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BIOTECHNOLOGY AND GENETIC ENGINEERING OF PLANTS AND MICROBES

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Basic Concepts

Although "cloning of DNA" is considered as the most important event in the history of biotechnology, but, the origin of biotechnology may have occurred in prehistoric times where microbes were used in fermentation like processes. Chaim Weizmann used Clostridium acetobutylicum for converting starch into butanol and acetone in 1920. This indicated that chemicals can be commercially produced through biological processes or biotechnology. Commercial production of penicillin by Penicillium notatum was another important event during World War II. And of late development of recombinant-DNA technology has revolutionized sciences and now biotechnology has assumed special significance.

Biotechnology can be defined as the symbiosis between science and technology. It means the production of compounds or substance of human economic value by the technology based on biological organisms or principles. Products of biotechnology e.g., pharmaceutical drugs, new food sources etc are playing important role in the society. Biotechnology includes fermentation by microorganisms, formation of yogurt, cheese, vinegar, antibiotics, baking, brewing as well as recombinant biotechnology all come under the area of Biotechnology.

Principle and Scope

According to British Biotechnologists, Biotechnology is "the application of biological organisms, system or processes to manufacturing and service industries". While, according to US National Science Foundation - its the controlled use of biological agents such as micro-organisms or cellular components for beneficial use.

Biotechnology has applications in various areas and it has a wide scope. Important areas are described below:

1. Tissue Culture and Biotechnology

Tissue culture is an important part of biotechnology. Plant cells and tissues are used for variety of purposes eg. anther culture, gameto- clonal and somaclonal variations, artificial seeds, production of transgenic plant by protoplast transformation etc.

Animal cell tissue culture has also large-scale applications production of superior live stock, invitro fertilization etc.

2. Medicine and Biotechnology

Biotechnology has enormous application in the field of medicine eg. production of insulin, interferons, vaccines and antibodies. Probes are also made of detection of various diseases. Gene therapy can be used for curing genetic diseases and DNA fingerprinting has application in forensic medicine.

3. Industrial Microbiology and Biotechnology

A large number of drugs and chemicals are produced using industrial microbial biotechnology. Acetone, butanol, enzymes, amino-acids etc. are commercially produced by biotechnology.

4. Gene technology and biotechnology

Recombinant DNA technology and gene cloning is used in most of the biotech companies to produce recombinant products. PCR is also used in gene technology.

5. Hybridoma and monoclonal antibodies and Biotechnology

It is an important area where progress is occurring. Antibodies are used for producing diagnostic kits.

6. Protein Engineering and Biotechnology

By use of this better enzymes and proteins are produced. Use of immobilized enzymes is also an important source of cutting cost during product formation in various industries.

7. Agriculture and Biotechnology

Use of biotechnology has revolutionized agriculture plant tissue culture, transgenic plants (eg BT cotton), somatic hybrids and transgenic animals have contributed to this. But still lot of progress is going on in these areas. Biofertilizers and Biopesticides are common now days.

8. Environment and Biotechnology

Biotechnology has application in - pollution control, extraction of universals, source of energy, restoration of degraded lands and biodiversity conservation. Use of biopesticides, bioinsecticides and biosensor provide cleaner environment.

But questions are being raised that there are concerns regarding the release of recombinant products. In this view rules have been made.

9. Intellectual Property Rights and Biotechnology

IPR include patents, trade secrets, trademarks and copyrights. It is thought that developments in biotechnology should be protected by IPR but some areas are exempted e.g. medical sciences, developments cannot be patented. But products like house hold products, modified antibiotics etc can be patented.

Biotechnology in India

Like the developed countries, USA, Japan and Europe, India is also giving priority to development of biotechnology. National Biotechnology Board has been made under the Department of Science and Technology to develop this area. A separate "Department of

Biotechnology" in the Ministry of Science and Technology was established in 1986. International Center for Genetic Engineering and Biotechnology (ICGEB) was established in 1987.

Important centers for Biotechnology in India are - IARI (Indian Agricultural Research Institute, Delhi), NDRI (National Dairy Research Institute, Karnal), IVRI (Indian Veterinary Research Institute, Izatnagar (U.P.). Besides these researches are being done at various other places.

Important centers for Bioinformatics are Indian Institute of Information Technology, I.I.Sc., Maduri Kamraj University, JNU (Delhi), C.C.M.B. (Center for Cellular and Molecular Biology), Hyderabad.

DBT has provided infra-structural facilities for - Germplasm banks, animal houses, DNA synthesizes, enzyme production, radio labeled compounds, protein sequencing etc.

A large number of institutes are providing training in the area of Biotechnology from graduation to research level. Important achievement have been made in following areas:

- Embryo transfer to develop improved buffalo calves.
- DNA finger printing (Dr. Lalji Singh of CCMB)
- Diagnostic kit for hepatitis etc.
- Birth control
- Vaccines have been developed
- Micropropagation of plants
- Biopesticides and biofertilizers
- Aquaculture technology
- Sericulture

Besides these, work is going on in large number of areas and hopefully India will emerge as a country with Biotechnology know how and trained manpower in years to come.

Plant Cell and Tissue Culture

General Introduction, History and Scope

Plant tissue culture deals with techniques of protoplast, cell, tissue and organ culture in order to regenerate whole plants from cultured plant materials.

Development in this field is historically linked to discovery of cell and cell theory. More than 234 years ago Henri-Louis Duhamd du Monceau's (1756) demonstrated spontaneous callus formation during wound healing in elm plants. Tre'cule (1853) observed callus formation in number of plants and Wiesner (1884) that organ forming substances are distributed in a polar fashion. These historical findings paved way for studying the role of in vitro cultures. The major historical events in development of tissue culture were;

1. Concept of Cell Culture

German botanist G. Haberlandt (1902) developed the concept of invitro tissue culture. He first time cultured, isolated fully differentiated cells in nutrient medium. He is regarded as father of tissue culture.

2. Development of Tissue Culture

Hanning (1904) started culture of embryogonic tissue. He excised embryo from crucifers and grew them to maturity on lab media.

3. Root Tip Culture

Kotte and Robbins in 1922 started root tip culture. They could grow tips for short time but White (1934,37) developed continuously growing root tip cultures. Bonner (1937) demonstrated the importance of thiamine and Gantheret (1953) demonstrated the importance of Copper, Manganese, iodine and chelating ions on root metabolism.

4. Embryo Culture

Laibach (1925) demonstrated practical applications of zygotic embryo cultures in plant breeding. Hanning raised zygotic embryos from non-viable seeds, Van Overbeck (1941) used coconut milk for embryo development and callus formation in Datura.

5. Stem Tip Culture

Loo (1945) first time obtained cultures from stem tips of dodder and Asparagus. Ball (1946) developed a method of identifying exact part of shoot meristem which gives rise to whole plant.

6. Role of Auxin

Gautheret demonstrated that addition of auxin (IAA) enhanced the proliferation of cambial cultures. White reported similar results in cultures of tumor tissues and Nobe'court established

continuously growing cultures of carrot slices. Finally Role auxins in tissue cultures was independently announced by Gautheret, White and Nobe'court in 1939.

7. Discovery of Cytokinin

Skoog (1944), Skoog and Tsui (1951) demonstrated that adenine stimulates cell division and induces bud formation in tobacco tissue even in the presence of IAA. Skoog and Millar finally isolated kinetin (a derivative of adenine) from autoclaved yeast extracts. Letham (1963) isolated similar substance from young maize endosperm and called it zeatin. Later on these substances were called cytokinins.

8. Hormonal Control of Organ Formation

Skoog and Miller (1957) gave the concept of hormonal control of organ formation. They found that in tobacco pith cultures, high concentration of auxin promoted rooting whereas more cytokinin initiated shoot formation.

9. Media improvement

Initially Knop's mineral solution was used for tissue culture. White supplemented the medium with different trace elements. Murashige and Skoog (1962) proposed a solution 25 times more concentrated than Knop's medium. This medium gave better growth. Later on special media were developed for special purposes.

10. Preparation and cloning of single cell cultures

Stanford et al. (1948) started studies on single cell cultures of animal cells. Muir (1953) demonstrated that if callus of N. tabacum is agitated in shake cultures then single cells can be obtained. Muir et al (1954) induced cell divisions in these single cell shake cultures. On transfer to filter paper over a callus, cells multiplied and formed colonies, plating technique was developed by Bergmann 1960. Jones et al (1960) designed microculture method using hanging drop method. Vasil and Hildebrandt (1965) changed media, added coconut milk and NAA and observed cell divisions in isolated tobacco hybrid cells.

11. Regeneration of whole plant

Vasil and Hildebrandt (1965) first saw colonies arising from single cells which formed plantlets. Steward et al observed somatic embryogenesis for first time. These embryos later developed into plants.

Recent Advances in Tissue Culture (Scope and applications)

Efforts have been made to develop practical applications of tissue culture technology. Important applications have been made in the areas of morphology, biochemistry, pathology and genetics. Some of the areas where work is done and more scope exists are described below.

1. Morphology

Tissue culture is an important technique by which role of substances in cell differentiation and organ formation can be studied.

Another morphological application of tissue culture is micropropagation. By this process hundreds of plants can be raised by small amount of tissue. About 4 million genetically identical plants can be obtained from single bud. This is used extensively for rapid propagation of orchids and other rare endangered plants.

2. Production of Secondary Metabolites

Various important secondary metabolites are commercially produced by tissue culture e.g. Anthocyanins, steroids, pisatin etc. They can be used as pharmaceuticals, medicines, or in other industries.

3. Production of Pathogen-free Plants

Tissue culture can be used to generate virus free (White, 1934) and other pathogen free plants.

4. Genetics

Role of tissue culture in genetics is very important. The principle aspects are :

a. *Genetic variability:* Long term cultures are unstable and produce different kinds of cells. If regenerated separately they provide a direct source of genetic variability.

b. *In vitro pollination*: Reproductive organs of flowering plants can be cultured in vitro. Intra ovarian pollination and test tube fertilization can be easily done. By this, barriers at sexual level can be overcome. This is extensively used to overcome self incompatibility in different plants. This was first done by Zenkteler et al (1975).

c. *Induction of haploidy:* Tissue culture can be used to develop haploid plants. This was first done by Guha and Maheshwari (1966) in Datura innoxia for developing embryoids and plantlets from immature anthers. Later Bourgin and Nitsch (1967) developed haploids from pollen culture. San Noeum (1976) raised haploids from ovaries and ovules. These findings led to vast development in this area.

d. *Somatic hybridization:* Isolation, regeneration and fusion of protoplasts is also important area of genetic manipulation. Somatic hybridization is used as new tool in plant breeding. Pioneering work in this area was done by Cocking (1960), who isolated protoplasts using cellulose enzyme. 1st somatic hybrid was obtained by Carlson et al (1972). This area also has lot of scope.

e. *Genetic transformation:* Using recombinant DNA technology foreign genes can be introduced in plant protoplasts. Usually *Agrobacterium* is used as vector. This has lot of potential for crop improvement.

Thus tissue culture is an important area in biotechnology with great scopes and applications.

Concept of Cellular Differentiation and Totiopotency

Totiopotency can be deferred as the potential of a cell to grow and develop as a multicellular or multiorganed higher organism. The potential for this lies mainly in cellular differentiation. This shows that al genes needed for differentiation are present in individual cells, but many of them remain inactive in undifferentiated tissues and are expressed only under appropriate conditions. The integration of cell division and differentiation results in development of an adult organism from a single cell (zygote).

Cells isolated from differentiated tissue are generally non-dividing and quiescent. They undergo dedifferentiation and then redifferentiation in order to express totiopotency.

Dedifferentiation can be defined as the phenomenon of reversion of mature cell to meristmatic cell and formation of undifferentiated callus tissue. While, the ability of a dedifferentiated cell to form whole plant or plant organs is called redifferentiation. But cell differentiation is a basic event in development of higher organisms and is termed as cyto differentiation. The scheme of cyto differentiation is shown Figure 1.1:

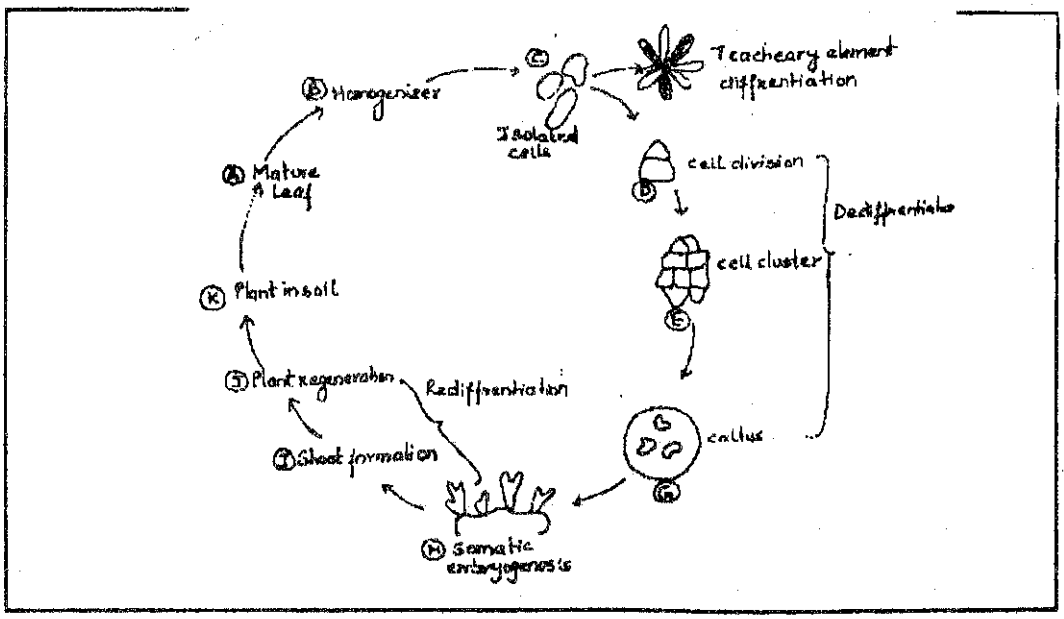


Figure 1.1

In contrast to plants the differentiation is irreversible in animals. By tissue culture factors responsible for differentiation can be studied.

Vascular Differentiation

Xylogenesis can be defined as differentiation of parenchyma into cells which have localized secondary wall thickening as in xylem of vascular plants. These xylem like cells are called wound vessels, vessels elements, tracheids and tracheary elements. They help in understanding mechanism of differentiation by following methods:

1. *Culture systems:* In cultures tracheary elements can be induced by wounding the internodes of plant cells. Callus culture : Vascular or tracheary elements can be induced in callus culture.
2. *Suspension culture:* Such studies show that in tobacco suspension culture enzymes in shikimate and cinnamate pathways are co-ordinately enhanced during trachery element differentiation.
3. *Single cell culture:* They are a very good system for studying cellular differentiation. Addition of kinetin and low ammonium concentration improves differentiation.

4. *Protoplast culture:* Vascular element formation has been reported in mesophyll tissue of Zinnia. But this system needs further improvement as rate of formation was low. But this is a good system to study fundamental process of cytodifferentiation.

Cytological and Cytochemical Aspects

Such changes during differentiation have been studied using electron microscope. The important changes are following:

a. Volume of cytoplasm and number of organelles (except chloroplast) increase when cells are cultured in differentiation inducing medium.

b. Chloroplasts gradually get disordered, so large space appears between chloroplasts and plasma membrane.

c. Cells synthesize metabolites and elongate, reticulate and helical thickening appear.

d. Secondary thickenings of cellulose micro fibrils appear.

e. ER, dictyosome and microtubules present between thickenings. Liquification occurs by cell wall bound enzymes.

f. General feature of secondary wall thickenings is cell autolysis which occur due to loss of nucleus and cytoplasmic contents in cultured cells.

g. This process of cell death is programmed at beginning of secondary cell wall thickening.

h. Vacuoles in trachery elements function as lysosomes, which lead to disruption of intracellular structures.

Physiological Aspects of Differentiation

Phytohormones have qualitative as well as quantitative effect on differentiation.

a. *Auxin and cytokinin:* Endogenous auxin is essential for cytodifferentiation. Cytokinin is absolutely required for vascular differentiation. But the concentrations may vary depending upon plants. Auxin is the principle limiting factor of vascular differentiation and cytokinin is essential in the next process of cytodifferentiation.

b. *Gibberellic acid, abscisic acid, ethylene and cAMP:* The requirement of GA in medium for callus differentiation is not absolutely necessary. Abscisic acid has inhibitory effect. Ethylene production is closely involved with vascular differentiation. Camp acts as secondary messenger after hormonal stimulus and plays a major role.

Other Chemical Factors

Sucrose acts as carbon source but also act as regulator of xylem differentiation. Reduction of total Nitrogen also increases vascularization.

Physical Factors

Light, temperature and pH effect differentiation and all should be present at optimum levels.

Cell Division and Cytodifferentiation

Cell division is closely associated with cytodifferentiation. Some workers reported that cell replication precedes the differentiation of xylem. Same chemical factors regulate both the processes so invitro systems are good for studying relationships between them.

Organogenic Differentiation

It is the outcome of process of dedifferentiation followed by redifferentiation of cells. Unorganized growth is favored by dedifferentiation and it results in randomly dividing callus. These can redifferentiate into shoots and buds under appropriate conditions. The cultured cells can regenerate into whole plants either by shoot-bud differentiation or by somatic embrygenesis. This also prove totioponency of somatic cells.

Factors Affecting Shoot-bud Differentiation

1. *Chemical factors:* Auxin/cytokinin ratio effects shoot bud differentiation. Cytokinin in the medium promotes bud differentiation and development. A relatively higher auxin concentration leads to cell proliferation and root differentiation. High amounts of phosphate in the medium remove inhibition of auxin and promote bud formation in absence of cytokinin. Casein hydrolyase or tyrosine also has similar effect. Polyamines help in induction of cell division, growth and differentiation of bacterial, animal and plant cells.

2. *Physical factors:* Light intensity has important role in organogenesis. High light intensity is inhibitory in tobacco. The light quality influences organogenic differentiation. Blue light promotes shoot bud differentiation in tobacco callus and red light promotes rooting. Alternate light and dark periods (15-16 hu) give good differentiation.

Temperature is also a critical factor. Temperature up to 33°C increases callus growth but for shoot-bud differentiation (18°C) low temperatures are better.

State of medium is also critical. Solid medium favours bud formation.

Genome and explant's physiological state also influence differentiation.

Anatomy of Shoot-bud Differentiation

During unorganized growth meristems in callus are scattered. Organized growth leads to formation of meristemoids (nodules/growth centers). These are localized clusters of cells which become vascularised by development of tracheidal cells in center. They act as sink of organ formation and lead to root or shoot formation.

Totipotency of Epidermal Cells

A single epidermal cell of flax can form shoot and buds when cultured under appropriate conditions. This also occurs in other species like *Daccus carota, Nicotiana tabacum* etc. For this purpose epidermal cells of hypocotyls of young seedlings are excellent materials.

Totipotency of Crown-gall Cells

Usually crown-gall cells lack organogenic differentiation. But they have unlimited growth capacity. A special type of crown-gall tumors can be induced in some plants by infection of *Agrobacterium tumefaciens*. Cells of these tumors have high potential to differentiate shoot-buds and leaves in in-vitro cultures. But shoots derived from these teratomas (crown-galls) are abnormal in growth and morphology. But with improved methods whole plants have been recovered. This system is of great use in genetic manipulation.

Organogenesis and Adventive Embryogenesis

Fundamental Aspects of Morphogenesis

Cell differentiation does not occur in isolation but its a coordinated part of the development of an organism as a whole. The plant development does not just require differentiation of cells into specialized cell types, but it also requires organization of differentiated cells into tissues and organs. This process of "development of forms" is called morphogenesis. Thus, organogenesis is an outcome of the process of dedifferentiation followed by redifferentiation of cell.

As early as 1939, White observed that submerged callus of tobacco hybrid could be induced to form leaf buds and shoot like structures. The whole plant regeneration from cultured cell may occur through:

1. Shoot-bud differentiation
2. Somatic embryogenesis

Organogenesis refers to the induction of morphologically well-defined organs such as shoot or root from callus cultures. Skoog and Miller (1957) pointed out that a balance between the relative amounts auxins and cytokinin plays a great role in initiation of shoot and root.

Embryogenesis refers to the development of somatic bipolar adventive embryoid from callus culture under certain nutritional and hormonal conditions. This development follows a sequence through pro-embryoid, globular and torpedo stages.

The tissue culture techniques can be used to generate whole plants from single cells and this potential is called totiopotency.

Somatic Embryogenesis

Somatic embryogenesis is a process in which a single cell or a group of cells initiate a developmental pathway which lead to reproducible regeneration of non-zygotic embryos which can germinate to form complete plants. This is not a natural pathway but occurs frequently in tissue cultures (alternative to organogenesis) and leads to whole plant formation. According to Sharp et al (1982) somatic embryogenuesis is initiated either by "Pre-Embryogenic Determined Cells" (PEDCs) or by Induced Embryogenic Determind Cells (IEDCs).

In PEDCs the embryogenic pathway is predetermined. The presence or removal of inducer activates the cell to start mitotic divisions e.g. callus and embryosac. While in IEDCs the exposure to specific growth the regulator eg. 2,4-D redetermines the embryonic state of cells. These cells differentiate in anther and callus culture. On reaching the embryogenic state both types of cells proliferate in a similar way i.e., the Embryogenic Determined Cells (EDCs). These cells follow embryogenic pathway and produce plantlets.

Some time cells escape and form embryoids or nodular embryogenic cell, which consists of proembryoids - these are embryo like structure with bipolar unit, which can form full plantlet under suitable conditions.

Embryos formed in culture are called - Accessory embryos, adventive embryos, embryoids and supernumerary embryos.

Classification of embryos (according to Kohlenbach, 1978)

1. Zygotic embryos - those formed by fertilized egg or zygote.
2. Non-zygotic embryos-formed by cells other than zygote.
   i. Somatic embryos-formed by sporophytic cells (except zygote).
   ii. Adventives embryos- somatic embryos arising from embryos or organs e.g. stem embryos in carrot.
   iii. Parthenogenesis embryos -formed by unfertilized cells.
   iv. Androgenic embryos -formed by male gametophyte

Somatic Embryogenesis in Dicotyledonous Cultures

Explants from embryonic cells or seedling tissues can be used to generate totipotent embryogenic cells. Explants from other places can also be used. Like from inforescence, scutellum etc. Somatic embryos germinate in situ or when they are excised and cultured on fresh semi-solid medium.

Basic requirement for this are:

i. *Auxin supply*: The presence of auxins in the medium is essential for somatic embryogenesis.

ii. *Nitrogen source*: A nitrogen source usually in the reduced form is required for embryo initiation and maturation.

iii. *Other constituents:* Presence of high concentration of potassium, dissolved oxygen are critical.

iv. *Establishment of suppertime culture:* Spinning, stirred cultures or bioreactors can be used.

Somatic Embryogenesis in Monocotyledonous Cultures

Best explants are from embryogenic or meristematic tissues. First an embryogenic suspension is prepared, somatic embryos can be obtained without 2,4-D.

Embryo Maturation and Plantlet Development

Somatic embryos can only germinate when its mature to develop functional shoot and root apices. In presence of high auxin development and growth of shoot, meristem can be inhibited. Addition of cytokinin and ABA in low level is beneficial. Various physical factors also effect maturation. For example species, which grow in cold, require chilling for embryo maturation. Somatic embryos germinate on agar medium without growth regulators. After a number of leaves are formed, the small plantlets are transferred to jiffy pots, or vermiculite, for subsequent growth and development.

Loss of Morphogenic Potential in Embryogenic Cultures

The callus or suspension cultures due to aging or long period of subculturing looes morphogenetic ability. This could be to:

1. Genetic - Nuclear changes like polyploidy, aneuploidy etc.
2. Physiological changes : Altered hormonal balance.
3. Competitive growth : Competition between morphogenetic and non-totiopotent cells.

Practical Applications of Somatic Embryogenesis

Clone Propagation: somatic embryos can be used to proliferate and generate clones. Somatic embryogenesis can be combined with recombinant DNA technology for plant improvement.

1. Cloning Zygotic embryos for repetitive embryogenesis

During cloning of zygotic embryos many genotypes undergo auxin-stimulated somatic embryogenesis. They can form somatic embryoids under proper nutritional conditions.

2. Raising somaclonal variants in tree species

Embryos from PEDCs produce clonal embryos while embryos from IEDCs generate high frequency of somaclonal variants. Mutations occur during adventive embryogenesis which can generate a new strain of plant. Nucellar embryos like, shoot tips are free of virus and can be used for raising virus free clones, specially from tree species. For colonal propagation of tree species, somatic embryogenesis from nuclear cells is a good process.

3. Preservation of genotype

Somatic embryos are convenient organs for cryopreservation and germplasm storage.

4. Synthesis of Artificial seeds

Artificial seeds consisting of somatic embryos enclosed in a protective coating are "low-cost-high-volume" propagation system, two types of artificial seeds have been developed- (i) Hydrated (ii) Desiccated Hydrated seeds are formed by mixing somatic embryos with sodium alginate followed by dropping in a solution of calcium chloride to form calcium alginate beads. Coating with synthetic material makes Dessicate seeds; a 5% solution of polyethylene oxide (polyox WSR N-750) is mixed with equal volume of embryo suspension. This water-soluble resin is later dried to form polyembryonic desiccated seeds.

Embryo hardening treatment with 12% sucrose or 10-16M ABA followed by chilling increases survival of encapsulated embryos.

Fluid drilling is also used to obtain transgenic plants from somatic embryos. The embryos are suspended in viscous carrier gel, which extrudes into the soil.

5. Source of Regenerable Protoplast system

Embryogenic callus, suspension cultures and somatic embryos are used as source of protoplast isolation. As these cells have regeneration capacity, their protoplasts are capable of forming whole plants.

6. Genetic Transformation

By the advent of leaf-disc transformation systems it is possible to engineer species in which tissues are capable of regeneration by somatic embryogenesis. Repetitive embryogenesis from Agrobacterium transformed cells has been used to obtain multiple crops of somatic embryos without employing the callus phase.

Transformation technique applied to primary somatic embryo instead of zygotic embryo should give rise to transgenic somatic embryos.

7. Synthesis of metabolites

The repetitive embryogenesis system is useful in the synthesis of metabolites e.g. oils of pharmaceuticals. Somatic embryos of Borage by repetitive embryogenesis give continuous supply of g-linolenic acid.

Androgenesis, Mechanism, Techniques and Uses

Androgenesis can be defined as production of a haploid individual by development of an egg cell containing the male nucleus. The elimination or inactivation of egg nucleus occurs before fertilization. This occurs in vivo. On the other hand in vitro haploids can be induced by using anther androgenesis (also called androgenesis) or from cultures of individual pollen grains. These in vitro methods are very successful for haploid production. Guha and Maheshwari published the first report of anther culture from Datura innoxia.

Technique of Androgenesis

For androgenesis important requirements are:

1. Healthy plants grown in controlled environment.
2. Knowledge of pollen ontogenesis.
3. Temperature treatment - arrests exiting metabolism and shifts to new pathway of embryogenesis.
4. When androgenic embryos are formed, then their development into plants depend on various factors like - media composition, light, temperature, differentiation of embryo primordial and transfer to green house.

Thus, young plants grown in controlled environments are used to select bud of right stage. According to some workers best anthers are ones in which uninucleate microspores midway between release from tetrad and first pollen grain mitosis are present.

The selected buds are surface sterilized and anthers are removed from them along with their filaments. There are dipped in ethyl alcohol and tested for correct stage of pollen. If the stage is correct then other anthers from bud are removed and placed horizontally on medium. Care should be taken not to injure anthers.

In a different approach pollen from anthers are used to prepare pollen suspension for cultures.

The cultures are maintained in alternating periods of light (12-18 hr, 5000-10,000 lux/m2) at 28oC and darkness (12-6 hr).

The wall tissues turn brown and burst after 3-8 weeks due to pressure of callus or plants. Individual plants or shoots are transferred to rooting medium and rooted plants are transplanted to pot with soil in green house.

Factors Affecting Anther Culture

Number of factors influence anther culture:

1. *Genotype of donor plant:* Thus highly responsive genotypes should be selected. The genotype can be improved by breeding.
2. *Anther wall factors:* Wall factors effect anther culture. So many workers use nursing effect of whole anthers for androgenic development of isolated pollen of number of species.
3. *Culture medium:* The requirement of medium is different for different genotypes and for different age of anther. For most solanaceous species complete nutrient medium of Nitsch or MS medium is required. Iron plays an important role in pollen- embryo development. For non-solanaceous plants addition of growth adjuvant (growth regulators, complex nutrient mixtures coconut milk or yeast extract) is required. N6 medium of Yu-Pei is also a good androgenesis medium. Sucrose is essential and activated charcoal stimulates androgenesis in some systems
4. *Stage of microspore or pollen development:* The stage varies with the species.

5. *Effect of temperature and light:* Temperature shock enhances androgenesis (cold at 3-5°C for 72 hours). Frequency of haploids is better in light but isolated pollen are sensitive to light.
6. *Physiological state of donor plant:* Anthers of plants grown in short day and high light intensity, perform better. Seasonal changes also change the physiology of donor plant.

Differentiation of Pollen into Gametophytic or Sporophytic Tissue: Mechanism of Androgenesis

1. *Morphological:* The pollen which form haploid is smaller and stains less. Such embryogenic pollen are present in low quantities so frequency of haploid formation is low.
2. *Physiological:* Elaborate ER, abundant ribosomes and normal mitochondria are present in gametophytic pollen. Quiescence and repression of organelles in microspores leads to differentiation of embryogenic pollen. The differences between two are shown below:

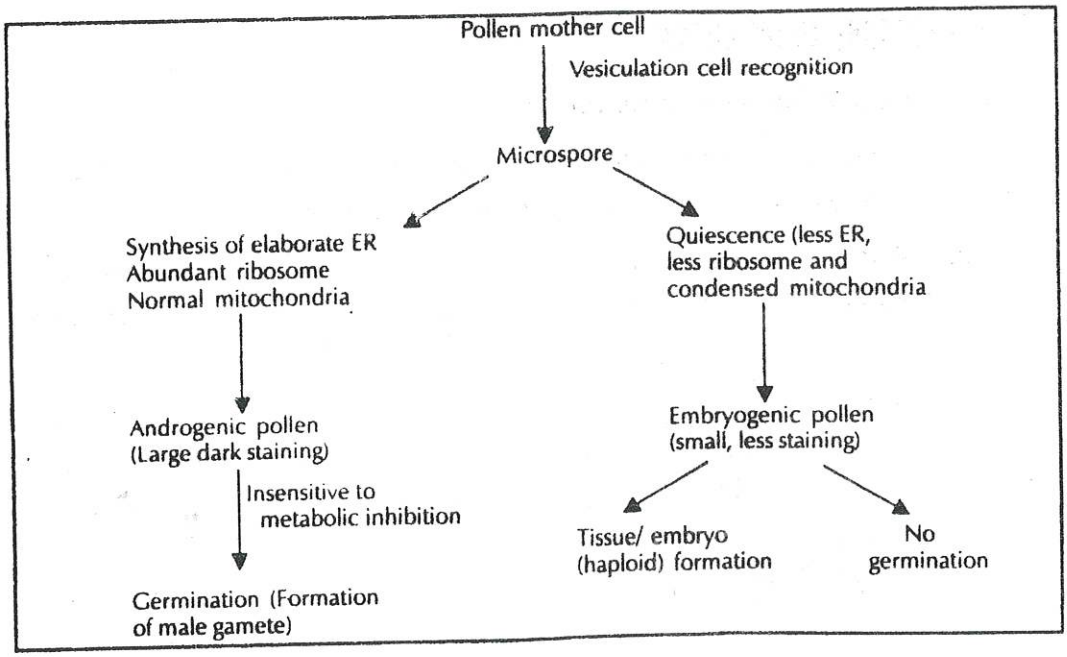


Figure 1.2

Pathways for development of androgenic haploids are following:

The four pathways based on initial divisions in microspore have been identified which lead to in-vitro androgenesis.

Pathway I: Microspore divide equally to form two identical daughter cells. Both contribute to sporophyte development. Eg. *Datura innoxia*.

Pathway II: Microspore divide to form unequal vegetative and generative cells. Vegetative cell forms sporophyte and generative cell degenerates eg. *Triticum*.

Pathway III: Microspore divides to form unequal cells but embryos develop mostly from generated cells eg. *Hyoscyamus niger*.

Pathway IV: Microspore divides unequally but both vegetative and generative cells contribute to sporophyte eg. *Datura innoxia*.

Later development: After any of the above pathway, the embryogenic pollen becomes multicellular and bursts open. It assumes globular shape and after normal stages form plant.

All stages and development is shown in Figure 1.3.

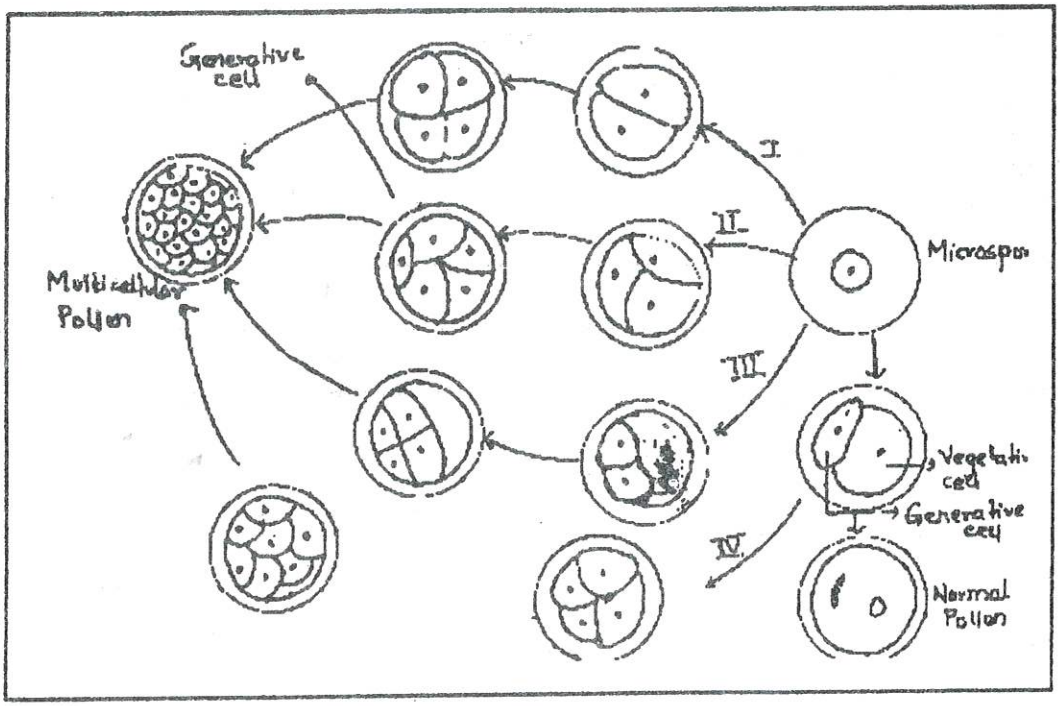


Figure 1.3: Formation of Multicellular Pollen

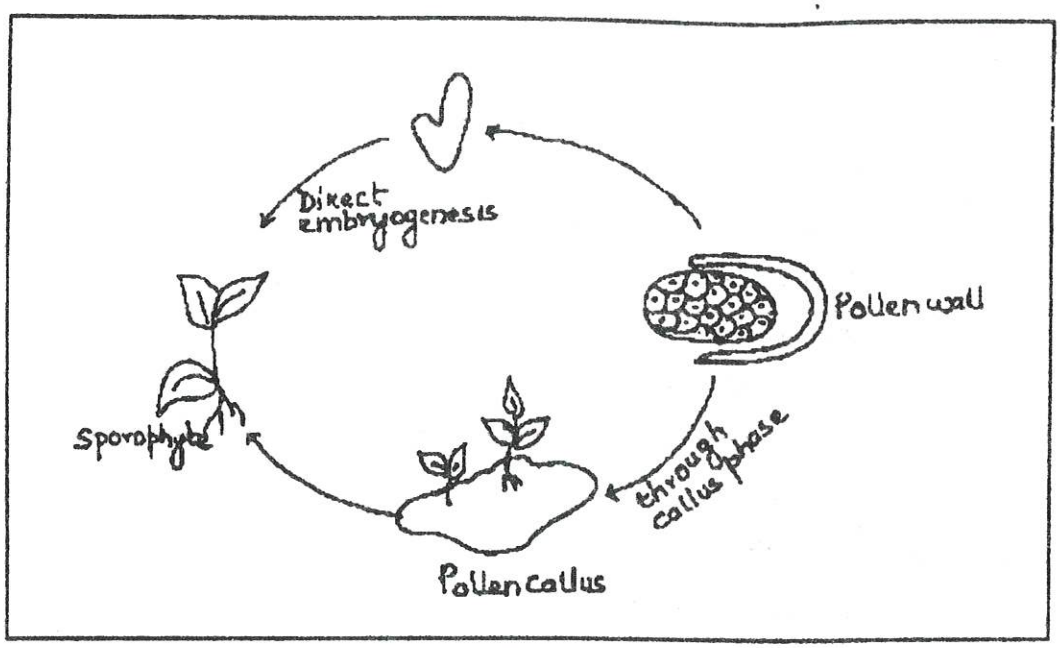


Figure 1.4: Development of Multicellular Pollen into Sporophyte

Haploids from Pollen or Microspore Culture

Anthers contain heterogenous population of pollen grains. Thus, development of haploids from isolated pollen has advantage as only plant of single genotype will develop. This was first reported by Tuleche in gymnosperms and later Kameya and Hinata in angiosperms. Nurse culture technique is suitable for pollen culture. This is shown in figure below.

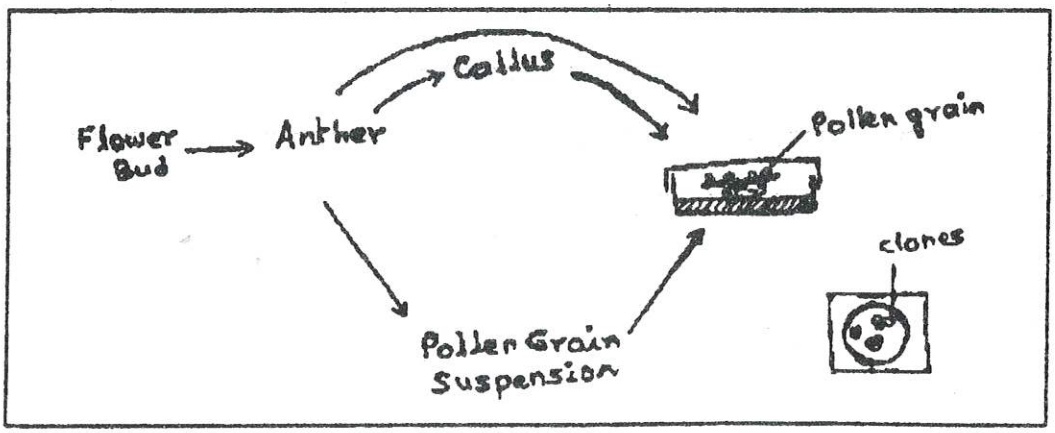


Figure 1.5: Diploidization of Haploid Plants

Haploid plants can grow normal but do not produce viable gametes and no seed. So its necessary to diplodise the haploids. This is done by using colchicines (0.4%).

Applications of Androgenesis

1. Use in Plant breeding
   a. Hybrid sorting in plant breeding.
   b. Production of diploid hybrids for breeding.
   c. Releasing new varieties through F1 double haploid system.
   d. Selection of mutants resistant to diseases.
   e. Development of asexual lines of Trees/perennial species.
   f. Transfer of desired alien genes.
   g. Establishment of haploid and diploid cell lines of pollen plant.
2. Other uses
   a. In mutation research.
   b. Gametoclonal variation.
   c. Cytogenetic research.
   d. Evolutionary studies.
   e. Genetic studies.

CHAPTER 2

SOMATIC HYBRIDIZATION

LEARNING OBJECTIVES

- Isolation of Protoplasts
- Applications of Plant Tissue Culture

The process of fusion between isolated somatic protoplasts under in-vitro conditions and development of fused heterokaryon to hybrid plant is called somatic hybridization and the cybridization is fusion of nuclear genome from one parent and cytoplasm from both, and the development into cybrid.

Isolation of Protoplasts

There are three methods of protoplast isolation:

1. *Mechanical:* The plasmolysed tissue is cut by sharp knife and protoplast is released by deplasmolysis. It is generally not used now a days.

2. *Sequential Enzymatic (2-step):* Macerated plant tissue is first treated with pectinase and after this treated with cellulose to release protoplasts.

3. *Mixed Enzymatic (simultaneous):* Plant tissues are treated with a mixture of cellulose and pectinase to release protoplasts in one step. This is less time consuming and decreases chances of contamination.

Source of Protoplasts: The protoplasts can be made from leaves, callus cultures, cell suspension cultures or from pre conditioned plant material.

Viability of Protoplasts: The viability can be determined by plating efficiency or by observation of cyclosis, measurement of oxygen uptake or by photosynthetic activity. Exclusion of Evan's blue dye by intact membrane and staining with FDA are commonly used to determine viability.

Culture of Protoplasts (or hybrid cells): The first step is development of cell wall around membrane of isolated protoplasts. This is followed by induction of divisions to form small colony. By culture manipulations this can be induced to form callus or regenerate whole plants.

Protoplasts can be cultured in liquid or agar medium.

Liquid Medium: Small drops or layer of liquid medium is placed inside a petri dish which is covered by other petri plate and sealed with parafilm. Its kept in low light or dark at 25–28°C.

Solid Medium: For culturing on agar medium Bergmann's plating techniques is used. 2 ml of isolated protoplasts (ca 10^3 – 10^5 cells/ml) are mixed with equal volume of agar nutrient medium (45°C) on solidification of agar plates are sealed and kept inverted at 25-28°C.

Culture Media: Generally media suitable for callus or suspension culture can be used. But the concentration of Fe, Zn, and NH_4 should be decreased and calcium should be increased. Mostly salts of B_5 and MS media are suitable. 3.5% sucrose is used, vitamins, auxius and cytokinins are used in various combinations, inclusions of osmoticum are sorbitol, mannitol, glucose or sucrose. Protoplasts are more stable in a slightly hypertonic solution. Ionic substances (KCl and $MgSO_4$) improve the viability of protoplasts.

Multiple Drop Array (MDA) Screening

Potrykus et al developed this in 1997. It is designed for systematically screening a large number of multiple combinations of media constituents for protoplast culture. In this technique hanging droplets of 40 ml are used. One droplet has one combination of factors to be tested. The droplets are arranged in 7 × 7 drops on lid of petridish. By this, quick screening of correct media can be done.

Plating Density: A density of 1×10^4 to 1×10^5 protoplasts per ml is optimal.

Feeder Layer Technique: This is another technique for protoplast culture. Protoplasts are exposed to X-rays at certain dose so that cells are metabolically active but unable to divide. They are washed to remove toxic substances and plated on soft-agar. This acts as feeder layer and over this non-irradiated protoplasts are plated.

Co-culture of Protoplasts

Metabolically active and dividing protoplasts of two types are mixed in a liquid medium and plated together so that there is cross feed between the two. This is generally used if Calli of two types can be morphologically distinguished.

Microdrop Culture

Specially desiqued Cuprak dishes are used. It has outer chamber filled with water for maintaining humidity and protoplasts in drops of media are placed in the wells of inner chamber. The dish is sealed and incubated under optimum conditions.

Protoplast Regeneration

Important steps in protoplast regeneration are:

1. Formation of Cell Wall: It starts within few hours but its completed in days. Regeneration can be demonstrated by calcolfluor dye. Ion osmoticum in media supports wall formation.
2. Development of Callus/whole Plant : After wall formation, there is increase in size and the first division occurs within a week. By divisions a small cell colony is formed. After 2-3 weeks macroscopic colonies are formed which are transferred to an osmoticum free medium where callus is formed. The callus can be induced to undergo organogenic differentiation i.e. whole plant regeneration. The steps of isolation, culture and regeneration of protoplasts are shown in Figure.

Protoplast Fusion

Because protoplasts lack cell walls, they are easy to fuse in-vitro. Interspecific, intergeneric or even interkingdom incompatibility barriers do not exist in this fusion. Thus, this a great tool for somatic cell genetics and crop improvement. There are various ways of fusion:

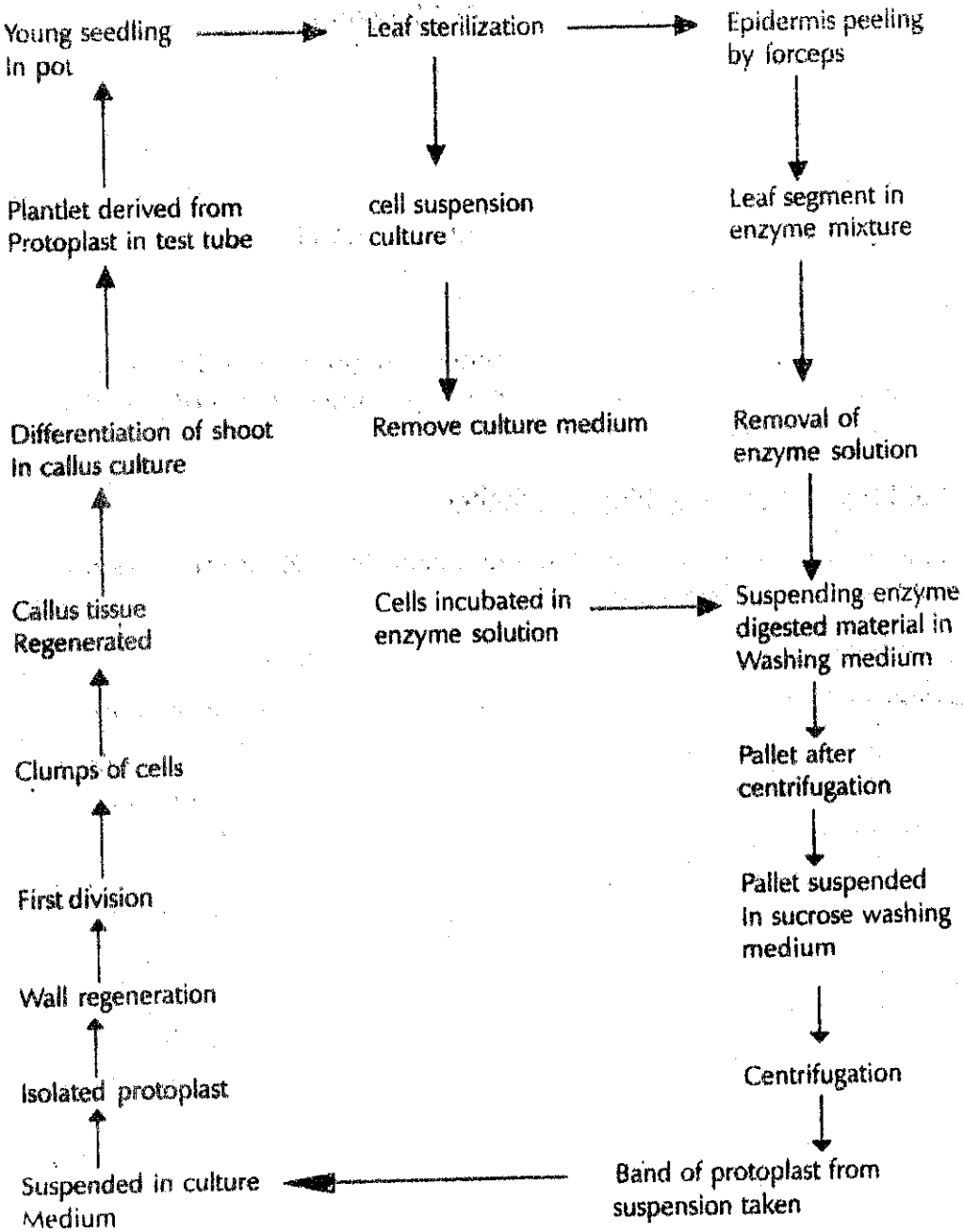


Figure 2.1

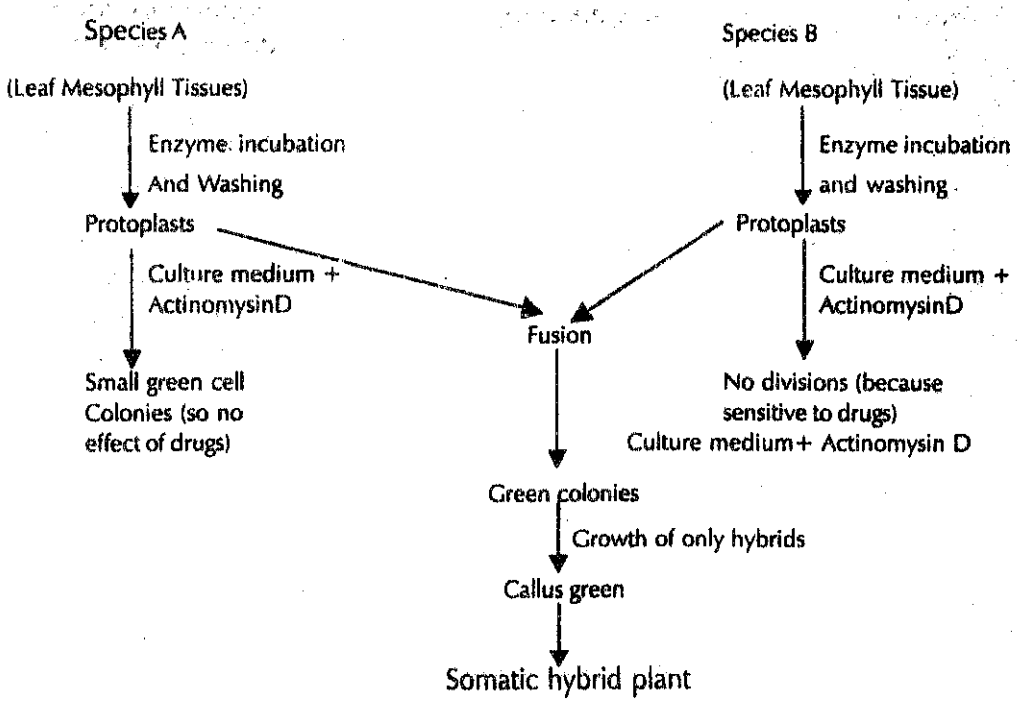
1. *Spontaneous Fusion:* These occur during protoplast isolation and lead to formation of homokaryons.
2. *Mechanical Fusion:* This fusion is achieved by pushing together two protoplasts.
3. *Induced Fusion:* Large number of fusogens are used to induce fusion eg. $NaNO_3$, artificial sea-water, lysozyme, PEG (Poly ethylene glycol) etc. Important are:
   i. $NaNO_3$
   ii. High pH/Ca^{++} treatment
   iii. PEG treatment
   iv. Electro poration - use of electric fields for protoplast fusion.

Hybrid Selection and Regeneration

Generally only a percentage (20-25%) of protoplasts undergo fusion and heterokaryon formation. So selection of hybrids is important from a population of unfused protoplasts. Different procedures are described below:

Biochemical Basis for Complementation and Selection

Biochemical markers like sensitivity to drug and auxotrophic mutants can be used for selection of hybrids as shown below:



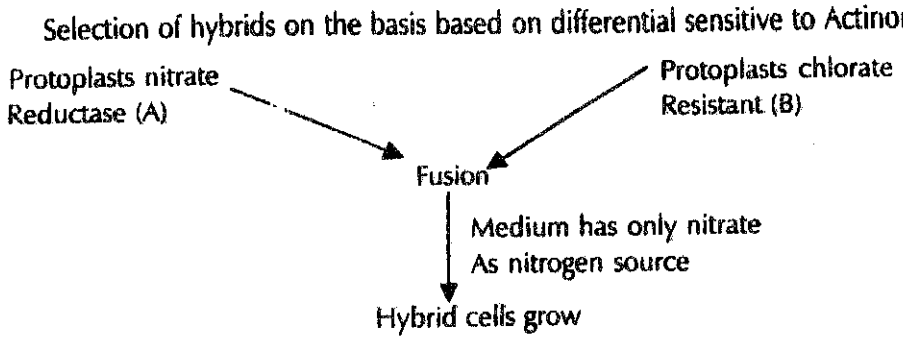


Figure 2.2

Visual Selection

Chloroplast deficient (non-green) protoplasts of one parent are fused with green protoplasts of other parent. The hetrokaryon formed can be visually identified under microscope as it will be half green and half white. Further selection of heterokaryons to develop somatic hybrids can be done by various ways:

i. Complementation selection coupled with differential media growth: It is shown in figure below:

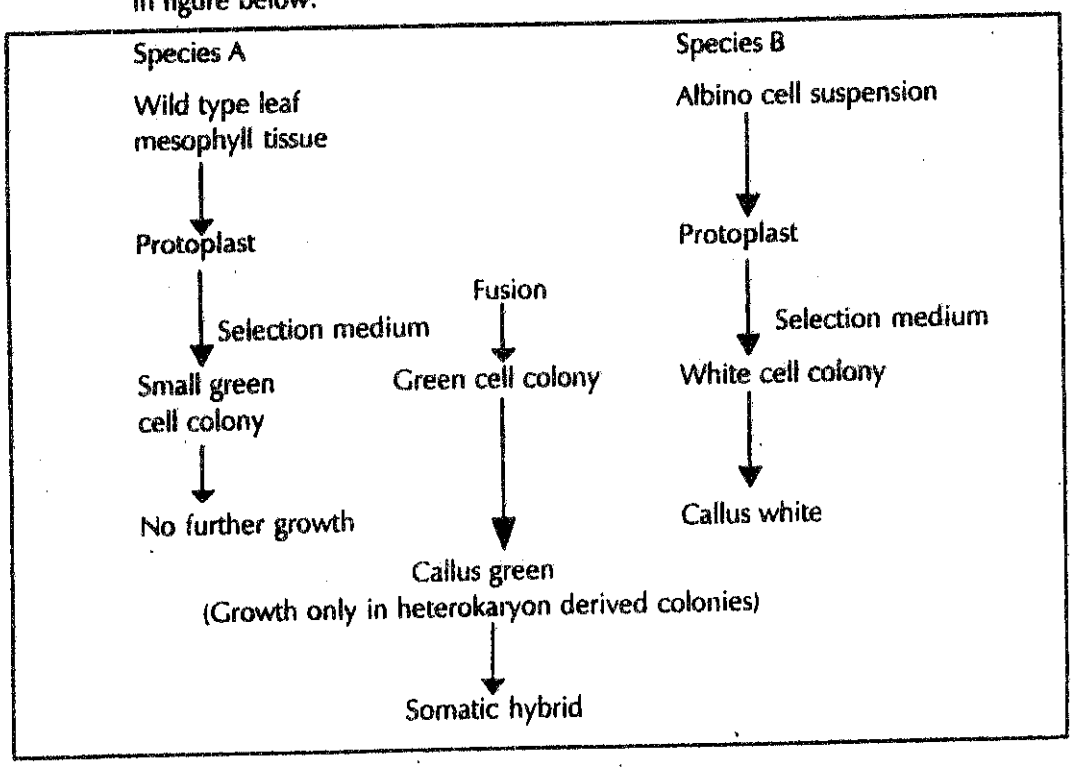


Figure 2.3

ii. Mechanical Isolation: Individual heterokaryons can be isolated mechanically under microscope using drummoud pipett.

Use of Non-allelic Mutants for Complementation Selection

Chlorophyll deficient chloroplasts of one species are fused with light sensitive protoplasts of other species and cultured in presence of light. After some time green colonies are observed as a consequence of complementation between two mutants.

Flow Cytometric Analysis

Flow cytometry and fluorescent activated cell sorting can be used to sort and differentiate heterokaryon from homokaryons.

Achievements and Limitations of Somatic Hybridization

Many achievements in science have been made using somatic hybrids and protoplasts. The important ones are:

1. Somatic hybridization is a means of genetic recombination in asexual or sterile plants.
2. Somatic hybridization is done as a means of overcoming sexual incompatibility, where conventional breeding cannot be done for crop improvement due to sexual incompatibility. Hybrids have been produced by somatic hybridization between sexually incompatible *Brassica juncea* and *Diplotaxis muralis*.
3. It can be used for cytoplasm transfer.
4. Protoplasts of sexual sterile plants can be fused to produce fertile diploids and polyploids.
5. Somatic cell fusion is useful in the study of cytoplasmic genes and their activities.
6. It can be used to do hybridization between juvenile plant.

Inspite of great potential there are some limitations also like some crops do not give satisfactory results through conventional media and techniques, so this has to be refined. Moreover the basis of genetic diversity generated by somatic cell fusion is still poorly understood. The degree of species-specific chromosome elimination in wide or distant somatic hybridization needs to be mastered.

Applications of Plant Tissue Culture

Clonal Propagation or Micropropagation

A variety of plant species can be easily propagated through the techniques of cell, tissue or organ culture. This is described as clonal propagation or micropropagation. The important advantages of this method are:

a. Helps in rapid multiplication of superior clones and maintenance of uniformity.

b. Multiplication of disease free plants.

c. Multiplication of sterile hybrids generated by sexual method.

Methodology

A sterilized shoot, tip or auxiliary bud is placed on a culture medium which is suitable to induce formation of multiple buds. The following stages are involved in micropropagation:

1. *Stage I:* Establishment of tissues in vitro.
2. *Stage II:* Multiplication of shoots (Usually same media is used in stage I and II).
3. *Stage III:* Root formation and conditioning of propagules before transfer to green house. In stage high intensity and media change for rooting is required.
4. *Stage IV:* Growth in pots followed by field trials.

The important stages are shown in figure below. The stages can be reduced or increased depending on the requirement.

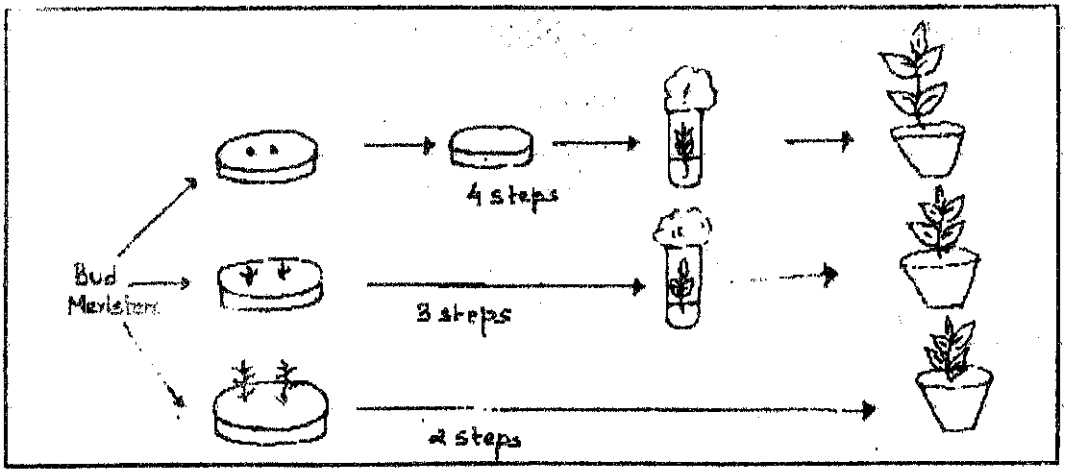


Figure 2.3: Three Different Methods of Clonal Propagation of Plants

A large number of plants have been regenerated through tissue culture technique. This have been very useful for propagation of tree species. Few examples of tissue propagated plant are: Cardamon, Bamboo, Coffee, *Ficus* sp. etc.

Artificial Seeds

In recent years a concept of synthetic or artificial seeds has been developed. This has gained lot of popularity also.

In such seeds somatic embryos are encapsulated in a suitable matrix. Sodium alginate is a good encapsulating material. Along with the embryos, other substances like mycorrhizae, insecticides, fungicides and herbicides are also included in such seeds. Such seeds have many advantageous features like:

1. They can be stored for long time (upto a year) without loss of viability.
2. They are easy to handle and deliver.
3. They are easy to transport.
4. They can be directly sown in soils like natural seeds and require no hardening in green houses.

Limitations of artificial seeds

1. Cost of production is high

Thus, artificial seeds have solved the problems of storage and transport of propaguels of tissue culture.

Cytopreservation and Germ Plasm Storage

Germ plasm and plant genetic resource conservation is an important thrust area of biotechnology. Several national and international institutes are involved in it like - NBPGR (National Bureau of Plant Genetic Resources), IBPGRI (International Plant Genetic Resources Institute).

For storage and conservation of germ plasm various strategies have been developed. The most important aspect here is that germ plasm does not loses its viability.

Germ plasm can be stored in various forms like - seeds, buds, protoplasts, cells, tissues etc. Well organized parts like shoot tips and plant lets in culture can also be used for storage.

The following different techniques in which the growing stage and growth of germ plasm is suspended are generally used for storage and conservation of germ plasm. These are:

1. Lowering the temperature.
2. Addition of chemical retardants or hormones.
3. Reduction in oxygen concentration.

In such methods limited growth is allowed, so they are effective for about a year and periodic renewal is required.

But the most popular and most effective method of long term storage of cell cultures, is cryopreservation. This storage is done at low temperature using liquid N2. By this method storage can be done virtually for infinite periods.

The steps in typical cryopreservation procedure for cell culture are shown below:

Cell culture under standard conditions

↓

Pre grow for 5 days in medium containing mannitol

↓

Cryoprotect with 0.5% to 10% DMSO + 1M sucrose (or glycol) in culture medium

↓

Transfer cells and cryoprotectant solution in ampule: Freeze at 1°C for 1 min to -35°C: Hold for 40 min, transfer to liquid N_2

↓

Store the ampule in liquid N_2 can. To revive the germ plasm.

↓

Thaw ampule in warm water.

↓

Pour into semisolid medium.

↓

Transfer recovered cells to liquid medium.

Storage of plant protoplasts is recommended in many cases and survival level is upto 75%.

The steps for the cryopreservation of shoot tips are described below:

Plants under standard conditions

↓

Dissect shoot lip

↓

Pre grow for 2 days on medium containing 5% DMSO.

↓

Cryoprotect with 10% DMSO in culture medium.

↓

Pick shoot tip by a hypodermic needle and dip into liquid N_2 immediately, transfer to ampule containing cryoprotectant : Freeze at 0.5°C, 1 min in liquid N_2.

↓

Thaw in warm water.

↓

Pick tip and dip into medium at 25°C → place on semisolid medium.

↓

Revived

Pollen needed for experiments can also be cryopreserved. The best stage of cells for cryopreservation is early exponential phase. Lag phase and stationary phase cells are susceptible to freezing injury, callus cultures, cell suspensions, zygotic embryos and somatic embryos can all be cryopreserved.

Production of Secondary Metabolites/Natural Products

Plants are important sources for variety of chemicals used for different purposes eg. Pharmacy, medicine and industry. In recent years plant cell suspension cultures and immobilized cells are being utilized for the production of such chemicals on a commercial basis. Such compounds have following advantages over compounds extracted from whole plants:

1. The yield and quality is more consistent as there is no influence of environmental conditions.
2. The time of production can be predicted and controlled in laboratory.

The most important chemicals produced by cell cultures are secondary metabolites. These are those cell constituents which are not essential for survival. They include-alkaloids, glycosides (steroids and phenolics), terpenoids and different flavoures, perfumes, agrochemicals etc.

The yield of these compounds in cultures can be increased by changing physiological and biochemical conditions. Sometimes cell cultures accumulate 2-10 times higher levels of secondary metabolites than those found in mother plants (from which cell culture is prepared).

Important secondary metabolites (and their uses) obtained by tissue culture of plant species are :

1. *Papavar somniforum* — Morphine — Analgesic, sedative.
2. *Catharanthus roseus* — Vincristine — Antileukaemic
   Serpentine — Tranquilizer
3. *Cinchona officinalis* — Quinine — Anti malarial
4. *Chrysanthemum cinnerariaefolium* — Pyrethrin — Insecticide
5. *Datura stramonium* — Scopolamine — Anti hypertension
6. *Jasminum sp.* — Jasmine — perfume
7. *Coptis japonica* — Berberine — Anti bacterial.

In order to decrease the cost of plant tissue culture either for micropropagation for production of bio active compounds automatic bioreactors having bioprocessors are being developed. By these production rates can be increased 10,000 times.

CHAPTER

3 RECOMBINANT DNA TECHNOLOGY

LEARNING OBJECTIVES

- Gene Cloning Principles and Techniques
- Construction of Genomic/cDNA Library
- Choice of vectors
- DNA Synthesis
- DNA Sequencing
- Polymerase Chain Reaction
- DNA Finger Printing

Recent advancements in DNA technology has enabled scientist to manipulate both prokaryotic as well as eukaryotic genomes, at a very fast rate. But the history of rDNA (recombinant DNA) technology goes back to 1973, when Stanley Cohen et al could transfer the DNA (genes) of interest from one organism to another organism. The product obtained by this procedure of rDNA technology is known as rDNA. rDNA technology and genetic engineering are of two types, which are used interchangeably in today`s times. rDNA technology has emerged as a powerful tool in the area of biotechnology.

Gene Cloning Principles and Techniques

rDNA technology or gene cloning or molecular cloning are the terms relating to procedure or protocols comprising:

1. Construction of rDNA molecule.
2. Introduction of the rDNA molecule into a host cell by transformation.
3. Screening or selection of cells that take up the rDNA construct, from those cells that do not.

The details of technique is following:

Construction of rDNA Molecule

Vectors

The primary requisite of gene cloning is to have a DNA molecule capable of taking up the gene of interest and replicate in to a host. This DNA molecule is primarily a vector or cloning vehicle. These vectors or cloning vehicles could be PLASMID, BACTERIOPHAGE, COSMID or PHAGEMID, TRANSPOSON or even a virus.

Plasmid

Plasmids are extra-chromosomal, autonomous genomic elements, which are circular and self-replicating duplex DNA molecules, present in a specific number in bacterial or yeast cells or eukaryotic organelles. It shows 2 conditions:

Single copy plasmids : One copy per cell

Multicopy plasmids : 10 – 20 copies per cell

Bacteriophages

Bacteriophages are having linear DNA molecules, which generate two sub-fragments upon breakage. A foreign DNA molecule can be inserted into phage sub-fragments to generate a chimeric phage construct, capable of infecting bacteria and making progeny particles after the lytic cycle. But the major draw back of this method lies in the limited capacity of the phage head, which will not take up foreign DNA, which is too long. To alleviate this problem, a phage lambda can be created as a vector, which is devoid of non-coding regions of DNA. Some examples are λgt 10, λgt 11, EMBL 3, etc.

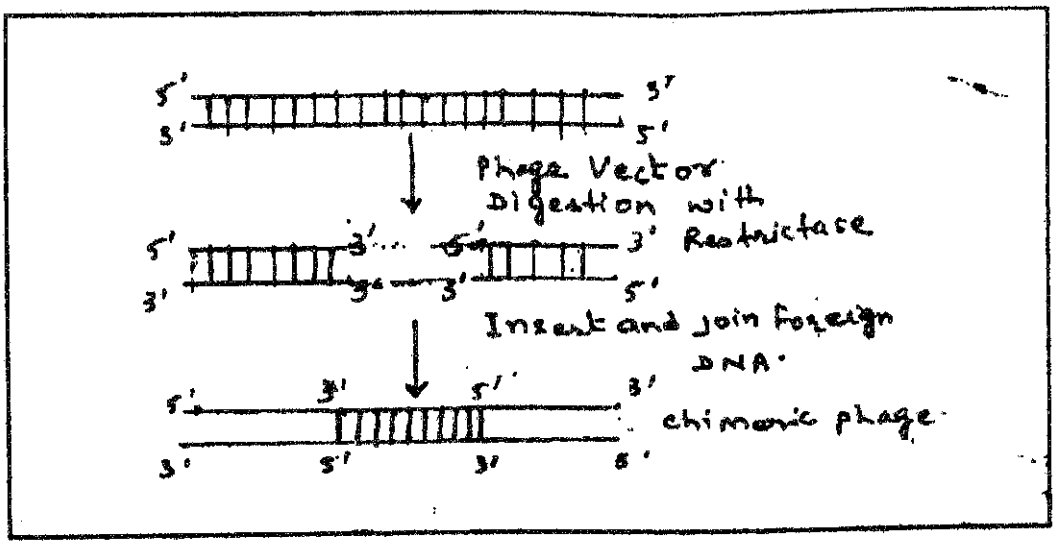


Figure 3.1: Cloning of Foreign DNA into a Phage Vector

Cosmid

Cosmids are plasmids in which cos sites, i.e., specific DNA sequences are inserted. Cosmids are useful for in vitro packaging of DNA into phage. Cosmids perpetuate in bacteria like plasmids and have a very high cloning efficiency.

Phagemids

Phagemids are artificial constructs possessing features of both phages and palsmids. One very widely used phagemid in molecular biology or biotechnology is pBluescript II KS, derived from pUC 19 (plasmid vector construct at University of California).

Transposons

Transposons are insertions of repeats of a specific DNA fragment into the genome. Each fragment posses a gene for the enzyme, transposase, which induces the phenomenon of transposition. Like P element in drosophila and Ac-Ds systems in corn (Activator - Dissociation system).

Animal and Plant Virus

Many a times, viruses have been used as vectors to introduce foreign genes of interest into cells for amplification and expression studies. Like Tobbacco Mosaic Virus (TMV), Cauliflower Mosaic virus (CaMV) etc.

Once the choice of vector is determined, the next step becomes the construction of a recombinant DNA molecule employing genetic engineering or gene manipulation or molecular cloning or gene cloning.

Enzymatic Cleavage of DNA Molecules

Restriction enzymes or restriction endonucleases are enzymes that cleave or cut specific sequences in double stranded DNA duplex. Without the availability of these enzymes, it would be very difficult to visualize rDNA technology as it exists today.

One basic premise for gene cloning is that both the source DNA or foreign DNA or passenger DNA containing the target sequence and the cloning vector should be consistently cut into discrete and reproducible fragments. These enzymes recognize a specific palindromic sequence (which read the same in forward and reverse directions) and cut at a specific position in both the strand of DNA. Some examples of these enzymes are following.

	Enzyme	Symmetry	Recognition site
1.	Eco RI	Staggered/sticky	G — A — A — T — T — C
2.	Bam HI	Staggered/sticky	G — G — A — T — C — C
3.	Hae III	Blunt/Flush ended	G — G — C — C
4.	Hpa I	Blunt/Flush ended	G — T — T — A — A — C

The length of the recognition site can be 4, 6, 8 or more nucleotide pairs and on the basis of this property, they are called as 4 cutters and 8 cutters.

Ligation of Target DNA into Vector

The target DNA and the vector which have been cut with the similar restriction enzyme are treated with DNA ligase in the presence of ATP. Upon treatment a number of different ligation combinations may be produced, including an original closed circular plasmid DNA.

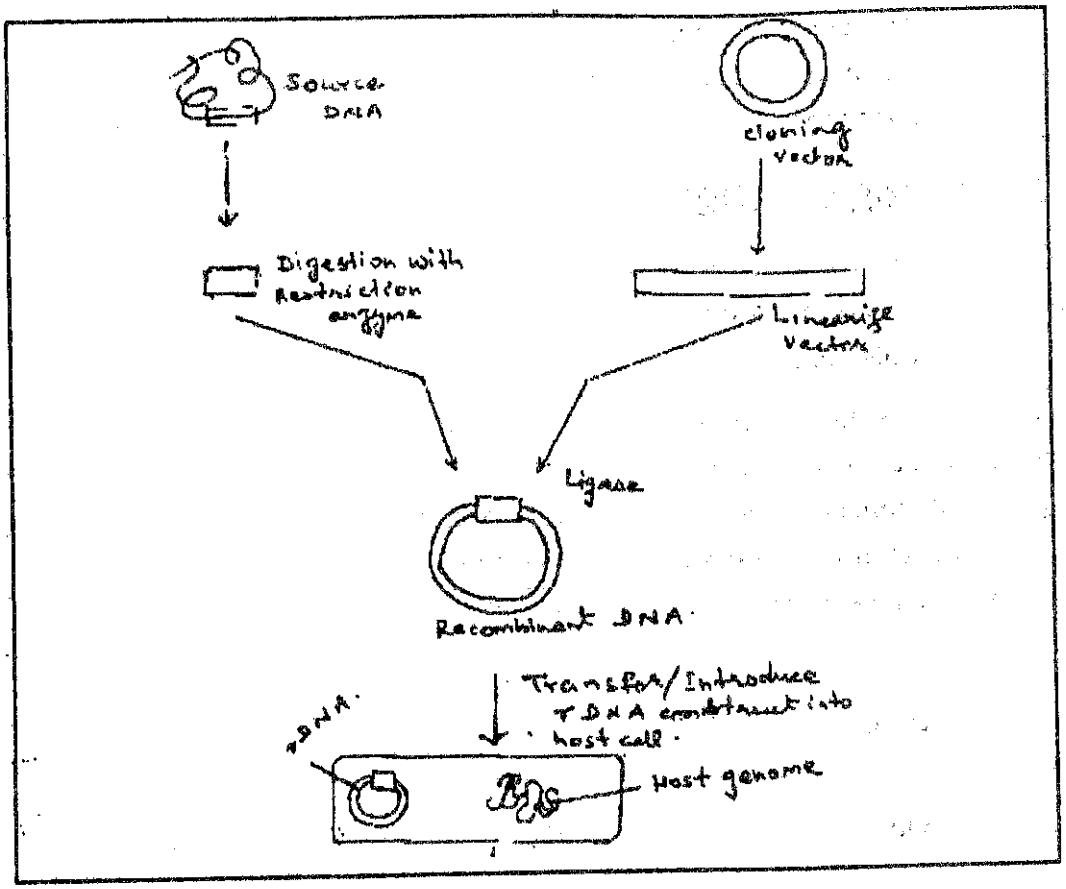


Figure 3.2: Grow Host Cell and Isolate the Cells Containing the rDNA Molecule

Introduction of rDNA Molecule into Host Cell

After a rDNA construct is successfully made, the next step involves the uptake of such rDNA or chimeric construct by bacteria or eukaryotes. The procedure for introducing this rDNA construct into a bacterial and host cell is called as transformation. But transformations requires that the cells be chemically heat-treated, so as to allow the proper uptake of the DNA molecule. The most widely used procedure employs a regimen of high temperature and calcium chloride. But transformation is an inefficient process; therefore, the transformed cells need to be differentiated from those that have not acquired the new foreign rDNA construct.

The host cells can be either bacteria or eukaryotic cell.

1. **Bacteria**

   These are two types:

A. Gram negative:

It can be used as hosts for plasmids, cosmids, and phages. Like, E. coli, *Pseudomonas* sp.

B. Gram positive:

It can be used as hosts for plasmids only. Like Bacillus sp., *Streptomyces* sp.

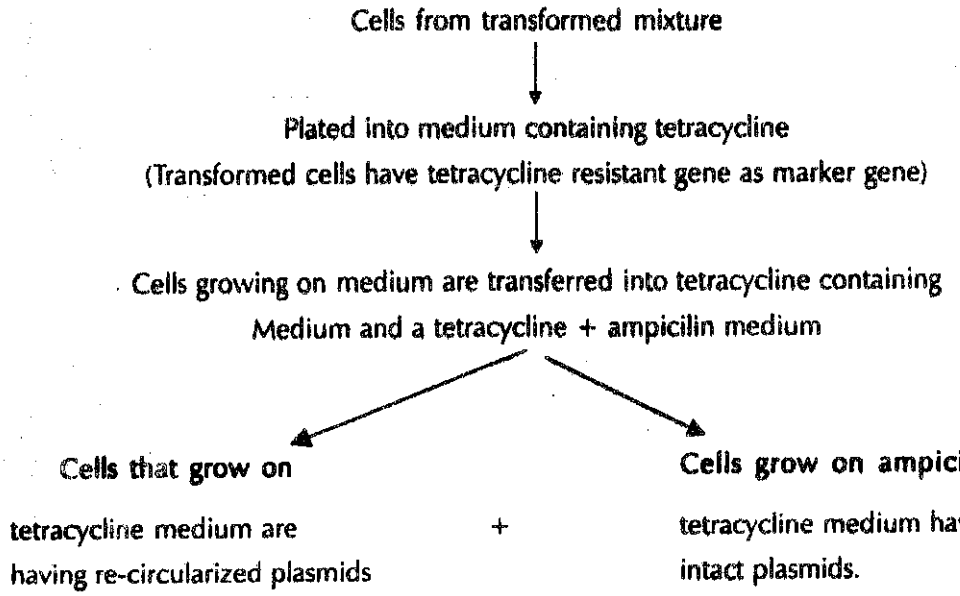
2. Eukaryotic Cell

Gene cloning has been accomplished in yeast, mouse cells and a variety of human cell lines.

When eukaryotic genes are cloned and expressed in prokaryotes, the problem arises, as the prokaryotes do not posses the able assembly of carrying out gene expression correctly. Hence, for successful and efficient cloning of eukaryotic genes, eukaryotic cells are required.

Screening or selection of transformed cells

Identification of transformed cells containing the rDNA construct of interest is the step following the transformation step. The simplest method involves the antibiotic sensitivity test. It is based on following steps:



The above experiment can be explained if the genetic map of pBR 322 cloning vector is studied. This vector has both the tetracycline and ampicilin resistant genes. If the vector is

unable to uptake rDNA construct, it will re-circularize and hence will become resistant to both these antibiotics. On the other hand if rDNA construct is inserted within the ampicilin resistant gene; the coding region of this antibiotic is disrupted, hence the ampicilin resistant will be lost. So the cell carrying the rDNA construct will be the ones resistant to tetracycline and sensitive to ampicilin.

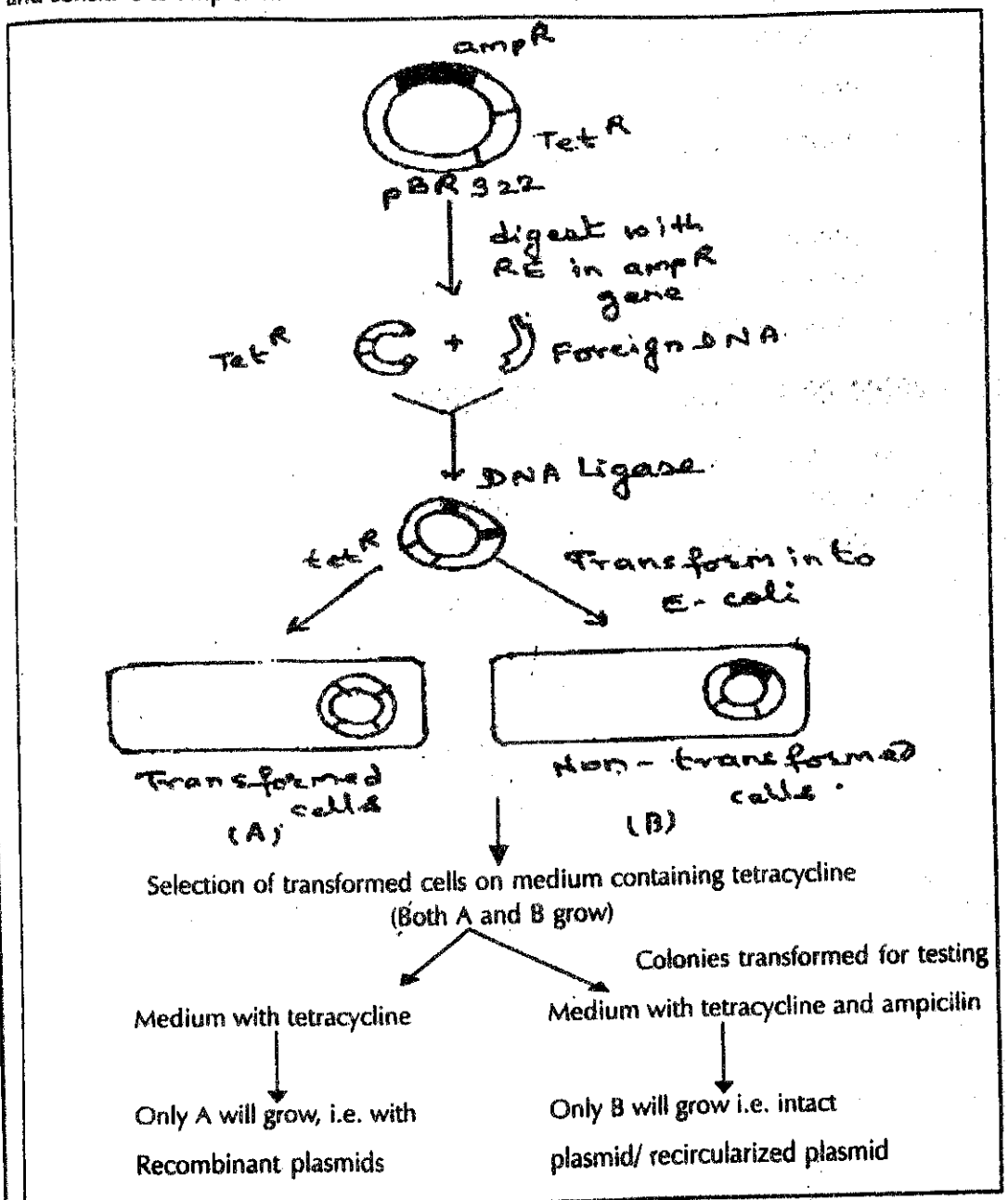


Figure 3.3

Construction of Genomic or cDNA Library

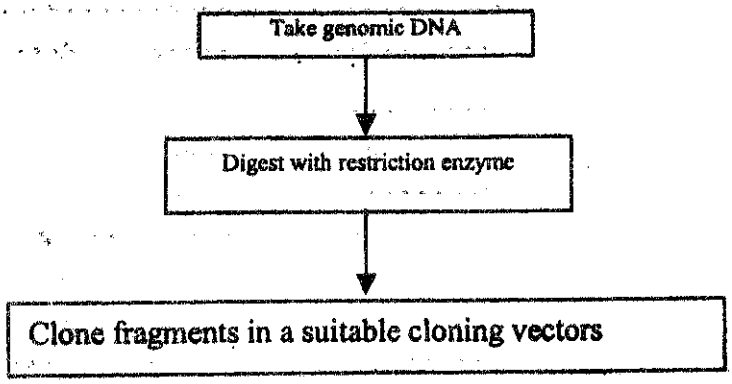
In order to study a particular gene or DNA fragment of interest; the genomic DNA of a particular organism can be cleaved with a restriction enzyme and the population of a DNA fragment cloned into a suitable vector. Such a collection of clones containing at least one copy of every DNA sequence in the genome is called as genomic library. cDNA library or complementary DNA libraries are collection of clones of DNA copies of mRNA's, collected from the cells.

Chromosome libraries are those having a collection of clones of fragments of individual chromosomes.

Genomic Libraries

Cloning procedures are employed for the successful construction of genomic libraries, are following:

1. First Process



Limitations

If the gene of interest contains more than restriction site, the gene will be split into two or more fragments; the genes will be cloned in two or more pieces.

2. Second Process

To overcome the limitation of process one; the problem of gene split into fragments can be minimized if long DNA fragments can be cloned in to a suitable vector. This can be achieved i.e. long DNA fragments can be generated by mechanical shearing of HMW DNA (High Molecular Weight DNA).

Limitation

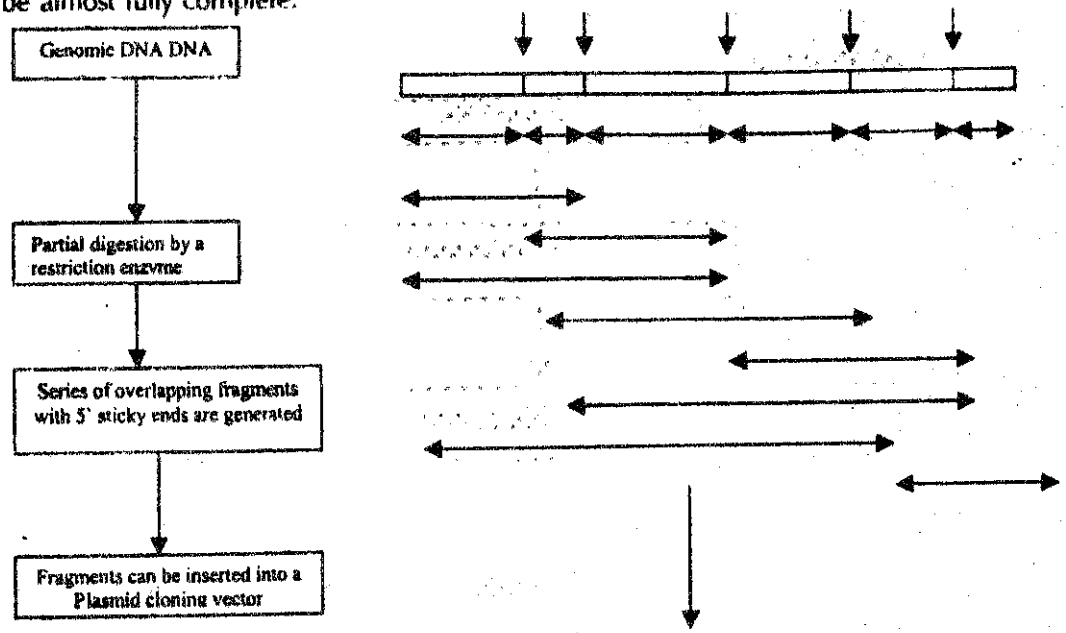
Since the ends of mechanically sheared DNA are not generated via restriction enzyme cleavage, additional enzymatic manipulations are required before their insertions into proper sites into the cloning the vector.

3. Third Process

The best suitable approach would be to generate DNA fragments of appropriate size for the fruitful construction of a genomic library by partial digestion with restriction enzyme. Partial digestion refers to only a portion of the restriction site being readily available for cleavage due to either:

- Limiting amount of enzyme or,
- Limited time of incubation of enzyme and DNA.

This way the entire genome will be available as overlapping fragments and the library will be almost fully complete.



Screening of a Genomic Library

After a genomic library is created; the clones of the target sequences can be identified by:

1. DNA hybridization with a labeled DNA probe,
2. Immunological screening of the protein product
3. Screening for protein activity.

The details of each step is following:

1. Screening by DNA Hybridization

A DNA probe is used to detect a target nucleotide sequence in a DNA sample and the technique is called DNA hybridization. This procedure depends on the formation of stable base pairs between the probe and target sequence.

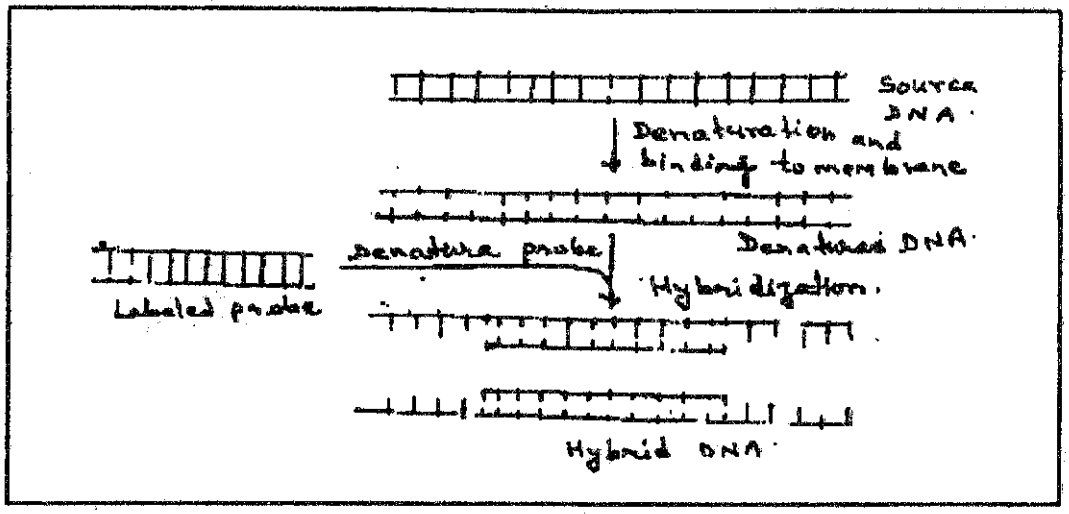


Figure 3.4: Detection Labeled Probe with Autoradiography

2. Immunological Screening of the Protein Product

If DNA probe is not available; alternatively; a cloned DNA sequence can be transcribed and translated and the protein can be screened immunologically. Briefly; the clones of the library are grown and a sample of each colony is transferred to a matrix; cells are lysed and the released proteins attach to the matrix. The protein on the matrix is treated with an antibody, which is specific for the protein encoded by the specific gene of the interest. Following this interaction, the matrix is treated with a tagged secondary antibody specific for the earlier one. The tag is an enzyme, which hydrolyses a colorless substrate to give a color. Hence, for all the specific interactions; the clones of the genomic DNA library can be characterized and screened.

3. Screening by Protein Activity

For screening a target gene that encodes for a specific enzymes; plate assays are used to screen members of a library carrying the functional gene.

cDNA Libraries

Complementary DNA (cDNA) is made from all the mRNA molecules present in a population at a particular time in cells via a procedure called as reverse transcription. If these cDNA molecules are cloned; then the entire library produced is known as cDNA library. The cDNA library is useful when gene expression studies are performed for comparing gene activities in different cell types or at different times as in cell differentiation and development.

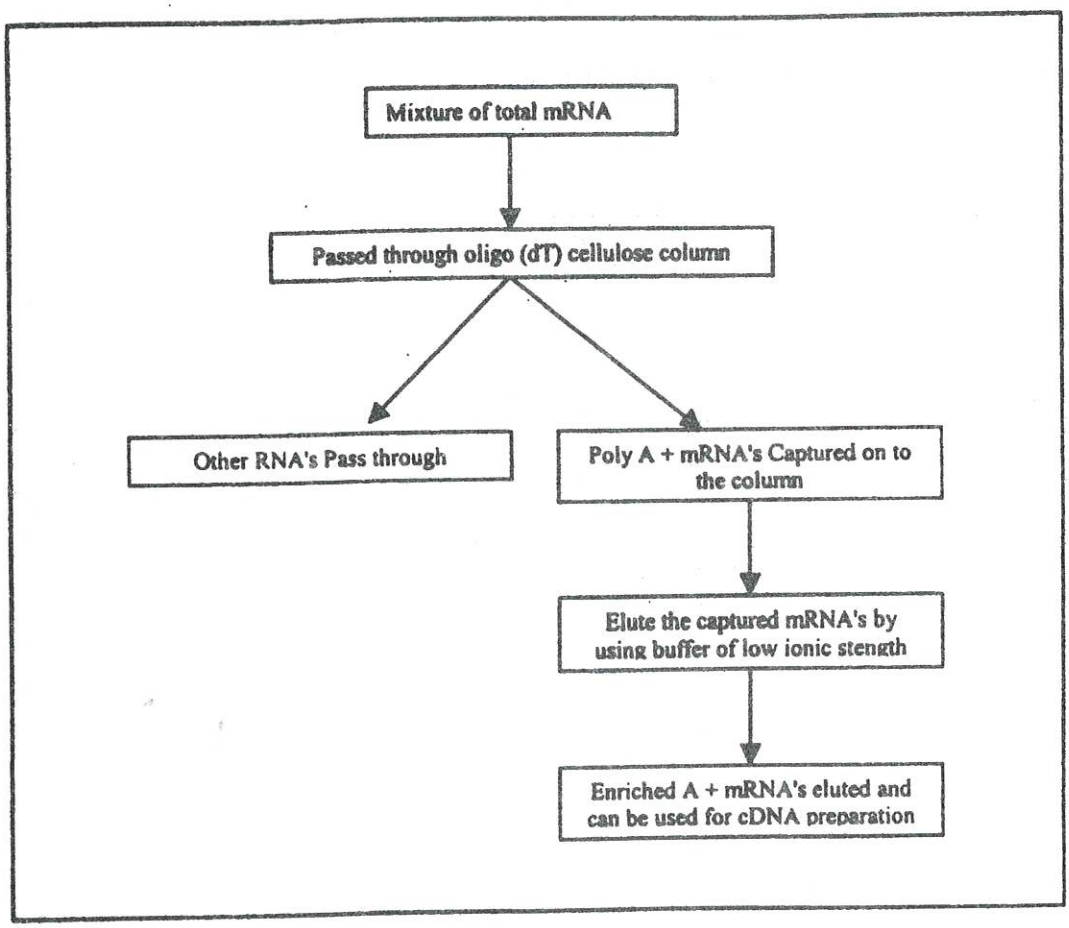


Figure 3.5: Separation of Poly A mRNA's

The primary difference in the gene clones and cDNA clones lies in the fact that intron sequence will not be present in cDNA clones and the mRNA`s are mature and processed molecules are obtained after splicing of the introns.

cDNA libraries are uniquely made from those RNA`s possessing a poly A tail; a unique feature of mRNA.

Construction of a cDNA Molecule

mRNA posses a 3` poly A tail, hence a short primer of oligo (dT) is synthesized to prime the first step. The primer is extended by an enzyme reverse transcriptase (RNA dependent DNA polymerase) to make a cDNA copy of the mRNA strand. The resultant is a DNA - mRNA hybrid. RNase H is a ribonuclease, which degrades the RNA strand in the hybrid and then the DNA polymerase I synthesizes new DNA fragments to make a complete chain. Hence, a double stranded cDNA molecule can be formed.

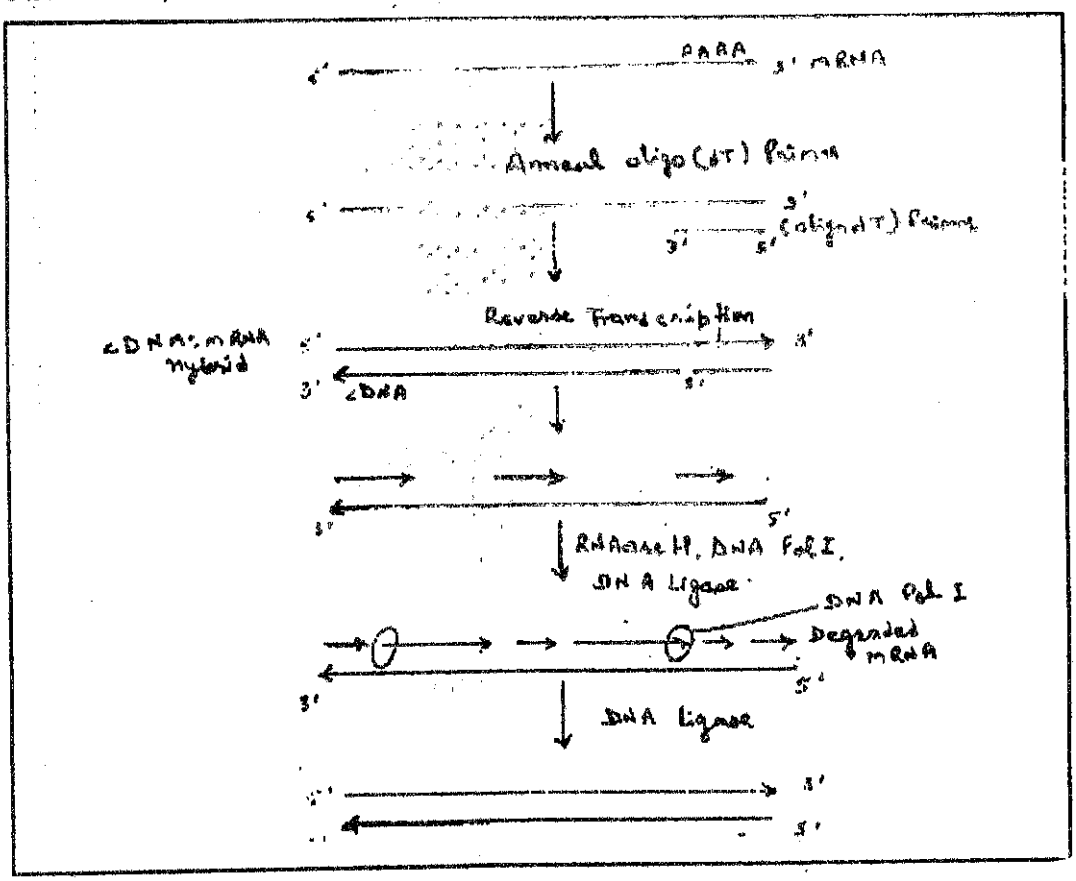


Figure 3.8: Double Stranded cDNA

Cloning of a cDNA Population

One very popular method of cloning of cDNA is using shoet double stranded pieces of DNA (8 -12 bases long) that includes a restriction sequence. Such DNA pieces are called as restriction site linker or linkers.' Since both the cDNA linker have blunt ends, they can be ligated together with DNA ligase. The resulting DNA molecule can be inserted into a cloning vector and the rDNA molecule can be transformed into a host cell (E.coli) for further cloning.

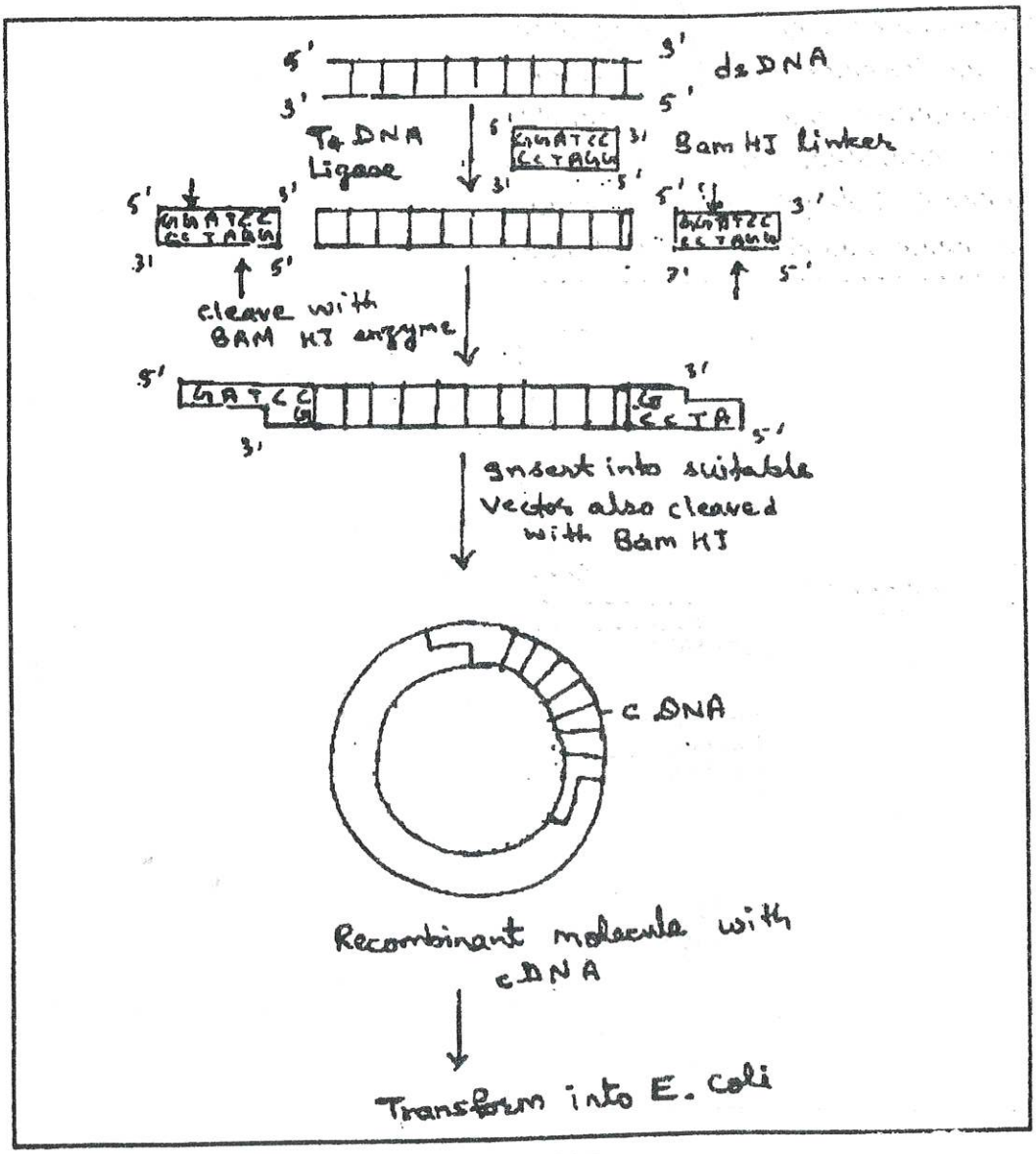


FIGURE 8.7

Screening of cDNA library

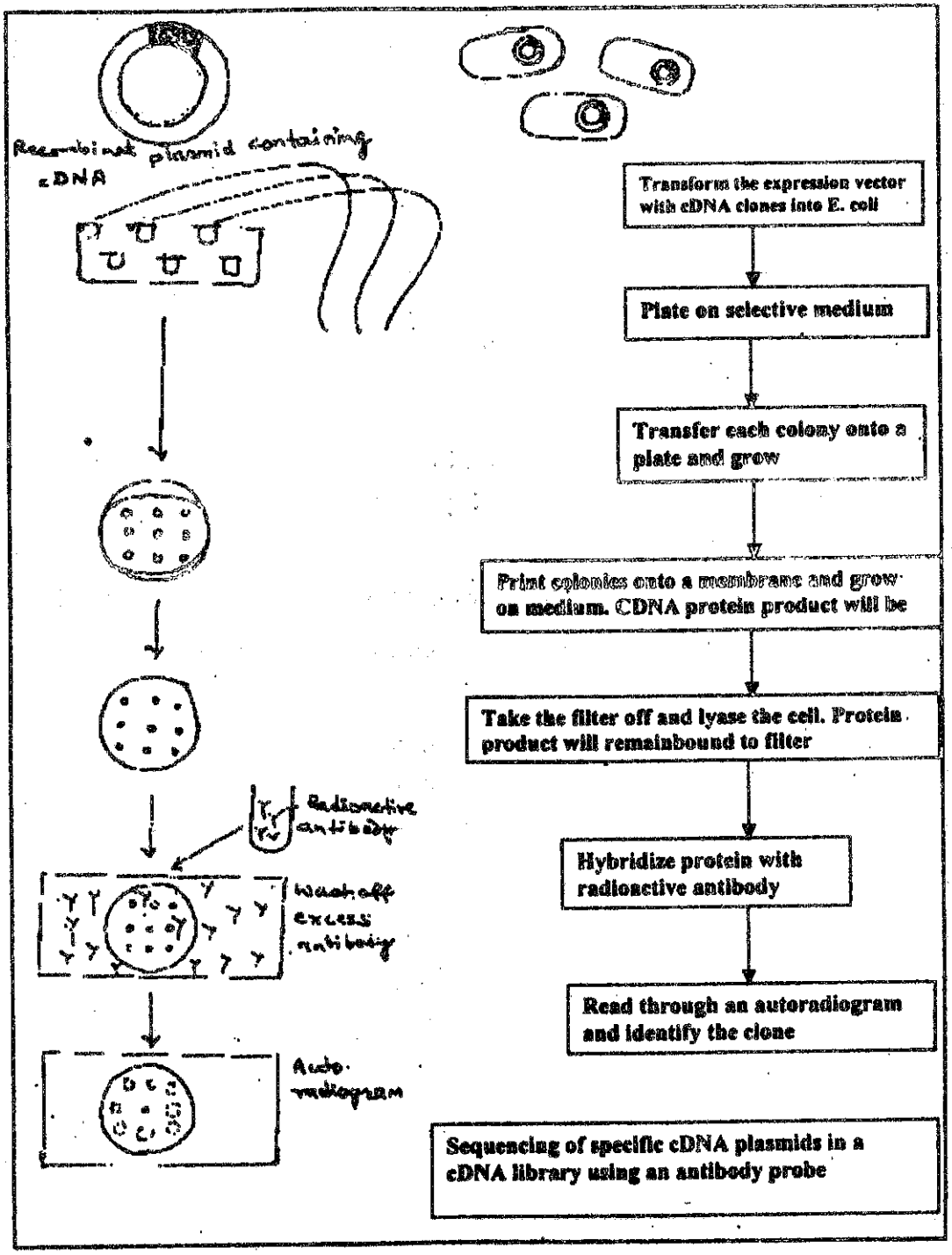


Figure 3.9

The cDNA can be selected if it encodes a specific protein. Then antibodies specific for that protein can be used for screening. For this purpose cDNA must be cloned in a specific type of vector called as expression vector. The antibodies used for such an approach can be radioactively labeled and then the protein of interest can be identified through an autoradiogram, by the process called as autoradiography.

Choice of Vectors

Several kinds of vectors are used in DNA or gene cloning. Different vectors have different molecular properties and also have different capacity to accommodate the DNA or gene of interest. These vectors are of following types:

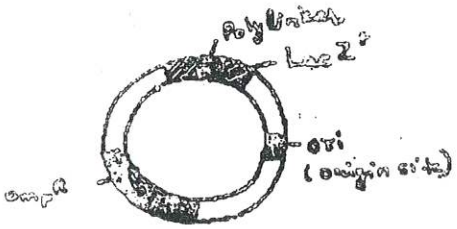
Plasmids

The DNA is circular; double stranded and has sequences (Ori) required for plasmid replication. Like pUC 19 (puck 19°). The key features required by an E. coli plasmid-cloning vector are:

A. An Ori (origin of replication) sequence, needed for plasmid to replicate in E.coli.

B. A selectable marker like antibiotic resistant gene, which can easily distinguish cells that lack the plasmid.

C. Restriction enzyme cleavage sites must be present in vector, so that the DNA fragment of interest can be appropriately inserted.

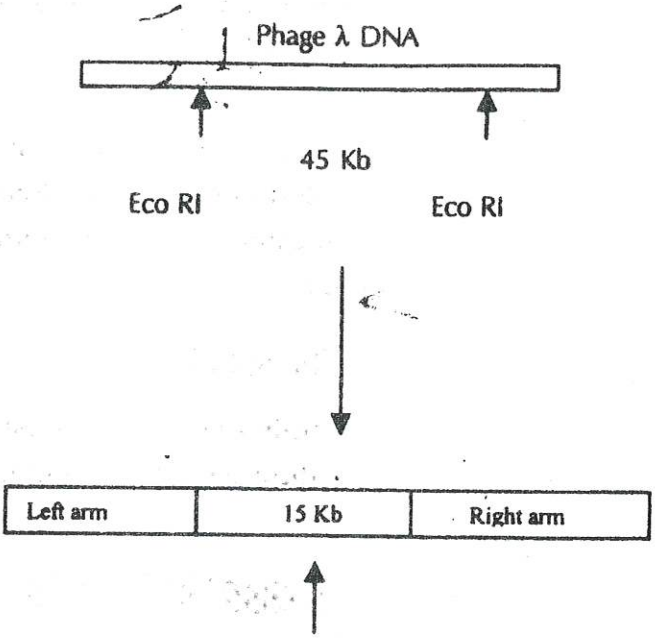


pUC 19 Vector
(2.68 kb)

Figure 2.9

Phage λ Cloning Vectors

These vectors are derivatives of bacteriophage λ so that lytic cycle is possible, but lysogenic is not. Like λ replacement vector.



Not required for λ replication
(Arms contain all genes essential for replication)

Cosmid Vector

These are not naturally occurring and have been derived from constructing features of both plasmid and phage λ.

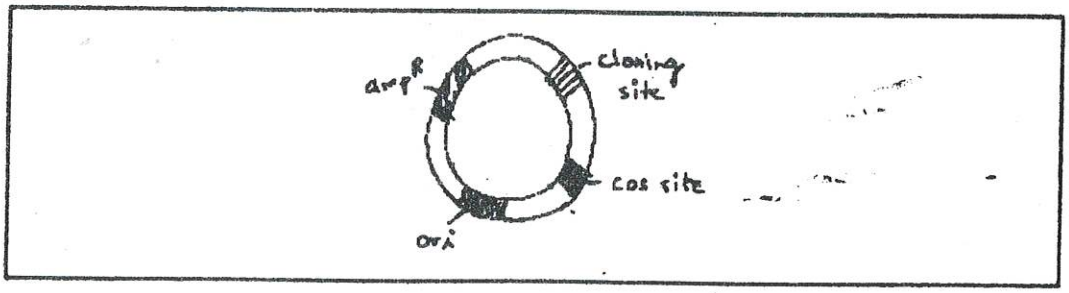


Figure 3.10: Cosmid Cloning Vector

Cos sites are derived from phage λ and these sites are those at which λ genome is present in multiple copies attached as à concatemer. The λ genome is cleaved into 48 Kb pieces to be packaged into phage heads. The cos sites permits the packaging of DNA into a λ phage and facilitates the introduction of large DNA molecules into a bacterial cell.

Shuttle Vectors

The vectors used for transforming mammalian cells in culture, or to transform other animal, plant, yeast or other cells are called as shuttle vectors. More specifically, these can be introduced into two or more different host organisms. Like these are some shuttle vectors, that can be transformed into *E. coli* and Yeast.

Yeast Artificial Chromosome (YACs, Yaks)

These enable artificial chromosomes to be made and cloned in yeast cells. These can accommodate DNA fragments up to 500 Kb. These are useful in creating physical maps of large genomes such as human genome.

Bacterial Artificial Chromosomes (BAC`s, `Backs)

These vectors are useful in cloning large DNA fragments up to 200Kb in size in E. coli. BAC`s can be handled like regular plasmid vectors and have been widely used for sequencing for the human genome in the **human genome project**.

DNA Synthesis and Sequencing

Biochemical advancement aided DNA technology has come up with means of chemical synthesizing oligonucleotides of given sequence. This has resulted in case in molecular cloning and DNA characterization. Automated machines, that enables the chemical reactions in a DNA synthesis reaction to be carried out in a step wise manner is called as DNA synthesizers or gene machines. Chemical synthesis is the chemical process by which each incoming nucleotide is coupled with to the 5`-OH terminus of the growing chain.

Phosphoramidite method

This is the procedure widely used. Before the introduction of bases into the reaction column, the amino groups of the bases A, G and C are deactivated by addition of benzoyl, isobutyryl and benzoyl groups respectively, to prevent undesirable side reactions during chain growth.

Thymine lacks amino groups, so it is not treated. Solid phase synthesis is used so that all the reactions can be conducted into one reaction vessel.

Flow diagram for chemical synthesis of oligonucleotides:

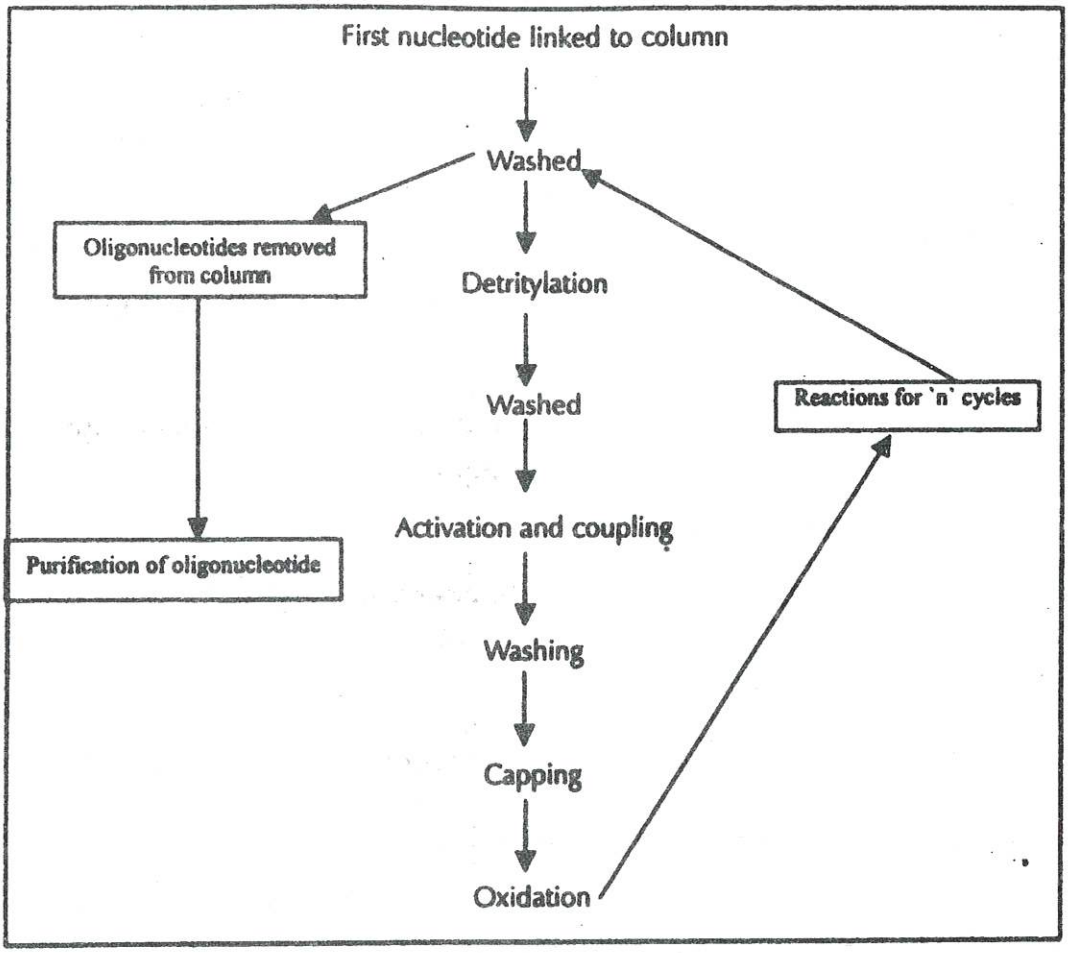


Figure 2.11

The brief sequence of process is following:

Initial nucleotide (base + sugar) attached to an inert solid support like CPG beads

↓

First nucleotide attached to spacer molecule by 3` terminus and spacer is attached to the support material

↓

5` terminus will have a dimethoxy trityl (DMT) group
(to prevent 5` - OH group from reacting nonspecifically)

↓

Each added nucleotide to the growing chain has a 5` DMT
and a di-isopropyl amine group at the 3` phosphite group,
protected by a methyl residue. This is called a phosphoramidite.

↓

Once the first nucleotide attaches to the CPG beads, the cycle begins.

↓

Column is washed with acetonitril and then flushed with argon to purge
the acetonitril.

↓

The 5` DMT group is removed by detritylation by treating with TCA
(Trichloroacetic Acid) to yield a 5` - OH group.

↓

Column washed with acetonitril to remove TCA and then flushed with
argon to purge the acetonotril

↓

Machine is programmed to introduce the next base (phosporamidite)
and tetrazole. Tetrazole activates phosphoramidite and then washings are done.

↓

The unlinked residues are prevented from linking to next nucleotide
by capping followed by oxidation to form stable groups.

↓

This cyclic process continues till new DNA strand is made.

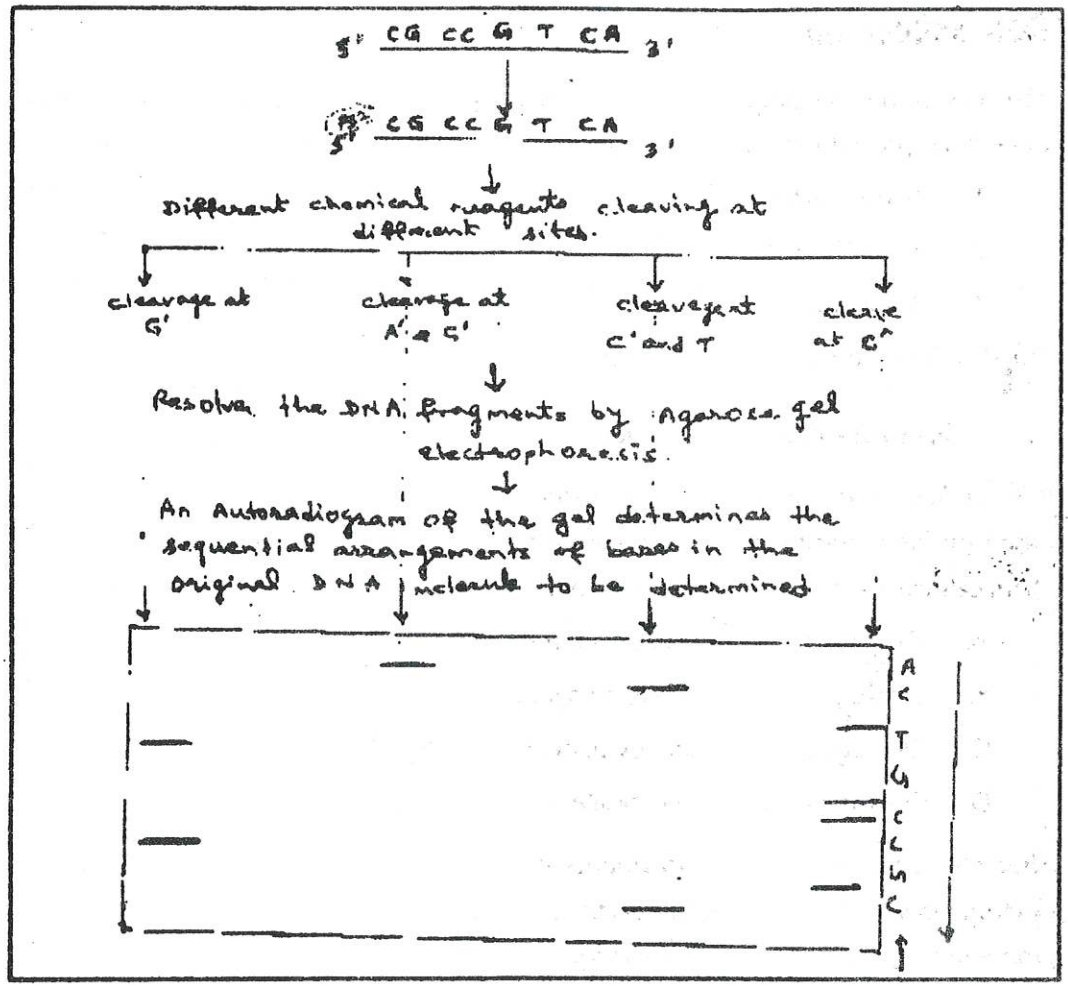


Figure 3.12: An Autoradiogram Showing Different Fragments of DNA of Different Sizes

It`s disadvantages are following:

A. Only single sequence can be determined from a single set of reactions by the Maxam - Gilbert method.

B. Since the chemical degradation reaction all require different conditions, so rate of reaction becomes slow.

As a consequence, the chemical degradation method is now used only where it is important to sequence the DNA directly, rather than via an enzymatic copy. This includes applications such as studies of DNA modification, secondary structure and protein interaction.

2. Chain Termination Method

It is also known as Sanger`s dideoxy method, as it is proposed by Sanger. In this method dideoxy nucleotides are utilized, which lack a 3` -OH group. It stops the incorporation of further nucleotides in the DNA synthesis. Here 4 normal deoxynucleotide substrate plus low concentration of one of the four dideoxynucleotides is used in reactions. Once a dideoxynucleotide is incorporated, the DNA chain will be prematurely terminated and a series of fragment of varying size will be produced.

Resolution of fragments is carried out with the help of agarose gel electrophoresis. After resolution an autoradiogram of the gel is obtained, which determines the sequential arrangements of bases in the original DNA molecules.

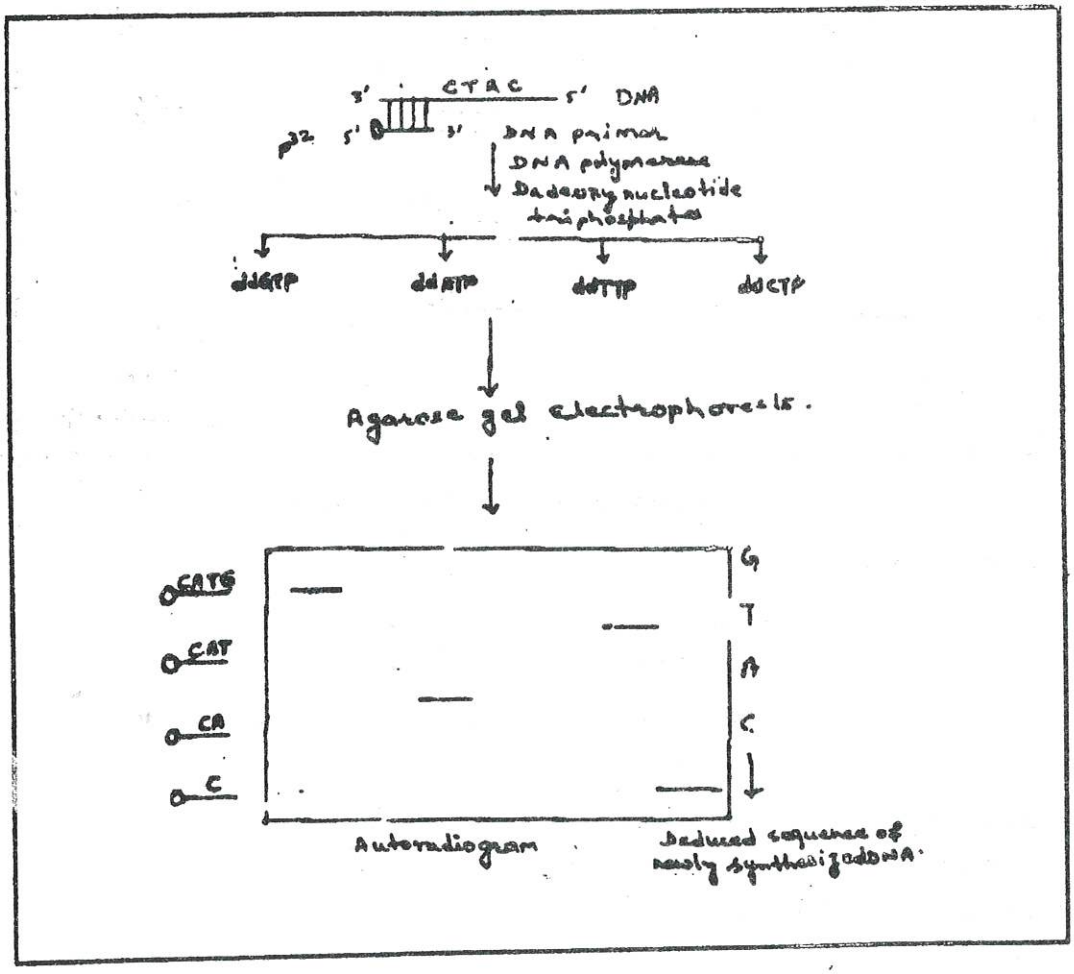


Figure 2.10

3. Automated DNA Sequencing

This method is a slight deviation from the senger`s method as the dideoxynucleotides used for each reaction are labeled with a differently colored fluorescent tag. This is also called as high throughput DNA sequencing, as this technology allows DNA sequencing containing thousands of nucleotides to be determined in a few hours. The human genome project comprising approximately three billion base pairs was completed in 2001 employing this powerful technique.

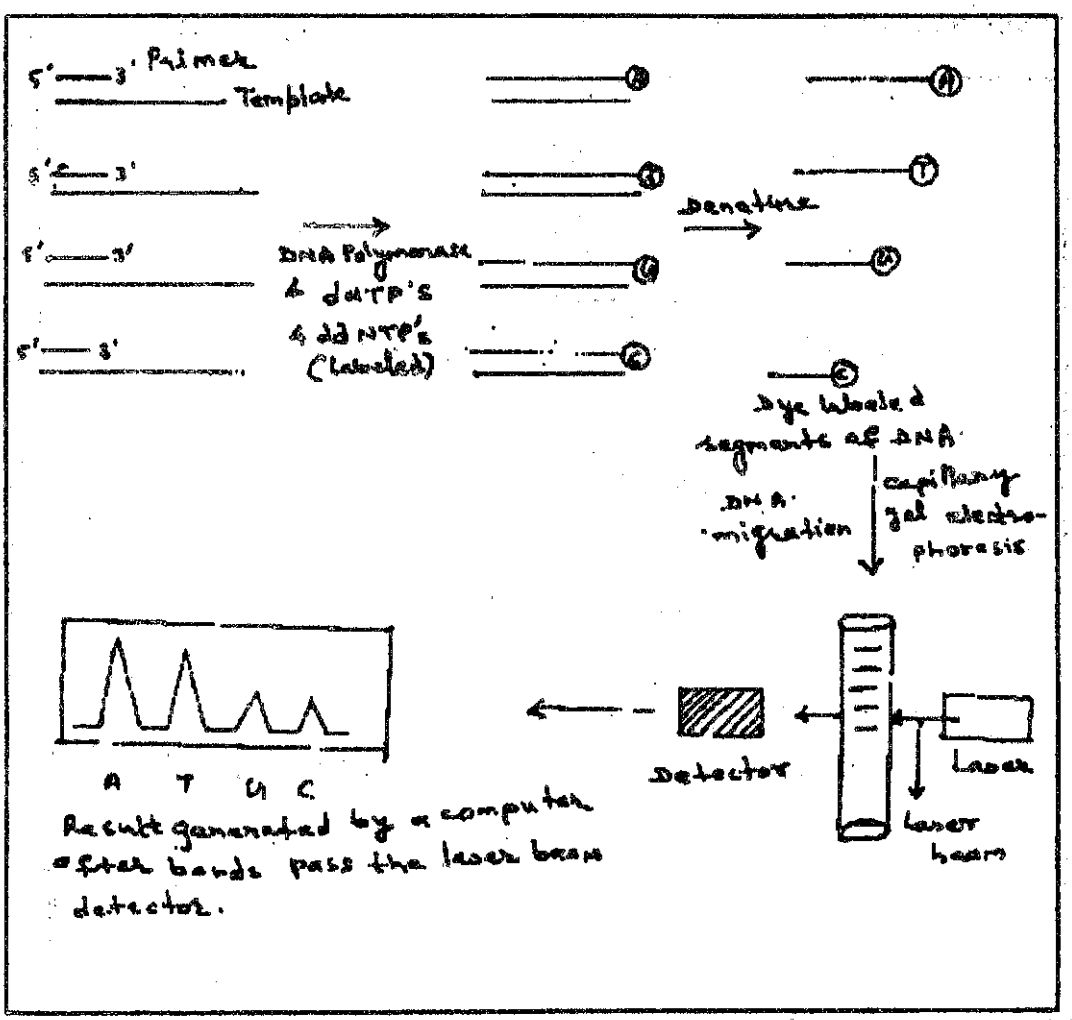


Figure 2.14

All the four labeled ddNTP`s are added to a single tube and the resulting colored DNA fragments are separated by size in a single electrophoretic gel contained in a capillary tube. All fragments of a given length migrate through the capillary gel in a single peak and one associated with each peak is detected using a laser beam. Hence, the DNA sequence is read once they the pass the detector and the information is fed directly to a computer and the DNA sequence is determined.

Polymerase Chain Reaction

In 1987, Kary Mullis of the Cetus Corporation developed an in vitro method for the amplification of a specific DNA sequence without the need of gene cloning. This in vitro method used for amplification employing a heat resistant (thermostable) DNA polymerase enzyme, i.e. Taq polymerase, is called as polymerase chain reaction (PCR). This term is derived from two branches of science:

Polymerase: Chemistry ⟶ Polymerization

Chain reaction: Physics ⟶ Nuclear fission resulting into daughter molecule from a parent molecule.

The amplified products are called as amplicous or amplimers. The important steps involved in a typical PCR are:

1. PCR cycle
2. PCR products

The details of the processes are following:

1. PCR Cycle

A PCR cycle is based on following three steps:

A. Denaturation:

- The starting point of PCR is a double stranded DNA containing the gene or sequence of interest and a pair of oligonucleotide primers that flank that DNA sequence.
- Heating at 94 to 95 oC denatures the double stranded DNA.

B. Annealing:

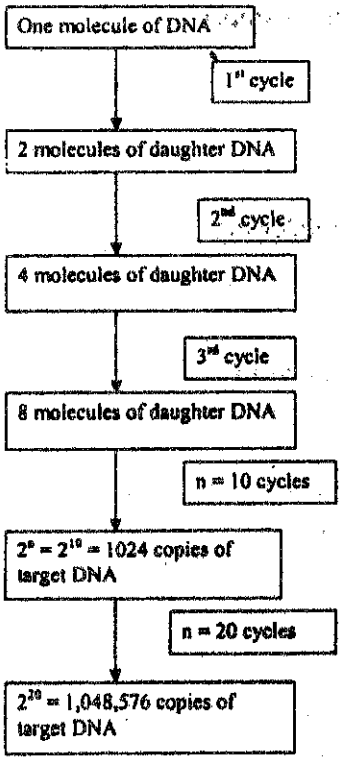
- Once the denatured DNA cools; the oligonucleotides primers anneal at their specific sites on the target DNA.
- The primers are designed in such a way that they bind to both the strands of DNA template, at temperatures ranging from 37 to 65 oC.

C. Extension:

- The primers are then extended by a thermostable Taq polymerase at temperature ranging from 72 - 75 oC.
- Taq polymerase is isolated from a thermophilic bacterium Thermus aquaticus.

All the aforementioned steps are repeated in cyclic manner and by this procedure; the newly generated DNA sequence of interest increases geometrically in amount. These steps are carried out in a machine called thermal cycler which automatically cycles the steps of specific temperatures in a programmed way.

2. PCR Products



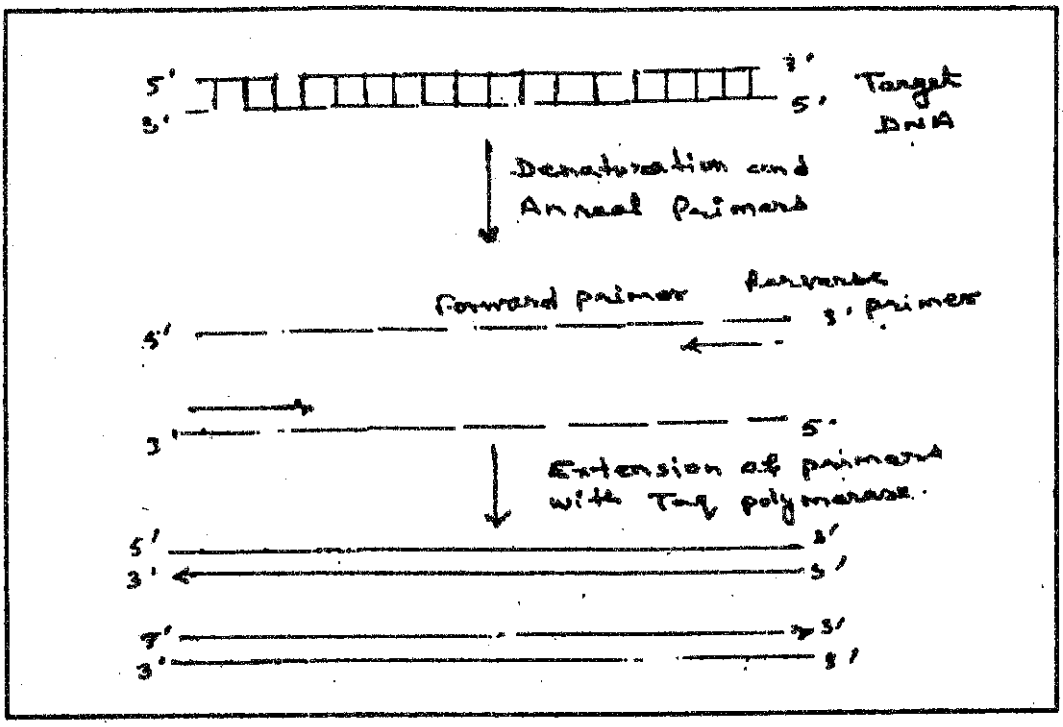


Figure 3.16: Repeat Steps 1, 2 and 3 for a Number of Cycles Till the Desired Sequence is Amplified Selectively.

Types of PCR

Variations in the basic PCR technique have been developed which allow various applications of this powerful amplification technique:

1. Inverse PCR

In this process amplification takes place in those regions which are away from the primers and not that which is flanked by the primers. Inverse PCR can also be used for amplifying the border sequences of a gene(like the regulatory sequences), if the gene sequence is known.

2. Anchored PCR

If only the sequence of the end of the target is known; then anchored PCR is used which utilizes only one primer in the 1st step.

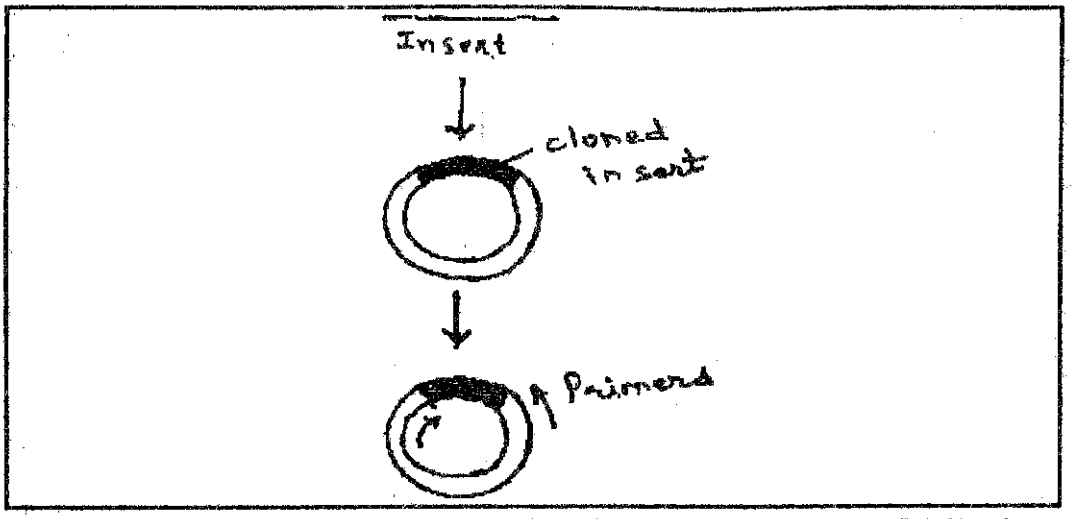


Figure 3.16: Cloning of Foreign DNA by Inverse PCR Utilizing Vector Border Sequences for Amplification of Insert

3. RAPD PCR

Random Amplified Polymorphic DNA's is used for developing molecular markers.

4. Competitive PCR

This modification of PCR allows the quantitation of the genomic DNA, utilizing an internal standard called as a competitor or standard.

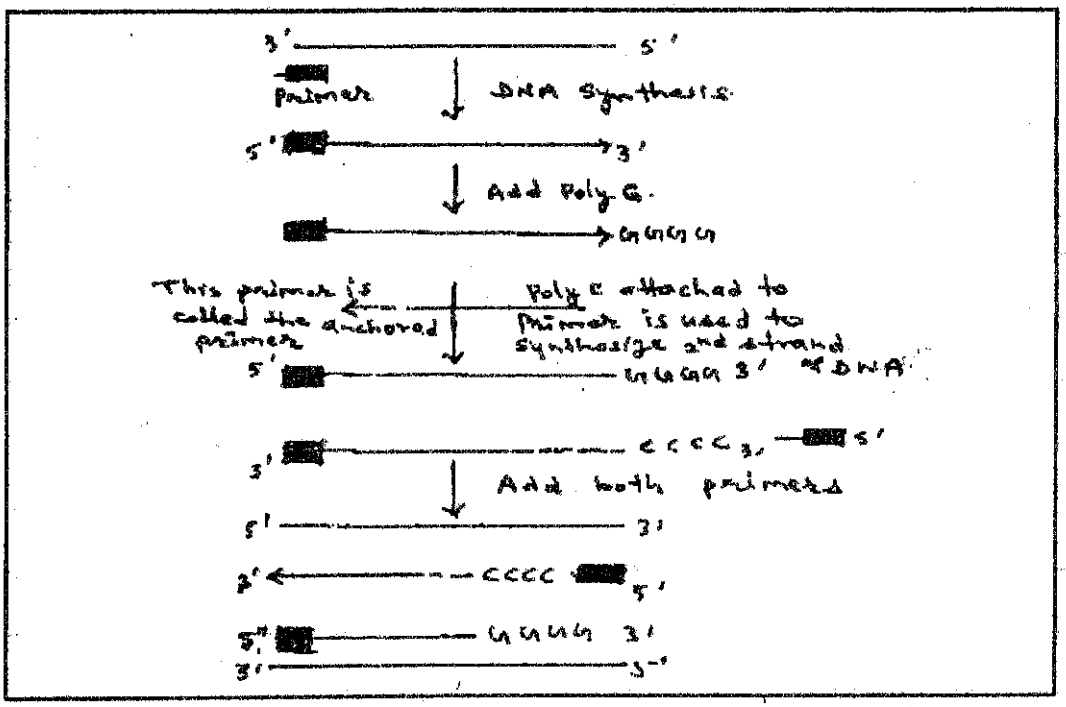


Figure 3.17

5. Multiplex PCR

It allows the amplification of a number of sequences in a single tube utilizing many pairs of primers.

6. RT - PCR

It allows an amplification of a specific transcript by cDNA synthesis followed by PCR.

7. RACE (Rapid amplification of cDNA Ends)

These PCR protocols generate complementary DNA (cDNA) from either a 5` or 3` end of a specific messenger RNA. Here Gene Specific primers (GSP) are used for cDNA synthesis and cDNA amplification during PCR cycles.

Application of PCR

1. Amplification of DNA for cloning.
2. Amplification of DNA from genomic DNA preparations.
3. DNA mapping.
4. Disease diagnosis by amplifying DNA of a pathogen causing the disease.
5. Sex determination of embryos.
6. Forensics of identification of individuals who participated in a crime by identifying DNA in samples found at the scene of a crime such as hair, blood, semen etc.
7. Molecular evolution studies: The DNA sequence amplified from samples of tissue preserved for hundreds and thousands of years allows those sequences to be amplified, analysed and compared with contemporary DNA samples.

DNA Fingerprinting

The father of DNA fingerprinting is Sir Alec J. Jeffrey, who first developed this technique in 1985. Important facts of this technique are following:

- No two human individuals (except identical twins) have a similar genome; hence this led to development of a new technique called DNA fingerpriniting or DNA typing or DNA profiling.

- This technique relies on molecular markers such as RFLP's (Restriction Fragment Length Polymorphism) for DNA analysis followed by PCR and agarose gel electrophoresis.

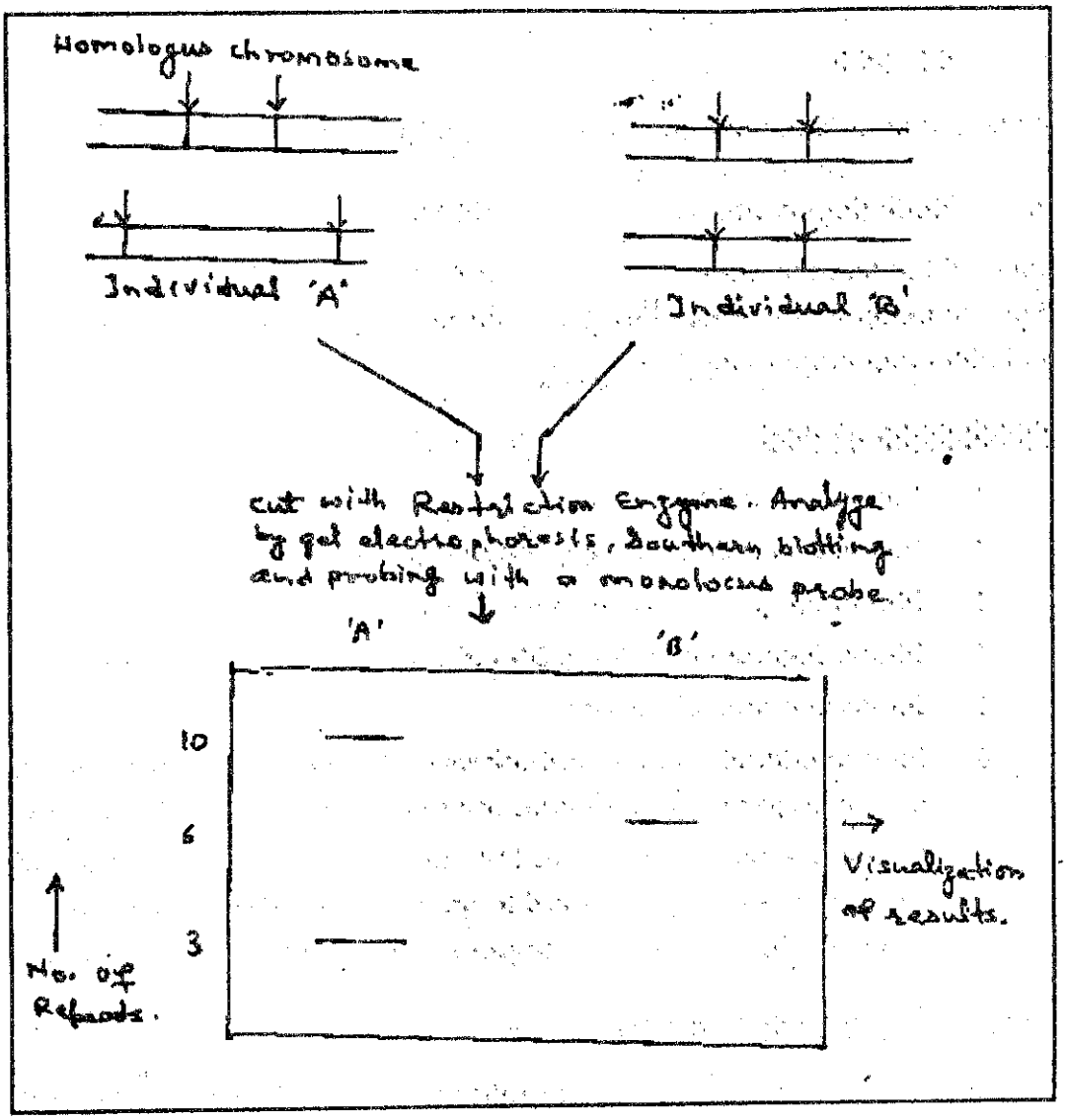


Figure 3.18

- The molecular markers are highly polymorphic and hence there are lots of differences among individuals in a population like short identical segments of DNA tandemly arranged head to tail are called tandem repeats.

- If the repeating unit are 2, 3 or 4 bases long it is called a Short Tandem Repeats (STR) or a micro-satellite.
- If the units are 5, 10 or 20 bases long; it is called as Variable Number of Tandem Repeats (VNTR) or a mini-satellite.
- Now a days a single base change polymorphism in a specific sequence is widely used for polymorphism studies, which is popularly known as Single Nucleotide Polymorphism (SNP or snips).
- STR`s and VNTR`s are used as markers in RFLP based DNA typing.

Application of DNA Fingerprinting or Typing

1. **DNA Fingerprinting in a Paternity Case**

DNA from mother, baby and alleged father

↓

All three types of DNA are treated with restriction enzymes in separate tube

↓

Resolving of fragments by agarose gel electrophoresis

↓

Southern blot prepared from gel

↓

Radio-labeled probe used for southern hybridization

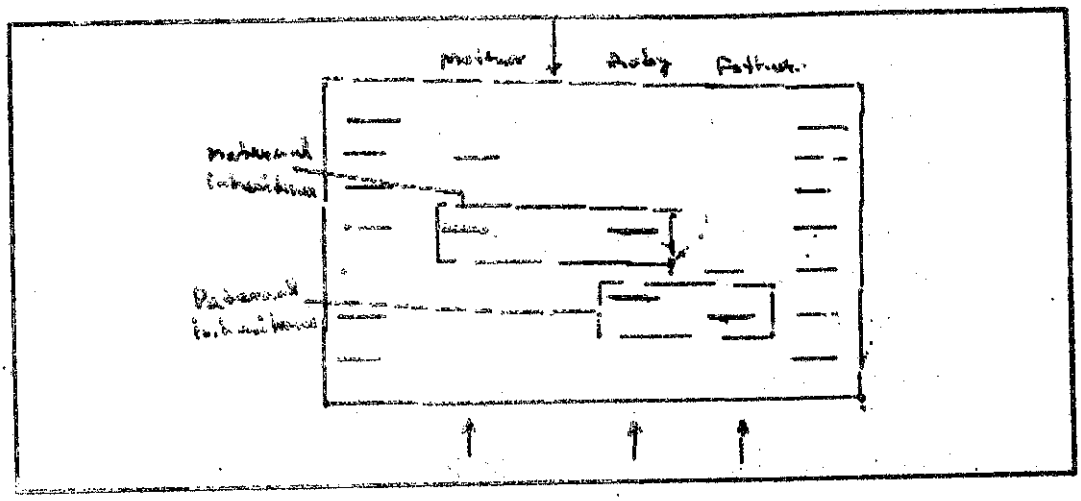
↓

Washing of excess probe

↓

Autoradiogram is prepared

(Banding pattern of each sample is a DNA fingerprint).



2. Forensics: murder, rape, violent crimes. For minimum amount of DNA, PCR can be employed for amplification of target DNA for typing experiments.
3. Population genetics studies.
4. Pedegree analysis of horses and other animals.
5. Study of conservation biology of endangered species.
6. Forensic analysis of wild life crimes.
7. Detecting genetically modified organisms (GMOs).
8. Molecular evolution studies.
9. Study of fossils.
10. Many historical controversies and mysteries have been solved by DNA typing.

CHAPTER 4

GENETIC ENGINEERING OF PLANTS

LEARNING OBJECTIVES

- Introduction
- Strategies for Development of Transgenic Plants
- Plant Cell Suspension Method
- Agrobacterium the Natural Genetic -DNA
- Transposon Mediated Gene Tagging
- Chloroplast Transformation and its Utility
- Intellectual Property Rights
- Possible Ecological Risks and Ethical Concerns

Introduction

Plant genetic engineering is a quality that's becoming a reality. Genetic engineering involves manipulation of the genetic material towards a desired goal in a directed and predetermined manner. It basically involves recombinant DNA technology or gene cloning. In this, DNA molecule of 2 organisms are isolated, recombined in desired combination and then transferred to cell for replication and expression. A model genetic engineering of plant has following steps:

1. Selection of plant gene whose introduction in other plants is of positive value.
2. Identification and isolation of such gene.

3. Transfer of isolated genes to plant cell.
4. Regeneration of complete plants from transferred cells or tissues.

Important goals of plant genetic engineering are:

1. Production of plants resistant to herbicide, insects, fungal and viral pathogens.
2. Improved protein quality and amino acid composition.
3. Improved photosynthetic efficiency.
4. Improved post harvest handling.

Aims, strategies for development of transgenic plants (With suitable example)

A cell that has a gene introduced into plants by artificial means is called a "transgenic cell" and the plant, which develops from such a cell, is called transgenic plant. The aims for developing transgenic plants are:

1. To increase efficiency of crop production systems e.g. to produce plants resistant to herbicide, insects, viruses and large number of other stresses.
2. To produce plants suitable for food processing e.g. bruise resistance and delayed ripening in tomato.
3. Use of transgenic plants as bioreactors or factories for production of chemicals and pharmaceuticals.
4. For identification of gene functions and researches.
5. Production of plants of agricultural, horticultural, or ornamental value and creating plants with novel characteristics.
6. Introduction of increased photosynthetic efficiency.
7. Development of nitrogen fixing cultivars in non-leguminous crops.
8. Improvement in nutritional quality of seed storage proteins.

Strategies for Development of Transgenic Plants

For introduction of foreign gene into plants an efficient and reliable plant gene vector is required. A vector is an agent that helps in placing foreign gene into plants or plant parts.

Besides the vector mediated DNA transfer, direct uptake of DNA by protoplasts also occurs. Both the methods are described below:

Vector Mediated Gene Transfer

These are three major groups of gene vectors for plants e.g. Ti plasmid of Agrobacterium tumefaciens, the Caulimoviruses and geminiviruses. Some other vectors can also be used e.g. transposon. All are described below:

1. *Agrobacterium Tumefaciens:* A. tumefaciens is a soil microorganism that causes crown gall disease in dicots (few monocots) when it invades a wound.

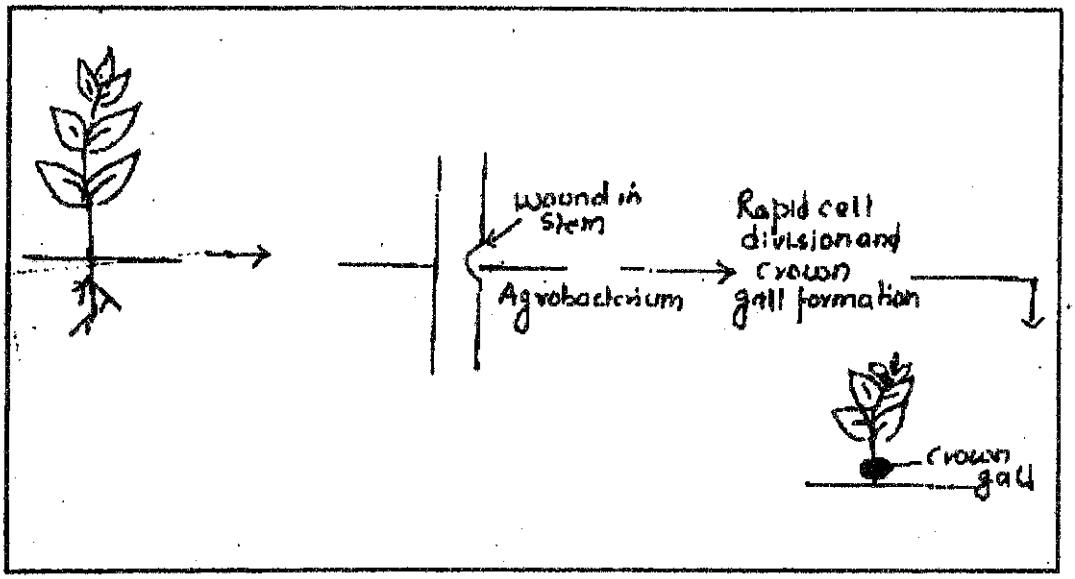


Figure 4.1: Agrobacterium Tumefaciens

The bacterium transfers a part of its Ti plasmid into cells of host plants. The plasmid integrates into genome of plant and alters its metabolism.

Ti plasmid is circular DNA molecules with molecular weights of about 1.2 X 108. It's about 200 Kb in size. After infection part of this plasmid is integrated into plant chromosomal DNA. This segment is called T-DNA and it varies between 15-30 Kb in size. It carries about 8 genes, which are responsible for cancerous properties of transformed cells.

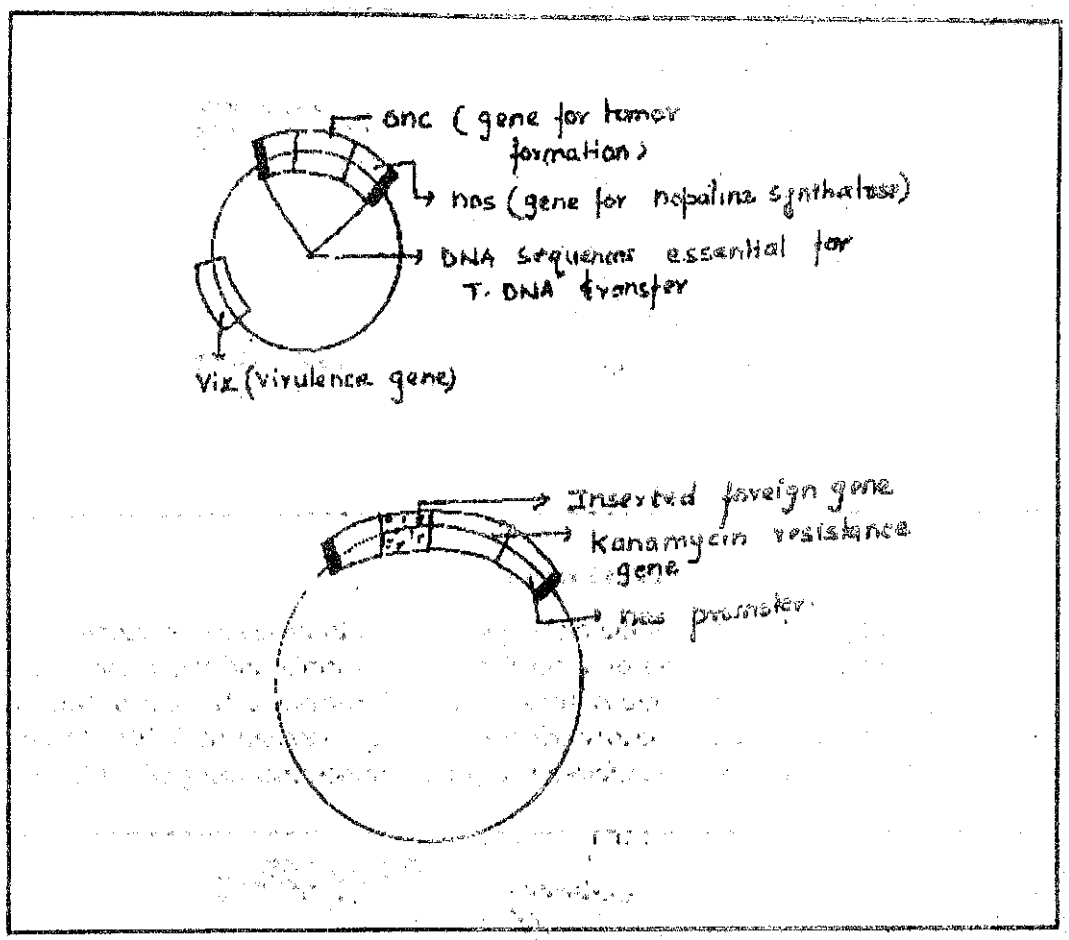


Figure 4.2: Diagram of Ti Plasmid

2. *Use of Ti plasmid as vector:* Ti plasmid and T-DNA contains a very useful vector for introducing new DNA sequences into plant cells. There are 2 problems with Ti plasmid.
   - Large size.
   - It infects only dicots (but major crops are monocots).

Thus inserting new DNA in it in the following manner has modified it.

Any gene placed between 25 bp borders will integrate into host genome.

3. *Binary vector system:* This is a two-plasmid system in which T-DNA is on relatively small molecule and rest of plasmid is in normal form. Both are used for transformation. The T-DNA plasmid is small and possesses unique restriction sites.

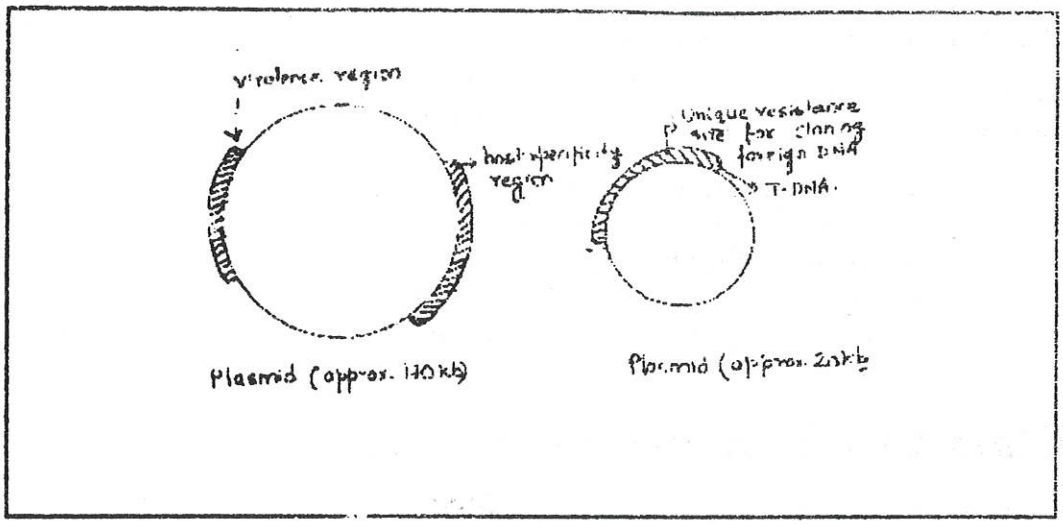


Figure 4.3: Binary Vector System

4. *Cointegration system:* In the technique the gene to be cloned is inserted into a unique restriction site on a small pBR 322 plasmid and this recombinant plasmid is introduced into A. tumefaciens cells carrying a Ti plasmid. Natural recombination occurs due to which foreign gene gets inserted into T-DNA. During infection new gene is transferred into plant chromosomes along with T-DNA.

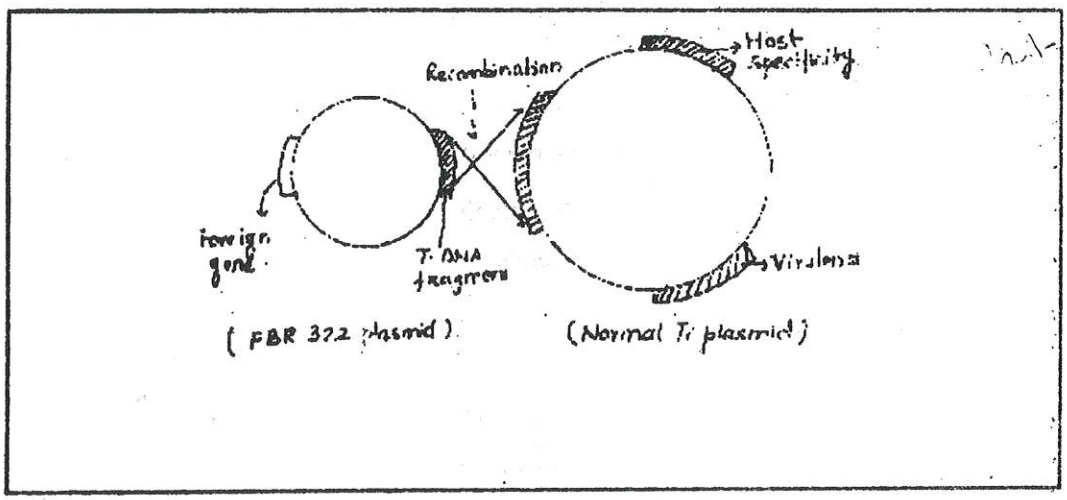


Figure 4.4: The Cointegration System

Methods for introducing new gene into plant cell. e.g. binary vector system and cointegration system are shown in figure 4.4.

Both these methods have been effectively used e.g. Malus spp transgenic have been produced by binary system and transgenic of Pranus spp have been produced by cointegration system.

5. *Transgenic plants from transformed cells:* A culture of plant cells in liquid medium is infected by A. tumefaciens containing engineered Ti plasmid. The transformed cells are selected by their ability for kanamycin resistance. These cells also contain foreign DNA. The cells are used for regeneration into plants. Protoplast as well as leaf discs are also used for this. The strategies are shown below:

Plant Cell Suspension Method

Plant cell suspension inoculated with recombinant

A.tumefaciens

↓

Plant cell containing recombinant bacterium

↓

Selection of transformed cells and plating on solid media.

↓

Callus of transformed cells

↓

Transfer to different media

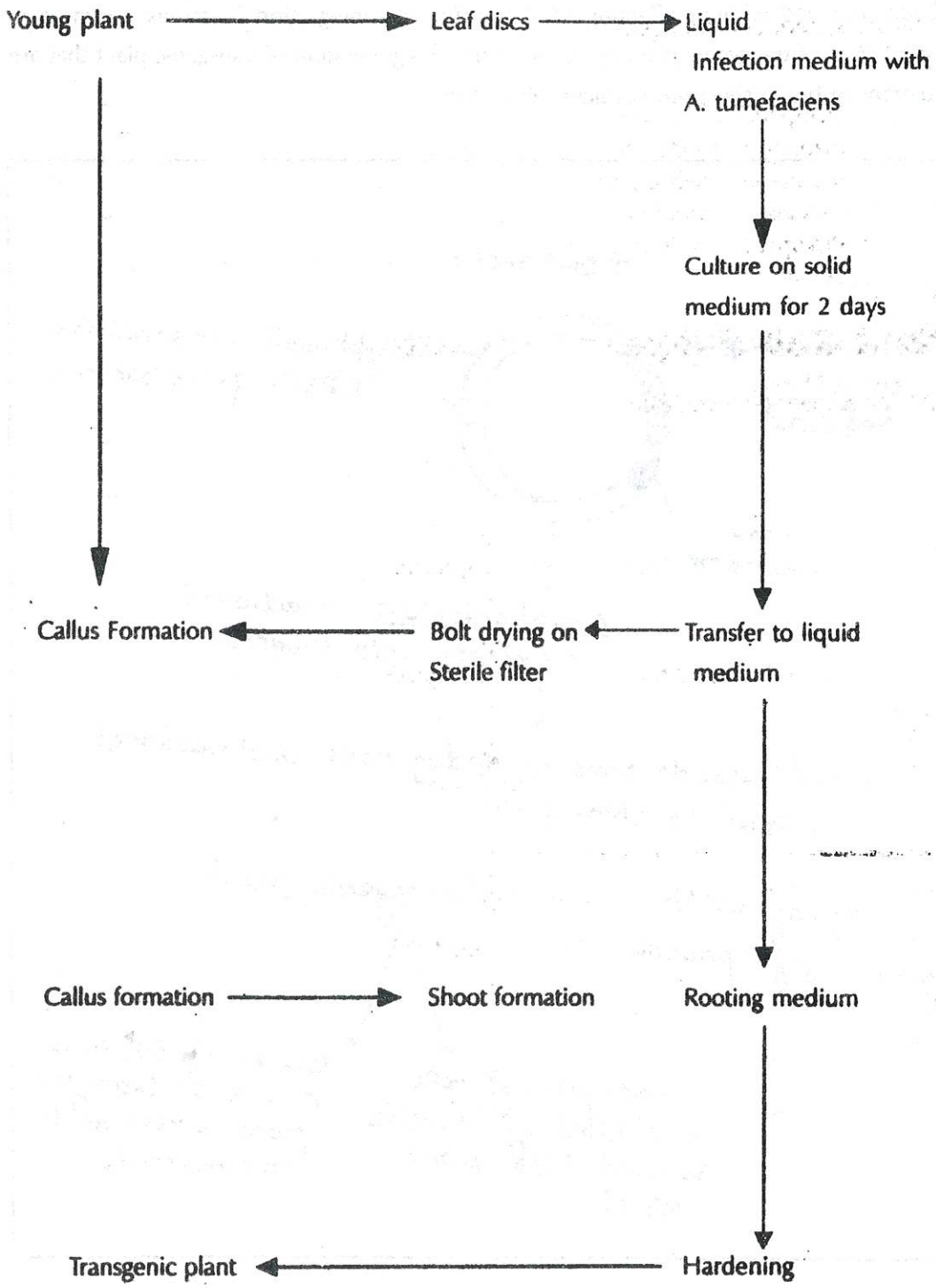
↓

Root and shoot formation

↓

Transgenic plant

Leaf disc transformation system:



Now "disarmed" plasmid have been created in which genes responsible for crown gall have been removed without effecting DNA transfer and integration functions. Example of production of transgenic plant by Agrobacterium is generation of transgenic plant that are tolerant to broad-spectrum herbicide Round upTM.

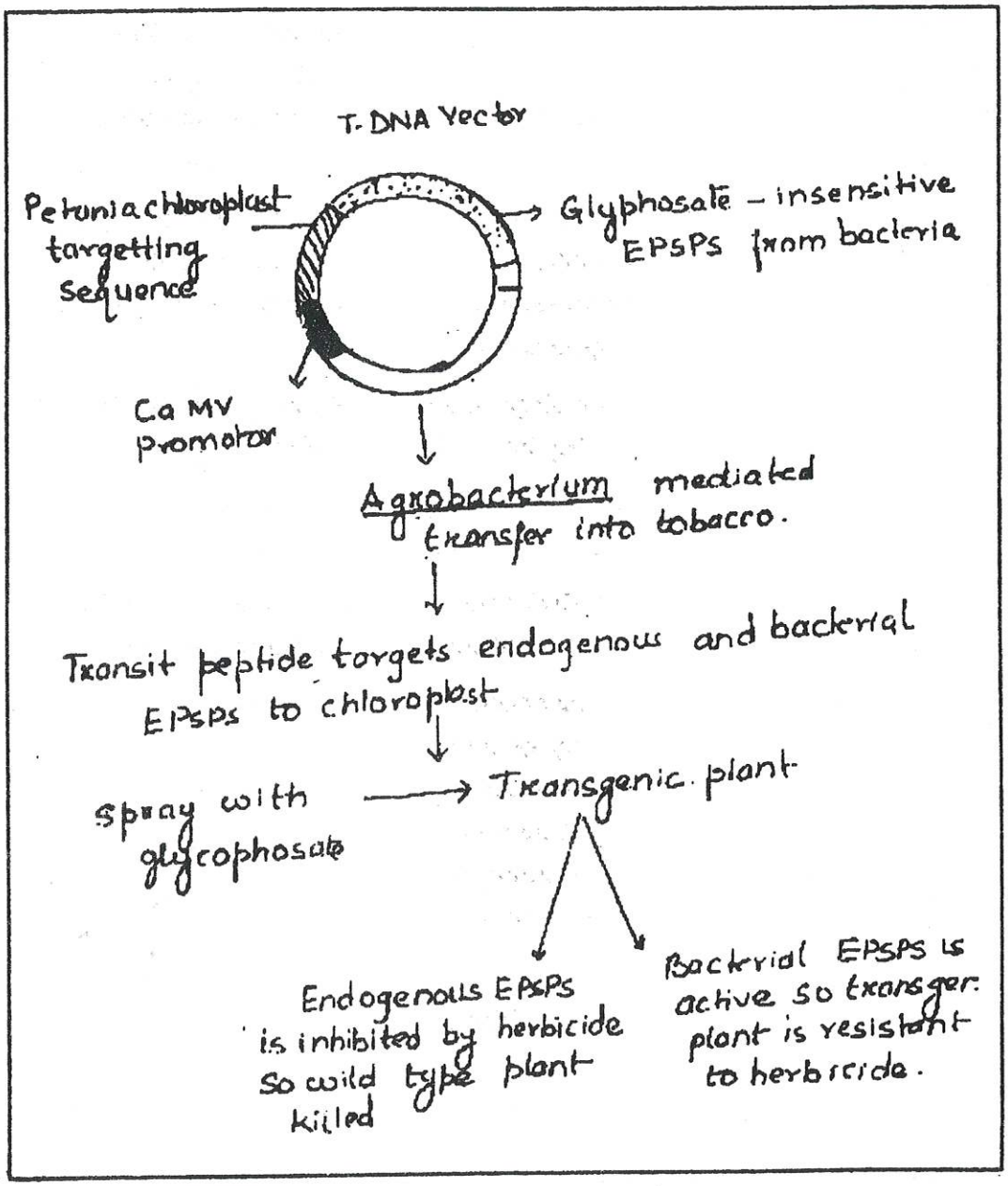


Figure 4.5

Round up contains glyphosate which kills plants by inhibiting EPSPS (5-enol- pyruvyl-shikimat-3-phosphate synthase), which is a chloroplast enzyme required for biosynthesis of essential amino acids.

In another example scientists have devised a way to block tomato from making normal amount of polygalacturonase (PG), a fruit-softening enzyme. A copy of the PG gene with backward orientation with respect to promoter has been introduced. So when it is transcribed, it produces antisence mRNA for gene. This binds to normal "sense" mRNA in cell thus preventing its translation. Thus vector less PG enzyme is produced and ripening of tomato is slowed.

(a) *Caulimoviruses:* e.g. CaMV, These are unique plant viruses having double stranded genomes (about 8Kb), foreign DNA can be inserted without affecting infectivity. They can also spread in whole plant body. So they are also used as vectors. Using them has generated transgenic turnips.

(b) *Geminiviruses:* They have single stranded DNA and replicate via double stranded intermediated. They infect wide range of crops. So can be used as vectors.

(c) ***Transposable elements:***

Besides vectors mediated DNA transfer transgenic can also be produced by direct DNA replace. Different methods for this are described below:

(i) ***Microprojectile bombardment:*** This is also called biolistics and most promising DNA delivery system in plants. Gold or tungsten particles are coated with DNA. The particles are accelerated at high speed by a particles gun, so that they penetrate plant cell walls and membranes. Once inside cell they integrate into genome. This can be used to introduce foreign genes into, monocots, conifers, dicots, cellsuspensions, callus, and pollen etc.

(ii) ***Direct gene transfer into plant protoplasts:*** It can only be used with cell protoplasts, which can be regenerated into viable plants.

(iii) ***Microinjection:*** It has limited use because only one cell can be injected at one time and very skilled individual is required.

(iv) ***Electroporetion:*** It is also generally limited to protoplast.

(v) *Liposome fusion:* Nucleic acid is entrapped in a liposome, so it's not attacked by nuclease. This is also receiving great attention. It is generally used with protoplast.

Use of Reporter genes

These are used to detect the foreign DNA, which is integrated in plant genome so that transformed cells can be identified. These are also used to quantity level of gene expression. The reporter genes usually encode an activity that can be assayed. The important plant cell reporter and selectable marker gene systems are:

E.coli ß-D-glucouronidase (GUS) gene: It encodes a stable enzyme that is not normally present in plants and that catalyzes the cleavage of a range of ß-D- glucouronidase. Observing the blue colour that is formed after hydrolysis of uncoloured substrate 5-bromo-4-chloro-3 indoyl ß-D-glucuronic acid can localize the GUS activity in transformed plant tissues. GUS activity in plant extracts can be quantatively assayed also by flurometric analysis in which hydrolysis of the substrate 4-methylumbelliferyl ß-D-glucuronide to form a fluorescent product. But this cannot be used as selectable marker.

Hygromycin phosphotransferase can be used as selectable marker and also as reporter gene.

Production of marker free transgenic plants

The introduction of marker genes has raised concerns e.g. they can be toxic or allergic. Thus marker free transgenic plants have been developed. For this either co-transformation of plants with two separate DNAs are carrying marker and one carrying foreign DNA is done. The DNAs integrate at different places and marker can be removed by breeding methods. In other way marker is cloned in transposable element (Ds) so it's excised later on.

Agrobacterium the Natural Genetic -DNA

A.tumefaciens is a gram-negative soil bacterium; it is a plant pathogen, which genetically transforms cells during its normal course of lifecycle. Due to transformation crown gall tumors develop in the plant at wound sites. This disease (crown gall) affects dicots plants including grapes, stone fruit trees and roses. Crown gall formation occurs due to integration and expression of genes of specific segment of bacterial plasmid DNA-called T-DNA

(transferred DNA) into plant genome. The T-DNA is a part of Ti (tumor inducing) plasmid, which is present in most of the strains of A. tumefaciens. Ti plasmid is a large plasmid of about 200Kb.

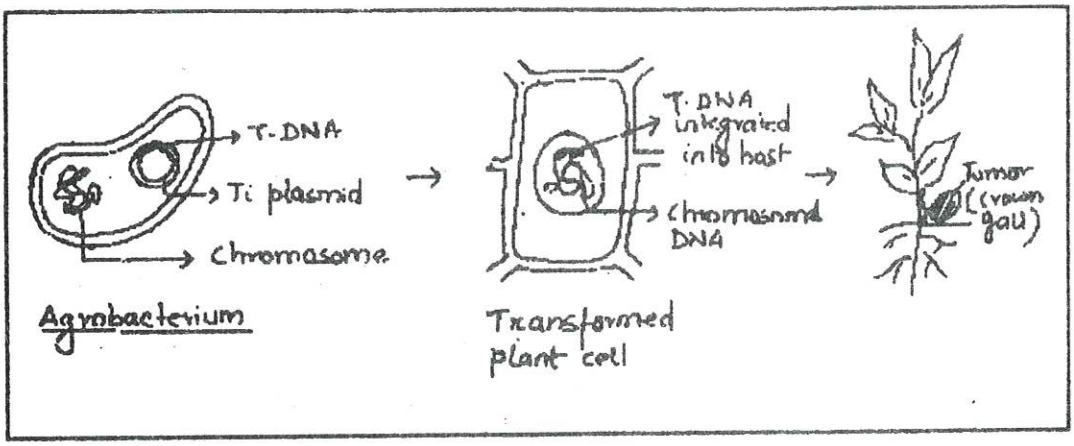


Figure 4.6

In the first step the Agrobacterium attaches to the plant a wound site usually near base of plants stem. The wounded plants produce certain phenolics like acetosyringone +hydroacetosyringone. These chemicals induce the activity of vir (virulence) genes present on Ti plasmid. The vir genes are not found on T-DNA but products of vir genes are required for transfer and integration of T-DNA in plant genome. These are about 7-8 different vir genes.

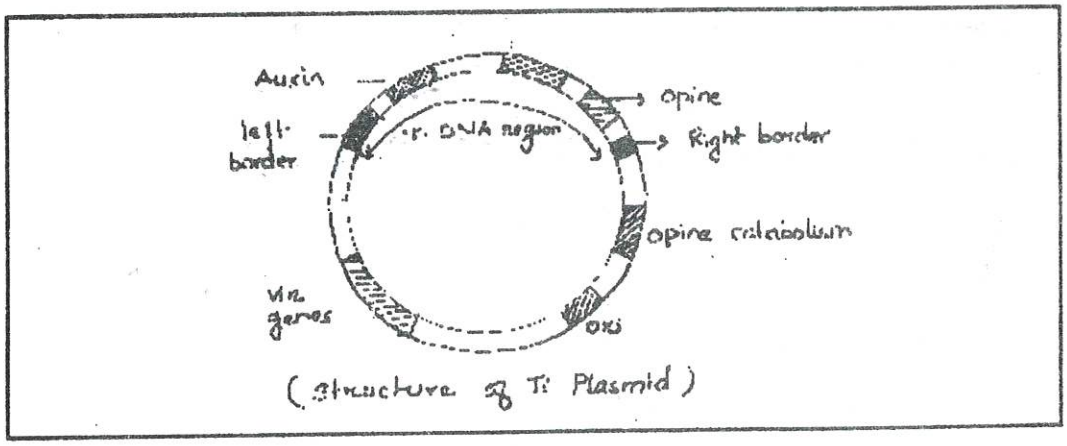


Figure 4.7

After the induction of vir genes a process similar to conjugation transfers the T-DNA. T-DNA is transferred as linear single stranded molecule and it gets integrated in plant

chromosomal DNA. The 5' end carries the right border and 3`end carries the left border. The genes present on T-DNA get activated after insertion in plant genome. The products of these genes are responsible for crown gall formation. The genes and their products are:

1. IaaM and iaaH (also called tms1and tms2 respectively), they synthesize plant hormone auxin iaaM codes for indole-3- acetoamide and iaaH codes for indole-3-acetoamide into auxin.

2. tmr (or ipt) genes codes for isopentyl transferase. Which is involved in synthesis of cytokinins called transzeatin and transribosylzeatin.

   These two components in excess lead to growth and development of tumors.

3. *Gene for opine synthesis:* Opines are condensation products of amino acid and sugar or amino acid and betosugar, e.g. condensation product of arginine and pyruvic acid is called octopine and condensation product of arginine and 2- Ketoglutraldehyde is nopaline. Agropine is derivative of glutamic acid. These opine are made in crown gall and then secreted. The Agrobacterium uses them as carbon source and nitrogen source.

   (Which infected the plant) which has genes for opine catabolism on Ti plasmid (not part of T-DNA). Different plasmids have specific genes for catabolism of particular gene.

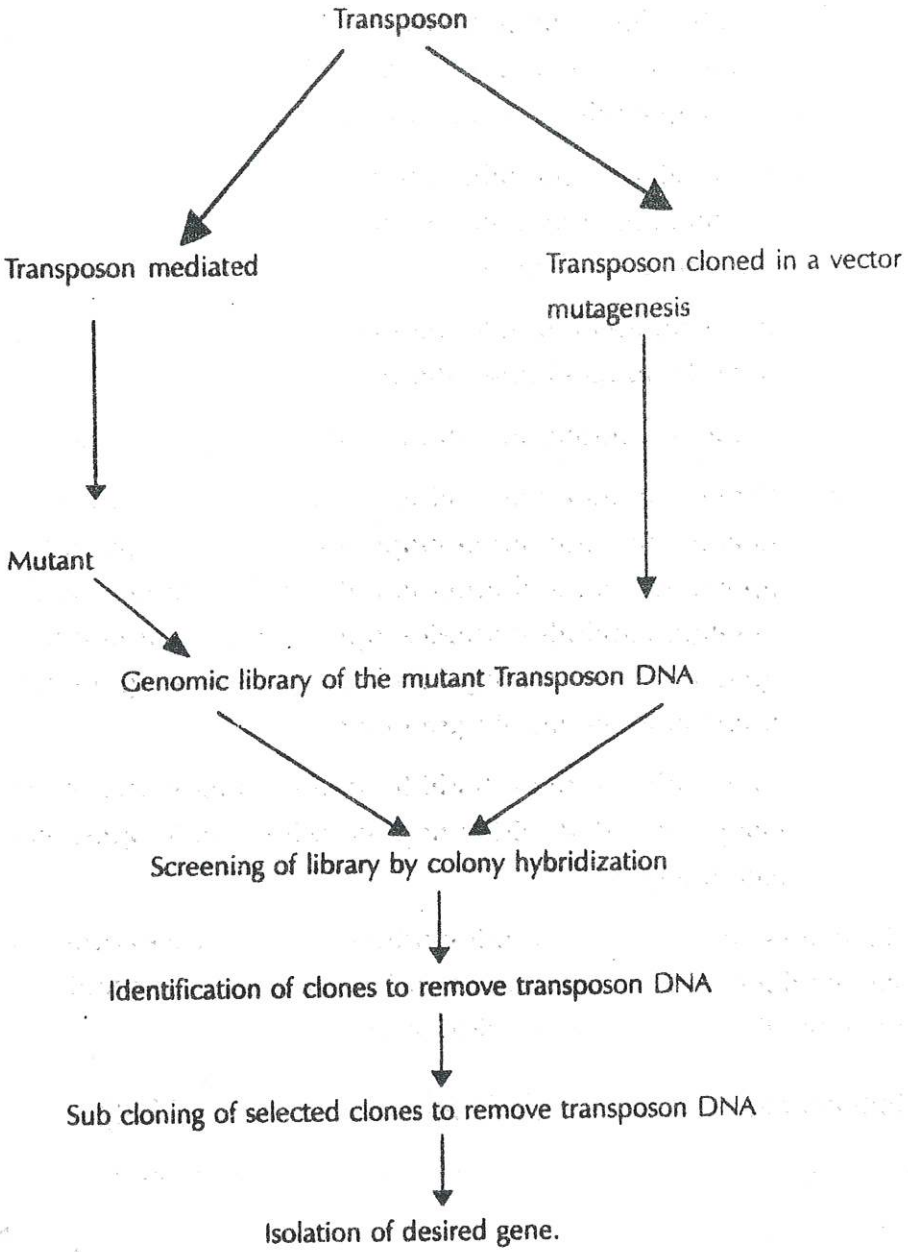
So a unique set of mechanism is there in which each strain of A.tumefaciens makes genetically engineered plants and which function as biological factories for production of carbon (and sometimes nitrogen) compounds which can be used by it.

Transposon Mediated Gene Tagging

The Transposable Elements (TEs) can be used for isolation genes when gene product is known.

Transposon Tagging

The transposable elements have been used in some cases for isolation of genes when gene product is unknown. In such cases transposon works as mutagen and tags a gene. Following steps are involved in this:



In maize transposon like Ac/Ds, En/spm and have been isolated. From Antirrhinum majus Tam3 and Tam7 have been isolated. In maize several genes like B21, P, Al, tagging with different transposon has successfully isolated Cl and C2. Few are listed below:

1. B21 (Enzyme for anthocyanin biosynthesis) isolated from maize by tagging with Ac.
2. C1 (Regulatory gene) isolated by tagging with En/spm in maize.

3. Knotted (leaf development) gene has been isolated from maize by tagging with Ds.

4. Pallida (Enzyme required for anthocyanin biosynthesis) has been isolated from snapdragon (A.majus) by tagging with transposon Tam3.

Usually for tagging endogenous, transposon are used. But sometimes transposon from one plant species can be invaded to genome of other plant species for isolating its gene,e.g. Ac element of maize has been transferred to tobacco where it can be integrated at any gene, which can be tagged and isolated.

Chloroplast Transformation and its Utility

Chloroplast genetic engineering means changes at the level of plastid proteins. Genes for plastid proteins may be located either on nuclear genome or in the chloroplast genome. In the former case proteins are synthesized in cytoplasm and targeted to plastids while in latter case the proteins are synthesized in chloroplast can be introduced in two ways:-

1. Insertion of foreign gene into chloroplast for expression by chloroplasts protein synthesis machinery.

2. Insertion of chimeric foreign gene construct (gene of interest + plastid transit peptide) into nuclear genome. The protein is expressed in cytoplasm and targeted to chloroplast.

Transformation of chloroplast in Chlamydomonas

Chlamydomonas have single large chloroplast and large number of available photosynthetic mutants, thus transformation of chloroplast is easy. Two different methods are used.

1. Bombardment of cells with DNA coated tungsten particles

2. Agitation of cells with DNA and glass beads.

Selectable markers are:

1. Streptomycin resistance and spectinomycin resistance, encoded by 16SrDNA.

2. Erythromycin resistance encoded by 23SDNA, these are 4.2 Kb apart in genome

3. atpB gene (codes for subunit of ATPase) essential for photosynthesis.

Transformation of Chloroplast of Higher Plants

Chloroplast transformation has been achieved in higher plants but efficiency is low. For transgenic plastome term transplastomic lines is used. There are various genes for utility, so efforts are being made for transfer of foreign genes. For transformation Agrobacterium mediated transformation or bombardment with DNA coated tungsten particles is used. Coding sequence of (CAT) Chloramphenicol and Acetyltransferase and APH II, gene for amino-glycoside phosphotransferase have been transferred in chloroplast to tobacco by Agrobacterium tumefaciens.

Important Methods of DNA Introduction in Chloroplasts

1. By Ti plasmid of Agrobacterium tumefaciens
2. Particle gun method- by bombarding DNA coated tungsten particle
3. Direct DNA introduction by microinjection or by electroporation.

Utility of Chloroplast Transformation

Various important metabolic processes are carried out in chloroplast:

1. Synthesis of amino acids, proteins and lipids
2. Light capture and carbon fixation
3. Photorespiration

For their operation nuclear encoded proteins are targeted to outer membrane, inner membrane, intermembrane space, stroma, thylakoid membrane or thylakoid lumen. The chloroplast encoded proteins remain in stroma or is targeted to thylakoid membrane or thylakoid lumen.

Improvement in the efficiency of any of these processes can be achieved by chloroplast transformation, e.g. photosynthetic efficiency can be improved by modification RuBP carboxylase and photorespiration can be reduced by introducing recombinant RuBP carboxylase with high affinity for CO_2, More over variants can be generated by bringing together chloroplast and nuclear genes from different species, chloroplast transformation can also be used for following other studies:

1. Understanding mechanism for targeting
2. Identification for signal peptides and receptor molecules.

3. Understanding integration interaction between signal peptides and receptor molecules.

Chloroplast transformation has been successfully achieved for following uses.

1. Agene for NPT (Neomycin Phosphotransferase -a bacterial protein) with small subunit gene of rubisco was introduced in plants chloroplast.
2. Bromo mosaic virus protein, ß-glucouronidase, yeast mitochondria supreoxide dismutase and light harvesting chlorophyll-binding protein etc all have been successfully targeted to chloroplast.
3. One other example is targeting of a mutant bacterial EPSPS (5-enol-pyruvyl-shikhimat-3 synthase) synthase imparting resistance to herbicide glyphosate. This herbicide inhibits the chloroplast ESPS synthase resulting in inhibition of plant growth due to inhibition of the synthesis of aromatic amino acids. Transfer of EPSPS bacterial gene, which overproduces this enzyme, developed the resistant plant.

Thus, by chloroplast transformation enormous implications novel can be introduced and targeted efficiently.

Intellectual Property Rights

Biotechnology processes and products come under intellectual property and the protection (Intellectual property protection = IPP) and rights (intellectual property rights) available for their protection are assuming great importance in recent years.

Intellectual property includes "patents", "trade secrets", "copy rights" and "trade marks". The IPR protects these properties and prohibits others from using, copying, making or selling these.

Under biotechnology important examples of intellectual property are products and processes of genetic engineering. New plant varieties also come in this category and are protected by 'Plant Breeders Rights (PBR). Various properties are described below:

1. Patents

IN USA patent means right to exclude other from making, using or selling an invention for a 17-year period. India allows process patents and not product patents, the duration is years.

2. Trade Secrets

They include private information or physical material, which is advantageous for the owner, e.g. hybridization conditions, cell lines etc. They have unlimited duration in its disclosure and unlimited duration. Its disclosure and unauthorized use is punishable by law.

3. Copyright

They include books or audio-video cassets, which cannot be, reproduced without permission e.g. DNA sequence data, which may be published.

4. Trade marks

It is a word or symbol used by manufacturers or merchants for identification of his goods from others e.g. every lab equipment has a trademark.

Biotechnological products can be protected by any of the four IPR discussed above. Selection can depend on various factors e.g. cost, security etc.

IPR and PGR (Plant Genetic Resources): In most of the countries crop varieties come under PBR (Plant Breeder's Right).

GAAT (General Agreement of Tariff's and Trade) and TRIP (Trade Related Intellectual Property): GAAT was formed in 1948 to settle disputes regarding share of worlds trade. TRIP governs the trade related intellectual property.

Of all the four IPR, patents are most important in biotechnology and are described below:

Obligations with Patent Application

Following conditions must be met for patenting-novely, inventive, should be discloved property, there should be scope for protection. It should be patentable.

Current issues: The current issue regarding patenting line forms is still debatable but the resistance has been overcome in most countries. Some important patents have been Factor VII, Erythropoietin, ß-NGF and Hybridoma technology.

Among higher plants most important example is patent for tryptophan-overproducing maize. Transgenic animals like 'Oncomouse' have also been patented. Genes and DNA sequence are also being patented.

Possible Ecological Risks and Ethical Concerns

Although it's exciting and important for man to advance, for which biotechnology provides great benefits. But there are some concerns and risks associated with development of recombinant DNA technology. Important are:

1. Some genetically engineered organisms may be harmful either to other organisms or to the environment.
2. Developmental and use of genetically engineered organisms may reduce natural genetic diversity.
3. Concerns regarding production of genetically engineered humans.
4. Diagnostic procedure can undermine individual privacy.
5. There is question mark regarding patenting of genetically engineered animals.
6. Financial support for molecular biotechnology may lead to shortage of funds for development of other technologies.

Environmental and Ecological Risks

The release of genetically engineered and manipulated organisms is considered to pose a threat to humanity and to the environment. The major environmental and ecological concerns are discussed below.

The major concerns regarding release of genetically engineered microorganisms as they multiply rapidly and there is no way to destroy them. But concerns regarding release of genetically engineered plants and animals also exist. Some scientists fear that introduction of genetically engineered plants may disturb ecological balance in an unpredictable manner. There are two major concerns. The first concern is that cloned genes may be transferred to use species by cross-pollination, which may result in many hardy and fit weeds. The other concerns is that vector, used if is of pathogenic origin then it may lead to disease transmission.

Social and Ethical Concerns

Some people able if humans have right to "play god" by altering genome of living organisms and thus altering their evolutionary future. From our perspective we may be improving varieties but it may have unforeseen consequences e.g., we can create plants that are likely

to evolve ways to infect these resistant plants. So we may end up with even deadlier pathogens. There is also a danger of spreading of recombination genes. There is also fear that recombination could give rise to new potentially dangerous viruses. There is also safety concern regarding genetically engineered food as they may cause allergic reactions to some individuals. Some insect resistant plants when eaten also inhibit certain human enzymes.

Genetically engineered has also raised legal issues like who should have right to clone human gene? - The scientist or the individual whose genome is cloned.

Thus we may be creating a perfect world by biotechnology but it has its darker side also.

CHAPTER 5

MICROBIAL GENETIC MANIPULATION

LEARNING OBJECTIVES

- Genetic and Physical Mapping of Genes
- Molecular Markers for Introgression of Useful Traits
- Artificial Chromosomes
- High Throughput Sequencing
- Genome Projects
- Bioinformatics
- Functional Genomics
- Microarrays
- Protein Profiling and its Significance

Genetic and Physical Mapping of Genes

A genetic or linkage map is used to identify the distance between mutations in terms of recombination frequencies. This map can be constructed by measuring recombination between sites in genomic DNA. The sites have specific sequence variations and can be cleaved by specific restriction enzyme. A restriction map is generally the distance measured between cleavage sites for specific restriction enzymes when DNA is cleaved into fragments with restriction enzymes. Hence, a restriction map provides the physical map of genetic material. In general terms if the sequence of wild type DNA is compared with that of a

mutant allele, the nature of mutation and it`s exact sites of occurance can be established. Therefore, genetic map is entirely based on sites of mutations where as the physical map comprises the sequence of DNA.

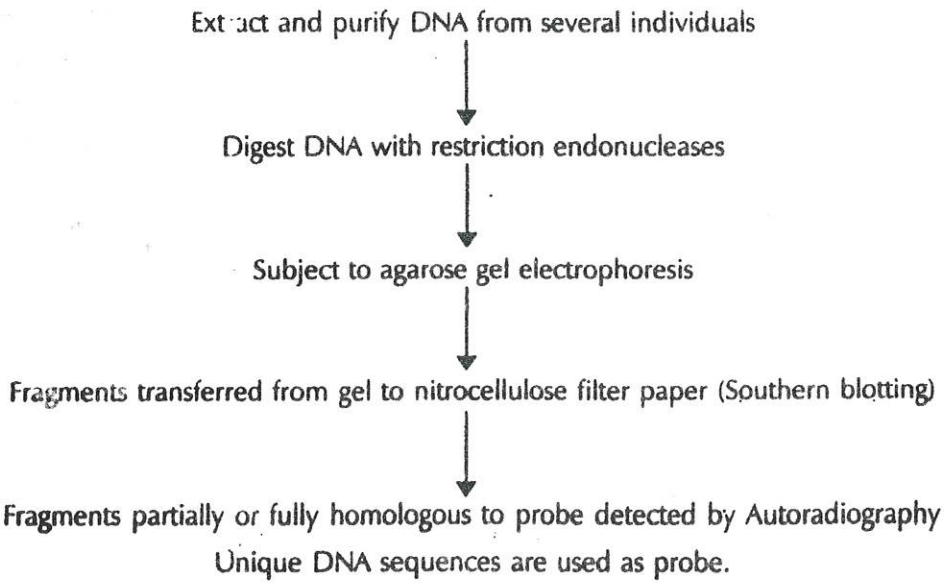
Genetic Map

Two diverse parents belonging to a species are used to generate a mapping population either by selfing to produce F2 or by back crossing to produce BC1. Several molecular markers have been used to prepare molecular genetic maps. These markers are first screened for detecting polymorphism between the parents. These markers are further used for recording, later on the two parents one used. This data finally is used to prepare a genetic map. The different markers used to prepare genetic maps are the following:

RFLPs (Restriction Fragment Length Polymorphisms)

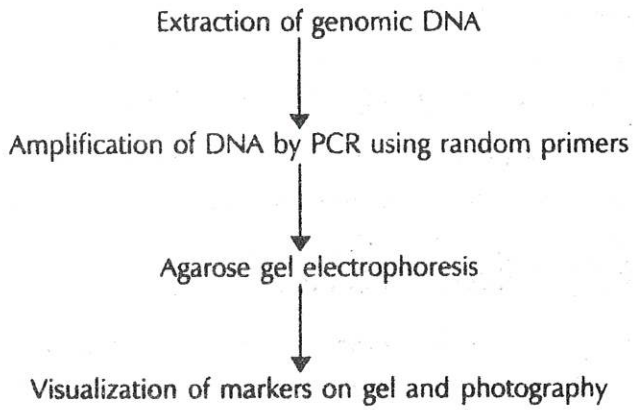
If the genomic DNA is subjected to restriction enzyme digestion and electrophoresed, fragments of different sizes arise, which are difficult to identify. Hence specific DNA sequences are used to identify the variations at the DNA level. These variations are RFLPs, which can be explained as variation monitored as changes in the length of defined individual DNA fragments. These polymorphic RFLPs have been used for genetic mapping in humans, fruitfly, mice etc.

RFLP experiment:



RAPD (Random Amplified Polymorphic DNA)

Random primers (arbitrary sequences of oligonucleotides approx 20 bases long) are used for PCR amplification in this technique. This amplification results in random sample of DNA markers which are called as random amplified polymorphic DNA (RAPD) technique. RAPD technique can be performed approx 5 times more quickly than RFLPs. The steps of this technique are following:



Minisatellites and Microsatellites (Variable Number of Tandem Repeats)

Polyallelic markers are useful in mapping both simple Mandelian traits and polygenic traits. VNTRs are an example of polyallelic markers, which exhibit highly polyallelic fragment length variation. VNTRs include minisatellite and microsatellites.

Minisatellites are useful in DNA finger printing, but not genetic mapping, since they are not uniformly distributed throughout the genome. These are a member of tandem repeats associated with rRNA genes in rDNA concentrated at Nucleolar Organizing Regions (NORs). Microsatellites or simple sequence repeats represent variation in the repeat member of sequences, 1 - 6 bp long such as (AC)n or (AAC)n , mouse, humans, barley, wheat etc.

Sequence Tagged Sites (STS) and Expressed Sequence Tags (ESTs)

STS sequences are amplified using STS primers and help in converting RFLP maps into STS maps. If cDNA generated from mRNA, is used for the preparation of molecular genetic map, this map will have markers of all expressed sequences. These sites are described as Expressed Sequence Tags (ESTs).

Linkage and Recombination between Molecular and Phenotypic Markers

For this RFLP/PCR patterns and the phenotypes of two parents that differ morphologically as well as for molecular markers are considered. When the two parents are crossed, the recombination fragments between molecular and phenotypic markers are estimated. A linkage between the genetic and molecular markers allows identification of genetic loci at the molecular level and a tight linkage of such markers with a disease can be useful for diagnosis of the disease and isolation of its gene.

SNPs are Molecular Markers in Genetic Mapping

Single Nucleotide Polymorphism (SNPs) is the single base pair difference as between the DNA base sequences of two individuals, approximately every 500 - 1000 bp. At a particular position on the DNA, one base pair will be common one present in most individuals and another base pair at the same position will be the less common variant. If the less common variant is present in at least 1% of the human population; the genomic base pair location is defined to be the site of an SNP. There are approx three million SNPs in the human genome and this kind of polymorphism represents approx 98% of all DNA polymorphism.

Typing of hundreds of SNPs can be achieved using DNA microarrays, discussed later in this chapter, for SNP typing on a probe array. Oligonucleotides that match the common and all possible variant alleles of each SNP to be analyzed are synthesized on the chip and the target DNA is labeled and hybridized.

Molecular Physical Maps

The distances between markers in a genetic map is represented in units of Centi Morgans (cMs) but are not proportionate to physical distances. Physical distances are usually represented either in megabase pairs (106 bp), or as a proportion of a whole chromosome arm.

Physical mapping using chromosome deletions

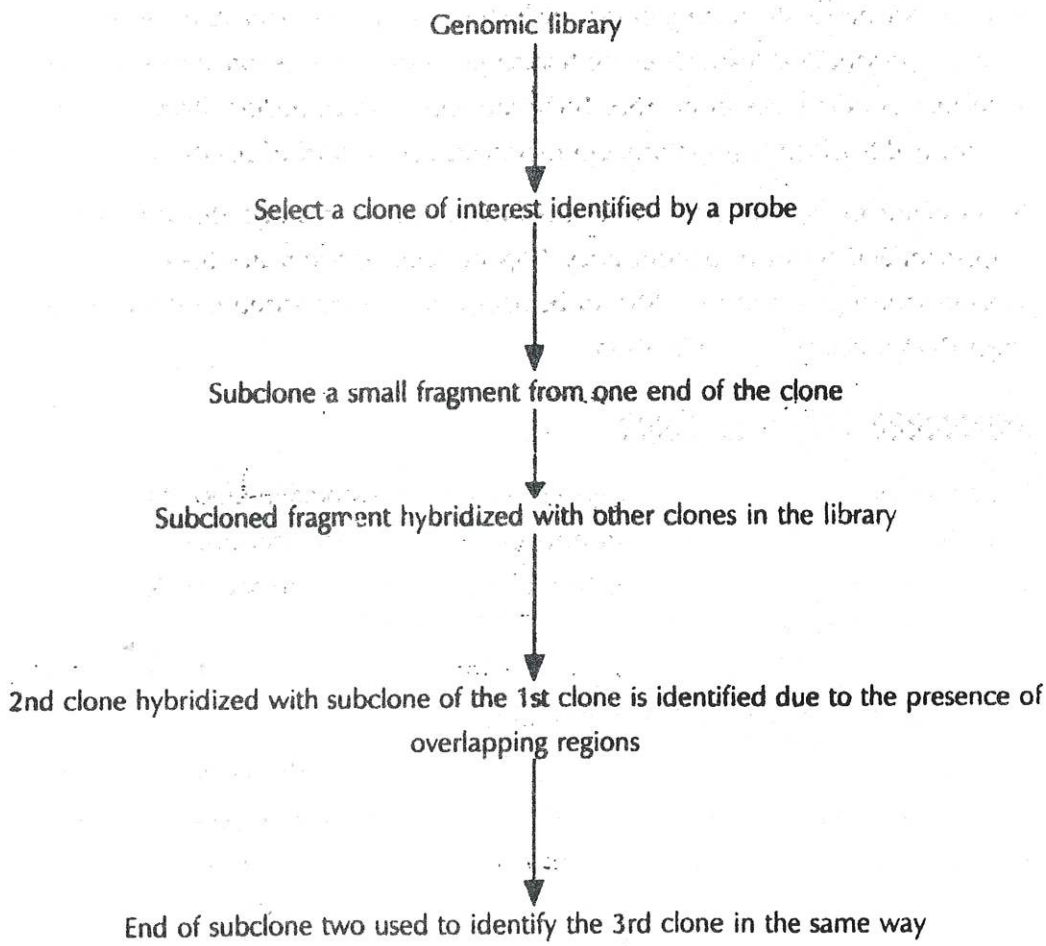
If genomic DNA is extracted from normal organism and a deletion stock and the same marker is used as a probe then absence of the markers or its homologous sequences in the deletion stock will indicate the presence of the markers in the deleted segments. Hence deletion stocks have been widely used for physical mapping of various molecular markers in bread wheat etc.

In situ Hybridization

Probe for molecular markers are used in in situ hybridization studies in the chromosomes. The probes are radioactively labeled or fluorescent labeled and used for hybridization with DNA sequences on well spread metaphase chromosomes. Physical location of rDNA on chromosomes has been accomplished by this technique

Chromosome Walking

When a probe is used for identification of a gene sequence in a genomic library. The probe normally hybridizes with a number of clones, each carrying a part of large gene fragmented during preparation of a genomic library. These fragments may be present as overlapping sequences. These overlapping sequences are used to construct the original genomic sequence, by a technique called as chromosome walking.



Finally a restriction map of each selected clone is prepared and compared

This technique is equivalent to overlapping regions walking along the chromosome or along a chromosome segment

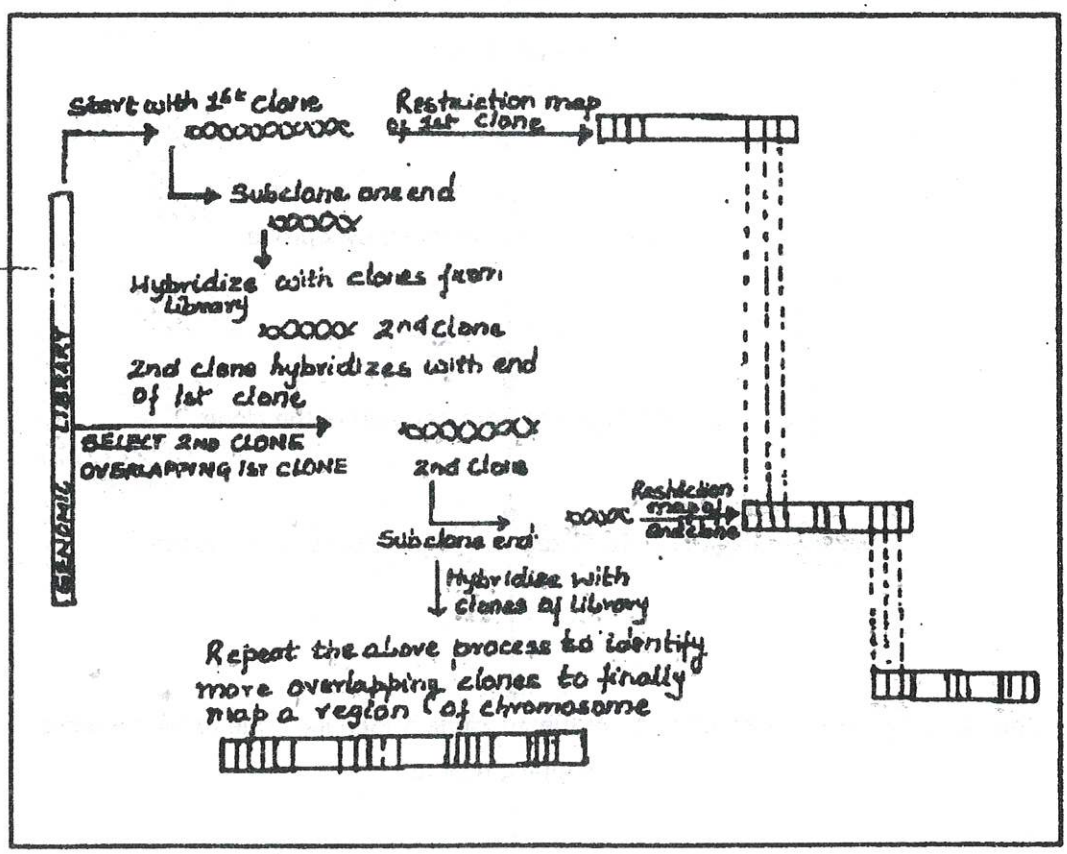


Figure 5.1

Chromosome Jumping or Hopping

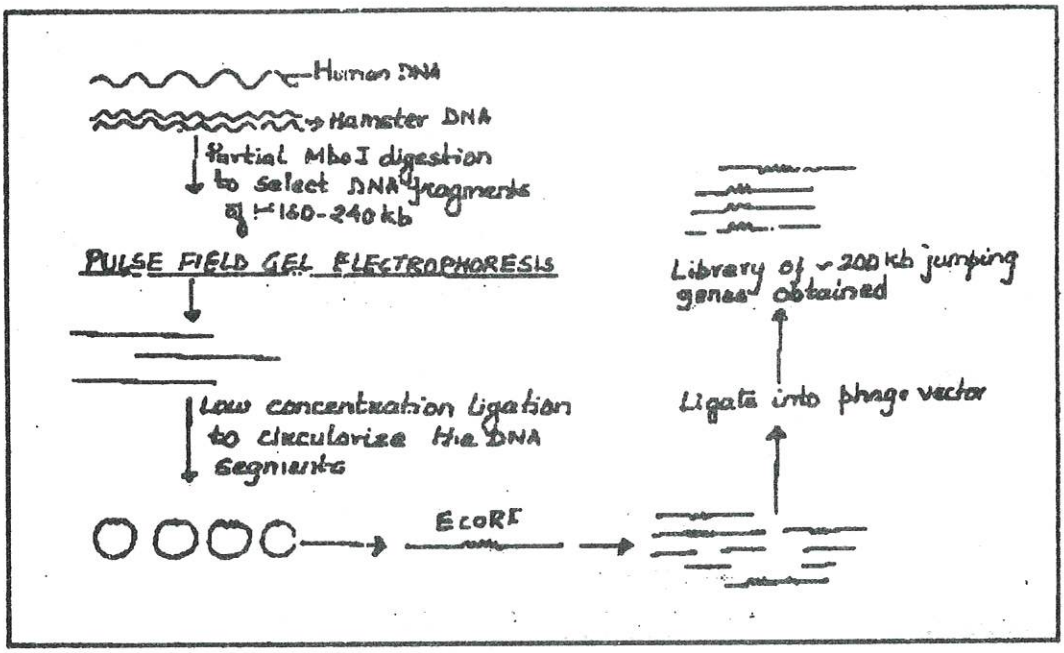


Figure 8.2: Chromosome Jumping

These are used for physical maps and map based cloning of specific genes. The gene segment to be cloned is linked to the molecular markers and brought close to the gene by chromosome jumping.

Molecular Markers for Introgression of Useful Traits

Once the genetic and physical mapping is achieved with the help of molecular markers; the next step is the use of these marker for the introgression of useful traits.

RFLP's in Gene Tagging

These markers are used to map positions of major genes as well as polygenic traits. Once the genes are mapped on RFLP maps; RFLPs linked to specific genes/ gene clusters (gene tagging) can be used in selection schemes for plant breeding studies. If marker is close to a known gene; the genes can be effectively isolated.

Near Isogenic Lines (NILs) for Gene Tagging

NILs are produced when conventional markers, like disease resistance markers, is transformed from a donor parent to a recurrent parent and the genotype of the recurrent parent is restored through 57 back crosses, retaining the conventional marker in each such back cross. This kind of study helps in estimation of linkage distance between the conventional and molecular markers. NILs have been successfully used for tagging genes of economic value in several crops like potato, tomato, maize, barley, rice etc.

Mapping of Quantitative Trait Loci (QTLs) using RFLPs

The quantitative traits under polygenic control are selected but since the effect of each gene is very small and also influenced by the environment these selections are often ineffective. Hence linkage between qualitative traits and major molecular markers should be known. RFLPs have been used to identify and map QTLs in crop plants like tomato and maize.

RFLPs in Identification of Breeding Lines and Varieties

DNA fingerprint can distinguish closely related individuals utilizing VNTRs and STRs (Short Tandem Repeats) as probes. Genomic specific RFLP pattern have been detected and used for identification of breeding lines and varieties. DNA finger printing using SSRs or dinucleotide repeats like (CT)n and (AC)n have been used in plants.

RAPD Markers for Mapping and Crop Improvement by PCR

RAPDs have been need in preparation of linkage maps. Procedure is simple, quick to perform and requires minute amounts of DNA.

RAPDs and SSRs

PCR can be used for molecular mapping using RAPD and SSR marker loci. These polymorphic markers are mapped in a similar way as RFLP`s genetic maps of maize, rice, tomato etc have been prepared using RAPD and SSR loci.

PCR in mapping STS and EST

Most of the molecular markers like RFLPs and RAPDs are used in a variety of plant materials and are converted to sequence tagged sites (from genomic DNA) and ESTs (from cDNA probes).

RAPD for Gene Tagging, Tagging of QTLs and Identification of Somatic Hybrids

In tomato, PCR has been used to tag major genes using NILs like two NILs, which differed for the presence or absence of the gene (pto), which conferred resistance. Pseudomonas were screened using random primer and lead to the identification of three RAPDs, tightly linked with pto gene.

In tomato, a high-density linkage map was used to select two intervals, each containing the gene responsible for pedicel abscission and fruit ripening. Each interval was flanked by RFLP markers. A continuous range of variation characterizes the QTLs. The plants exhibiting these variations are screened for RAPDs using random primers and the markers linked to to the loci controlling these quantitative traits are identified.

RAPDs have been used for identification of somatic hybrids among the developing regenerants. In this method, DNA from the developing regenerant is used to determine hybridity. Primers are used which gene polymorphic PCR products from each parent involved in fusion, or order to identify the hybrid by their molecular profile, which is infact a combination of the profile of the two parents.

SNPs as Molecular Markers for DNA Polymorphism Studies

The SNPs explained earlier can be used as molecular markers for introgression of traits/ genes.

Artificial Chromosomes

Artificial chromosomes are cloning vectors that enable these chromosomes to be made and cloned in a variety of cells like-

Yeast — YACs — Yeast artificial chromosomes

Bacteria — BACs — Bacterial chromosomes

Mammal — MACs — Mammalian artificial chromosomes

Plants — PACs — Plant artificial chromosomes

Human — HACs — Human artificial chromosomes

YACs

YACs are linear vectors with following features:

1. A yeast telomere (TEL) at each end.
2. Yeast centromere (CEN)
3. A selectable marker on each arm (TRP 1 or URA 3)
4. Origin of replication (ARS): autonomously replicating sequence.
5. Restriction sites

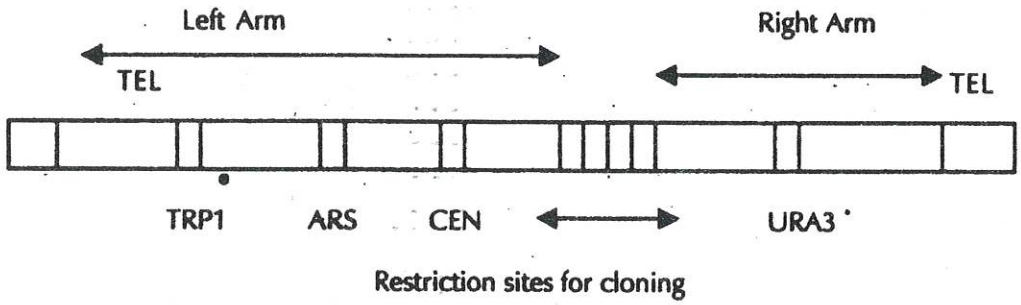


Figure 5.2: YAC Cloning Vector

High Throughput Genome Sequencing

Haemophilus influenzae bacterium was the first cell whose whole genome was sequenced by the direct shotgun approach. The whole genome was broken into partially overlapping fragments; cloned and sequenced and sequence assembled using a computer. High throughput method is the usage of sophisticated computer algorithms to assemble sequence of hundreds and thousands of 300 - 500 bp sequences. The human genome was sequenced in this way by J. Graig Venters, Celera Genomics Company.

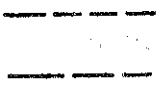
Cell of a particular organism

↓

Extraction of DNA

↓

Fragmented DNA



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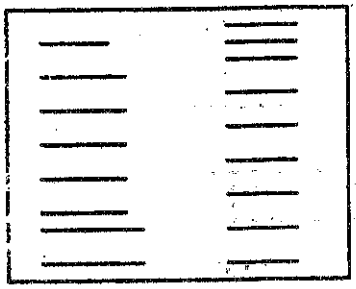
Agarose Gel Electrophoresis



Marker Cellular DNA

↓

Purify 1.5 - 2.0 Kb DNA from gel

↓

Prepare a library of the DNA clones

↓

The end sequences of DNA inserts are acquired

↓

Enter the sequences into a computer

↓

Short segments decoded

Computer assembles sequence into contigs with the help of overlaps

Shotgun Approach for Obtaining Genomic DNA Sequence

Automated sequencing plays a key role in this methodology. The raw sequences are assembled into sequence contigs, which is -bases priced together in the absolutely correct order as they are found in the genome.

Genome Projects

In 1981 the first genome completely sequenced was the circular chromosome of the human mitochondrion, which was 16,159 bp long. Since then researchers have gone a long way in sequencing the genomes of bacteria, archaea, yeast, fungi, and protozoans.

Bacterial Genomes

1. ***Haemophilus influenzae:*** This was the first cellular organisms to have it's genome sequenced. This organism had a genome size equivalent to that of bacteria, but G- C content is close to that of humans. The institute for genomic research (IGR) completed this project in 1995. The genome of H. influenzae is 1.83 Mb in size, with 38% overall G - C content. The genome analysis predicted protein coding genes, tRNA genes, rRNA genes with roughly 1,743 protein coding genes comprising 85% of the genome of these predicted genes 736 have not been assigned any functional role.
2. ***Mycoplasma genitalium:*** This bacterium was also sequences by IGR 1995. These bacterium lack a cell wall and have low G - C content. The genome was sequenced by the direct shotgun approach and the genome is 580 Kb long. G - C content was to be found 32%. 470 genes were estimated from the sequence, which comprised 88% of the genome.
3. ***E. coli:*** In 1997 genome center at the University of Wisconsin USA, reported the annotated genome sequence of E. coli K12. The genome of E. coli is 4.6 Mb long with 50.8% G - C content.
4. Other bacteria whose genome has been completely sequenced are Treponema pallidum, Rickettsia prowazekii, and Deinococcus radiodurans etc.

Eukaryotic Genomes

1. *Yeast:* Saccharomyces cerevisiae (yeast) genome was the first eukaryotic genome to be sequenced completely; in 1996 collaboratively by more than 100 laboratories. The 16 chromosome genome was 12.067,280 bp with 230 Kb in chromosome I and 1.52 Mb in chromosome fourth.

2. ***Coenorhabtidis elegans (Nematode worm):*** The C. elegans genome project was carried out by an international consortium initiated Sydney Brenner. In 1998, the complete genome sequence of 97 Mb was reported.

3. ***Drossophilla malenogaster (Fruit fly):*** In 2000 the genome sequence was reported by University of California, Berkeley and J. Craig Venter`s Celera Genomic Company collaboratively. The Drossophilla genome is estimated to be about 180 Mb in size and 120 Mb is approximately. The euchromatic part with more than 99% of the genes.

4. *Arabidopsis thaliana:* In December 2000, 115 Mb sequence of the estimated 125 Mb total genome was reported. About 25,900 genes have been estimated being distributed over the plant`s five chromosomes.

5. ***Homo sapiens(Human genome project):*** The working draft of the genome of Homo sapiens was released in a joint press conference in 2000 by National Human Genome Research Institute(NHGRI) and J. Craig Venter`s Celera Genomics. The sequencing was carried out by the Human Genome Project Consortium of scientists at 16 institutions in USA, UK, France, Germany and China.

The complete sequencing of the human genome was proposed in 1986, and the sequencing project called Human Genome Project (HGP) was started formally in USA in 1990 with joint fundings from:

A. NIH: National Institute of Health

B. DOE: Department of Energy

The estimated time of the project was 15 years.

The goals of the project were following:

A. Identify all genes in human DNA.

B. Determine the sequence of 3 billion bp of human DNA.

C. Store this information in public databases.

D. Develop tools for data analysis.

E. Transfer related technologies to the private sector.

F. Address the Ethical, Legal and Social Issues (ELSIs), which arise due to project.

13 International Institutions were involved Globally

A. Baylor college of Medicine, USA.

B. Genoscope, France.

C. Kelo University, Japan.

D. RIKEN Genomics Sciences center, Japan.

E. University of Washington Genome Center, USA, (Seatle).

F. Gesells chaft fur Biotechnologische, Germany.

G. Washington University Genome sequencing center (St. Louis) USA.

H. Institute of Molecular Biotechnology, Jena, Germany.

I. Stanford DNA sequencing and Technology Development Center, USA.

J. University of Washington, Multi Megabase Sequencing Center, USA.

K. Genome Therapeutics Corporation Waltham, USA

L. The Sanger Center, UK.

M. Max Planc Institute for Molecular Genetics, Germany

N. Beijing Human Genome Center, China.

O. White Head Institute for Biomedical Research, MIT, Ujjian.

P. Joint genome Institute, USA.

Major Land Marks During Human Genome Project

A. Publication of Human Genetic Map

B. Sequence Tagged Sites (STS) based map

C. Transcript map

D. Break point map - chromosomal rearrangements that recur frequently

E. STS based radiation hybrid map

F. Physical map of Human Genome

G. Sequencing and mapping of MHC on chromosome VI

H. Completion of sequencing of chromosome 22

I. Completion of sequencing of chromosome 21

J. 66% completion @ of 1000 bp/sec

K. 1st final draft of 22 billion bp covering total DNA sequence almost 7 fold

L. Pvt Company Celera Genomics Inc founded by Craig Venter - pioneer of shotgun Technology aimed to complete project within 10 months September 1999 and June 2000

M. 2 groups jointly announced rough draft on 26th June 2000 - Human Genome Consortium and Celera Genomics Inc.

86% of the total human genome has been evaluated and classified (99.9% accurate). Sequencing without evaluation is complete for 97% of genome.

Whose Genome was Sequenced

DNA (substrate) for sequencing was obtained by elaborate process. Samples of DNA (Blood from female donors and sperms from male donors) were collected from multiple anonymous donors in accordance to IRB (International Review Board) protocol. After screening few samples (approx 20 - 50 individuals) are combined and used for library construction followed by sequencing name of the donors are kept anonymous.

Methodology of Sequencing

Two methodologies have been used for complete genome projects, which are following:

1. Top down approach

   Genome is segregated into smaller fragments in stepwise manner. The small fragment is inserted into vectors and then sequenced. After sequencing the fragments are pieced together back tracking and their point of origin in individual chromosome is formed. This was used in initial step of project. Plotting cross over frequencies between different genetic loci generated genetic maps. These loci are served as markers to identify a given fragment and to determine the order of different fragments.

2. Bottom up approach (shotgun approach)

   Genomic DNA is broken into small fragments and all are sequenced in unbiased manner. Because this procedure has degeneracy (total fragments 10 times original size of genome) and many overlaps are present and by this feature the sequences are assembled. A computer program is used for this purpose.

In both approaches the basic technique is dideoxy method of Senger. Specially designed high-speed sequencer does most sequencing, which need very little human help and high throughput. Each of the four dideoxy terminators is tagged with different fluorescence markers, which are read by automated instruments.

Tools of the Trade

For sequencing plasmids, cosmids, YAC, BACS are used. After sequencing is completed, a computational procedure-

1. Aligns fragments
2. Do error checking
3. Detect potential coding regions
4. Features of genes are analyzed (eg. CpG islands etc)
5. Genes are identified

Some programs, which are used, are following:

1. TRNASCAN: detects RNA coding regions
2. FGENEH: identify presence of genes
3. GENSCAN: predicts coding regions and intron/exon splice site
4. MUMMER: Aligns whole genome
5. REPEATMASCAR: searches for low complexity regions
6. Annotator: Genome annotation tool
7. PSI - BLAST: for homology search
8. Genefinder: coding regions and splice sites
9. AAT: Analysis and annotation tools etc.

It has been predicted that total number of genes like to be present may be 33,000 - 44,000, but other groups predicts about 1,20,000. The report from consortium suggests about 38,000. Large numbers of genes are unclassified and have not been reported before. Till June 2000 OMIM (Ovline Mendalian Inheritance in Man) database had 11,741 gene loci mapped on specific sites on the human chromosome.

Implication of HGP

- Sequence information forms a starting point at best for scientific investigations.
- Sequence availability will result in rapid characterization of any gene and its protein one is characterized by established protocol of molecular biology and biochemistry.
- Important issues will be generation of user-friendly databases so those scientists are not lost in digital amazons of the sequence date. Same date bases have been generated e.g. mutation databases for p53, cystic fibrosis CDC receptors. Information of such databases is given at national center for biotechnological information.
- Standardized protocol for annotations of newly generated sequence and accessing these databases from web-based front-end interface like browsers in current area of activity.

Bioinformatics

Bioinformatics is the symbiotic relationship between computational and biological sciences. The ability to sort and extricate genetic codes from a human genome database of 3 billion base pairs of DNA in a meaningful way is perhaps the simplest form of Bioinformatics. Bioinformatics can be defined as the study of two information flaws in molecular biology. The first class of Bioinformatics applications can address the transfer of any information at any stage in the central dogma, including the organization and control of genes in the DNA sequence, the identification of transcriptional units in DNA, the prediction of protein structures from its primary structures and the analysis of molecular functions. The second information flaw is based on the scientific method like creating hypothesis regarding biological activity, design experiment to test the hypothesis, evaluate to resulting data for compatibility with the hypothesis and extend or modify the hypothesis in response to the data. It addresses the transfer of information within this protocol including system that generate hypothesis, design experiment, store and organize the data from these experiments in databases, test the compatibility of data with models and modify hypothesis.

Development of databases that store the information regarding the sequence and the structure of the nucleic acids and of protein is a major task in Bioinformatics. Most of the biological databases are interlinked to get the vast information related to a single sequence or structure. Regarding the biological databases they are of four types. In which three are for the sequence and one for the structural data. Primary sequence data base contains information regarding the primary structure of the protein directly submitted to the authors to the respective database e.g. PIR, MIPS, SWISSPROT, TrENBL etc. Composite databases are the amalgamation of the two or more databases so as to get the maximum information regarding a sequence that might have got missed in other databases, like NRDB, OWL. Secondary databases contain the fruits of analysis of the sequences in the primary sources. They store the data in the form of regular expressions, aligned motifs etc, like PROSITE, PROFILE, Pfam etc. Structure classification databases were created to classify the existing protein structures on the basis of their evolutionary relationships with the other proteins, presence of other motifs, domains and the sequence similarity like SCOP, CATH, and PDBsum. Protein Data Bank (PDB), is the well-known repository of the 30 structure of proteins determined by X-ray crystallography and NMR techniques. In case of nucleic acids, EMBL, GenBank and DDBJ are the well-known databases. Matching and finding the similarity between the unknown and the sequences with the known functions can find out

function of an unknown protein or nucleic acid sequence. This can be done with the help of heuristics like BLAST, FASTA etc. The cardinality of the sequences in the sequence database (SWISSPROT) is growing exponentially to some lakhs whereas the number of structure is in thousands. Hence there is an urgent need of methodologies and software to predict and model the 3D structure of the protein from its sequence. Abinitio methods, homology modeling and threading procedures are being done to model the unknown proteins. Data mining and data warehousing are the other important branches of Bioinformatics. Data mining is, basically the Knowledge Discovery in Databases (KDD). Pharmacogenomics (devising drug based on ones genetic make up) and computer aided drug designing are the two more aspects of Bioinformatics is in neonatal stage and it has to resolve too many biological problems both at DNA and protein levels. Too many algorithms have to be developed and evaluated to solve the biological problems.

Functional Genomics

Functional genomics deals with the assessment of the function of genes identified by between genome comparisons. Introducing mutations into the gene and then examining the resultant mutant organism for an altered phenotype taste the function of the newly identified gene.

Genome analysis depends to a larger extent on sequence analysis methods that identify gene function based on similarity between proteins of unknown functions and protein of known function. Known functions are derived from experimental evidence in molecular biology and genetic studies with model organisms. Orthologous genes between biologically distinct species (for example yeast and fruit flies) can be identified. The high sequence similarity between them is strong evidence for a related function. Given the more complex multicellular biology of flies, the fly genes could have an additional function that is not predictable by the yeast model. In other cases, the occurrence of families of paralogous genes that share common domains can make a precise guess of function of one of these proteins more difficult because all match a protein to some degree.

Sequence based methods of gene prediction can be augmented by the types of genome comparisons as mentioned above that are designed to identify related genes based on common patterns of expressions, evolutionary profiles, chromosomal locations and other features. However all of the above methods can fail to provide a precise determination of gene function. Hence, methods have been devised for directing mutations into specific genes that inactivate or modify the gene function and the effect is then analyzed in the mutant organism.

Two general types of approaches are used:

A. A genetic construct is made that interferes with the expression of a particular gene (and some times a related set of genes).

B. Large numbers of random mutations are generated in a population of organisms.

The individual with a mutation in a particular gene is then identified. Once mutations are obtained, the effect of mutant genes on phenotype is determined. The gene function may then be predicted on the basis of the observed alterations. Because such extreme genetic experiments cannot be performed with humans, the mouse model for the human genome serves the same purpose. An orhtologous gene is identified in the mouse genome; the sequence or expression of the gene is disrupted in some fashion and a transgenic mouse homozygous for the mutant gene is then produced. Using this technology, one can systematically go through genes that regulate cell division, for example and determine the significance of these genes in normal verses abnormal (tumor) growth.

Important web sites that compare human and more genome:

a. Celera Informatics: The company that assembles genome sequences by automated fragment assembly www.celera.com

b. Human genome resources at NCBI. www.ncbi.nlm.nih.gov/genome/guide

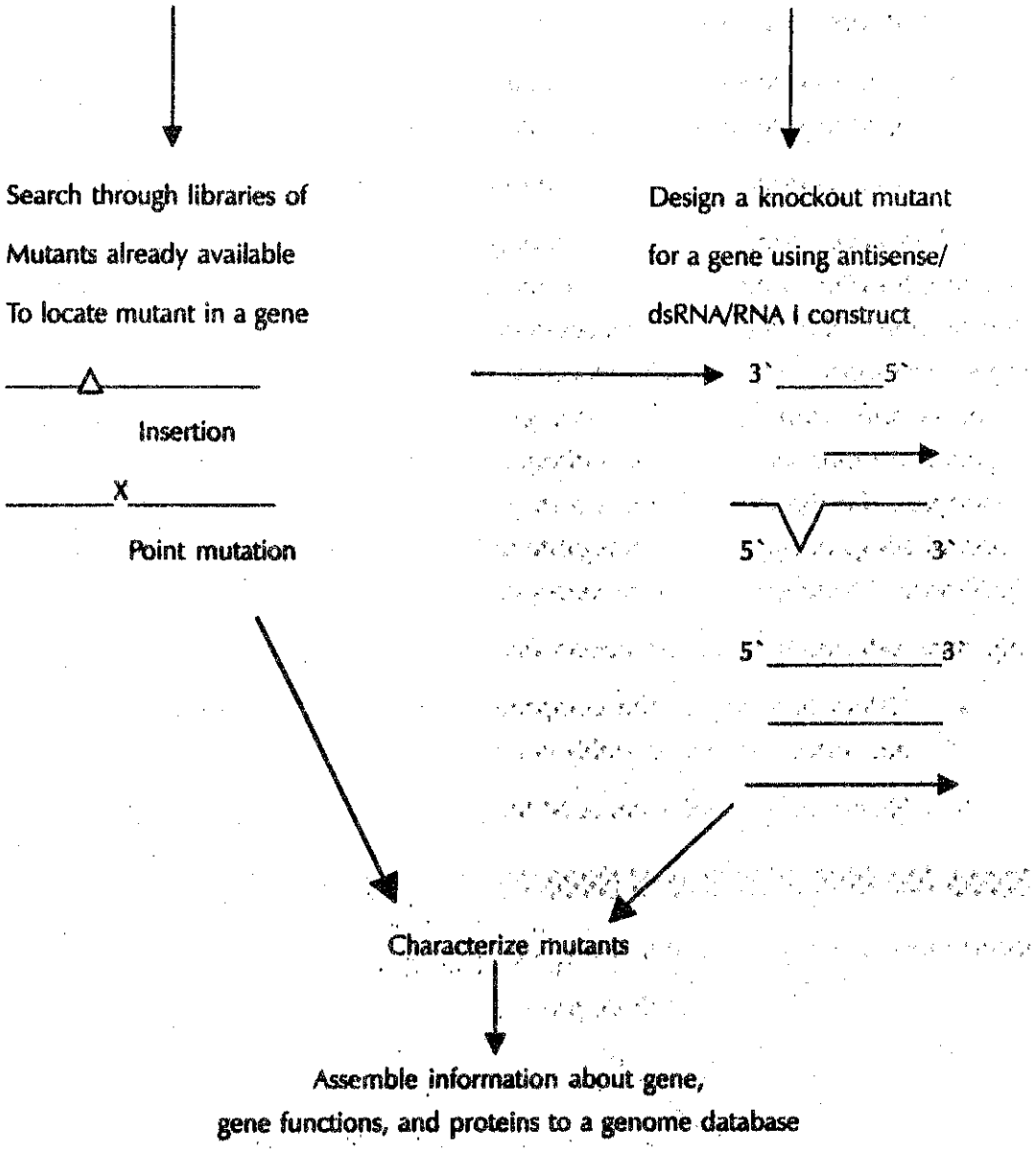
Steps for Identification of Gene Function in an Organism

Identify gene of known function in model organism e.g. human, drosophila

(cDNA, protein sequence)

Problem search for similar protein sequences (Protein sequence, translation of EST sequences, redicted protein)

Sequence mRNA of similar gene (cDNA and protein)



DNA Micro Array/DNA Chips/Gene Chip Arrays/Oligonucleotide Arrays

The DNA microarray technology was developed in early 1990`s by affymatrix Inc. DNA microarray is an ordered arrangement of knov.n DNA sequences in a grid pattern on a solid substrate like silicon chip, glass or nylon membrane. This technique is extremely powerful for studies of whole genome expression profiling in a single experiment. A typical microarray experiment comprises:

1. Preparation of DNA probe/ chip.
2. Preparation and labeling of target.
3. Hybridization of probe with the microarray.
4. Statistical analysis and interpretation of results.

Preparation of DNA probe/chip

There are two major types of microarray technology:

Mechanical Microspotting

It is developed at Stanford University. In this method DNA molecule is genomic DNA, PCR products, oligos etc are spotted into glass by mechanical microspotting. For this spotting pins uploaded the DNA by capillary action and a small volume of DNA is released into the glass when the pin touches the surface. After washing the pin the next sample of DNA is loaded and spotted on the adjacent spot. To increase the efficiency of a microarray a multipin print head under rolsotic* control is used. The microarrays prepared by this method can have approx 10,000 DNA molecules spotted in an area of 3.6 cm^2.

Ink Jetting

This technology utilizes miniature nozzles similar to ink jet printers to deliver drops of DNA into glass. Affymatrix Inc, USA came with another method by which oligonucleotides could be synthesized on in-situ on the substrate at defined positions. They put thousands of transistors into a surface integrated chip to make gene chips or oligo arrays of approx 1.6 cm^2 with a density of approx 1-million oligonucleotide/ cm2. For these oligos approx 25 bases long and synthesized by a light directed chemical synthesis process i.e. a glass substrate has covalent linker molecule terminated with a photolabile-protecting group at every defined array position. A series of photolithographs masks define the oligonucleotide synthesis process:

First mask is designed for addition of Ts at the first position

↓

Light shining through masks deprotects and activates the sites and the programmed nucleotide with an attached photolabile protecting group is chemically added to those sites.

↓

The mask is removed and replaced with next mask defining the subsequent nucleotide to be added like C:

↓

Again light deprotection and activation step is repeated and the protected C is added

↓

Repetition of this process leads to synthesis of desired oligos at their appropriate programmed position on the chip

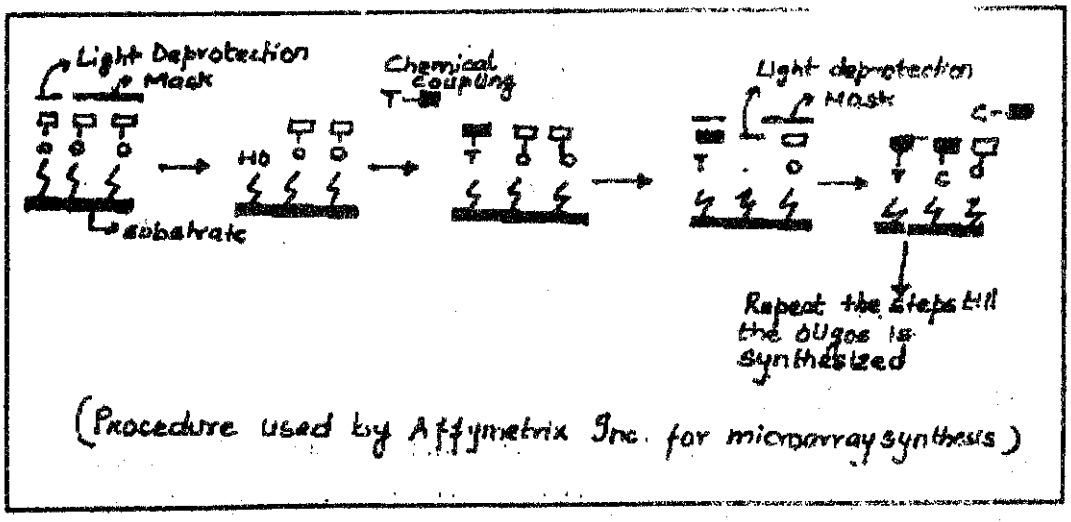
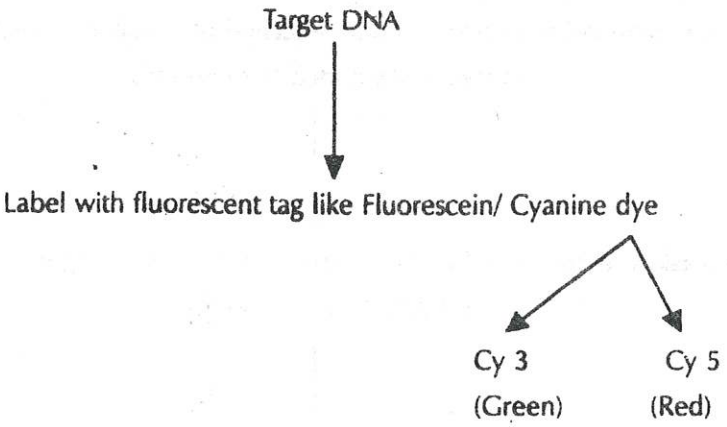


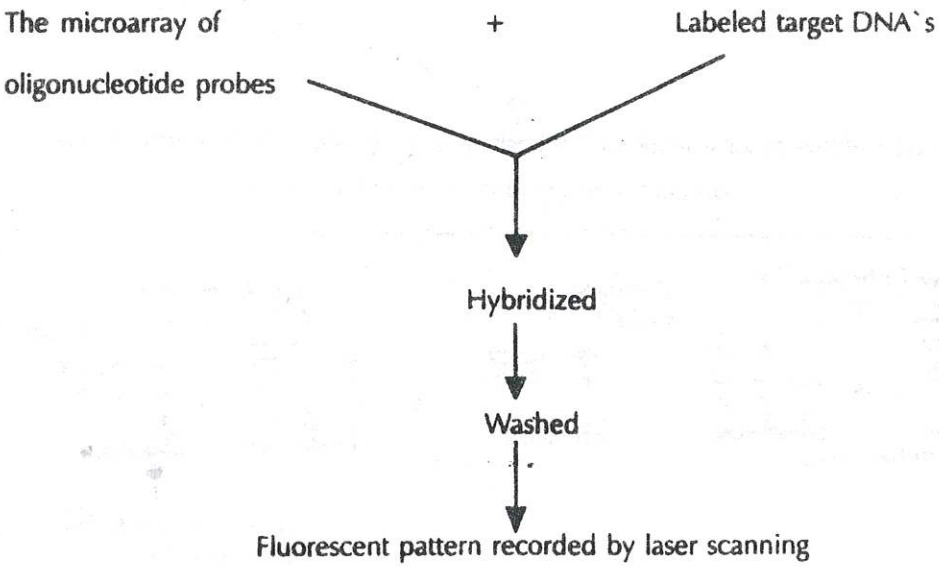
Figure 8.3

Preparation and Labeling of Probes

A microarray, typically also requires labeled probes as in a southern/ northern hybridization experiments. But here the probes are DNA molecules fixed into the chip and the target DNA are labeled DNA whose identities or quantities have to be analyzed.



Hybridization



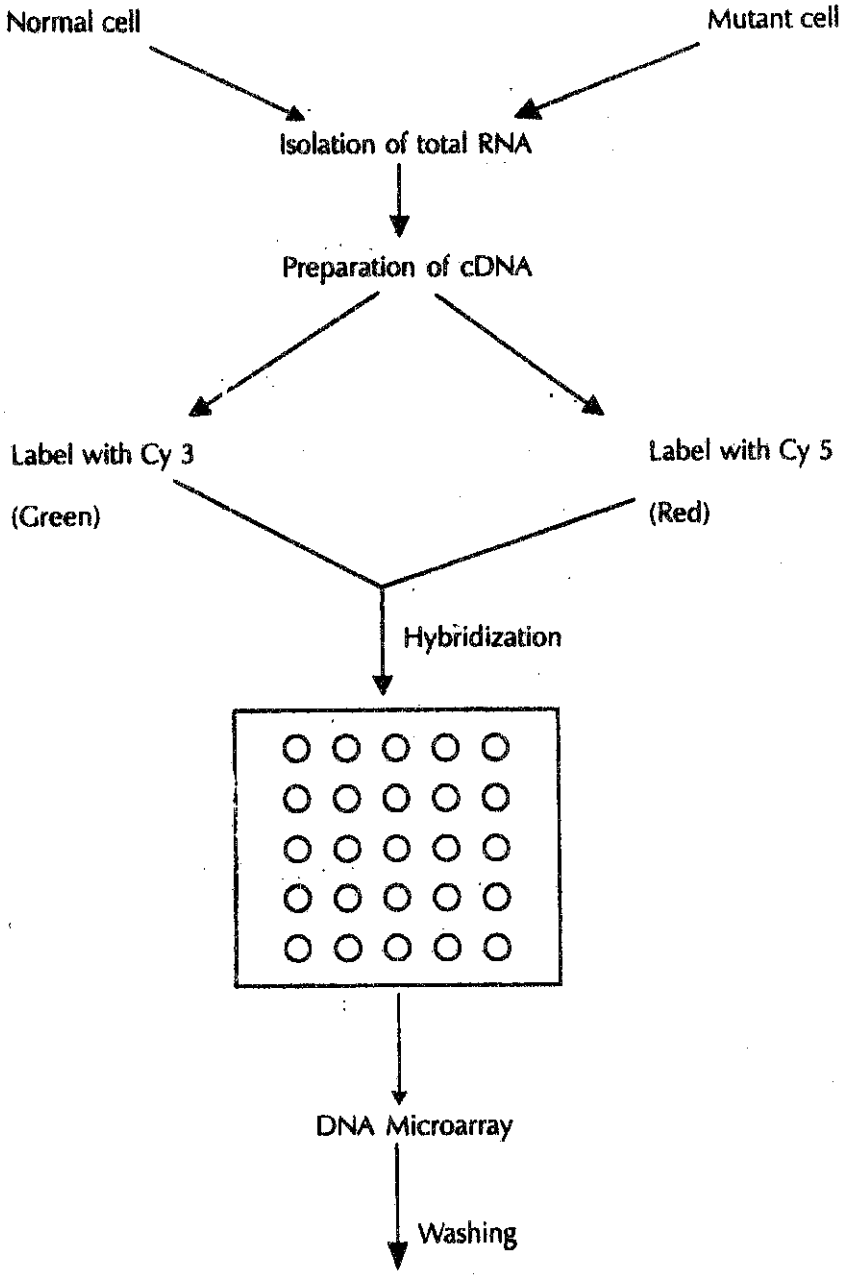
Analysis and Interpretation

The fluorescent pattern is detected by a laser detector, which can distinguish the fluorescence emitted by the different wavelengths of the red and green dye efficiently. The background fluorescent is subtracted from the spotted arrays fluorescence and the results are interpreted.

Application of Microarrays

cDNA microarrays

The microarray contains ESTs (Expressed Sequence Tags) spotted on to a glass slide and the cDNA expression patterns of two different cells; wild verses mutant strains; tumor verses normal cells are compared.



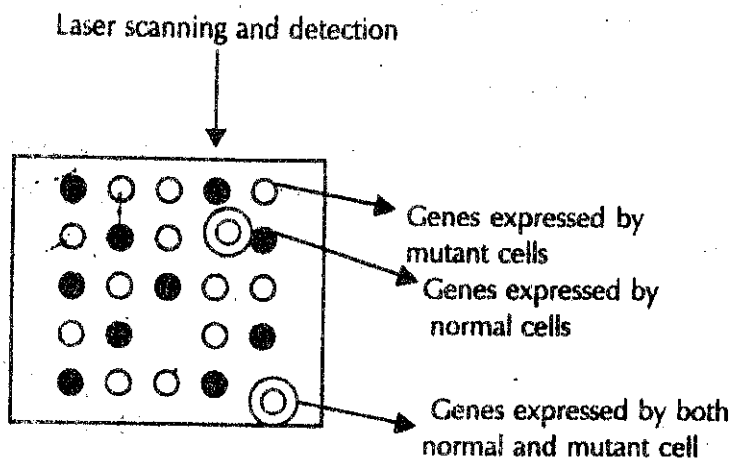


Figure 6.6: Typical cDNA Microarray Experiment

SNP Microarray

SNP microarray are used for SNP typing on a probe array for studying all possible variant alleles of each single nucleotide polymorphism to be analyzed.

Whole Genome Microarray for Comparative Genomics

Whole genome microarray for comparative genomics are used to compare the genomic sequences of all groups, sub groups of a phylum, systems of different organisms/species to study the phylogeny.

Microarray and Diagnostics

The genomes of infectious diseases causing pathogens can also be detected using microarrays.

Protein Profiling and its Significance

Protein in today's terms is analogous to proteomics. Proteomics deals with the cataloging and analysis of a set a proteins, expressed at a particular time, to determine when it is expressed, how much it is made and how does it interact with other proteins. The complete set of expressed proteins, at particular time is called the proteome of that organism. Biochemical and molecular approaches are mostly used in proteomics to:

1. Identify every protein in the proteome by isolating and analyzing the protein by mass spectrometry.
2. Determine the sequences of every protein and entering the data into databases.
3. Analysis of protein levels in different cell types or stages of development in an organism.

The first step towards protein profiling is to identify and sequence all the proteins from a cell. This is much more complex than mapping and sequencing the genome. Keeping this in mind, the human proteome organization (HuPO) was launched to increase the awareness of proteomics research at scientific, political and financial levels. Primarily, the field of proteomics is gaining importance as it focuses on functional products of the genes upon which the phenotype of a cell is determined.

For protein profiling following steps are used:

Purification of Protein from a Mixture

A cell/tissue homogenate typically contains 10,000 - 20,000 different proteins. It becomes necessary to purify the protein of interest from this mixture. Basically only four different fractionation steps are needed to purify a given protein. In exceptional circumstances; proteins have been purified in a single chromatographic step. The degree of purity depends upon the purpose for which it will be used. For sequence determination; 90% pure protein is enough but for therapeutic purposes; protein obtained should be of highest purity. During protein purification; it is necessary to determine the protein concentration for which a successful fractionation step is involved in protein purification. This is accomplished by an increase in specific activity of the sample. The specific activity of the protein relates to the total activity of the protein to the total amount of protein present in the preparation. All protein purification techniques exploit the properties of the proteins on the basis of which they differ from one another like solubility, stability, charge, size, affinity and hydrophobicity.

Chromatographic Techniques Commonly used in Protein Purification

S.No.	Technique	Property exploited	Capacity	Resolution
1	Hydrophobic interaction	Hydrophobicity	High	Medium
2	Ion exchange	Charge	High	Medium
3	Affinity	Biological function	Medium	High
4	Chromatofocussing	Charge and PI	High – medium	High – medium
5	Gel filtration	Molecular size	Medium	Low

Once the first step of purification is accomplished, the next step is to analyze the mixture obtained. This can be done by the following method:

1. 1-D PAGE
2. 2-D PAGE
3. Mass spectrometry

Separation and Characterization of Proteins by Electrophoresis: 1 D and 2 D PAGE

The separation of protein based on the migration of charged proteins in an electric field is called electrophoresis. By this method proteins can be visualized and separated and the property of the protein like its approximate molecular weight and isoelectric point can be determined. This technique is carried out in gels made up of a polymer polyacrylamide which aids in the migration of proteins in proportion to their charge to mass ratio, hence this technique is called Polyacrylamide Gel Electrophoresis (PAGE). If a detergent like SDS (Sodium Dodecyl Sulphate) is used which binds to most of the proteins contributing a net negative charge to the proteins electrophoresis is called as SDS-PAGE, which separates proteins exclusively on the basis of mass.

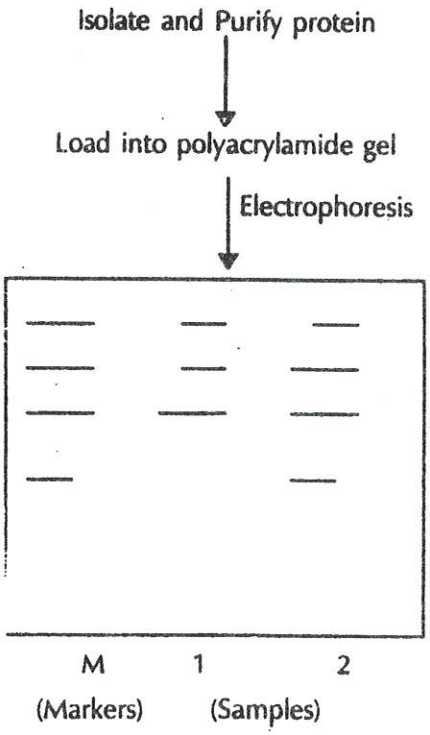


Figure 6.6

Isoelctric Focusing

Isoelctric focusing is a technique used to determine the isoelctric point (pI) of a protein. A pH-gradient is used to allow a mixture of low molecular weight organic acids and bases (ampholytes) to distibute themselves in an electric field generated across the gel. Each protein migrates till it reaches a pH that matches pI.

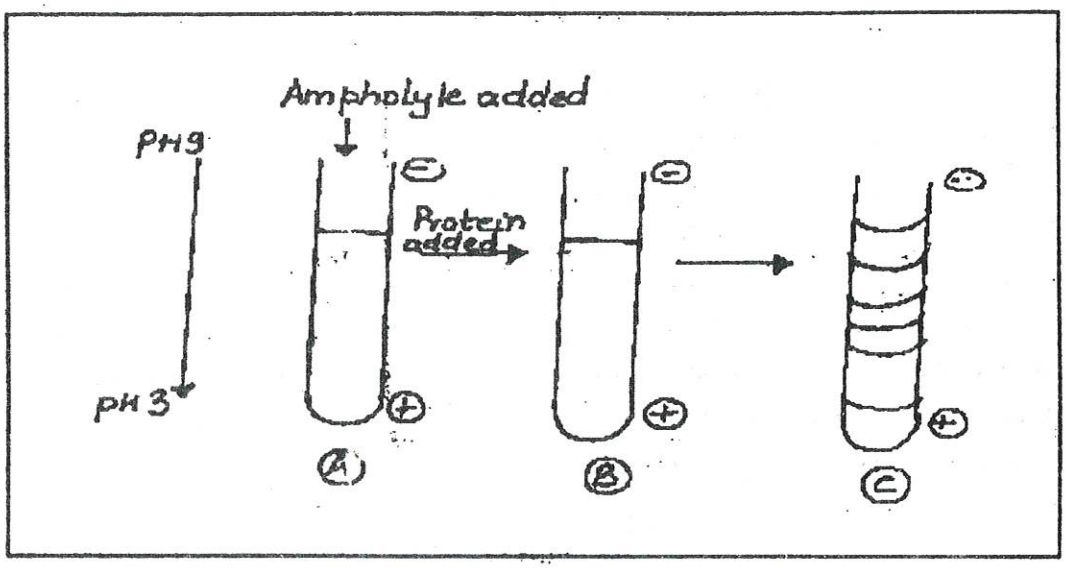


Figure 5.8

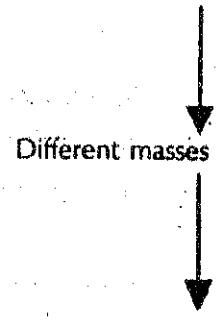
A. Stable pH gradient is established in the gel after application of an electric field.

B. Protein solution is added and electric field is reapplied.

C. After staining protein bands are seen to be distributed along pH gradient according to their pI values.

2-D PAGE

If IEF and SDS-PAGE are sequentially performed it is called 2-D PAGE and this permits the resolution of a complex mixture of proteins. This allows the separation of proteins of identical molecular weight that differ in pI or proteins with similar pI values but different molecular weights.



Steps Towards Mass Spectroscopy Analysis

Mass spectrometry of a peptide mixture shows a number of molecular wins relating to peptides. With the knowledge of the relative molecular masses of each of the 20 naturally occurring amino acids; it is possible to determine the presence of a particular residue at any point within the peptide. The position of the assigned amino acid is deduced by virtue of the M/Z ratio of the two ions. By reading several amino acids, the sequence of amino acids will be assembled. All the paptides can be analyzed in the same manner, confirming the identity of the protein. Hence the protein can be characterized by the mass spectrometric method.

Significance

Once a preliminary profile of the protein is obtained and a sequence is obtained; the following can be established:

1. To search the sequence databases to see whether the protein of interest has already been isolated and hence can be identified.
2. To search for sequence homology using computerized databases to identify the function of that protein.
3. The sequence can be used to design an oligonucleotide probe for selecting appropriate clones from complementary DNA libraries. Hence the DNA coding for the protein can be isolated and DNA sequence can be determined.
4. The protein sequence data can be used in quality control check in the pharmaceutical industry, which produce antibodies, enzyme and synthetic peptides. The identity and structural integrity of the protein can be confirmed by this method.

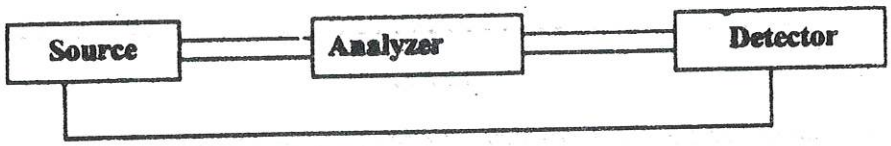
Peptide Sequencing

Peptide sequencing is done by Edman degradation method. This method is a chemical method, which ensures the stepwise removal of amino acid (aa) residues from the N-terminus of a peptide or protein. For this technique a free amino acid group at N-terminum of the peptide is required. But 50% to 70% of all proteins have their N terminal amino group blocked. Such proteins need to be cleaved to produce peptides and then purified and sequenced. Peptide mass profiling is based on the observation that accurate peptide masses obtained by mass spectrometric analysis of a protein digest and provides a characteristics fingerprint of that protein. For mass spectrometry, it is essential to disintegrate the peptide and produce fragment ions, each of which is represented by a peak in the resultant spectrum. A typical mass spectrometer has

A. An ionization chamber

B. A mass analyzer

C. A detector



High Vacuum

Peptides

Ionise to produce ions in the gas phase

Accelerate them to a specific velocity using electric fields

Project them to a mass analyzer

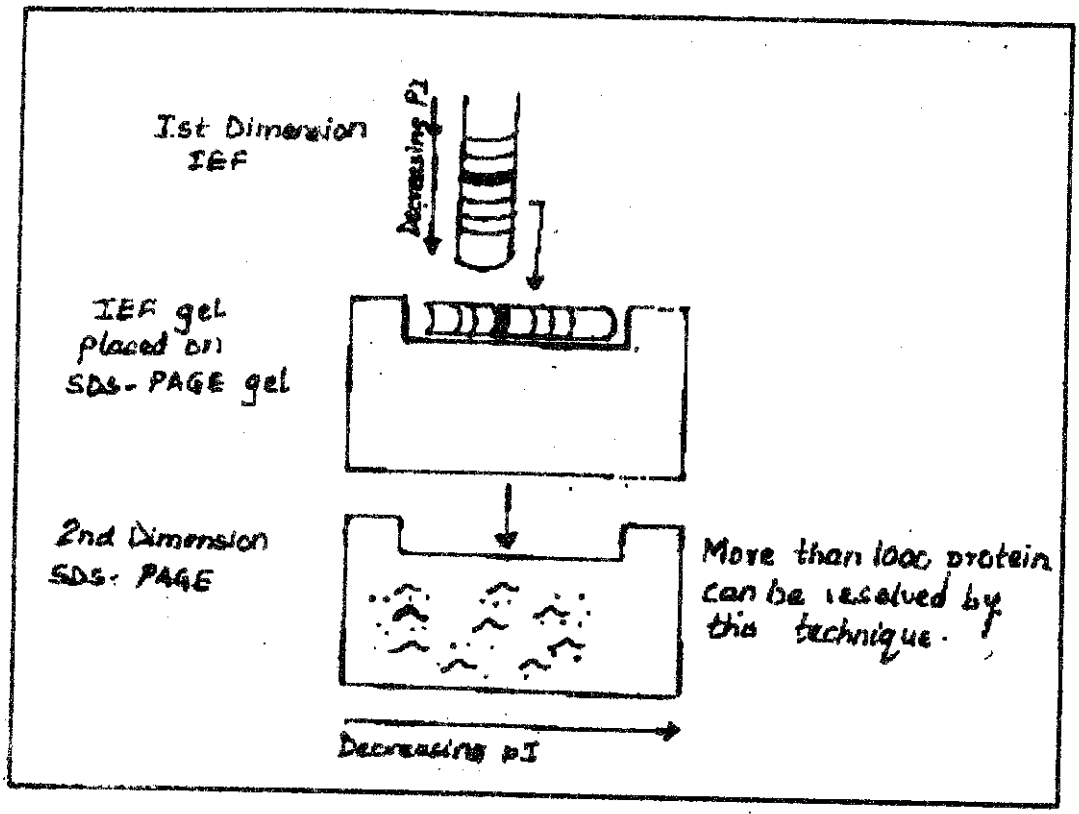


Figure 8.7

The individual proteins are then identified by partial sequence analysis.

Knowledge of the complete primary structure of the protein is essential to determine the 3-D structure of the protein, which in turn can help in understanding the function of a protein.

Amino acid sequence on protein primary structure

3-D structure

Association of protein in different cell types and regulation of gene expression

A 2-D PAGE pattern is useful, as a preliminary diagnostic tool by comparing the 2-D pattern of the protein produced under normal and diseased conditions. This also can be used to check the action of the drug after its administration.

The differential production of proteins in different conditions (of a normal vs diseases case) can be effectively analyzed and determined using protein microarrays. In this technique, the array is an ordered arrangement of known specific antibodies and a mixture of isolated protein (tagged with fluorescent molecules) is allowed to interact with the protein microarray. The proteins specific for a certain antibody will bind and the fluorescent signal is obtained. Hence for high throughput protein profiling, researchers are trying to actively develop this technique.

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