds are synthesized industrially; they also are produced by plants and microorganisms, with variation between and within species. Although similar to alcohols, phenols have unique properties and are not classified as alcohols. They have higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. The acidity of the hydroxyl group in phenols is commonly intermediate between that of aliphatic alcohols and carboxylic acids.

### Classification of Phenols

They can also be classified on the basis of their number of phenol groups. They can therefore be called simple phenols or monophenols, with only one phenolic group, or di- (bi-), tri- and oligophenols, with two, three or several phenolic groups respectively.

The largest and best studied natural phenols are the flavonoids, which include several thousand compounds, among them the flavonols, flavones, flavan-3-ol (catechins), flavanones, anthocyanidins and isoflavonoids. The phenolic unit can be found dimerized or further polymerized, creating a new class of polyphenol. For example, ellagic acid is a dimer of gallic acid and forms the class of ellagitannins, or a catechin and a gallocatechin can combine to form the red compound theaflavin, a process that also results in the large class of brown thearubigins in tea.

Two natural phenols from two different categories, for instance a flavonoid and a lignan, can combine to form a hybrid class like the flavonolignans.

### Naturally occurring Phenolic compounds

Cannabinoids	the active constituents of cannabis
Capsaicin	the pungent compound of chili peppers
Carvacrol	found in, antimicrobial and neuroprotectant
Cresol	found in coal tar and creosote
Estradiol	estrogen - hormones
Eugenol	the main constituent of the essential oil of clove
Gallic acid	found in galls
Guaiacol	found in roasted coffee, whisky, and smoke
Methyl salicylate	Main constituent of the essential oil of wintergreen
Raspberry ketone	a compound with an intense raspberry smell
Salicylic acid	precursor compound to Aspirin (chemical synthesis is used in manufacturing)
Serotonin / dopamine / adrenaline / noradrenaline	natural neurotransmitters
Thymol	(2-Isopropyl-5-methyl phenol) - found in thyme; an antiseptic that is used in mouthwashes
Tyrosine	an amino acid
Sesamol	in sesame seeds

### Synthetic Phenolic compounds

Phenol	the parent compound, used as a disinfectant and for chemical synthesis
Bisphenol A	and other bisphenols produced from ketones and phenol / cresol
BHT	(butylated hydroxytoluene) - a fat-soluble antioxidant and food additive
4-Nonylphenol	a breakdown product of detergents and nonoxynol-9
Orthophenyl phenol	a fungicide used for waxing citrus fruits
Picric acid	(trinitrophenol) - an explosive material
Phenolphthalein	pH indicator
Xylenol	used in antiseptics & disinfecticides

Phenols often have chiral centers. An example of such a molecule is catechin. Cavicularin is an unusual macrocycle because it was the first compound isolated from nature displaying optical activity due to the presence of planar chirality and axial chirality.

### Biological roles

Phenols are aromatic benzene ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. Phenolics play important roles in plant development, particularly in lignin and pigment biosynthesis. They also provide structural integrity and scaffolding support to plants. Importantly, phenolic phytoalexins, secreted by wounded perturbed plants, repel or kill many microorganisms, and some pathogens can counteract or nullify these defenses or even subvert them to their own advantage. Phenolic compounds are defined by the presence of one or more aromatic rings bearing a hydroxyl functional group. Many are synthesized from the amino acid phenylalanine. Simple phenolic compounds,

such as salicylic acid, can be important in defense against fungal pathogens. Salicylic acid concentration increases in the leaves of certain plants in response to fungal attack and enables the plant to mount a complex defense response. Interestingly, aspirin, a derivative of salicylic acid, is routinely used in humans to reduce inflammation, pain, and fever. Other phenolic compounds, called isoflavones, are synthesized rapidly in plants of the legume family when they are attacked by bacterial or fungal pathogens, and they have strong antimicrobial activity.

Lignin, a complex phenolic macromolecule, presents in cell walls and is the main component of wood. It is a very important structural molecule in all woody plants, allowing them to achieve height, girth, and longevity. Lignin is also valuable for plant defense: Plant parts containing cells with lignified walls are much less palatable to insects and other animals than are non woody plants and are much less easily digested by fungal enzymes than plant parts that contain only cells with primary cellulose walls. Anthocyanins and anthocyanidins are phenolic pigments that impart pink and purple colours to flowers and fruits. This pigmentation attracts insects and other animals that move between individual plants and accomplish pollination and fruit dispersal.

### **(1) Role in soils**

In soils, it is assumed that larger amounts of phenols are released from decomposing plant litter rather than from through fall in any natural plant community. Decomposition of dead plant material causes complex organic compounds to be slowly oxidized lignin-like humus or to break down into simpler forms, which are further transformed into microbial biomass.

### **(ii) Role in survival**

Phenolic compounds act as protective agents, inhibitors, natural animal toxicants and pesticides against invading organisms, i.e. herbivores, nematodes, phytophagous insects, and fungal and bacterial pathogens. The scent and pigmentation conferred by other phenolics can attract symbiotic microbes, pollinators and animals that disperse fruits.

**(iii) Defense against predators**

Volatile phenolic compounds are found in plant resin where they may attract benefactors such as parasitoids or predators of the herbivores that attack the plant. In the kelp species *Alaria marginata*, phenolics act as chemical defence against herbivores. In tropical *Sargassum* and *Turbinaria* species that are often preferentially consumed by herbivorous fishes and echinoids, there is a relatively low level of phenolics and tannins. Marine allelochemicals generally are present in greater quantity and diversity in tropical than in temperate regions. High phenolic concentrations occur in brown algae species from both temperate and tropical regions, indicating that latitude alone is not a reasonable predictor of plant phenolic concentrations.

**(iv) Defense against infection**

In *Vitis vinifera* grape, trans-resveratrol is a phytoalexin produced against the growth of fungal pathogens such as *Botrytis cinerea* and delta-viniferin is another grapevine phytoalexin produced following fungal infection by *Plasmopara viticola*. Pinosylvin is a pre-infectious stilbenoid toxin, contrary to phytoalexins, which are synthesized during infection. It is present in the heartwood of Pinaceae. It is a fungitoxin protecting the wood from fungal infection. Sakuranetin is a flavanone, a type of flavonoid found in *Polymnia fruticosa* and rice. It acts as a phytoalexin against spore germination of *Pyricularia oryzae*. In Sorghum, the SbF3'H2 gene, encoding a flavonoid 3'-hydroxylase, seems to be expressed in pathogen-specific 3-deoxyanthocyanidin phytoalexins synthesis, for example in Sorghum-Colletotrichum interactions. Stilbenes are produced in *Eucalyptus sideroxylon* in case of pathogens attacks. Such compounds can be implied in the hypersensitive response of plants. High levels of phenolics in some woods can explain their natural preservation against rot.

**(v) Role in allelopathic interactions**

Natural phenols can be involved in allelopathic interactions, for example in soil or in water. Juglone is an example of such a molecule inhibiting the growth of other plant species around walnut trees. The aquatic vascular plant *Myriophyllum spicatum* produces ellagic, gallic and pyrogalllic acids and (+)-catechin, allelopathic phenolic compounds inhibiting the growth of blue-green alga *Microcystis*

aeruginosa. Besides, phenolics, and in particular flavonoids and isoflavonoids, may be involved in endomycorrhizae formation.

#### **(vi) Human metabolism**

In animals and humans, after ingestion, natural phenols become part of the xenobiotic metabolism. In subsequent phase II reactions, these activated metabolites are conjugated with charged species such as glutathione, sulphate, glycine or glucuronic acid. These reactions are catalysed by a large group of broad-specificity transferases. UGT1A6 is a human gene encoding a phenol UDP glucuronosyltransferase active on simple phenols. The enzyme encoded by the gene UGT1A8 has glucuronidase activity with many substrates including coumarins, anthraquinones and flavones.

### **18.9 Glycoside**

Glycoside is molecule in which a sugar group is bonded through its anomeric carbon to another group via a glycosidic bond. Glycosides can be linked by an O-(an O-glycoside), N- (a glycosylamine), S-(a thioglycoside), or C- (a C-glycoside) glycosidic bond. According to the IUPAC, the name "C-glycoside" is a misnomer; the preferred term is "C-glycosyl compound". In addition to this sugar can be bonded with non-sugar molecule as a glycoside, thus excluding polysaccharides. The sugar group is then known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside.

The first glycoside ever identified was amygdalin, by the French chemists Pierre Robiquet and Antoine Boutron-Charlard, in 1830. Molecules containing an N-glycosidic bond are known as glycosylamines. Glycosylamines and glycosides are grouped together as glycoconjugates; other glycoconjugates include glycoproteins, glycopeptides, peptidoglycans, glycolipids, and lipopolysaccharides.

#### **Classification of Glycoside**

Glycosides can be classified by the glycone, by the type of glycosidic bond, and by the aglycone.

##### **1. by glycone/presence of sugar**

If the glycone group of a glycoside is glucose, then the molecule is a glucoside; if it is fructose, then the molecule is a fructified; if it is glucuronic acid, then the

molecule is a glucuronide; etc. In the body, toxic substances are often bonded to glucuronic acid to increase their water solubility; the resulting glucuronides are then excreted.

## **2. by type of glycosidic bond**

Depending on whether the glycosidic bond lies "below" or "above" the plane of the cyclic sugar molecule, glycosides are classified as  $\alpha$ -glycosides or  $\beta$ -glycosides. Some enzymes such as  $\alpha$ -amylase can only hydrolyze  $\alpha$ -linkages; others, such as emulsin, can only affect  $\beta$ -linkages.

There are four types of linkages present between glycone and aglycone:

C-linkage/glycosidic bond, "non hydrolysable by acids or enzymes"

O-linkage/glycosidic bond

N-linkage/glycosidic bond

S-linkage/glycosidic bond

## **3. By aglycone**

Glycosides are also classified according to the chemical nature of the aglycone. For purposes of biochemistry and pharmacology, this is the most useful classification.

Alcoholic glycosides

An example of an alcoholic glycoside is salicin, which is found in the genus salix. Salicin is converted in the body into salicylic acid, which is closely related to aspirin and has analgesic, antipyretic, and antiinflammatory effects.

Anthraquinone glycosides

These glycosides contain an aglycone group that is a derivative of anthraquinone. They have a laxative effect. They are mainly found in dicot plants except the Liliaceae family which are monocots. They are present in senna, rhubarb and Aloe species. Antron and anthranol are reduced forms of anthraquinone.

**Coumarin glycosides**

Here, the aglycone is coumarin or a derivative. An example is apterin which is reported to dilate the coronary arteries as well as block calcium channels. Other coumarin glycosides are obtained from dried leaves of *Psoralea corylifolia*.

### **Flavonoid glycosides**

Among the important effects of flavonoids are their antioxidant effects. They are also known to decrease capillary fragility. Examples of this large group of glycosides include: Hesperidin (aglycone: Hesperetin, glycone: Rutinose), Naringin (aglycone: Naringenin, glycone: Rutinose), Rutin (aglycone: Quercetin, glycone: Rutinose), Quercitrin (aglycone: Quercetin, glycone: Rhamnose)

### **Phenolic glycosides**

The aglycone is a simple phenolic structure. An example is arbutin found in the Common Bearberry *Arctostaphylos uva-ursi*. It has a urinary antiseptic effect.

### **Saponins**

Saponin glycosides are found in liquorice. Their medicinal value is due to their expectorant, and corticoid and anti-inflammatory effects. Steroid saponins, for example, in *Dioscorea* wild yam the sapogenindiosgenin—in form of its glycoside dioscin—is an important starting material for production of semi-synthetic glucocorticoids and other steroid hormones such as progesterone. The ginsenosides are triterpeneglycosides and Ginseng saponins from *Panax Ginseng* C. A. Meyer, (Chinese ginseng) and *Panax quinquefolius* (American Ginseng). Steroidal glycosides or cardiac glycosides.

### **Thioglycosides**

These compounds contain sulphur. Examples include sinigrin, found in black mustard, and sinalbin, found in mustard. Glycoside, any of a wide variety of naturally occurring substances in which a carbohydrate portion, consisting of one or more sugars or an uronic acid (i.e., a sugar acid), is combined with a hydroxy compound. The hydroxy compound, usually a non-sugar entity (aglycon), such as a derivative of phenol or an alcohol, may also be another carbohydrate, as in cellulose, glycogen, or starch, which consist of many glucose units.

### **Biological roles**

Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. These can be activated by enzymehydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications. In

animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body.

Various medicines, condiments, and dyes from plants occur as glycosides; of great value are the heart-stimulating glycosides of Digitalis and Strophanthus, members of a group known as cardiac glycosides. Several antibiotics are glycosides (e.g., streptomycin). Saponins, widely distributed in plants, are glycosides that lower the surface tension of water; saponin solutions have been used as cleansing agents. Glycosides derived from glucuronic acid and steroids are constituents of normal animal urine. Compounds (nucleosides) derived from the partial breakdown of nucleic acids are also glycosides.

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## 18.10 Summary

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Primary metabolites have functions that are essential for growth and development and are therefore present in all plants. In contrast, secondary metabolites are variously distributed in the plant kingdom, and their functions are specific to the plants in which they are found. Secondary metabolites are often coloured, fragrant, or flavourful compounds and they typically mediate the interaction of plants with other organisms. Such interactions include those of plant-pollinator, plant-pathogen, and plant-herbivore.

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## 18.10 Glossary

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- **Alkaloids :** are a large group of nitrogen-containing compounds occur in certain families of flowering plants.
- **Metabolites :** are compounds synthesized by plants for both essential functions, such as growth and development (primary metabolites), and specific functions, such as pollinator attraction or defense against herbivory (secondary metabolites).
- **Glycoside :** is molecule in which a sugar group is bonded through its anomeric carbon to another group via a glycosidic bond. Glycosides can be linked by an O- (an O-glycoside), N- (a glycosylamine), S-(a thioglycoside), or C- (a C-glycoside) glycosidic bond.

- **Antioxidant :** inhibits the oxidation of other molecules by terminating the chain reactions of oxidation process by removing free radical intermediates, and inhibit other oxidation reactions.

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## 18.11 Self-Learning Exercise

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### Section A : (Very Short Answer Type Questions)

1. Alkaloids are \_\_\_\_\_ compounds.
2. Ascorbic acid is also known as \_\_\_\_\_.
3. Two naturally occurring phenolic compounds are \_\_\_\_\_, \_\_\_\_\_
4. Saponin glycosides are found in \_\_\_\_\_.
5. Red colour of Tomato is due to \_\_\_\_\_.

### Section B : (Short Answer Type Questions)

1. What are Antioxidants? Briefly describe their role.
2. What are Glycosides?

### Section C : (Long Answer Type Questions)

1. Write notes on –  
(1) Phenols (2) Proteins
2. What are Secondary metabolites? Describe the role of Flavonoids and Terpenoids.
3. What are Alkaloids? Describe their roles.

### Answer key of Section – A

1. Nitrogen containing compounds
2. Vitamin C
3. Capsaicin and Salicylic acid
4. Liquorice
5. Lycopene

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## 18.12 References

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- J L Jain - “Biochemistry”
- Voet & Voet - Biochemistry
- Salisbury & Ross - Plant physiology and Biochemistry
- Lehninger - Biochemistry.

## Unit - 19

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# Intellectual Property Rights and Biosafety

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### Structure of the Unit:

- 19.0 Objectives
  - 19.1 Introduction
  - 19.2 Intellectual Property Rights
    - 19.2.1 History
    - 19.2.2 Types of Intellectual Property
    - 19.2.3 Intellectual Property Organization
  - 19.3 Biosafety
    - 19.3.1 History
    - 19.3.2 Biosafety Committees
    - 19.3.3 Norms of Biosafety
    - 19.3.4 Fields of Biosafety Applications
  - 19.4 Bioethics
  - 19.5 Summary
  - 19.6 Glossary
  - 19.7 Self-Learning Exercise
  - 19.8 References
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### 19.0 Objectives

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After studying this unit you will understand about –

- Intellectual Property Rights,
- types of Intellectual Property, Intellectual Property Organization, Patents and Copy Right; Biosafety,
- its Norms, Fields of Biosafety Applications and Bioethics

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## 19.1 Introduction

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Intellectual property is the area of law that deals with protecting the rights of those who create original works. It covers everything from original plays and novels to inventions and company identification marks. The purpose of intellectual property laws is to encourage new technologies, artistic expressions and inventions while promoting economic growth. When individuals know that their creative work will be protected and that they can benefit from their labour, they are more likely to continue to produce things that create jobs, develop new technology, make processes more efficient, and create beauty in the world around us.

Moreover, Intellectual property (IP) is creations of the intellect for which a monopoly is assigned to designated owners by law. Some common types of intellectual property rights (IPR) are copyright, patents, and industrial; and the rights that protect trademarks, trade dress, and in some jurisdictions trade secrets: all these cover music, literature, and other artistic works; discoveries and inventions; and words, phrases, symbols, and designs.

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## 19.2 Intellectual Property Right

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Intellectual property rights are the rights given to persons over the creations of their minds. They usually give the creator an exclusive right over the use of his/her creation for a certain period of time. Intellectual property rights are customarily divided into two main areas. Copyright and rights related to copyright. The rights of authors of literary and artistic works, such as books and other writings, musical compositions, paintings, sculpture, computer programs and films are protected by copyright, for a minimum period of 50 years after the death of the author. Another is Industrial property can usefully be divided into two main areas: One area can be characterized as the protection of distinctive signs, in particular trademarks which distinguish the goods or services of one undertaking from those of other undertakings and geographical indications which identify a good as originating in a place where a given characteristic of the good is essentially attributable to its geographical origin.

### **19.2.1 History**

The Statute of Anne came into force in 1710. Modern usage of the term intellectual property goes back at least as far as 1867 with the founding of the North German Confederation whose constitution granted legislative power over the protection of intellectual property to the confederation. When the administrative secretariats established by the Paris Convention (1883) and the Berne Convention (1886) merged in 1893, they located in Berne, and also adopted the term intellectual property in their new combined title, the United International Bureau for the Protection of Intellectual Property.

The organization subsequently relocated to Geneva in 1960, and was succeeded in 1967 with the establishment of the World Intellectual Property Organization (WIPO) by treaty as an agency of the United Nations. According to Lemley, it was only at this point that the term really began to be used in the United States and it did not enter popular usage until passage of the Bayh-Dole Act in 1980.

Moreover, the term intellectual property can be found used in an October 1845 Massachusetts Circuit Court ruling in the patent case *Davoll et al. v. Brown.*, in which Justice Charles L. Woodbury wrote that "only in this way can we protect intellectual property, the labours of the mind, productions and interests are as much a man's own...as the wheat he cultivates, or the flocks he rears." Until recently, the purpose of intellectual property law was to give as little protection possible in order to encourage innovation. Historically, therefore, they were granted only when they were necessary to encourage invention, limited in time and scope.

### **19.2.2 Types of Intellectual Property Right**

Intellectual property rights include patents, copyright, industrial design rights, trademarks, plant variety rights, trade dress, and in some jurisdictions trade secrets. There are also more specialized or derived varieties of rights, such as circuit design rights and supplementary for pharmaceutical products and database rights.

#### **(A) Patents**

A patent is a form of right granted by the government to an inventor, giving the owner the right to exclude others from making, using, selling, offering to sell, and importing an invention for a limited period of time, in exchange for the public

disclosure of the invention. An invention is a solution to a specific technological problem, which may be a product or a process and generally has to fulfill three main requirements: it has to be new, not obvious and there needs to be an industrial applicability.

Patents protect an invention from being made, sold or used by others for a certain period of time. There are three different types of patents.

- (i) Utility Patents - these patents protect inventions that have a specific function, including things like chemicals, machines, and technology.
- (ii) Design Patents - these patents protect the unique way a manufactured object appears.
- (iii) Plant Patents - these patents protect plant varieties that are asexually reproduced, including hybrids.

## **(B) Copyright**

A copyright gives the creator of an original work exclusive right to it, usually for a limited time. Copyright may apply to a wide range of creative, intellectual, or artistic forms, or "works". Copyright does not cover ideas and information themselves, only the form or manner in which they are expressed.

Moreover, copyright is a form of protection provided to the authors of "original works of authorship" including literary, dramatic, musical, artistic, and certain other intellectual works, both published and unpublished. The 1976 Copyright Act generally gives the owner of copyright the exclusive right to reproduce the copyrighted work, to prepare derivative works, to distribute copies or phonorecords of the copyrighted work, to perform the copyrighted work publicly, or to display the copyrighted work publicly. The copyright protects the form of expression rather than the subject matter of the writing. For example, a description of a machine could be copyrighted, but this would only prevent others from copying the description; it would not prevent others from writing a description of their own or from making and using the machine. Copyrights are registered by the Copyright Office of the Library of Congress.

**(C)Industrial design rights**

An industrial design consists of the creation of a shape, configuration or composition of pattern or colour, or combination of pattern and colour in three-dimensional form containing aesthetic value. An industrial design can be a two- or three-dimensional pattern used to produce a product, industrial commodity or handicraft. It can protect the visual design of objects that are not purely utilitarian.

**(D) Trademarks**

A trademark is a word, name, symbol or device which is used in trade with goods to indicate the source of the goods and to distinguish them from the goods of others. Moreover, a trademark is a recognizable sign, design or expression which distinguishes products or services of a particular trader from the similar products or services of other traders.

The terms "trademark" and "mark" are commonly used to refer to both trademarks and service marks. Trademark rights may be used to prevent others from using a confusingly similar mark, but not to prevent others from making the same goods or from selling the same goods or services under a clearly different mark. Trademarks which are used in interstate or foreign commerce may be registered with the Patent and Trademark Office. The registration procedure for trademarks and general information concerning trademarks is described in a separate pamphlet entitled "Basic Facts about Trademarks".

**(E)Trade dress**

Trade dress is a legal term of art that generally refers to characteristics of the visual appearance of a product or its packaging (or even the design of a building) that signify the source of the product to consumers.

**(F)Trade secrets**

A trade secret is a formula, practice, process, design, instrument, pattern, or compilation of information which is not generally known or reasonably ascertainable, by which a business can obtain an economic advantage over competitors or customers.

### **(G) Industrial design rights**

An industrial design right or "design right" protects the visual design of objects. An industrial design consists of the creation of a shape, configuration or composition of pattern or color, or combination of pattern and color in three-dimensional form containing aesthetic value. An industrial design can be a two- or three-dimensional pattern used to produce a product, industrial commodity or handicraft.

### **(H) Plant varieties**

Plant breeders' rights or plant variety rights are the rights to commercially use a new variety of a plant. The variety must amongst others be novel and distinct and for registration the evaluation of propagating material of the variety is examined.

## **19.2.3 Intellectual Property organization**

The objective of most intellectual property law is to "Promote progress." By exchanging limited exclusive rights for disclosure of inventions and creative works, society and the patentee/copyright owner mutually benefit, and an incentive is created for inventors and authors to create and disclose their work.

### **(i) World Intellectual Property Organization (WIPO)**

Every nation has its own intellectual property laws. World Intellectual Property Organization (WIPO) was constituted in 1967 and based in Geneva administer treaties in the field of intellectual property. There are 184 members in World Intellectual Property Organization (WIPO) along with India. Some common convention/treaty/protocol is Paris convention for protection of Industry and related to patents, trademarks and designs. Another is PCT (Patent cooperation treaty) which facilitates obtaining of patent in several countries. Madrid protocol administered by WIPO is a simple, facilitative and cost effective method for registration of International Trademarks. Besides, WTO (World trade organization) contains an agreement on IP called TRIPS (Trade related aspect of Intellectual property. Similarly, there is African (ARIPO), Organization (OAPI) or African Intellectual Property Organization.

### **(ii) Intellectual Property Organization in India**

India became a member of World Intellectual Property Organization (WIPO) in 1975. India has been a member of the World Trade Organization (WTO) since

1995. Pursuant to its obligations under the TRIPs Agreement, the Indian Parliament introduced various amendments in the Indian Patent Act and the corresponding Patent Rules. All Indian IP related laws are WIPO and TRIPS compliant and the Government has taken a comprehensive set of initiatives to streamline the intellectual property administration in the country in view of its strategic significance. In the Ministry of Commerce and Industry, the office of the 'Controller General of Patents, Designs and Trade Marks (CGPDTM)' has been set up under the Department. A range of statutory, civil, criminal and administrative laws are in place to support IP rights holders. As part of the 'Make in India' campaign, there is renewed focus on improving India's IP regime to best in the world standards by establishing an IP regime which maximizes the incentive for generation and protection of intellectual property for all type of inventors.

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### **19.3 Biosafety**

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Biotechnology refers to any technique that uses living organisms or substances from these organisms to modify or improve quality and product of crops and food, drugs and health care products, vaccines, industrial chemicals and its products. It consists of gradient of technologies ranging from the widely used techniques of traditional biotechnology through modern biotechnology which is based on the use of new techniques of Recombinant DNA (r- DNA) technology, known as Genetic Engineering.

Biosafety is one term that is used to describe the policies and procedures adopted to ensure the environmentally safe application of modern biotechnology. This is helpful in application of modern science in agriculture, medicine, and the environment, without endangering public health or environmental safety. Biosafety is the prevention of large-scale loss of biological integrity, focusing both on ecology and human health. These prevention mechanisms include conduction of regular reviews of the biosafety in laboratory settings, as well as strict guidelines to follow. Besides, biosafety is used to protect us from harmful incidents.

#### **19.3.1 History**

The UNIDO, WHO, FAO, UNEP have built up an Informal Working Group on Biosafety due to growing concerns arising from GMOs throughout the world. In 1991, this group prepared the "Voluntary Code of Conduct for the Release of

Organisms into the Environment". The ICGEB has also played an important role in issue related to biosafety and the environmentally sustainable use of biotechnology. The ICGEB organizes annual workshops on biosafety and on risk assessment for the release of GMOs. It collaborates with the management of UNIDO's BINAS (Biosafety Information Network and Advisory Service), aimed at monitoring the global development in regulatory issues in biotechnology. Since September 1998, the ICGEB has provided an on-line bibliographic data-base on biosafety and risk assessment for the environmental release of GMOs. This database which is accessible through the website of ICGEB also provides informations on biosafety to its Member States. The ICGEB is also assisting to its Member States in developing the national biosafety framework, since February 1999, it has also adopted a legally binding biosafety protocols by the signatory countries.

### **19.3.2 Biosafety Committees**

The first step in developing appropriate policies and procedures for the regulation of biotechnology is to establish a national biosafety advisory committee. The national committee should then move quickly to establish policies and procedures to govern the use of modern biotechnology in the country.

#### **1. National Biosafety Committee (NBC)**

An Egyptian National Biosafety Committee is being established, comprising policy makers and designers, scientific experts in Agriculture, Health, Industry and Environment from government and academic research institutes.

The purpose of the national committee is to establish policies and procedures to govern the use of modern biotechnology in the country. This includes publishing the National Biosafety Committee guidelines (NBC Guidelines) to be followed at the national level. The committee would also provide technical advice to the regulatory authorities and the institutions responsible for the development of biotechnology in the country.

#### **Activities of NBC**

##### **a) Formulate, implement and update safety codes**

In order to establish safety research policies, NBC shall formulate guidelines for both contained and uncontained applications to cover laboratory practices,

greenhouse facilities, small scale field trials, and finally commercial release. This will include guidelines for research with natural organisms that are exotic to the host country.

**b) Risk assessment and license issuance**

NBC takes initiatives to evaluate the benefits and potential risk of conducting research with modified organisms to the environment and to human community. If a license is issued after performing risk assessment analysis, NBC should periodically review containment measures and facilities to ensure that adequate safety guidelines are being followed.

**c) Coordination with international and national organizations**

NBC would establish contact and maintain communication with international and national organizations, taking into account new scientific and technical knowledge as they evolve. It would also monitor changes in intellectual property rights issues at the national and international level.

**d) Provide training and technical advice**

NBC is responsible that all personnel involved in biosafety issues receive adequate training on the most recent developments in safety procedures. It would also provide technical advice to the Institutional Biosafety Committees.

**e) Report at least annually to governmental authorities**

An annual progress report would be submitted to governmental authorities covering NBC activities throughout the year.

**2. The Institutional Biosafety Committee (IBC):**

The National Biosafety Committee should request that all institutions conducting R-DNA research assemble an Institutional Biosafety Committee.

The IBC is responsible for ensuring that the r-DNA research is carried out in full conformity with the Provisions of the NBC Guidelines. As part of its general responsibilities for implementing the NBC Guidelines, the IBC may establish additional.

### **19.3.3 Biosafety Norms**

Biosafety norms includes Primary containment like standard lab practices ,use of safety equipment like Biosafety cabinets, sharp containers, ,sealed rotors and wearing of personal protective equipment-lab coats, gloves, goggles etc., use centrifuge with Biosafety covers, aerosol, sharp and needle precautions, limited access, biohazard warning sign, Sops for decon,waste,medicals.

The main "topic of concern" related to the environmental release of GMOs are given below:

- (i) Risks for human health toxicity and food quality/safety allergies, pathogens' drug resistance i.e. antibiotic resistance
- (ii) Risks for the environment persistence of gene or transgene or transgene products resistance of target organisms or susceptibility of non-target organisms increased use of chemicals in agriculture transgene instability unpredictable gene expression
- (iii) Risks for agriculture weeds or superweeds alteration of nutritional value reduction of cultivars and loss of biodiversity
- (iv) Risks of interaction with non-target organism's genetic pollution through pollen or seed dispersal horizontal gene transfer transfer of foreign gene to microorganism i.e. DNA uptake generation of new line viruses by recombination.
- (v) General concerns higher cost of agriculture loss of familiarity ethical issues, etc.

With the potential future creation of man-made unicellular organisms, some are beginning to consider the effect that these organisms will have on biomass already present. Scientists estimate that within the next few decades, organism design will be sophisticated enough to accomplish tasks such as creating biofuels and lowering the levels of harmful substances in the atmosphere. Scientist that favour the development of synthetic biology claim that the use of biosafety mechanisms such as suicide genes and nutrient dependencies will ensure the organisms cannot survive outside of the lab setting in which they were originally created. Organizations like the ETC Group argue that regulations should control the creation of organisms that could potentially harm existing life. They also argue that

the development of these organisms will simply shift the consumption of petroleum to the utilization of biomass in order to create energy. These organisms can harm existing life by affecting the prey/predator food chain, reproduction between species, as well as competition against other species (species at risk, or act as an invasive species). Synthetic vaccines are now being produced in the lab. These have caused a lot of excitement in the pharmaceutical industry as they will be cheaper to produce, allow quicker production, and as well enhance the knowledge of virology and immunology.

The establishment of a National Biosafety System has become a necessity due to the introduction and the rapid increase of biotechnology applications in Egypt.

Establishing a National Biosafety System and assuring compliance with biosafety regulations would:

1. Ensure that Biotechnology continues to be safe and does not expose employees, the community and the environment to any avoidable ill effects.
2. Facilitate access to modern biotechnology generated abroad, as many international institutions and companies will not test genetically engineered organisms unless the tests have been approved by a responsible governmental body.
3. Result in faster public acceptance and further development of modern biotechnology.

#### **19.3.4 Fields of Biosafety Applications**

Biosafety as currently discussed in the International “Convention on Biological Diversity” (CBD) and designed to create internationally binding protocols on biosafety. The application of biotechnology to food and agriculture can bring not only potential risks and benefits as any technology can, but also concerns about the human dimensions coupled with biotechnology. These include both positive and negative impacts on stake holders, social institutions, economy and communities.

Biosafety is related to several fields: In ecology it refers to imported life forms from beyond ecoregion borders, In agriculture it is reducing the risk of alien viral or transgenic genes, genetic engineering or prions such as BSE/"MadCow", reducing the risk of food bacterial contamination. In medicine (referring to organs or tissues from biological origin, or genetic therapy products, virus; levels of lab

containment protocols measured as 1, 2, 3, 4 in rising order of danger), In chemistry i.e., nitrates in water, PCB levels affecting fertility. In exobiology i.e., NASA's policy for containing alien microbes that may exist on space samples - sometimes called "biosafety level 5" and in synthetic biology (referring to the risks associated with this type of lab practice).

The international Cartagena Protocol on Biosafety deals primarily with the agricultural definition but many advocacy groups seek to expand it to include post-genetic threats: new molecules, artificial life forms, and even robots which may compete directly in the natural food chain. When biological warfare or new, currently hypothetical, threats (i.e., robots, new artificial bacteria) are considered, biosafety precautions are generally not sufficient. The new field of biosecurity addresses these complex threats. Biosafety level refers to the stringency of biocontainment precautions deemed necessary by the Centers for Disease Control and Prevention (CDC) for laboratory work with infectious materials.

Typically, institutions that experiment with or create potentially harmful biological material will have a committee or board of supervisors that is in charge of the institution's biosafety. They create and monitor the biosafety standards that must be met by labs in order to prevent the accidental release of potentially destructive biological material.

Biosafety fields include Assistance with Pathogen Acquisition, Biohazards Approval (New Grant/Subsidiary Grant/Instructional) Biosafety Registry (Application/Instructional).

Different areas associated with biosafety include:

- (i) Agriculture and food system issues
- (ii) Market and consumer issues
- (iii) Institutional issues, business issues and
- (iv) Social issues

Agriculture and food system issues- These include the impact of biotechnology on the organization, structure and behavior of agricultural industry; further, the coexistence of conventional organic and biotechnology oriented agriculture; the

capacity of the food system to segregate genetically-modified commodity and product of specific markets; impacts on competitions involved, trade in agricultural commodities and the economical impacts of establishing oversight, standard regulations and public policies concerning biotechnology.

**Market and consumer issues**-These include various limitations which come to the rescue of consumer demand for overall against the products of agricultural biotechnology, the needs, desires and concerns of consumers in domestic and international markets; the influence of culture, advertising, product labeling, scientific information and recent new events on consumer decision making about the use of biotechnology products; different methods for most effectively increasing the understanding on which publication and primary decision making concerning biotechnology is based.

**Institutional issues and business issues**-These include the impacts of biotechnology on individual forms or group of forms about buying or selling biotechnology products and processes; changes in business practices, alliances and domestic and international markets including markets in Third World countries.

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## **19.4 Bioethics**

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Bioethics is the study of controversial ethical issues emerging from new situations and possibilities brought about by advances in biology and medicine. It is also moral discernment as it relates to medical policy, practice, and research. Bioethicists are concerned with the ethical questions that arise in the relationships among life sciences, biotechnology, medicine, politics, law, and philosophy. It also includes the study of the more commonplace questions of values which arise in primary care and other branches of medicine. The field of bioethics has addressed a broad swath of human inquiry, ranging from debates over the boundaries of life (e.g. abortion, euthanasia), surrogacy, the allocation of scarce health care resources (e.g. organ donation, health care rationing) to the right to refuse medical care for religious or cultural reasons. Bioethics often disagree among themselves over the precise limits of their discipline, debating whether the field should concern itself with the ethical evaluation of all questions involving biology and medicine, or only a subset of these questions.

The scope of bioethics can expand with biotechnology, including cloning, gene therapy, life extension, human genetic engineering, astroethics and life in space, and manipulation of basic biology through altered DNA, RNA and proteins. These developments will affect future evolution, and may require new principles that address life at its core, such as biotic ethics that values life itself at its basic biological processes and structures, and seeks their propagation.

Similarly, medical ethics is the study of moral values and judgments as they apply to medicine which encompasses its practical application in clinical settings as well as work on its history, philosophy, theology, and sociology.

Medical ethics tends to be understood narrowly as an applied professional ethics, whereas bioethics appears to have worked more expansive concerns, touching upon the philosophy of science and issues of biotechnology. Still, the two fields often overlap and the distinction is more a matter of style than professional consensus. Besides, it also shares many principles with other branches of healthcare ethics, such as nursing ethics.

The term Bioethics (Greek bios, life; ethos, behavior) was coined in 1926 by Fritz Jahr, who "anticipated many of the arguments and discussions now current in biological research involving animals" in an article about the "bioethical imperative," as he called it, regarding the scientific use of animals and plants. In 1970, the American biochemist Van Rensselaer Potter also used the term with a broader meaning including solidarity towards the biosphere, thus generating a "global ethics," a discipline representing a link between biology, ecology, medicine and human values in order to attain the survival of both human beings and other animal species.

The National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research was initially established in 1974 to identify the basic ethical principles that should underlie the conduct of biomedical and behavioral research involving human subjects. However, the fundamental principles announced in the Belmont Report (1979) namely, autonomy, beneficence and justice have influenced the thinking of bioethicists across a wide range of issues. Others have added non-malificence, human dignity and the sanctity of life to this list of cardinal values.

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## 19.5 Summary

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Applications of biotechnology are on the verge of great expansion in this decade. The production and release of the resulting Genetically Engineered organisms (GEOs) have raised concern about possible risks to human and to the environment; accordingly, all biotechnology research has to be carried within a Regulatory Biosafety Framework. The establishment of such a system will ensure that Biotechnology continues to be safe and does not expose employees, the community and the environment to any possible hazards. Genetically altered microorganisms are useful in controlling pests and weeds in agriculture, producing vaccines, cleaning up toxic chemicals at waste sites, microbial leaching of mineral ores, and enhancing oil recovery. Plants are being genetically engineered to enhance many traits: increasing pest and herbicide resistance, tolerating draught or other environmental stresses, decreasing loss of food during storage and transport and increasing nutritional value of food products.

Intellectual property (IP) is a legal term that refers to creations of the mind which includes music, literature, and other artistic works; discoveries and inventions; and words, phrases, symbols, and designs. Some common types of intellectual property rights (IPR) are copyright, patents, and industrial design rights; and the rights that protect trademarks, trade dress, and in some jurisdictions trade secrets. Besides, it is a form of property, called intangible property.

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## 19.6 Glossary

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- **Accessible environment:** refers to the environment that can be reached by the organism and its progeny if introduced at the research site.
- **Biosafety:** refers to the policies and procedures adapted to ensure the environmentally safe application of biotechnology.
- **Contained facility:** refers to a structure, (e.g., a laboratory or greenhouse) which surrounds and encloses the organism to effectively restrict its movement outside the structure.
- **Genetically Modified Organism:** is operationally defined as an organism whose hereditary traits have been modified by human intervention using any

method that results in the introduction, rearrangement or removal of genetic material from the genome of an organism.

- **Managed or natural ecosystem:** refers to all plants, animal's microorganisms, and their interactions, in domesticated and wild environment.
- **Organism:** refers to any biological entity, cellular or non-cellular with the capacity for self-perpetuation and response to evolutionary forces.
- **Parental organism:** refers to the initial organism which is to be the recipient of introduced genetic material or whose genome is to be altered by removal or rearrangement of genetic material.
- **Research involving planned introduction into the environment:** refers to research outside a contained facility at a designated site(s) with appropriate confinement.
- **Risk Assessment:** refers to assessment of the risks of introducing R-DNA engineered organism into the environment, to human and natural or managed ecosystem.

## **19.7 Self-Learning Exercise**

### **Section A : (Very Short Answer Type Questions)**

1. IPR includes \_\_\_\_\_ , \_\_\_\_\_
2. Industrial design right protects \_\_\_\_\_
3. Full name of NBC is \_\_\_\_\_
4. Bioethics term was coined by \_\_\_\_\_
5. Medical ethics is the study of \_\_\_\_\_

### **Section B : (Short Answer Type Questions)**

1. Briefly describe Bioethics?
2. What do you mean by Trademark?

### **Section C : (Long Answer Type Questions)**

1. Write notes on –
  - (1) Patent

- (2) Copy right
2. Describe Intellectual property Rights and its various branches?
  3. What do you mean by Biosafety? Describe its norms and field of Application.

**Answer key of Section – A**

1. Patent, copy right, trademark and plant varieties
2. Visual design of object.
3. National Biosafety Committee
4. Fritz Jahr ,1926
5. Moral values and judgements apply to medicine

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## **19.8 References**

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## Unit - 20

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# Biotechnology and Genetics Engineering in Human Welfare

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### Structure of the Unit:

- 20.0 Objectives
  - 20.1 Introduction
  - 20.2 History of Biotechnology
  - 20.3 Branches of Biotechnology
  - 20.4 Applications of Biotechnology
    - 20.4.1 Agricultural Applications
    - 20.4.2 Medicinal Applications
    - 20.4.3 Environmental Applications
    - 20.4.4 Industrial Applications
  - 20.5 Achievements and Prospects in Biotechnology
  - 20.6 Summary
  - 20.7 Glossary
  - 20.8 Self-Learning Exercise
  - 20.9 References
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### 20.0 Objectives

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After studying this unit we understand about -

- biotechnology, application of Biotechnology and Genetics Engineering in Agriculture, Medicine, Environmental, Industry;
- Biotechnological Achievements and Prospects

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## 20.1 Introduction

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Biotechnology is the use of living organisms to develop or make products, or "any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use". Depending on the tools and applications, it often overlaps with the fields of bioengineering, biomedical engineering, etc. For time immemorial, humankind has used biotechnology in agriculture, food production, and medicine. In the late 20th and early 21st century, biotechnology has expanded to include new and diverse sciences such as genomics, recombinant gene techniques, applied immunology, and development of pharmaceutical therapies and diagnostic tests.

The concept of "biotech" or "biotechnology" encompasses a wide range of procedures for modifying living organisms according to human purposes, going back to domestication of animals, cultivation of plants, and "improvements" to these through breeding programs that employ artificial selection and hybridization. It also includes genetic engineering as well as cell and tissue culture technologies. According to American Chemical Society biotechnology is the application of biological organisms, systems, or processes by various industries to learning about the science of life and the improvement of the value of materials and organisms such as pharmaceuticals, crops, and livestock.

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## 20.2 History of Biotechnology

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Agriculture has been speculating to have the dominant way of producing food since the Revolution. Through early biotechnology, the earliest farmers selected and bred the best suited crops, having the highest yields, to produce enough food to support a growing population. As crops and fields became increasingly large and difficult to maintain, it was discovered that specific organisms and their by-products could effectively fertilize, restore nitrogen, and control pests. Throughout the history of agriculture, farmers have inadvertently altered the genetics of their crops through introducing them to new environments and breeding them with other plants.

Biotechnology has also led to the development of antibiotics. In 1928, Alexander Fleming discovered the mold *Penicillium*. His work led to the purification of the antibiotic compound formed by the mold by Howard Florey, Ernst Boris Chain and

Norman Heatley - to form what we today know as penicillin. In 1940, penicillin became available for medicinal use to treat bacterial infections in humans. The field of modern biotechnology is generally thought of as having been born in 1971 when Paul Berg's (Stanford) experiments in gene splicing had early success. Herbert W. Boyer and Stanley N. Cohen significantly advanced the new technology in 1972 by transferring genetic material into a bacterium, such that the imported material would be reproduced.

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## 20.3 Branches of Biotechnology

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Biotechnology is the integration of natural science and organisms, cells and molecular analogues for products and services. Moreover, it also includes pure biological sciences like- animal cell culture, biochemistry, cell biology, embryology, genetics, microbiology, and molecular biology.

Bioinformatics is an interdisciplinary field which deals with biological problems using computational techniques, and makes the rapid organization as well as analysis of biological data possible. The field may also be referred to as computational biology, and can be defined as, "conceptualizing biology in terms of molecules and then applying informatics techniques to understand and organize the information associated with these molecules, on a large scale." Bioinformatics plays a key role in various areas, such as functional genomics, structural genomics, and proteomics, and forms a key component in the biotechnology and pharmaceutical sector.

**Blue biotechnology** is a term that has been used to describe the marine and aquatic applications of biotechnology, but its use is relatively rare.

**Green biotechnology** is applied to agricultural processes, such as the selection and domestication of plants via micro propagation, designing of transgenic plants to grow under specific environments in the presence (or absence) of chemicals. Green biotechnology might produce more environmentally friendly solutions than traditional industrial agriculture. An example of this is the engineering of a plant to express a pesticide, thereby ending the need of external application of pesticides. An example of this would be *BT* corn. Whether or not green biotechnology products such as this are ultimately more environmentally friendly is a topic of considerable debate.

**Red biotechnology** is applied to medical processes. Some examples are the designing of organisms to produce antibiotics, and the engineering of genetic cures through genetic manipulation.

**White biotechnology**, also known as industrial biotechnology, is biotechnology applied to industrial processes. An example is the designing of an organism to produce a useful chemical. Another example is the using of enzymes as industrial catalysts to either produce valuable chemicals or destroy hazardous/polluting chemicals. White biotechnology tends to consume less in resources than traditional processes used to produce industrial goods. The investment and economic output of all of these types of applied biotechnologies is termed as "bioeconomy".

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## 20.4 Applications of Biotechnology

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Biotechnology holds many advantages in the fields of Environmental, Industrial, Agriculture and medicines. Environmental biotechnology includes Clean technology, Clean coal technology, Ecological design, Ecological engineering, Ecotechnology, Environmental engineering, Environmental engineering science, Green building, Green nanotechnology, Landscape engineering, Renewable energy, design, Sustainable. Similarly, Industrial biotechnology includes Automation, Business informatics, Engineering management, Enterprise engineering, Financial engineering, Industrial biotechnology, Industrial engineering, Metallurgy, Mining engineering, Productivity improving technologies, Research and development and in Agriculture biotechnology, Agricultural engineering, Aquaculture, Fisheries science, Food chemistry, Food engineering, Food microbiology, Food technology, GURT, ICT, Nutrition. At the same time, Medical biotechnology includes Bioinformatics, Biological, Biomechatronics, Biomedical, Biotechnology, Cheminformatics, Genetic engineering, Healthcare science, Medical research, Medical technology, Nanomedicine, Neuroscience, Neurotechnology, Pharmacology, Reproductive.

### 20.4.1 Agricultural Applications

Genetically modified crops ("GM crops") are plants used in agriculture .In most cases the aim is to introduce a new trait to the plant which does not occur naturally in the species. Examples in food crops include resistance to certain pests, diseases, stressful environmental conditions, resistance to chemical treatments i.e. resistance

to herbicide, reduction of spoilage, or improving the nutrient profile of the crop. Similarly in non-food crops include production of pharmaceutical agents, biofuels, and other industrially useful goods, as well as for bioremediation.

Genetically modified foods are produced from organisms that have specific changes introduced into their DNA with the methods of genetic engineering. These techniques have allowed for the introduction of new crop traits as well as a far greater control over a food's genetic structure than previously afforded by methods such as selective breeding and mutation breeding. Besides, There is broad scientific consensus that food on the market derived from GM crops poses no greater risk to human health than conventional food. GM crops also provide a number of ecological benefits,. However, opponents have objected to GM crops per se on several grounds, including environmental concerns, whether food produced from GM crops is safe, whether GM crops are needed to address the world's food needs, and economic concerns raised by the fact these organisms are subject to intellectual property law.

#### **20.4.2 Medicinal applications**

Biotechnology finds applications in areas such as pharmaceutical drug discovery and production, pharmacogenomics, and genetic testing and DNA microarray chip – some can do as many as a million blood tests at once. Pharmacogenomics is the technology that analyses how genetic makeup affects an individual's response to drugs. It deals with the influence of genetic variation on drug response in patients by correlating gene expression or single-nucleotide polymorphisms with a drug's efficacy or toxicity. By doing so, pharmacogenomics aims to develop rational means to optimize drug therapy, with respect to the patients' genotype, to ensure maximum efficacy with minimal adverse effects. Such approaches promise the advent of "personalized medicine"; in which drugs and drug combinations are optimized for each individual's unique genetic makeup.

Biotechnology has contributed to the discovery and manufacturing of traditional small molecule pharmaceutical drugs as well as drugs that are the product of biotechnology-biopharmaceutics. Modern biotechnology can be used to manufacture existing medicines relatively easily and cheaply. The first genetically engineered products were medicines designed to treat human diseases. To cite one example, in 1978 Genentech developed synthetic humanized insulin by joining its

gene with a plasmid vector inserted into the bacterium *Escherichia coli*. Insulin, widely used for the treatment of diabetes, was previously extracted from the pancreas of abattoir animals (cattle and/or pigs). The resulting genetically engineered bacterium enabled the production of vast quantities of synthetic human insulin at relatively low cost. Biotechnology has also enabled emerging therapeutics like gene therapy. The application of biotechnology to basic science has also dramatically improved our understanding of biology and as our scientific knowledge of normal and disease biology has increased, our ability to develop new medicines to treat previously untreatable diseases has increased as well.

Genetic testing allows the genetic diagnosis of vulnerabilities to inherited diseases, and can also be used to determine a child's parentage or in general a person's ancestry. In addition to studying chromosomes to the level of individual genes, genetic testing in a broader sense includes biochemical tests for the possible presence of genetic diseases, or mutant forms of genes associated with increased risk of developing genetic disorders. Genetic testing identifies changes in chromosomes, genes, or proteins. Most of the time, testing is used to find changes that are associated with inherited disorders. The results of a genetic test can confirm or rule out a suspected genetic condition or help determine a person's chance of developing or passing on a genetic disorder. Since genetic testing may open up ethical or psychological problems, genetic testing is often accompanied by genetic counseling.

#### **20.4.3 Environmental Applications**

Environmental biotechnology is used in waste treatment and pollution prevention. Environmental biotechnology can more efficiently clean up many wastes than conventional methods and greatly reduce our dependence on methods for land-based disposal. Every organism ingests nutrients to live and produces by-products as a result. Different organisms need different types of nutrients. Some bacteria thrive on the chemical components of waste products. Environmental engineers use bioremediation, the broadest application of environmental biotechnology, in two basic ways. They introduce nutrients to stimulate the activity of bacteria already present in the soil at a waste site, or add new bacteria to the soil. The bacteria digest the waste at the site and turn it into harmless byproducts. After the bacteria

consume the waste materials, they die off or return to their normal population levels in the environment.

Bioremediation is an area of increasing interest. Through application of biotechnical methods, enzyme bioreactors are being developed that will pretreat some industrial waste and food waste components and allow their removal through the sewage system rather than through solid waste disposal mechanisms. Waste can also be converted to biofuel to run generators. Microbes can be induced to produce enzymes needed to convert plant and vegetable materials into building blocks for biodegradable plastics. In some cases, the byproducts of the pollution-fighting microorganisms are themselves useful. For example, methane can be derived from a form of bacteria that degrades sulfur liquor, a waste product of paper manufacturing. This methane can then be used as a fuel or in other industrial processes.

#### **20.4.4 Industrial Applications**

Industrial biotechnology includes the practice of using cells such as micro-organisms, or components of cells like enzymes, to generate industrially useful products in sectors such as chemicals, food and feed, detergents, paper and pulp, textiles and biofuels. In doing so, biotechnology uses renewable raw materials and may contribute to lowering greenhouse gas emissions and moving away from a petrochemical-based economy.

Industrial biotechnology applies the techniques of modern molecular biology to improve the efficiency and reduce the environmental impacts of industrial processes like textile, paper and pulp, and chemical manufacturing. For example, industrial biotechnology companies develop biocatalysts, such as enzymes, to synthesize chemicals. Enzymes are proteins produced by all organisms. Using biotechnology, the desired enzyme can be manufactured in commercial quantities. Commodity chemicals (e.g., polymer-grade acryl amide) and specialty chemicals can be produced using biotech applications. Traditional chemical synthesis involves large amounts of energy and often-undesirable products, such as HCl. Using biocatalysts, the same chemicals can be produced more economically and more environmentally friendly. Detergent proteases, which remove protein impurities, are essential components of modern detergents. They are used to break down protein, starch, and fatty acids present on items being washed. Protease

production results in a biomass that in turn yields a useful byproduct- an organic fertilizer. It is also used in the textile industry for the finishing of fabrics and garments. Besides, it also produces biotech-derived cotton that is warmer, stronger, has improved dye uptake and retention, enhanced absorbency, and wrinkle- and shrink-resistance. Some agricultural crops, such as corn, can be used in place of petroleum to produce chemicals. The crop's sugar can be fermented to acid, which can be then used as an intermediate to produce other chemical feedstocks for various products.

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## 20.5 Achievements and Prospects in Biotechnology

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Genetic engineering is a source of incalculable benefits to medicine and agriculture. All organisms are made up of cells that are programmed by the same basic genetic material, called DNA (deoxyribonucleic acid). Each unit of DNA is made up of a combination of the following nucleotides -- adenine (A), guanine (G), thymine (T), and cytosine (D) -- as well as a sugar and a phosphate. These nucleotides pair up into strands that twist together into a spiral structure call a "double helix." This double helix is DNA and its segments are genes. It is the presence or absence of the specific protein that gives an organism a trait or characteristic. More than 10,000 different genes are found in most plant and animal species. This total set of genes for an organism is organized into chromosomes within the cell nucleus. The process by which a multicellular organism develops from a single cell through an embryo stage into an adult is ultimately controlled by the genetic information of the cell, as well as interaction of genes and gene products with environmental factors.

Genetic engineering is the technique of removing, modifying or adding genes to a DNA molecule in order to change the information it contains. By changing this information, genetic engineering changes the type or amount of proteins an organism is capable of producing. Genetic engineering is used in the production of drugs, human gene therapy, and the development of improved plants. For example, an "insect protection" gene (*Bt*) has been inserted into several crops - corn, cotton, and potatoes - to give farmers new tools for integrated pest management. *BT* corn is resistant to European corn borer. This inherent resistance thus reduces a farmer's

pesticide use for controlling European corn borer, and in turn requires fewer chemicals and potentially provides higher yielding Agricultural Biotechnology.

Benefits can also be seen in the environment, major advances also have been made through conventional breeding and selection of livestock, but significant gains can still be made by using biotechnology. Traditional biotechnology such as cross-pollination in corn produces numerous, non-selective changes. Genetic modifications have produced fruits that can ripen on the vine for better taste, yet have longer shelf lives through delayed pectin degradation. Tomatoes and other produce containing increased levels of certain nutrients, such as vitamin C, vitamin E, and or beta carotene, and help protect against the risk of chronic diseases, such as some cancers and heart disease. Similarly introducing genes that increase available iron levels in rice three-fold is a potential remedy for iron deficiency, a condition that affects more than two billion people and causes anemia in about half that number.

Modern biotechnology has offered opportunities to produce more nutritious and better tasting foods, higher crop yields and plants that are naturally protected from disease and insects. Modern biotechnology allows for the transfer of only one or a few desirable genes. It also offers effective techniques to address food safety concerns. Biotechnical methods may be used to decrease the time necessary to detect food borne pathogens, toxins, and chemical contaminants, as well as to increase detection sensitivity. Enzymes, antibodies, and microorganisms produced using rDNA techniques are being used to monitor food production and processing systems for quality control.

Genetic engineering in agriculture has recognized that genetic engineering has an important contribution to make in medicine. This field of science permits scientists to regenerate or to grow body-part replacements in laboratories. Indeed, organs could be genetically engineered to correct a patient's own genetic deficiencies. Genetic engineers have also succeeded in developing processes that affect man's interaction with pests, e.g., insects, to prevent the spread of disease. Genetic engineering with respect to medical applications takes a number of forms. Gene Therapy, one of the areas of concentration involves the use of viruses as the vehicles for transferring preferred genetic characteristics to cellular DNA. This approach is being used to address diseases such as AIDS and various cancers. On

the cosmetic side, scientist is engaged in genetic engineering to obtain greater uniformity and symmetry in Christmas tree species. Genetically engineered agricultural products have demonstrated that they can boost yields of production, diminish the reliance on synthetic pesticides, prolong the shelf life of foods, and improve the healthful features of foods generally. One important development is the transfer through genetic technology of a natural insecticide known as *Bacillus thuringiensis (BT)* to the genetic make-up of certain plants. Other plants have been genetically altered so that is immune to the effects of certain herbicides used to kill weeds. These developments have reduced the need to use hundreds of thousands of gallons of pesticides in the United States.

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## 20.6 Summary

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The applications of biotechnology are so broad, and the advantages so compelling, that virtually every industry is using this technology such as diverse as pharmaceuticals, diagnostics, textiles, aquaculture, forestry, chemicals, household products, environmental cleanup, food processing, forensics etc. Biotechnology is enabling these industries to make new or better products, often with greater speed, efficiency and flexibility. Moreover, modern biotechnology offers opportunities to improve product quality, nutritional content, and economic benefits. The genetic makeup of plants and animals can be modified by either insertion of new useful genes or removal of unwanted ones. Biotechnology is changing the way plants and animals are grown, boosting their value to growers, processors, and consumers.

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## 20.7 Glossary

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- **Biomass:** Material produced by or remaining after the death of organisms (e.g., bacteria, plants, and animals).
- **Biotechnology:** A collection of technologies that use living cells and/or biological molecules to solve problems and make useful products.
- **Bioremediation:** The use of organisms, usually microorganisms, to break down pollutants in soil, air or groundwater.
- **DNA (Deoxyribonucleic acid):** The chemical molecule that is the basic genetic material found in all cells. Each unit of DNA is made of nucleotides:

adenine (A), guanine (G), thymine (T) and cytosine (C), as well as a sugar and a phosphate.

- **Enzymes:** A protein that accelerates the rate of chemical reactions. Enzymes are catalysts that promote reactions repeatedly, without being damaged by the reactions.
- **Gene:** A unit of hereditary information. A gene is a section of a DNA molecule that specifies the production of a particular protein.
- **Genetic Engineering:** The technique of removing, modifying or adding genes to a DNA molecule in order to change the information it contains. By changing this information, genetic engineering changes the type or amount of proteins an organism is capable of producing.
- **Genetically modified organism (GMO):** An organism that has been modified, or transformed, using modern techniques of genetic exchange is commonly referred to as a genetically-modified organism (GMO).
- **Gene therapy:** Altering DNA within cells in a living organism to treat or cure a disease. It is one of the most promising areas of biotechnology research. New genetic therapies are being developed to treat diseases such as cystic fibrosis, AIDS and cancer.
- **Genome:** The complete set of an organism's genetic information. In humans this corresponds to twenty-three pairs of chromosomes.
- **Molecular biology:** A branch of biology concerned with studying the chemical structures and processes of biological phenomena at the molecular level.
- **Recombinant DNA:** DNA that is formed by combining DNA from two different sources. Humans direct formation of recombinant DNA through selective breeding and genetic engineering.
- **Recombinant DNA (rDNA) technology:** The laboratory manipulation of DNA in which DNA, or fragments of DNA from different sources, is cut and recombined using enzymes. This recombinant DNA is then inserted into a

living organism. rDNA technology is usually used synonymously with genetic engineering.

- **Transgenic plant:** Genetically engineered plant or offspring of genetically engineered plants. Transgenic plants result from the insertion of genetic material from another organism so that the plant will exhibit a desired trait. Recombinant DNA techniques are usually used.

## 20.8 Self-Learning Exercise

### Section A : (Very Short Answer Type Questions)

1. Transgenic plants are formed by \_\_\_\_\_.
2. Green biotechnology is applied to \_\_\_\_\_.
3. Pharmacogenomics is a combination of \_\_\_\_\_.
4. Genome is a \_\_\_\_\_.
5. Two Industrial application of Biotechnology involves \_\_\_\_\_, \_\_\_\_\_

### Section B : (Short Answer Type Questions)

1. What do you mean by Bioinformatics?
2. What are GM crops?

### Section C : (Long Answer Type Questions)

1. Write an essay on Biotechnological achievements and Prospects.
2. Describe the application of biotechnology and genetic engineering?

### Answer key of Section – A

- 1 R-DNA technology
- 2 Agricultural process
- 3 Pharmacology and Genomics
- 4 Complete set of genetic material of a cell
- 5 Textile and Petroleum

## 20.9 References

- P K Gupta - Biotechnology
- Ramawat - Biotechnology
- Alberts - Molecular biology of cell