GENETICS AND PLANT BREEDING

Introduction to Plant Biotechnology

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(16-07- 2007)

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Keywords
Totipotency, haploid production, cryopreservation, somaclonal variation, somatic hybrids, Agrobacterium, recombinant DNA, transgenic plants, molecular breeding

History of Plant Tissue Culture and Biotechnology

Biotechnology is name given to the methods and techniques that involve the use of living organisms like bacteria, yeast, plant cells etc or their parts or products as tools (for example, genes and enzymes). They are used in a number of fields: food processing, agriculture, pharmaceutics, and medicine, among others. Plant tissue culture can be defined as culture of plant seeds, organs, explants, tissues, cells, or protoplasts on nutrient media under sterile conditions.

The science of plant tissue culture takes its roots from path breaking research in botany like discovery of cell followed by propounding of cell theory. In 1839, Schleiden and Schwann proposed that cell is the basic unit of organisms. They visualized that cell is capable of autonomy and therefore it should be possible for each cell if given an environment to regenerate into whole plant. Based on this premise, in 1902, a German physiologist, Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He isolated single fully differentiated individual plant cells from different plant species like palisade cells from leaves of *Laminum purpureum*, glandular hair of *Pulmonaria* and pith cells from petioles of *Eicchornia crassiples* etc and was first to culture them in Knop's salt solution enriched with glucose. In his cultures, cells increased in size, accumulated starch but failed to divide. Therefore, Haberlandt's prediction failed that the cultured plant cells could grow, divide and develop into embryo and then to whole plant. This potential of a cell is known as totipotency, a term coined by Steward in 1968. Despite lack of success, Haberlandt made several predictions about the requirements in media in experimental conditions which could possibly induce cell division, proliferation and embryo induction. G Haberlandt is thus regarded as father of tissue culture.

Taking cue from Haberlandt's failure, Hannig (1904) chose embryogenic tissue to culture. He excised nearly mature embryos from seeds of several species of crucifers and successfully grew them to maturity on mineral salts and sugar solution. In 1908, Simon regenerated callus, buds and roots from *Poplar* stem segments and established the basis for callus culture. For about next 30 years (upto 1934), there was very little further progress in cell culture research. Within this period, an innovative approach to tissue culture using meristematic cells like root and stem tips was reported by Kolte (1922) and Robbins (1922) working independently.

All these research attempts involving culture of isolated cells, root tips or stem tips ended in development of calluses. There were two objectives to be achieved before putting Haberlandt's prediction to fruition. First, to make the callus obtained from the explants to proliferate endlessly and second to induce these regenerated calluses to undergo organogenesis and form whole plants. It was in 1930s, when progress in plant tissue culture accelerated rapidly owing to an important discovery that vitamin B and natural auxins were necessary for the growth of isolated tissues containing meristems. This breakthrough came from White (1934) who reported that not only could cultured tomato root tips grow but could be repeatedly subcultured to fresh medium of inorganic salts supplemented with yeast extract. He later (1937) replaced YE by vitamin B namely pyridoxine, thiamine and proved their growth promoting effect.

In 1926, Fritz Went discovered first plant growth regulator (PGR), indoleacetic acid (IAA). IAA is a naturally occurring member of a class of PGRs termed 'auxins'. Roger J Gautheret (1934) reported the successful culture of cambium cells of several tree species to produce callus and

addition of auxin enhanced the proliferation of his cambial cultures. Further research by Nobecourt (1937), who could successful grow continuous callus cultures of carrot slices and White (1939) who obtained similar results from tumour tissues of hybrid *Nicotiana glauca x N langsdorffii*. Thus, the possibility of cultivating plant tissues for an unlimited period was independently endorsed by Gautheret, White and Nobecourt in 1939.

Adding to the ongoing improvements in the culture media, Johannes Van Overbeek (1941) reported growth of seedlings from heart shaped embryos by enriching culture media with coconut milk besides the usual salts, vitamins and other nutrients. This provided tremendous impetus for further work in embryo culture. Stem tip cultures yielded success when Ernest Ball (1946) devised a method to identify the exact part of shoot meristem that gives rise to whole plant.

After 1950, there was an immense advancement in knowledge of effect of PGRs on plant development. The fact that coconut milk (embryo sac fluid) is nutritional requirement for tobacco callus besides auxin, indicated the non auxinic nature of milk. This prompted further research and so other classes of PGRs were recognized. Skoog and Tsui (1957) demonstrated induction of cell division and bud formation in tobacco by adenine. This led to further investigations by Skoog and Miller (1955) who isolated 'kinetin'- a derivative of adenine (6-furyl aminopurine). Kinetin and many such other compounds which show bud promoting activities are collectively called cytokinins, a cell division promoter in cells of highly mature and differentiated tissues. Skoog and Miller worked further to propose the concept of hormonal control of organ formation (1957). Their experiment on tobacco pith cultures showed that high concentration of auxin prmoted rooting and high kinetin induces bud formation or shooting. However, now the concept is altered to multiple factors like source of plant tissue, environmental factors, composition of media, polarity, growth substances being responsible for determination of organogenesis. Besides PGRs, scientists tried to improve culture media by differing essentially in mineral content. In this direction, Murashige and Skoog (1962) prepared a medium by increasing the concentration of salts twenty-five times higher than Knops. This media enhanced the growth of tobacco tissues by five times. Even today MS medium has immense commercial application in tissue culture.

Having achieved success and expertise in growth of callus cultures from explants under *in vitro* conditions, focus now shifted to preparation of single cell cultures. Muir (1953-54) demonstrated that when callus tissues were transferred to liquid medium and subjected to shaking, callus tissues broke into single cells. Bergmann (1960) developed a technique for cloning of these single cells by filtering suspension cultures. This technique called Plating technique is widely used for cloning isolated single protoplasts.

Next step for realization of Haberlandt's objectives was development of whole plant from the proliferated tissue of these cells. Vasil and Hilderbrandt were first to regenerate plantlets from colonies of isolated cells of hybrid *Nicotiana glutinosa x N tabacum.* In 1966, the classical work of Steward on induction of somatic embryos from free cells in carrot suspension cultures brought an important breakthrough by finally demonstrating totipotency of somatic cells, thereby validating the ideas of Haberlandt. This ability of regenerating plants from single somatic cells through normal developmental process had great applications in both plant propagation and also

genetic engineering. For e.g. micropropagation where small amounts of tissue can be used to continuously raise thousand more plants. Morel utilized this application for rapid propagation of orchids and Dahlias. He was also the first scientist to free the orchid and Dahlia plants from virus by cultivating shoot meristem of infected plants.

The role of tissue culture in plant genetic engineering was first exemplified by Kanta and Maheshwari (1962). They developed a technique of test tube fertlization which involved growing of excised ovules and pollen grains in the same medium thus overcoming the incompatibility barriers at sexual level. In 1966, Guha and Maheshwari cultured anthers of Datura and raised embryos which developed into haploid plants initiating androgenesis. This discovery received significant attention since plants recovered from doubled haploid cells are homozygous and express all recessive genes thus making them ideal for pure breeding lines.

Next breakthrough in application of tissue culture came with isolation and regeneration of protoplasts first demonstrated by Prof. Edward C Cocking in 1960. Plant protoplasts are naked cells from which cell wall has been removed. Cocking produced large quantities of protoplasts by using cell wall degrading enzymes. After success in regeneration of protoplasts, Carlson (1972) isolated protoplasts from *Nicotiana glauca* x *N. langsdorfii* and fused them to produce first somatic hybrid. Since then many divergent somatic hybrids have been produced.

With the advent of restriction enzymes in early 1970s, tissue culture headed towards a new research area. The totipotent plant cells could now be altered by insertion of specific foreign genes giving rise to genetically modified crops. In 1970, Smith and Nathans isolated first restriction enzyme from *Haemophillus influenzae* which was later purified and named *Hind III*. Same year witnessed other nobel prize winning discovery by Baltimore who isolated Reverse transcriptase from RNA tumor viruses. This is a useful enzyme in genetic engineering which functions to convert RNA to DNA and hence useful in construction of complementary DNA from messenger RNA. Another pathbreaking discovery establishing potential of genetic engineering came in 1972 when Paul Berg working at Stanford University produced first recombinant DNA *in vitro* by combining DNA from SV40 virus with that of lambda virus. This led to construction of first recombinant organism by Cohen and Boyer in 1973. Genetic engineering's potential was first exploited when a man made insulin gene was used to manufacture a human protein in bacteria.

Agrobacterium tumefaciens plays a crucial role in plant genetic engineering. The involvement of this bacterium in crown gall disease in plants was recognized as early as 1907 by Smith and Townsend. However, it was in 1974, that Zaenen *et al* discovered that Ti plasmid is the tumor inducing principle of Agrobacterium. This was followed by its successful integration in plants by Chilton *et al* in 1977. Zambryski *et al* in 1980 isolated and studied the detailed structure of T-DNA and its border sequences. Soon thereafter in 1984, transformation of tobacco with Agrobacterium was accomplished to develop transgenic plants. Simultaneously, there was an upsurge in development of techniques of genetic engineering in mid 1970s. Sanger *et al*(1977) and Maxam and Gilbert (1977) reported techniques for large scale DNA sequencing. This was followed by complete genome sequencing projects on many prokaryotes and eukaryotes like *Haemophilus influenzae* in 1995, *E coli* in 1997. Human genome was sequenced successfully in 2001, thus laying foundation of genomics which is the main focus of present day biotechnology.

Some of the landmark discoveries are tabulated below:

Year	Discovery
1902	Haberlandt proposed concept of *in vitro* cell culture
1904	Hannig cultured embryos from several cruciferous species
1922	Kolte and Robbins successfully cultured root and stem tips respectively
1926	Went discovered first plant growth hormone –Indole acetic acid
1934	White introduced vitamin B as growth supplement in tissue culture media for tomota root tip
1939	Gautheret, White and Nobecourt established endless proliferation of callus cultures
1941	Overbeek was first to add coconut milk for cell division in Datura
1946	Ball raised whole plants of Lupinus by shoot tip culture
1954	Muir was first to break callus tissues into single cells
1955	Skoog and Miller discovered kinetin as cell division hormone
1957	Skoog and Miller gave concept of hormonal control (auxin: cytokinin) of organ formation
1959	Reinert and Steward regenerated embryos from callus clumps and cell suspension of *Daucus carota*
1960	Cocking was first to isolate protoplast by enzymatic degradation of cell wall
1960	Bergmann filtered cell suspension and isolated single cells by plating
1962	Murashige and Skoog developed MS medium with higher salt concentration
1962	Kanta and Maheshwari developed test tube fertilization technique
1966	Steward demonstrated totipotency by regenerating carrot plants from single cells of tomato
1966	Guha and Maheshwari produced first haploid plants from pollen grains of Datura
1970	Smith and Nathans discovered first restriction enzyme from *Haemophilus influenza* (*HindIII*)
1970	Baltimore isolated Reverse transcriptase from RNA tumour virus
1972	Carlson produced first interspecific hybrid of *Nicotiana* by protoplast fusion
1972	Berg produced first recombinant DNA , combining SV40 virus and λ virus
1974	Zaenen *et al* discovered Ti plasmid is tumour inducing principle of agrobacterium
1975	O'Farrel developed high resolution two dimensional gel electrophoresis system
1977	Chilton *et al* successfully integrated Ti plasmid DNA from *Agrobacterium tumefaciens* in plants
1977	Sanger, Maxam-Gilbert gave technologies for DNA sequencing
1980	Zambryski detailed structure of T-DNA and border sequences
1983	Kary Mullis invented Polymerase chain reaction (PCR), for amplification of DNA.
1984	Horsh *et al* developed transgenic tobacco by transformation with Agrobacterium
1987	Klien *et al* developed biolistic gene transfer method for plant transformation
1995	Fleischmann *et al* sequenced *Haemophilus influenzae*
1997	Blattner *et al* sequenced *E coli* genome
2001	Human genome Project consortium and Venter *et al* sequenced human genome successfully
2005	Rice genome sequenced under International Rice Genome Sequencing Project

Scope and Importance of Biotechnology

Biotechnology is controlled use of biological agents for beneficial use. It is integrated use of biochemistry, molecular biology, microbiology to achieve technological application of the capabilities of biological agents. Therefore, biotechnology has emerged as a science with immense potential for human welfare ranging from food processing, human health to environment protection. The importance of this field of science in different streams will be evident from following examples:

- **Biotechnology in Medicine*:*** Production of monoclonal antibody, DNA, RNA probes for diagnosis of various diseases; valuable drugs like insulin and interferon have been synthesized by bacteria for treatment of human diseases. DNA fingerprinting is utilized for identification of parents and criminals. Development of recombinant vaccines like human hepatitis B etc by genetically engineered microbes includes the list of notable achievements.
- **Industrial Biotechnology*:*** is an area with which biotechnology was initiated for large scale production of alcohol and antibiotics by microorganisms. Even today, a variety of pharmaceutical drugs and chemicals like lactic acid, glycerine etc are being produced by genetic engineering for better quality and quantity.Biotechnology has provided us with a very efficient and economic technique for production of variety of biochemicals i.e. immobilized enzymes. Protein engineering is other important area where existing proteins and enzymes are remodelled for a specific function or for increasing efficiency of their function.
- **Biotechnology and Environment*:*** Environmental problems like pollution control, depletion of natural resources for non renewable energy, conservation of biodiversity etc are being dealt with using biotechnology. For e.g. bacteria are being utilized for detoxification of industrial effluents, in combating oil spills for treatment of sewage and for biogas production. Bio-pesticides give environmentally safer alternative to chemical pesticides for control of insect pests and diseases.
- **Biotechnology and Agriculture*:*** In agriculture, plant cell, tissue and organ culture is used for rapid and economic clonal multiplication of fruit and forest trees, for production of virus free genetic stocks and planting material as well as in the creation of novel genetic variations through soma-clonal variation. Genetic engineering techniques are utilized to produce transgenic plants with desirable genes like disease resistance, herbicide resistance, increased shelf life of fruits etc. Also, molecular breeding has hastened the process of crop improvement for e.g. molecular markers like RFLP, SSRs provide powerful tools for indirect selection of both qualitative and quantitative traits and also for studying genotypic diversity.

With all the above inputs of biotechnology in every sphere, there is an increasing demand of research to explore more of its commercial potential in this area. Besides, government organizations, private sector are also becoming interested in biotechnological research. Therefore, 21st century will be century of biotechnology.

Tissue and Cell Culture

Tissue culture is a technique of growing plant cells by culturing explant aseptically on a suitable nutrient medium.

Media: Plant tissue culture is a technique of *in vitro* cultivation of plant cells and organs, which divide and regenerate into callus or particular plant organs. This technique relies on the following conditions:

- Explant
- Aseptic environment
- Nutrient media

- **A small tissue excised from any part of the plant is called explant** which is the starting point. It can be initiated from any part of plant- root, stem, petiole, leaf or flower, choice of explant varies with species. Meristems are more responsive and give better success as they are actively dividing. The physiological state of the plant also has an influence on its response to initiate tissue culture. Therefore, the parent plant must be healthy and free from obvious signs of disease or decay.
- **Aseptic environment during culture is required to avoid contamination from microorganisms**. Since plant cell division is slower compared to the growth of bacteria, fungi and even minor contaminants can easily overgrow the plant tissue culture. Therefore, all the materials like glassware, instruments, medium, explant etc to be used in culture work must be freed of microbes using several techniques available as listed in table 1. Laminar flow is a mandatory prerequisite for any tissue culture laboratory for contamination free work (Fig.1)

Fig. 1: Laminar flow (tissue culture work in sterilized conditions)

- **Nutrient media**: The type and composition of culture media very strongly govern the growth and morphogenesis of plant tissues. The choice of tissue culture medium largely depends upon the species to be cultured. For e.g. some species are sensitive to high salts or have different requirements for PGRs. Some tissues show better response on solid medium while others prefer a liquid medium. Therefore, development of culture medium formulations is result of systematic trial and experimentation considering specific requirements of a particular culture system. White's medium is one of the earliest plant tissue culture media originally formulated for root culture. Murashige and Skoog (MS) medium is the most suitable and commonly used medium for plant regeneration from tissues and callus. This is a high salt medium due to its content of potassium and nitrogen salts. B5 medium works well

for protoplast culture. It has lesser amounts of nitrate and particularly ammonium salts than MS medium. Nitsch's medium developed for anther culture contains salt concentration intermediate to MS and White.

Components of Tissue Culture Medium:

***1.*Inorganic Nutrients:** *In vitro* growth of plants also requires combination of macro and micronutrients like *in vivo* growth. **Macronutrients** are classified as those elements which are required in concentration greater than 0.5 mM/l. They include nitrogen, potassium, phosphorus, calcium, magnesium and sulphur in form of salts in media. Nitrogen is usually supplied in form of ammonium (NH_4^+) and nitrate (NO_3^-) ions. Nitrate is superior to ammonium as the sole N source but use of NH_4^+ checks the increase of pH towards alkalinity. Culture media should contain atleast 25mM/l nitrogen and potassium. Other major elements are adequate in concentration range of 1-3mM/l. **Micronutrients** are those elements which are required at a concentration less than 0.05mM/l. These include iron, manganese, zinc, boron, copper and molybdenum. These inorganic elements although required in small quantity are essential for plant growth, most critical of them being iron which is not available at low pH. Therefore, it is provided as iron EDTA complex to make it available at wide range of pH.

***2.*Carbon Source:** Sugar is very important part of nutrient medium as energy source, since most plant cultures are unable to photosynthesize effectively owing to inadequately developed cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels etc. Hence they lack auxotrophic ability and need external carbon for energy. The most preferred carbon or energy source is sucrose at a concentration of 20-60g/l. While autoclaving the medium, sucrose is hydrolysed to glucose and fructose which are then used up for growth. Fructose, if autoclaved is toxic. Other mono or disaccharide and sugar alcohols like glucose, sorbitol, raffinose etc may be used depending upon plant species. Carbohydrates also provide osmoticum and hence in anther culture higher concentration of sucrose (6-12%) is used.

3.Organic Supplements:

- **Vitamins:** are organic substances required for metabolic processes as cofactors or parts of enzymes. Hence for optimum growth, medium should be supplemented with vitamins. Thiamine (B1), nicotinic acid (B3), pyridoxine(B6), pantothenic acid(B5) are commonly used vitamins of which thiamine (0.1 to 5mg/l) is essentially added to medium as it is involved in carbohydrate metabolism. Rest vitamins are promontory.

- **Amino acids:** Addition of amino acids to media is important for stimulating cell growth in protoplast cultures and also in inducing and maintaining somatic embryogenesis. This reduced organic nitrogen is more readily taken up by plants than the inorganic nitrogen. L-glutamine, L-asparagine, L-cystein, L-glycine are commonly used aminoacids which are added to the culture medium in form of mixtures as individually they inhibit cell growth.

- **Complex organics:** are group of undefined supplements such as casein hydrolysate, coconut milk, yeast extract, orange juice, tomato juice etc. These compounds are often used when no other combination of known defined components produce the desired growth. Casein hydrolysate has given significant success in tissue culture and potato extract also has been found useful for anther culture. However, these natural extracts are avoided as their

composition is unknown and vary from lot to lot and also vary with age affecting reproducibility of results.

- **Activated charcoal:** acts both in promotion and inhibition of culture growth depending upon plant species being cultured. It is reported to stimulate growth and differentiation in orchids, carrot, ivy and tomato whereas inhibits tobacco, soybean etc. It absorbs brown-black pigments and oxidized phenolics produced during culture and thus reduce toxicity. It also absorbs other organic compounds like PGRs, vitamins etc which may cause the inhibition of growth. Another feature of activated charcoal is that it causes darkening of medium and so helps root formation and growth.

4. PGRs: stimulate cell division and hence regulate the growth and differentiation of shoot and roots on explants and embryos in semisolid or in liquid medium cultures. The four major PGRs used are auxins, cytokinin, gibberellins and abscissic acid and their addition is must to the culture medium.

- **Auxins:** induce cell division, cell elongation, apical dominance, adventitious root formation, somatic embryogenesis. When used in low concentration, auxins induce root initiation and in high, callus formation occurs. Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA). 2,4 dichlorophenoxyacetic acid (2,4-D), indole-3 acetic acid (IAA), indolebutyric acid (IBA) etc. Both IBA and IAA are photosensitive so the stock solutions must be stored in the dark. 2,4-D is used to induce and regulate somatic embryogenesis.
- **Cytokinins:** promote cell division and stimulate initiation and growth of shoots *in vitro*. Zeatin, 6- benzylaminopurine (BAP), kinetin, 2-iP are the frequently used cytokinins. They modify apical dominance by promoting axillary shoot formation. When used in high concentration, CK inhibits root formation and induces adventitious shoot formation. The ratio of auxin and cytokinin in the culture decides morphogenesis. If this ratio is high, leads to embryogenesis, callus initiation and root initiation whereas if ck/auxin is high, it gives rise to axillary and shoot proliferation.
- **Gibbrellins and abscissic acid:** are lesser used PGRs. Gibbrellic acid (GA3) is mostly used for internode elongation and meristem growth. Abscissic acid (ABA) is used only for somatic embryogenesis and for culturing woody species.

*5.***Solidifying agents:** are used for preparing semisolid tissue culture media to enable explant to be placed in right contact with nutrient media (not submerged but on surface or slightly embedded) to provide aeration. Agar is high molecular weight polysaccharide obtained from sea weeds and can bind water. It is added to the medium in concentration ranging from 0.5% to 1 %(w/v). Agar is preferred over other gelling agents because it is inert, neither does it react with media constituents nor digested by plant enzymes.

Agarose, a purified extract of agar is used for protoplast culture. Alternative gelling compounds like gelrite etc form clear gels (unlike agar which is translucent) and hence easier to detect contamination which might develop during culture growth. Mechanical support for cell or tissue growth can also be provided without using any gelling agent by filter paper bridge, perforated cellophane and polyurethane foam etc.

6. **pH:** pH affects absorption of ions and also solidification of gelling agent. Optimum pH for culture media is 5.8 before sterilization. Values of pH lower than 4.5 or higher than 7.0 greatly inhibit growth and development *in vitro*. The pH of culture media generally drops by 0.3 to 0.5 units after autoclaving and keeps changing through the period of culture due to oxidation and also differential uptake and secretion of substances by growing tissue.

Table 1 Sterilization techniques used in Plant Tissue Culture

Technique	Materials sterilized
Steam sterilization/Autoclaving (121°C at 15 psi for 20-40 min)	Nutrient media, culture vesels, glasswares and plasticwares
Dry heat (160-180°C for 3h)	Instruments (scalpel, forceps, needles etc.), glassware, pipettes, tips and other plasticwares
Flame sterilization	Instruments (scalpel, forceps, needles etc.), mouth of culture vessel
Filter sterilization (membrane filter made of cellulose nitrate or cellulose acetate of 0.45- 0.22μm pore size)	Thermolabile substances like growth factors, amino acids, vitamins and enzymes.
Alcohol sterilization	Worker's hands, laminar flow cabinet
Surface sterilization (Sodium hypochlorite, hydrogen peroxide, mercuric chloride etc)	Explants

Preparation of Media: This is a very crucial step for the experiment to be successful. While making the media taking individual constituents, each ingredient is separately weighed and dissolved before putting them together. After making up volume by water, pH is adjusted and then medium is autoclaved. Preferably, following four stock solutions are prepared:

- Major salts (20X concentration)
- Minor salts (200X concentration)
- Iron (200X concentration)
- Organic nutrients (200X concentration)

Separate stock solution for each growth regulator is prepared. Appropriate quantities are taken from stocks and mixed to constitute basal medium. Required quantity of agar, sucrose and organic supplements if needed are added separately.

Selection of a suitable new medium: A suitable medium may be devised by trying many combinations of different concentrations of major components like PGRs, salt, sucrose. Also, various combinations of low, medium and high concentration of the following four categories of components can be evaluated i.e. minerals, auxins, cytokinin, organic nutrients to get a suitable medium. The exact conditions required to initiate and sustain plant cells in culture or to regenerate intact plants from cultured cells, are different for each plant species. Each variety of species will have a particular set of cultural requirements. Despite all the knowledge that has

been obtained about plant tissue culture during the twentieth century, these conditions have to be identified for each variety through experimentation.

Modes of Culture: The plant cells if cultured on a solid surface will grow as friable, pale brown, unorganized mass of cells called callus. Tissues and cells of plant cultured in a liquid medium aerated by agitation grow as suspension of single cells and cell clumps. For growth, the cells need to divide, whereas, the cells breaking up from explant are mature, nondividing. Therefore, the differentiated tissue undergoes modifications to become meristematic. **This phenomenon of a mature cell reverting back to meristematic state to form undifferentiated callus tissue is called dedifferentiation**.

(i)Callus culture*:* The culture of undifferentiated mass of cell on agar media produced from an explant of a seedling or other plant part is called callus culture. For callus formation, auxin and cytokinins, both are required. Callus can be subcultured indefinitely by transferring a small piece of the same to fresh agar medium. Subculturing needs to be done every 3-5 weeks in view of cell growth, nutrient depletion and medium drying.

The rate of growth of callus grown on solid agar medium is relatively slow. The new cells are formed on the periphery of existing callus mass. Consequently, callus consists of cells which vary considerably in age. Since nutrients are gradually depleted from the agar, a vertical nutrient gradient is formed. Because of low degree of uniformity among cells in callus, slower growth rate and development of nutrient gradients, the usefulness of callus in experimental system is limited. The main use of callus culture is for purposes of maintaining cell lines and for morphogenesis.

(ii)Cell suspension culture*:* The culture of tissues and cells cultured in a liquid nutrient medium produce a suspension of single cells and cell clumps, this is called **suspension culture**. A callus mass friable in texture is transferred to liquid medium and vessel is incubated on shaker to facilitate aeration and dissociation of cell clumps into smaller pieces. Gradually, over several weeks by subculturing, cells of callus dissociate and a liquid suspension culture is obtained. Cell suspensions are also maintained by subculturing of cells in early stationary phase to a fresh medium. Their growth is much faster than callus cultures and hence need to be subcultured more frequently (3-14days). Cell suspension cultures when fully established consist of a nearly homogeneous population. This system has an advantage that the nutrients can be continually adjusted and hence it is the only system which can be scaled up for large scale production of cells and even somatic embryos.

The suspension cultures are broadly classified as:

- **Batch culture:** The culture medium and the cells produced are retained in the culture flask. These cultures are maintained continuously by subculturing i.e. by transferring a small aliquot of inoculum from the grown culture to fresh medium at regular intervals. The biomass or cell number of a batch culture follows a typical sigmoidal curve, where to start with the culture passes through **lag phase** during which cell number is constant, followed by brief exponential or **log phase** where there is a rapid increase in cell number because of culture cell division. Finally, the growth decreases after 3-4 generations which is the **doubling time** (time taken for doubling of cell number) and culture enters **stationary phase** during which cell number again becomes static. The cells stop dividing due to depletion of nutrients and accumulation of cellular wastes. Batch cultures undergo a constant change in

cell density and metabolism and hence, not used for studies related to aspects of cell behaviour. But batch cultures are convenient to maintain, hence used for initiation of cell suspension and scaling up for continuous cultures.

- **Continuous culture:** Here steady state of cell density is maintained by regularly replacing a portion of the used up medium with fresh medium. Continuous culture are further classified into two types: 1) **Closed** 2) **Open**

  1) In **closed** type, the used medium is replaced with fresh medium, hence, the cells from used medium are mechanically retrieved and added back to the culture and thus, the cell biomass keeps increasing.

  2) In **open** type, both cells and used medium are replaced with fresh medium thus maintaining culture at constant and submaximal growth rate. There are further two types of open continuous suspension culture: **turbidostat** and **chemostat.** In **turbidostat**, cells are allowed to grow upto a certain turbidity (decided on the basis of optical density) when a predetermined volume of the culture is replaced by fresh culture. On the other hand, in **chemostat**, the fresh culture medium to be added has one nutrient kept at a concentration so that it is depleted rapidly and becomes growth limiting while other nutrients are still in concentration higher than required. Increase or decrease in the concentration of growth limiting factor is correspondingly expressed by increase or decrease in growth rate of cells. Thus, the desired rate of cell growth can be maintained by adjusting the level of concentration of growth limiting factor with respect to that of other constituents. Chemostats are useful for the determination of effects of individual nutrients on cell growth and metabolism.

***(iii)*Single cell culture:** Free cells isolated from plant organs or cell suspensions when grown as single cells under *in vitro* conditions thus producing a clone of identical cells is called single cell culture.

Isolation of single cell from plant organs*:* Leaf tissue in particular is utilized as it has homogeneous population of cells using either of the following two methods:

1. **Mechanical:** Small pieces of leaves are cut and macerated in mortar and pestle in a grinding buffer. This homogenate is filtered through muslin cloth followed by centrifugation to finally pellet down the cells.
2. **Enzymatic:** Leaves are cut into moderate pieces after peeling the lower epidermis off. Cut pieces are then incubated with macerozyme or pectinase which degrade the middlelamella and cell wall of parenchymatous tissue. A suitable osmoticum like 0.3M mannitol is added to the culture which provides protection to cell wall from any damage to cells by enzymatic action.

Isolation of Single Cells from Cell Suspension*:* Suspension cultures are prepared from friable calli as described earlier from which isolation is carried out by filtering and harvesting the cells by centrifugation.

Culture of single cells*:* Isolated single cells are unable to divide in normal tissue media, therefore, they are cultured on nurse tissue where well grown callus cultures are made to diffuse their exudates through filter paper placed on them. The single cells placed on the filter paper derive their nutrition from these exudates and thus called **nurse tissue**. This technique of culturing single cell is known as **Filter paper-Raft nurse tissue technique.** Besides this, there

are other techniques for single cell culture like microchamber, microdrop, Bergmann's plating technique, thin layer liquid medium etc., out of which **Bergmann's plating technique** is widely used. In this technique, free cells are suspended in a liquid medium. Culture medium with agar (1%) is cooled and maintained at 35°C in a water bath. Equal volumes of liquid and agar medium are mixed and rapidly spread in a petridish. The cells remain embedded in the soft agar medium. These embedded cells in the soft agar are observed under inverted microscope. When cell colonies develop, they are isolated and cultured separately.

Other point to be taken care of here is that since light has detrimental effect on cell proliferation, single cells should be cultured in dark.

Synchronisation of suspension cultures*:* Cell suspension is mostly asynchronous, different cells of different size, shape, DNA, nuclear content and also in different stages of cell cycle (G1, S, G2, M). This is not desirable in cell metabolism studies. Hence, it is essential to obtain synchrony in suspension cultures and can be achieved by following methods:

- **Starvation:** Cells are starved of a nutrient like a growth regulator which is necessary for cell division resulting in arrest of cell growth during G1 or G2. After some time, when the nutrient is supplied, all arrested cells enter divisions synchronously.
- **Inhibition:** Using a biochemical inhibitor of DNA synthesis like 5 aminouracil, cells are arrested at G1 so that removal of inhibitor leads to synchreonous division of cells.
- **Mitotic arrest:** Colchicine is widely used to arrest cells at metaphase but only for short duration as longer colchicine treatment may induce anormal mitosis and chromosome stickiness.

Cell viability test: The objective of cell suspension culture is to achieve rapid growth rates and uniform cells with all cells being viable. The viability of cells can be determined by following approaches:

- **Phase contrast microscopy**: Live cells having a well defined healthy nucleus and streaming cytoplasm are easily observed under phase contrast microscope.
- **Reduction of tetrazolium salts**: When cell masses are stained with 1-2% solution of 2,3,5-triphenyl teterazolium chloride (TTC). The living cells reduce TTC to red coloured formazon which can be extracted and measured spectrophotometrically for quantification of viability. This approach is not used for single cells.
- **Fluorescein diacetate (FDA)**: Esterase present in live cells cleaves FDA to produce fluorescein which fluoresces under UV so that live cells appear green under UV.
- **Evan's Blue staining**: This is the only dye which is taken up by dead cells. Therefore, evan's blue is used usually to complement FDA.

Applications of Cell Culture

- **Mutant screening and selection:** Induced mutagens produce more frequency of mutants than spontaneous ones and screening them at cellular level also inhibits chimeric formation which is a drawback in mutation breeding.
- **Production of secondary metabolites:** Plants being important source of variety of chemicals used in pharmacy, medicine and industry, cell cultures are effectively utilized for production of these chemicals on a commercial scale for enhanced yield and better production control.

Micropropagation

In vitro propagation of plants vegetatively by tissue culture to produce genetically similar copies of a cultivar is referred to as **micropropagation** or **clonal propagation**. Sexually propagated plants (through generation of seeds) demonstrate a high amount of heterogeneity since their seed progenies are not true to type whereas asexual reproduction (by multiplication of vegetative parts) gives rise to genetically identical copies of parent plant. Thus, it permits perpetuation of the parental characters of the cultivars among the plants resulting from micropropagation.

Micropropagation proves useful for propagation of:

- Sexually sterile species like triploids, aneuploids which cannot be perpetuated by seeds.
- Seedless plants like banana
- Cross bred perennials where heterozygosity is to be maintained
- Mutant lines like auxotrophs which cannot be propagated *in vivo*
- Disease free planting material of fruit trees and ornamentals

Micropropagation involves following major stages:

Stage 0 (3 months) — ***Selection and maintenance of stock plants for culture initiation***

Stage I (3-24 months) — ***Preparation and establishment of explant on suitable culture medium*** (usually shoot tips and axillary buds used)

Stage II (10-36 months) — ***Regeneration: multiplication of shoots or somatic embryos on defined culture medium***

Stage III (1-6 weeks) — ***Rooting of regenerated shoots/ somatic embryo in vitro***

Stage IV — ***Transfer of plantlets to sterilized soil for hardening under greenhouse environment***

(Stage III can be skipped for in vivo rooting of stage II regenerated shoots)

Advantages of micropropagation over conventional propagation methods

- Genotype constitution maintained as there is lesser variation in somatic embryo
- Easier transport and storage is facilitated by small size propagules and their ability to grow in soil less medium.
- Control over growing conditions as the production of planting material is completely under artificial control *in vitro*
- Reduced growth cycle and rapid multiplication as shoot multiplication has short cycle and each cycle results in exponential increase in number of shoots
- Selective multiplication can be done for e.g. auxotrophs, aneuploids, selected sex in dioecious species
- Virus free plants can be raised and maintained through meristem culture which is the only method available for this

Disadvantages:

- Involves high cost
- **Somaclonal variation**- any variation if occurs during multiplication may go unnoticed
- **Recalcitrancy of species/ genotype**- many tree sp like mango etc do not respond to *in vitro* growth.

Application of micropropagation:

1. **Commercial production of secondary metabolites*:*** The compounds/ biochemicals which are not directly involved in primary metabolic processes like respiration, photosynthesis etc are secondary metabolites. These include a variety of compounds like alkaloids, terpenoids, phenyl propanoids etc with various biological activities like antimicrobial, antibiotic, insecticidal, valuable pharmacological and pharmaceutical activities. Therefore, micropropagation allows their commercial scale production from cell cultures *viz.* shikonin derivatives used in dyes, pharmaceuticals are produced from cell cultures of *Lithospermum erythrorhizon*. Also, cultured cells of many plant species produce novel biochemicals which have otherwise not been detected in whole plants.

2. **Production of synthetic seeds*:*** Synthetic seed is a bead of gel containing somatic embryo or shoot bud with growth regulator, nutrients, fungicides, pesticides etc needed for development of complete plantlet. These are better propagules as donot need hardening and can be sown directly in field.

3. **Raising somaclonal variants*:*** The genetic variability occurring in somatic cells, plants produced in vitro by tissue culture are referred to as somaclonal. When these variations involve traits of economic importance, these are raised and maintained by micropropagation.

4. **Production of disease free plants*:*** Most of the horticultural fruit and ornamental crops are infected by fungal, viral, bacterial diseases. Micropropagation provides a rapid method for production of pathogen free plants. In case of viral diseases especially, the apical meristems of infected plants are free or carry very low concentrations of viruses. Thus culturing meristem tips provides disease free plants.

Micro-propagation Methods: The ability of mature cell to dedifferentiate into callus tissue and the technique of cloning isolated single cell *in vitro* discussed earlier in this chapter have demonstrated that the somatic cells can differentiate to a whole plant under particular conditions. This potential of cell to divide and develop into multicellular plant is termed as **cellular totipotency**. To express totipotency, after **dedifferentiation**, the cell has to undergo **redifferentiation** or regeneration which is the ability of dedifferentiated cell to form plant or plant organs. This may occur through either of two processes:

- Organogenesis
- Embryogenesis

(i)Organogenesis is a process involving redifferentiation of meristematic cells present in callus into shoot buds. These shoot buds are monopolar structures which in turn give rise to leaf primordial and the apical meristem. The buds have procambial strands connected with preexisting vascular tissue present in the explant or callus. The stimulation of shoot bud differentiation in plants depends on many factors which differ for different plant species. In general, it is promoted by cytokinin and inhibited by auxins. The classical studies of Skoog and

Miller (1957) demonstrated that the relative ratio of CK and auxin is important in determining nature of organogenesis in tobacco pith tissue. In tobacco, high level of CK initiates bud formation while high concentration of auxin favours rooting. But there have been studies in other plant species which donot follow this concept of auxin/CK ratio. In most cereals, callus tissue exhibits organogenesis when it is subcultured from a medium containing 2,4-D to a medium whre 2,4-D is replaced by IAA or NAA. GA_3, which in general has inhibitory effect on shoot buds whereas many species show enhanced shoot regeneration due to abscissic acid. The variable responses of different plant species to the growth regulators is because the requirement of exogenous GRs depends on their endogenous levels which might differ in different plant species and also in different plant materials.

Other factors affecting organnogenesis are size and souce of the explant. The larger the explant (containing parenchyma, cambium and vascular tissue), more is likelihood of shoot bud formation. Also, genotype of explant affects shoot regeneration as explant taken from different plant varieties of same species show different frequencies of shoot bud differentiation. Light has been shown to have inhibitory effect. Even the quality of light has effect as blue light has been shown to induce shoot formation and root by red light in tobacco. The optimum temperature required may vary with plant species.

(ii)Somatic embryogenesis*:* is a process involving redifferentiation of meristematic cells into nonzygotic somatic embryo which are capable of germinating to form complete plants. Somatic embryos are bipolar structures with radical and plumule is contrast to monopolar shoot bud with only plumular end in organogenesis. While developing into somatic embryo, the mersitematic cells break any cytoplasmic or vascular connections with other cells around it and become isolated. Therefore, unlike shoot bud, the somatic embryos are easily separable from explants.

Somatic embryogenesis involves three distinct steps which are absent in organogenesis:

- **Induction:** is the initiative phase where cells of callus are induced to divide and differentiate into groups of meristematic cells called **embryogenic clumps** (ECs). These ECs develop into initial stages of somatic embryo i.e. globular stage.
- **Maturation: I**n this phase somatic embryos develop into mature embryos by differentiating from globular to heart shaped, torpedo to cotyledonary stages. The mature embryo here undergoes biochemical changes to acquire hardiness.
- **Conversion:** Embryos germinate to produce seedlings.

Somatic embryogenesis is influenced by following factors**:**

- **Growth regulators:** The presence of auxin (generally 2,4-D) in the medium is essential for induction phase. 2,4-D induces dedifferentiation of explant cells to form ECs. When auxin is removed or its concentration is reduced, ECs convert to somatic embryos. Once induced, cells don't need PGRs. Still some doses of CK at maturation and conversion make better plants. Maturation is achieved by culturing somatic embryos on high sucrose medium. Also, ABA is added as it gives hardening due to water loss which is important for embryo maturation.

  Ethylene inhibits both somatic embryogenesis and organogenesis. Therefore, silver nitrate is added to the medium as inhibitor of ethylene for plant regeneration.

- **Nitrogen source:** NH_4^+ form of nitrogen is essential for induction of somatic embryogenesis while NO_3^- form is required during maturation phase.
- **Other factors:** Like shoot bud differentiation, explant genotype has influence on somatic embryogenesis also. In cereals, use of maltose as carbohydrate source promotes both somatic embryo induction and maturation.

Anther Culture

Culturing anther on a suitable media to regenerate into haploid plants is called anther culture. First time, haploid plants were discovered in *Datura stramonium* by A.D. Bergner in 1921. Guha and Maheshwari (1964) pioneered the formation of embryos from anthers of *Datura innoxia* grown *in vitro.* After this, haploid plants have been produced via anther culture in more than 170 species. The anther culture technique is useful in haploid production.

Haploid production: Haploid plant is defined as a sporophyte with gametophytic chromosome number. The *in vitro* production of haploid plants can be achieved by many techniques like:

- **Delayed pollination** which may not result in fertilization and hence only female genome grows up to form a haploid plant.
- **Temperature shock –** Extremes of temperature (both high and low) are used to suppress syngamy or make pollen inactive, thus leading to induction of haploidy.
- **Irradiation effect -** X rays, UV rays induce chromosomal breakage in pollen cells thus making them sterile which in turn results in haploid production.
- **Chemical treatment –** Treatment with colchicines, maleic hydrazide and toluene blue etc also induces chromosomal elimination.
- **Genome elimination by distant hybridization –** In case of distant crosses like inter-generic and inter-specific crosses where during the developmental process, one of the parental genomes is selectively eliminated subsequently leading to formation of haploid plants.

Therefore, production of a haploid plant where egg cell is inactivated and only male genome is present is called **androgenesis**. Similarly, production of haploid by development of unfertilized egg cell due to inactivation of pollen is called **gynogenesis.**

Among all the methods illustrated above, anther culture is the most popular and successful for haploid production.

Anther culture procedure:

Step1

Experimental material: Young healthy plants grown under controlled conditions are used as experimental material from which flower bud of right stage (varies with species) is excised.

Step2

Disinfestation, excision and culture of anther: Flower buds are surface sterilized in laminar flow chamber followed by excision of anther from the bud. Stage of pollen

development is determined by squashing an anther in acetocarmine and observing it under microscope. While excising anthers from flower buds, care is taken that anthers are not injured as injury leads to callusing hence giving mixture of diploids, haploids and aneuploids.

Step3
Culture medium conditions: The anthers are generally cultured on a solid agar medium where they develop into embryoids for anther culture under alternate light and dark period. Medium should have sucrose for induction of embryogenesis.

Step 4
Haploid plants: In species following direct **androgenesis** i.e. which develop through embryoid formation, small plants emerge in 3-8 weeks after culturing which are then transferred on to a rooting medium with low salt and small amount of auxin. Those species undergoing indirect androgenesis involving callus formation, callus is removed from the anther and placed onto a regeneration medium with suitable ratio of cytokinin to auxin. The haploid plants thus produced in both cases are transplanted to soil in small pots and maintained under controlled conditions in greenhouse.

Diploidisation of haploid plants: Haploid plants produced from anther culture maintained *in vitro* can grow till the flowering stage but cannot be perpetuated. Since these plants are haploid and have only one set of homologous chromosomes of the diploid species, they cannot form viable gametes and hence no seed setting takes place for further perpetuation. Therefore, it is necessary to double the chromosome number of haploids to obtain homozygous diploids or dihaploid plants followed by their transfer to culture medium for further growth.

Application of haploid production: Diploidisation of haploid plants result in rapid achievement of homozygous traits in doubled haplouds, hence these anther derived haploid plants have been used in breeding and improvement of crop species.

1. **Production of homozygous lines:** The most important use of haploids is the production of homozygous lines which may be used directly as cultivars or may be used in breeding programme. For e.g. doubled haploids have been used for rapid development of inbred lines in hybrid maize programme.

The anthers from F_1 hybrids of selected or desirable cross are excellent breeding material for raising anther derived homozygous plants or doubled haploids in which complementary parental characteristics are combined in one generation. The doubled haploid plants are subjected to selection for superior plants (**F**ig.2). This approach is described as **hybrid sorting** where recombinant superior gametes are virtually being selected since the heterozygous gene combination in the F_1 hybrid is transformed into homozygous combinations. Hybrid sorting reduces the time required for haploid breeding by 4-5 years as in conventional breeding by pedigree/ bulk method, the same requires ten years. Also, selection among DH lines reduces the size of breeding population.

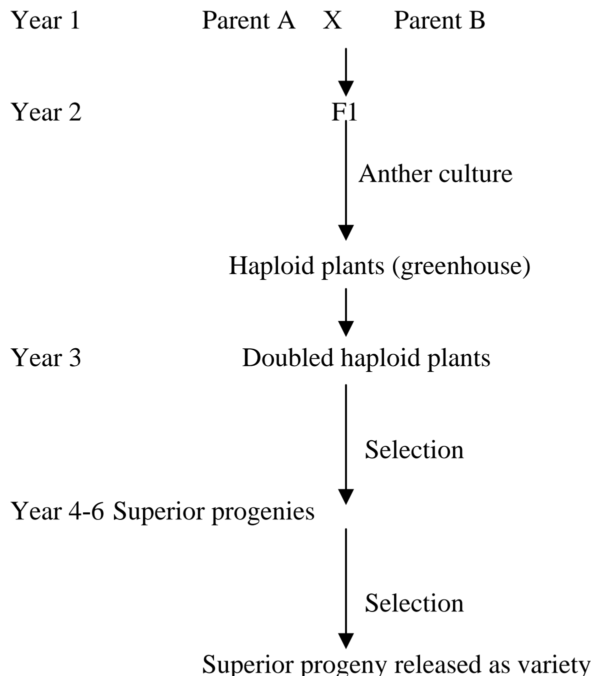

Fig. 2: Anther culture derived haploid plants for hybrid sorting

2. **Gametoclonal variation:** The variation observed among haploid plants having gametic chromosome number developing from anther culture is called gametoclonal variation. Such variations resulting in desirable traits are subjected to selection at haploid level followed by diploidisation to get homozygous plants which can be released as new varieties.
3. **Selection of desirable mutants:** Haploids offer a system where even recessive mutations are expressed unlike diploids where they express only in segregrating single plant progeny in M2 generation. Therefore, in several crops desirable mutants including traits like resistance to diseases, antibiotics, salts etc have been isolated from haploids derived from anther culture. For e.g. tobacco mutants resistant to black shank disease and wheat lines resistant to scab (*Fusarium graminearum*) have been selected and used as improved cultivars.

Problems associated with haploid plants:

- Many species are not yet amenable for haploid production
- Deleterious mutations may be induced during *in vitro* phase.
- Plants having more or lesser than gametic chromosome number is also obtained which necessitates cytological analysis first.
- Occurrence of gametoclonal variation limits the use of anther derived embryos for genetic transformation

The major **advantages of cell culture systems** over the conventional cultivation of whole plants are**:**

- Higher and quicker yields of product from very small amount of plant material needed to initiate the culture in contrast to large amounts of mature plant tissues processed to achieve

low yields of final product, for e.g. the dry weight of shikonin produced from cell culture is 20% more than from plants.

- In case of plant material facing threat of extinction or are limited in supply like *L erythrorhizon*, *in vitro* production of secondary metabolites is saving option.
- Controlled environmental conditions in cell culture ensure continuous supply of metabolites. In conventional system, source plant may be seasonal, location specific and also subject to environmental degradation. Also, *in vitro* culture of cells is more economical for those plants which take long to achieve maturity.
- **Bioconversion:** Low cost precursors are supplied as substrates to cell cultures for conversion to the high cost final product, thus minimizing labor, cost and time. Also, specific substrates can be biotransformed to more valuable product by single step reaction *in vitro.*
- **Production of novel compounds:** Mutants cell lines can be utilized to produce novel compounds which were not previously found naturally in plants *viz.*cell suspension cultures of *Rauwolfia serpentina* produce novel glucosides of ajmaline (alkaloid).

Production of Secondary Metabolites

Plants are the source of a large variety of biochemicals which are produced as both primary and secondary metabolites. Primary metabolites include nucleic acids, proteins, carbohydrates and fats which along with their intermediates function for survival of cell and organism.. Compounds like alkaloids, nonprotein aminoacids, terpenoids and phenolics are grouped under secondary metabolites which donot participate in vital metabolic function of cell. Primary metabolites essentially provide the basis for growth and reproduction, while secondary metabolites for adaptation and interaction with the environment. As secondary metabolites provide industrially important natural products like colour, insecticides, antimicrobials and fragrances, therapeutics etc, they are of great economic importance. Therefore, plant tissue culture is being potentially used as an alternative to plants for production of secondary metabolites. The first large scale production was successfully done for shikonin produced from *Lithospermum erythrorhizon.* It is used as antiseptic and as dye for cosmetics. Since then many valuable secondary metabolites like taxol, berberine etc. have been obtained using tissue culture.

Increasing productivity of secondary metabolites by cell cultures: In plants, most of the secondary metabolites are produced in differentiated cells or organized tissues. However, callus and cell suspension culture lack organ differentiation and hence produce low yields of these biochemicals. The yield of secondary metabolite by undifferentiated tissue or cell cultures can be increased by following techniques:-

- **Select proper cell line**: The heterogeneity within the cell population can be screened to select lines capable of accumulating higher level of metabolite.
- **Medium manipulation**: The constituents of culture medium like nutrients, phytohormones also the culture condition like temperature, light etc influence the production of metabolites.For e.g. if sucrose concentration is increased from 3% to 5%, the production of rosamarinic acid is increased by five times. In case of shikonin production, IAA enhances the yield whereas 2,4-D and NAA are inhibitory.

- **Elicitors**: Compounds that induce the production and accumulation of secondary metabolite in plants are known as elicitors. Elicitors produced within the plant cells include cell wall derived polysaccharides like pectin, pectic acid, cellulose etc. Product accumulation also occurs under stress caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, high salt concentration grouped under abiotic elicitors. These elicitors when added to medium in low concentration (50-250ng/l) enhance metabolite production.
- **Permeabilisation**: Secondary metabolites produced in cell are blocked in the vacuole. By manipulating the permeability of cell membrane, they can be elicited out to media. Permeabilisation can be achieved by electric pulse, UV, pressure, sonication, heat. Even charcoal is added to medium to absorb secondary metabolites.
- **Immobilisation**: Cell cultures encapsulated in agarose and Calcium alginate gels or entrapped in membranes are called **immobilised plant cell culture**. Here cell to cell contact is better while cells are also protected from high shear stresses. These immobilized systems effectively increase the productivity of secondary metabolites in number of species. Elicitors can also be added to these systems to stimulate secondary metabolism.

Limitations

- High production cost is involved.
- Lack of knowledge of biosynthetic pathways of many compounds is major bottleneck in improvement of their production.
- Cultured plant cells are often unable to produce high value compounds.

Protoplast Isolation and Fusion

A fundamental difference between plant and animal cells is that ‘plant cells are totipotent’ which formed the basis of plant tissue culture. Another important difference is that ‘presence of cell wall in plants’ paved way for the most significant development in the field of plant tissue culture which is isolation, culture and fusion of protoplasts. **Plant cells from which cell wall has been removed are termed protoplasts.** Figure 3 shows the microscopic view of protoplasts isolated from banana leaves. Cultured protoplasts besides being used for genetic recombination through somatic cell fusion can also be used for taking up foreign DNA, cell organelles, bacteria and virus particles. Therefore, protoplast culture has achieved important status in plant biotechnology.

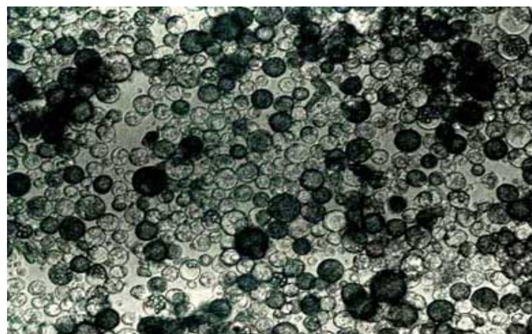

Fig. 3: Microscopic view of isolated protoplasts from banana

Isolation of Protoplast: Plant protoplasts were first isolated by Klercker in 1892 from onion bulb scales plasmolysed in hypertonic solution. This mechanical procedure gave low yield of protoplasts and could be utilized for only highly vacuolated and non meristematic cells. It was in 1960 when Cocking demonstrated the isolation of intact protoplasts by use of cell wall degrading enzyme, cellulase prepared from the fungus, *Myrothecium verrucaria*. By 1968, commercial preparations of purified cell wall degrading enzymes such as macerozyme, cellulase and hemicellulose became available that gave further progress to enzymatic isolation of protoplasts. Enzymatic method of protoplast isolation can be classified into two groups:

1. **Sequential enzymatic:** This involves two steps where first macerated plant tissues are incubated with pectinase to get single cells followed by cellulase treatment to get protoplast.
2. **Mixed enzymatic:** This involves simultaneous separation of cells and degradation of their walls to convert protoplast by immersing plant tissues in mixture of pectinases and cellulases.

Protoplasts have been isolated from all plant parts like shoot tips, cotyledons, flower petals, however, leaf mesophyll tissue is widely preferred for its high reproducible potential for regeneration. Generally, the youngest, fully expanded leaves from young plants or seedlings are used as source of protoplast. Preconditioning plants in darkness or cold (4-10°C) for 24-72h before protoplast isolation improves protoplast yield. The leaves are surface sterilized and cut into small pieces and floated on an osmotically adjusted solution at 20-25°C for 1-24h. During this step, plasmodesmatal complex is broken down, water moves out of cell which causes the cell contents to shrink and draw away from the cell wall. This allows cells to retain their integrity after the cell wall is removed otherwise they start fusing. This step is followed by incubation of leaf pieces with digestive enzymes in darkness on shaker (30-50rpm). Incubation time and temperature varies with species and time. The osmotic concentration of enzyme mixture and of subsequent media is elevated by adding sorbitol or mannitol to stabilize protoplasts or they will burst. Addition of 50-100mM/l $CaCl_2$ improves stability of plasma membrane. At acidic pH in the range of 5.0-6.0 is optimal. The buffer often contains phosphate at 3mM to minimize shifts in pH during digestion. In case of monocots, where mesophyll cannot be the source, cell suspension cultures are used for isolation of protoplasts. The released protoplasts are separated by centrifugation at low speed for about ten minutes followed by washing two to three times before transfer to culture medium to remove enzymes and debris.

Protoplast viability and Plating Density: Before culturing protoplast, their viability is estimated by staining with Fluorescein diacetate (FDA). Viable protoplasts exhibit green fluorescence under UV. It should be examined within 5-15 min after the FDA treatment after FDA dissociates.

Some protoplasts have leaky membrane through which metabolites are leached out. If sufficient density of protoplasts is there, metabolites can be absorbed back. Therefore, a minimum plating density is required for growth to begin and it is estimated by haemocytometer. For genetic engineering, single or fewer protoplast culture is required for which either conditional media or feeder layer are used. Conditional media is one where plant cells were already grown and so has metabolites leached into it. After filtering, this media is used for growing isolated protoplasts. Feeder layer is prepared by plating solid media with protoplasts followed by irradiation which

inactivates the nucleus but protoplasts are viable. Protoplasts at lower densty can now be plated on this feeder layer.

Protoplast Culture: Freshly isolated protoplasts are spherical because they are unbound by cell wall. Viable protoplasts regenerate a new cell wall within 48 to 96 h after isolation which can be determined by staining with calcafluor white. Protoplasts with new cell wall fluoresce bluish white under UV. Protoplasts that fail to regenerate a wall generally will not divide and die eventually. Also, all the healthy protoplasts may not divide and therefore, plating efficiency is calculated to estimate cell vigor. Plating efficiency is number of dividing protoplasts/total number of protoplasts plated. The protoplasts capable of dividing undergo first division within 2-7 days after isolation. The delicate nature of protoplasts demand modifications in MS and B5 media or any other culture medium used for organ regeneration from explants. Besides higher osmotica, the inorganic salt concentration is adjusted (Ammonium nitrate concentration is lowered and calcium level is increased), more of organic components, vitamins and PGRs are added to hasten and promote cell wall synthesis. Due to sensitivity to light, protoplasts are cultured in diffuse light for initial 4-7 days. After early culture, when protoplasts have regenerated new cell wall and divided, they are transferred back to normal medium where plants regenerate by shoot formation or somatic embryogenesis. Plant regeneration from protoplasts has been reported from alfalfa, tobacco, carrot, tomato etc but cereals still pose problems.

Protoplast Fusion and Somatic Hybridization: Purified protoplasts once obtained from any two different sources (can be different tissues, different plants or species or different genera), they can be fused together to form somatic hybrids. This non-conventional method of genetic recombination involving protoplast fusion under *in vitro* conditions and subsequent development of their product to a hybrid plant is known as somatic hybridization. First, somatic hybrid plant of *Nicotiana glauca* (+) *N. langsdorfii* was reported by Carlson in 1972.

Protoplasts can be induced to fuse by variety of fusogens or electrical manipulations which induce membrane instability. Most commonly reported **fusion inducing agents** are sodium nitrate (used by Carlson), high pH/Ca^{2+} concentration and Polyethylene glycol (PEG) treatment. Sodium nitrate treatment results in low frequency of heterokaryon formation, high pH and high Ca^{2+} concentration suits few plant species whereas PEG is the most favoured fusogen for its reproducible high frequency of heterokaryon formation and low toxicity. However, treatment with PEG in presence of high pH/ Ca^{2+} is reported to be most effective in enhancing heterokaryon formation and their survivability.

A more selective, simpler, quick and non toxic approach is **electrofusion** which utilizes electric shock or short pulse of high voltage to promote membrane fusion between two cells. Many useful somatic hybrid plants produced by electrofusion have been reported like *Nicotiana plumbaginifolia* (+) *N tobacum*, *Solanum tuberosum* (+) *S. charcoense* (resistant to Colorado potato beetle).

Somatic Hybrids

Protoplast population following fusion treatment is heterogeneous mixture of unfused parents, homokaryons, heterokaryons, fused protoplasts with independent two nuclei etc thus necessitating selection of stable hybrids. Therefore, some identification and selection system

should be incorporated into each parental cell line before fusion. Usually, two parental cell lines with differing requirements as selective screens make selection of hybrid convenient as only those fused cells that possess complementary traits of both parents will thrive. Resistance to antibiotics, herbicides and ability to grow on specific amino acid analogs are few of the selection methods used. Besides these, two different vital stains which donot affect viability of cells like fluorescein isothiocynate (FITC) and rhodomine isothiocyanate (RITC) have been successfully used. Under fluorescent microscope, FITC stained cells appear green and RITC stained protoplasts appear red whereas fused cells having both RITC and FITC fluoresce yellow.

Application of Somatic Hybridization:

- **Genetic recombination in asexual or sterile plants:** Protoplast fusion has overcome the impediment of reproduction in haploid, triploid and aneuploid plants. Also, genomes of asexually reproducing plants can be recombined using this approach *viz.*protoplasts isolated from dihaploid potato clones have been fused with protoplasts of *S. brevidens* to produce hybrids of practical breeding value.
- **Genetic recombination between sexually incompatible species:** The incompatibility barriers in sexual recombination at interspecific or intergeneric levels are also overcome by somatic hybrisation. Generally, somatic hybrids are used for transfer of useful genes such as disease resistance, abiotic stress resistance or genes of industrial use for e.g. Datura hybrids (*D. innoxia + D discolor*, *D. innoxia + D stramonium*) show heterosis for scopolamine (alkaloid) content which is 20-25% higher than in parent species and therefore has industrial application.
- **Cytoplasm transfer:** Somatic hybridization minimizes the time taken for cytoplasm transfer to one year from 6-7 years required in back cross method. Also, this method allows cytoplasm transfer between sexually incompatible species. Cybrids have cytoplasm from both parents but nucleus of only one. Nucleus of other parent is irradiated. This approach has been potentially used to transfer two desirable traits – cytoplasmic male sterility (CMS) and resistance to atrazine herbicide, both coded by cytoplasmic genes in Brassica to different crops like tobacco, rice etc.

Somaclonal Variation

The genetic variability present in somatic cells, plants or plant progenies derived from cells/tissue cultured in vitro is called somaclonal variation. Larkin and Scrowcroft (1981) coined this term for all plant variants derived from any form of cell or tissue culture. Some variants are obtained in homozygous condition in the plants regenerated from cells cultured *in vitro* (R_0 generation) but mostly variants are recovered in the selfed progeny of tissue culture regenerated plants (R_1 generation).

This variation includes aneuploids, sterile plants and morphological variants, some of which may involve traits of economic importance for crop plants. Somaclonal variation may be genetic or epigenetic. Since only gametic variation follow Mendelian inheritance pattern and transmitted to next generation, they are important for crop improvement. Therefore, in several crops, R_0, R_1 and R_2 progenies are analysed for transmission of variant trait to sexual progeny (R1) and 3:1 segregation leading to isolation of true breeding variants (R_2).

The significance of somaclonal variation in crop improvement was first demonstrated in sugarcane and potato where few somaclones with disease resistance against Fiji, downy mildew (sugarcane) and late and early blight in case of potato were recovered.

Major causes of Somaclonal Variation:

- **Physiological:** Variations induced by physiological factors in culture medium for e.g. prolonged exposure to PGRs (2,4-D; 2,4,5-T) results in variability among the regenerants. Often such variations are epigenetic and hence donot follow Mendelian inheritance.
- **Genetic:** All the alterations at chromosomal level are grouped under genetic cause of variation observed in regenerants. Chromosomal rearrangements such as deletion, duplication, translocation, inversion polyploidy, aneuploidy, have been reported to be the chief source of somaclonal variation. Meiotic crossing over involving symmetric and asymmetric recombination could also be responsible for variation observed among somaclones. Transposable elements like Ac-Ds in maize have been shown to get activated in *in vitro* culture. In maize (*Zea mays* L.) and broad beans (*Vicia faba* L), late replicating heterochromatin is the main cause of somaclonal variation. Single gene mutations in cultures also give rise to variations which are not detected in plants regenerated *in vitro* from any cell or tissue (R_0 plants) but express in R_1 plants (after selfing R_0 plants).
- **Biochemical:** The most common kind of biochemical variation is change in carbon metabolism leading to failure of photosynthesis *viz.* albinos is rice. Any variation in other cell processes like starch biosynthesis, carotenoid pathway, nitrogen metabolism, antibiotic resistance etc also lead to somaclonal varaiation.

Application in Crop Improvement: Somaclonal variation represent useful source for introduction of valuable variations to plant breeders. Cell culture systems are well defined controlled environments, away from limitations of availability of space, time and variations due to environmental effects which are major bottlenecks in conventional mutation breeding. Somaclonal variation occurs at much higher frequencies than induced mutants which are associated with undesirable features. Cell culture systems allow plant breeder to have greater control on selection process as here they have the option to select from large amount of genetically uniform material. Therefore, this is the only approach for genetic improvement in perennial species limited by narrow germplasm and long regeneration cycle, asexually propagated plants like bananas, for isolation of biochemical mutants like auxotrophs. Somaclonal variants have been isolated for variety of valuable traits like disease resistance, stress (salt, low temperature) resistance, improved yield and efficient nutrient uptake etc. Bio-13 is a somaclonal variant of *Citronella java,* a medicinal plant which yields 37% more oil and 39% more citronellol than the control varieties. Pusa Jai Kisan, with bolder seeds and higher yield developed at Indian Agricultural Research Institute is another successful application of somaclonal variation of *Brassica juncea* variety 'Varuna'. Somaclonal variants in Lathyrus with low toxin level have also been developed at Indian Agricultural Research Institute.

Germplasm Conservation

The genetic material especially its molecular and chemical constitution that is inherited and transmitted from one generation to other is referred to as germplasm. In other words, the sum total of all the genes present in a crop and its related species constitutes its germplasm. It is

generally represented by a collection of various strains and species. Germplasm is valuable because it contains diversity of genotypes that is needed to develop new and improved genetic stocks, varieties and hybrids. Therefore, germplasm is the basic indispensable ingredient of all breeding programmes and great emphasis is placed on collection, evaluation and conservation of germplasm. The continuing search for high yielding varieties of crop plants with resistance to biotic and abiotic stresses necessitates the conservation of germplasm of different crops and their wild and weedy relatives.

A) *In- situ* conservation: *In situ* (on-site) conservation refers to the maintenance and use of wild plant populations in the habitats where they naturally occur and have evolved without the help of human beings. The wild populations regenerate naturally and are also dispersed naturally by wild animals, winds and in water courses. There exists an intricate relationship, often interdependence, between the different species and other components of the environment (such as their pests and diseases) in which they occur. The evolution is purely driven by environmental pressures and any changes in one component affect the other. Provided that changes are not too drastic, this dynamic co-evolution leads to greater diversity and better adapted germplasm. The conservation of the forests and other wild plant species is often carried out through protected areas such as national parks, gene sanctuary and nature reserves. However, this mode of conservation has certain limitations such as there is risk of loss of material due to environmental hazards.

(B) *Ex-situ* conservation: Ex situ (off-site) conservation of germplasm takes place outside the natural habitat or outside the production system, in facilities specifically created for this purpose. This is the chief mode of preservation of genetic resources for both cultivated and wild material. The most convenient method of ex-situ germplasm conservation is in the form of seeds. Thus, majority of field crops and vegetables which produce **orthodox** (dessication tolerant) seeds are conserved in **gene banks** by reducing their moisture content (3-7%) and storing under low humidity and low temperature.

In case of crops with dessication sensitive or **recalcitrant** seeds (which lose their viability after being dried below a critical limit) and also in vegetatively propagated crops, ***in vitro*** methods are the most useful for germplasm conservation. This tissue culture based method has been mainly utilized for conservation of somaclonal and gametoclonal variations in cultures, plant material from endangered sp., plants of medicinal value, storage of pollen, storage of meristem culture for production of disease free plants and genetically engineered materials.

***In vitro* Germplasm conservation:** Germplasm can be stored in vitro in variety of forms including isolated protoplasts, cells from suspension or callus cultures, meristem tips, somatic embryos, shoot tips or propagules at various stages of development. Methods for *in vitro* germplasm conservation are classified into two groups based on culture growth:-

1. **Slow growth cultures:** where limited growth of culture is allowed. This is a simple, effective and economic method and can be used in all species where shoot tip/ nodal explant are available. In thses techniques, growth is suspended by either cold storage or lowering oxygen concentration. Such methods require serial subculturing for periodic renewal of cultures. The storage of germplasm by repeated cultures has some disadvantages like during subculturing there is risk of contamination by pathogen, genetic changes may also occur.

2. **Cryopreservation:** Any growth in plant cell and tissue culture is brought to a halt still retaining its viability in this technique by storing at ultra low temperature (-196°C) using liquid nitrogen. This method, also called freezepreservation, is most popular and effective for indefinite storage. Cryopreservation for germplasm purposes utilizes shoot tips and buds only but protoplasts, cells, tissues and somatic embryos are also cryopreserved for other tissue culture processes.

Factors affecting viability of cells frozen for cryopreservation:

- **Physiological state of material:** Cells in the late lag or exponential phase are considered ideal for freeze preservation. After thawing, these cytoplasm rich cells are able to retain their viability and grow again from the actively dividing meristematic cell component. But in shoot tips, embryos etc, tissue is large with highly vacuolated cells which get damaged by freezing and are unable to recover back.

- **Prefreezing treatment:** Conditioning treatment given to cells before freezing results in their hardening and increased survival rates. Such hardening treatments include growing culture in presence of **cryoprotectant** or growing at low temperature (4°C) (for cold dormant sp) or in presence of osmotic agents like sucrose. These treatments function by either changing the cell water content, metabolite content or membrane permeabilites.

- **Cryoprotectants:** are chemicals imparting protection to withstand low temperature. For plants, most frequently used cryoprotectant is Dimethyl sulphoxide (DMSO). About 5-10% of DMSO is prepared and added gradually to prevent plasmolysis of the cells. Other commonly used cryoprotectants include glycerol, polyvinyl pyrollidone, polyethylene glycol (PEG) etc.

- **Thawing rate and reculture:** For better survival of preserved samples, rapid thawing from -196°C to about 22°C is recommended. By thawing rapidly, the damaging effects of ice crystal formation (crystallization of cell water while freezing) are minimized. These thawed samples during reculturing require special growth conditions, for enhanced recovery rates like dim light, high osmoticum, gibbrellic acid, and activated charcoal in the medium.

Methods of Cryopreservation: The sensitivity of cells to low temperature varies with the species. However, usually the sample to be preserved are treated with suitable cryoprotectant and then frozen by any one of the following methods:

- **Rapid freezing**: The vials with plant materials are directly dipped in liquid nitrogen. The temperature lowers very fast at the rate of 200°C/minute. It is a very hard treatment and hence survival rate is low. However, this method has been successful for germplasm conservation of large number of species where plant material with small size and low water content has been chosen.

- **Controlled freezing**: The plant material is cooled stepwise from room temperature to intermediate temperature (-20°C) maintained at that temperature for thirty minutes followed with rapid freezing by dipping into liquid nitrogen. This is a reliable method and is applicable to wide range of plant materials including shoot apices, buds and suspension cultures.

Advantages of Cryopreservation:

- Indefinite preservation as metabolism comes to halt

- Low maintenance as only liquid nitrogen needs to be replenished
- No contamination
- Applicable to all species amenable to tissue culture

Limitations:

- Sophisticated equipment and facilities required
- Expertise needed
- Cells/tissues get damaged due to ice crystal formation or high solute concentration during dessication.

Genetic Engineering and Gene technology

Since the 1970s, there have been considerable developments in the methods and techniques used to study biological processes at the molecular level. The discovery of restriction enzymes, transformation technique within this period were breakthrough developments which allowed scientists to cut, splice and alter DNA, the molecule that carries the blueprints for life. This further led to isolation of single DNA sequence from one organism by breakage of DNA molecule at two desirable places and then inserts it at a desired position in another DNA molecule from completely different organism to form recombinant DNA and the technique involved is called **recombinant DNA technique**. This has also been termed as **genetic engineering** because of the potential for creating novel genetic combinations. Using this technique, single copy of a gene or DNA can be isolated and cloned into indefinite number of identical copies and this is known as gene cloning.

The basic steps involved in **recombinant DNA technique** can be outlined as follows:

1. Isolation of desired DNA fragment or gene of interest
2. Insertion of the isolated gene in a s suitable vector.
3. Introduction of this recombinant molecule into host cell by transformation
4. Selection of transformed host cells
5. Multiplication and expression of introduced gene in the host

To explain these steps of gene cloning, understanding of a few basic techniques is essential which are discussed below.

Gel Electrophoresis: Gel electrophoresis is a technique used to separate molecules based on physical characteristics such as size, shape, or isoelectric point. A gel is composed of agarose or polyacrylamide. Agarose is used for separating DNA fragments in size range of 100bp to 20kb. Polyacrylamide is preferred for smaller DNA fragments. Separation is achieved by moving the negatively charged nucleic acid molecules through agarose matrix with an electric field. Rate of migration of DNA molecules is inversely proportional to their molecular weight. Smaller molecules move faster than larger ones (Fig 4). But conformation of the DNA molecule is also an important factor. Conformations of a DNA plasmid that has not been cut with a restriction enzyme will move with different speeds such as: supercoiled plasmid DNA will move fastest followed by linearised DNA, while open circular DNA will move at the slowest pace. To visualise DNA, gels are stained with ethidium bromide which intercalates in DNA and fluorescse under UV.

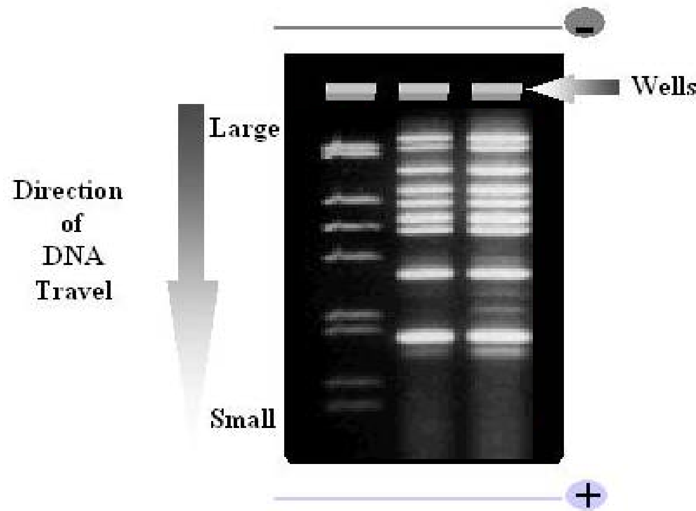

Fig. 4: Agarose gel electrophoresis of DNA
(ethidium bromide stained gel showing migration of DNA molecules)

Restriction enzymes: Restriction endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain. The chemical bonds that the enzymes cleave can be reformed by other enzymes known as ligases, so that restriction fragments generated from different genes can be joined together These enzymes cleave DNA at specific sites and their recognition sequences or have two fold axis of rotational symmetry or are palindrmic (i.e. the sequence on one strand reads the same in the same direction on the complementary strand).

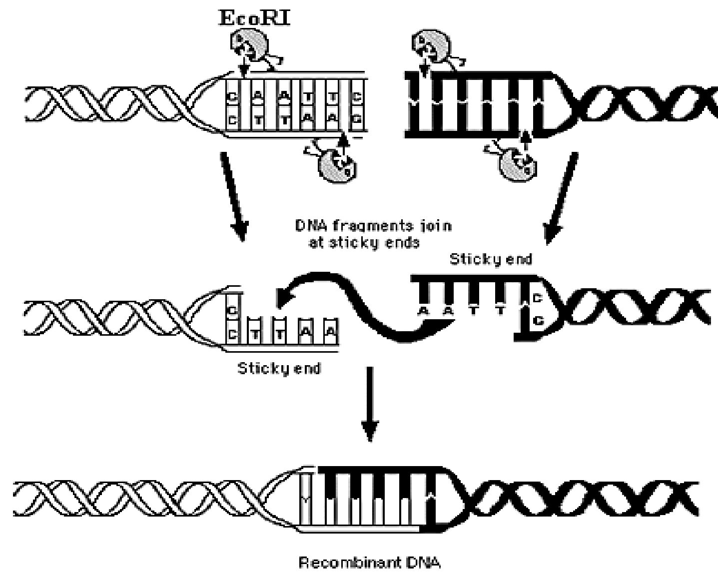

Fig. 5: Mechanism of restriction with *Eco*RI endonuclease

Fig. 5 explains the action of EcoRI restriction enzyme. These enzymes are often used in genetic engineering to make recombinant DNA for introduction into bacterial, plant, or animal cells.

Polymerase chain reaction: Polymerase chain reaction is an extremely versatile technique for copying DNA. In brief, PCR allows a single DNA sequence to be copied to millions of times in predetermined ways. It usually consists of a series of 20 to 35 cycles. Commonly, PCR is carried out in three steps such as **denaturation**, **primer annealing** followed by **primer extension.**

1. Prior to the first cycle, during an initialization step, the PCR reaction is often heated to a temperature of 94-96°C to ensure that most of the DNA template and primers are denatured. Also, some PCR polymerases require this step for activation. Following this hold, cycling begins, with denaturation step at 94-96°C for 20-30 seconds.
2. The denaturation is followed by the annealing step. In this step the reaction temperature is lowered so that the primers can attach to the single-stranded DNA template. The temperature at this step depends on the T_m of the primers and is usually between 50-64°C for 20-40 seconds.
3. The annealing step is followed by an extension/elongation step during which the DNA polymerase copies the DNA template, starting at the primers annealed to both of its strands. The temperature at this step depends on the DNA polymerase used. Taq polymerase has a temperature optimum of 70-74°C; thus, in most cases, during the extension a temperature of 72°C is used.

PCR can be used for lot of molecular biology experiments such as to introduce restriction enzyme sites, or to mutate (change) particular bases of DNA. PCR can also be used to determine whether a particular DNA fragment is found in a cDNA library. PCR has many variations, like reverse transcription PCR (RT-PCR) for amplification of RNA, and, more recently, real-time PCR (qPCR) which allow for quantitative measurement of DNA or RNA molecules

Southern bloting: It is a method routinely used in molecular biology to check for the presence of a DNA sequence in a DNA sample. Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size-separated DNA to a filter membrane for probe hybridization. The method is named after its inventor, the Edwin Southern. In this method, DNA samples are separated by gel electrophoresis and then transferred to a membrane by blotting via capilliary action. The membrane can then be probed using a labelled DNA complementary to the sequence of interest. Most original protocols used radioactive labels, however now non-radioactive alternatives are available. Southern blotting is less commonly used in laboratory science due to the capacity of using PCR to detect specific DNA sequences from DNA samples. However, these blots are still used for some applications, such as measuring transgene copy number in transgenic plants.

Similarly, **northern blotting** is a technique used in research to study gene expression. It takes its name from the similarity of the procedure to the Southern blot procedure but the key difference is, instead of DNA, RNA is used for blotting and analysis by electrophoresis and detection with a hybridization probe. A **western blotting** is a method to detect protein in a given sample of tissue homogenate or extract. The proteins transferred to membranes are "probed" using antibodies specific to the protein.

DNA sequencing

The term DNA Sequencing encompasses biochemical methods for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide. Currently there are two types of DNA sequencing methods:

1**. Maxam-Gilbert sequencing:**In 1976-1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. The method requires radioactive labelling at one end and purification of the DNA fragment to be sequenced. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T). Thus a series of labelled fragments is generated, from the radiolabelled end to the first 'cut' site in each molecule. The fragments are then size-separated by gel electrophoresis, with the four reactions arranged side by side. To visualize the fragments generated in each reaction, the gel is exposed to X-ray film for autoradiography, yielding an image of a series of dark 'bands' corresponding to the radiolabelled DNA fragments, from which the sequence is inferred.

2. Sanger and Coulson method: The chain-terminator method developed by Sanger was more efficient and rapidly became the method of choice. The Maxam-Gilbert technique requires the use of highly toxic chemicals, and large amounts of radiolabeled DNA, whereas the chain-terminator method uses fewer toxic chemicals and lower amounts of radioactivity. The key principle of the Sanger method was the use of dideoxynucleotides triphosphates (ddNTPs) as DNA chain terminators. The classical chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP). These dideoxynucleotides are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand therefore terminates DNA strand extension, resulting in various DNA fragments of varying length. The dideoxynucleotides are added at lower concentration than the standard deoxynucleotides to allow strand elongation sufficient for sequence analysis.

The newly synthesized and labeled DNA fragments are heat denatured, and separated by size (with a resolution of just one nucleotide), by gel electrophoresis on a denaturing polyacrylamide-urea gel. Each of the four DNA synthesis reactions is run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. The terminal nucleotide base can be identified according to which dideoxynucleotide was added in the reaction giving that band. The relative positions of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence.

Gene cloning

1. **Isolation of the desired gene:** The DNA fragment of interest can be obtained from any of the following:-

a. Genomic library: is a large collection of recombinants in plasmid or phage vector, so that sum total of DNA inserts in this collection represent the entire genome of the concerned organism. The genomic library is prepared by shotgun approach where the total genomic DNA of organism is extracted and subjected to partial digestion by any restriction endonuclease or by sonication. These partial digests are then separated on agarose gel by electrophoresis or by sucrose density gradient centrifugation for selection of appropriate size fragments. The selected fragments are then inserted into phage λ vector or cosmid vector since these vectors can take up DNA inserts of upto 23-25kilobase (kb) pairs. The recombinant molecules are then cloned in a suitable bacterial host thus constructing a genomic library. The clone having the desired DNA insert is identified by screening the library using suitable probe. The probe may be mRNA of the gene, complementary DNA (cDNA) of its mRNA, homologous gene from another organism or synthetic oligonucleotide representing the sequence of a part of the desired gene. Once identified, the desired clones are picked up from the library.

b. cDNA library: A cDNA library is a population of bacterial transformants or phage lysates in which mRNA isolated from tissue or organism is represented as its cDNA inserts. cDNA is complementary DNA produced by reverse transcriptase using mRNA as template. The isolation and identification of desired clone from cDNA library is done by screening in a similar manner to that of genomic library. However, cloned eukaryotic cDNAs have their own special uses since they lack intron sequences that are usually present in the corresponding genomic DNA. Introns are noncoding sequences that often occur within eukaryotic coding sequences (exons) and are sliced out while post transcriptional processing to produce mRNA. Since, bacteria donot possess the enzymes necessary for splicing of intron, eukaryotic cDNA clones become essential when the expression of eukaryotic gene is required in a prokaryote. Also, if the sequence of genomic DNA is known, the intron/exon boundaries can be assigned by comparison with the cDNA sequence.

c. Chemical synthesis of gene: The basic sequence of any gene can be deduced from the nucleotide sequence of mRNA or amino acid sequence of the protein coded by it. The polynucleotide of deduced base sequence can be synthesized chemically using automated DNA synthesizers.

d. Gene amplification through PCR: The polymerase chain reaction technique amplifies single copy of the desired DNA to billion copies in few hours. This PCR based approach is quicker and simpler than library construction and screening and hence preferred to all the aforesaid approaches.

2. **Insertion of the isolated gene in a suitable vector:** Vector is a DNA molecule into which exogenous DNA is integrated for cloning and that has the ability to replicate in a suitable host cell. Vectors are used to assist in the transfer, replication and sometimes expression of a specific DNA sequences in a target cell. Therefore, vector must have the following properties:
   - Vector must have origin of replication to replicate autonomously in the cell population as the host organism grows and divides. Their maintenance should not necessarily require integration into host genome.

- Vector must have unique sites for many restriction enzymes called multicloning site (MCS) into which DNA insert can be cloned without disrupting as essential function.
- Vector must be fairly small, low molecular weight DNA molecules to facilitate their isolation and handling.
- Vector must have some selectable marker that will enable the recombinant vector to be selected from large population of cells that have not taken up foreign DNA.

A variety of vectors have been developed to function as cloning vehicles:

i) Plasmids are self replicating circular duplex DNA molecules which are stably inherited in an extrachromosomal state. Plasmid vectors are used for cloning DNA of small size (upto 12kb). The circular plasmid DNA which is to be used as vector is first cleaved by restriction endonuclease (RE) to give linear DNA molecule. The foreign DNA to be inserted is also cut by same endonuclease foolowed by ligation (joining) of the linearised vector and insert DNA resulting in a bigger circular DNA that can now be separated by gel electrophoresis on the basis of its size. Selection of the chimeric DNA can also be done if insertion of foreign DNA at a endonuclease site inactivates a gene whose phenotype is readily scorable. Usually, the selectable marker is resistance to different antibiotics. The naturally occurring plasmids of *Escherichia coli* have been modified, shortened, reconstructed and recombined to create many different plasmids of enhanced utility as vectors for e.g. pBR322, pUC series(pUC18, pUC19, pUC8 etc)

ii) Bacteriophage vector: are viruses of bacteria thateither infect the cell and lyse them (lytic cycle) or integrate into bacterial chromosome and multiply with it as prophage (lysogenic cycle). They act as cloning vehicles for larger pieces of DNA (23-25kb) as compared to plasmid vectors. Also, screening of recombinants is easier as phages form plaques (a clear zone where lysis has occurred in the bacterial lawn) in contrast to plasmids where bacterial colonies are screened. Most commonly used phage vector are lambda (λ) and M13 phages.

iii) Cosmid vectors: are plasmids which contain a fragment of λ DNA including the *cos* site. Since cos site enables the DNA to get packed in λ particle *in vitro.*Recombinant cosmid DNA is injected and circularized like phage DNA which infects host cells which is more efficient than plasmid transformation. But it replicates as a plasmid without expression of any phage functions. With a cosmid vector of 5kb, large DNA inserts (32-47kb) are selected as the distance between two cos sites must be between 38 and 52kb for packaging. Therefore, cosmids can accommodate upto 45kb long DNA inserts which is much more than a phage vector. Because of their capacity for taking up large fragments of DNA, cosmids are particularly used as vectors for constructing libraries of eukaryotic genome fragments.

iv) Phagemid vector: Those vectors that have origin of replication derived from both a plasmid and phage are known as phagemids. Under normal circumstances, the plasmid *ori* is used for replication but following a phage infection the other ori is used and single stranded DNA is produced which is useful for sequencing. One such plasmid is pBluescript which has its MCS flanked by T3 and T7 promoters, enabling expression of the cloned insert to be obtained regardless of its orientation.

v) Phasmid vector: are plasmid vectors containing λ attachment site (λatt) and functional origins of replication of both plasmid and λ. λatt allows plasmid to insert into a phage λ genome

by means of site specific recombination, responsible for lysogen formation. This is a reversible recombinational insertion which generates phasmids. Phasmid vectors are preferred for the advantage that DNA may be cloned in plasmid vector and recombinant plasmid can be converted to phage which is easier to store, have long shelf life and screening by plaque hybridization gives cleaner results than colony hybridization. M13 is a highly developed phasmid vector being used widely.

With help of enzyme ligase, the isolated gene is inserted into linearized vector DNA, thus producing recombinant DNA as schematically shown in Fig 6**.**

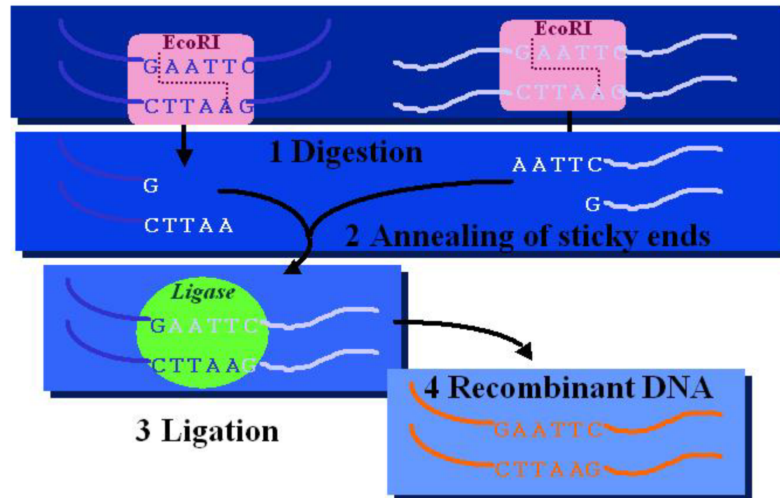

Fig. 6: A schematic diagram showing restriction and ligation to form recombinant DNA

3. **Introduction of the recombinant into suitable host:** The recombinant vector is now introduced into host cell for its multiplication and expression. The host cells are made competent or permeable by either Calcium chloride treatment for **transformation** or electrick shock for **electroporation**. Alternatively, cosmids, phasmids and λ particles are used which then infect *E. coli* cells.

4. **Selection of recombinant clones:** After transformation, there is a mixture of host cells which are not transformed or cells transformed with self ligated vector and transformed cells carrying recombinant vectors. So identification of recombinat transformed cells employs the reporter genes of vector. A **reporter gene** produces a phenotype which permits either easy selection or quick identification of cells in which it is present. For e.g. genes conferring drug resistance or nutritional deficiencies are selectable markers which allow only cells which possess it to survive under selective conditions. On the other hand, for easy identification of recombinants, scorable markers are used which produce phenotypes different from those that do not have them such as *lux* gene codes for luciferase which produces phosphorescence, *gus* gene codes for β-galactourinidase which produces blue colour in presence of substrate X-gluc.

The next step is to identify the clone having DNA of interest from a population of transformed cells with recombinants. This is achieved by nucleic acid hybridization methods by colony or plaque hybridization if a specific probe for the DNA insert is available. In cases where inserted

gene is expressed, clones synthesizing a foreign protein can be detected immunochemically using specific antibody.

Methods for Gene Transfer

The uptake of foreign DNA or the recombinant DNA by cells is called **gene transfer** or transformation. Conventionally, the gene transfer necessary for crop improvement is obtained through sexual and vegetative propagation. However, biotechnological approaches like somaclonal variation, protoplast fusion etc. has successfully speeded up the process of generating genetic variation and introgression of foreign genes. The most potential biotechnological approach for transferring recombinant DNA is based on genetic engineering which involves various techniques for gene transfer discussed ahead. **The transferred gene is called transgene and the plants that carry these stably integrated transgenes are called transgenic plants.** Various gene transfer techniques used are grouped into two broad categories:

- Direct gene transfer
- Agrobacterium mediated

(i) Direct Gene Transfer is a process where no vector is involved and can be applied to any species or genotype. The methods for direct gene transfer are further classified into two classes:- Physical where usually naked DNA is directly transferred .Therefore, also referred to as DNA mediated gene transfer. The various physical methods for gene transfer are-

1) **Electroporation:** is a process where the cells are exposed to electrical impulses of high voltage to reversibly make cell membranes permeable for uptake of DNA. Electroporation has been used extensively for transformation of protoplasts. Recently, transformation of intact plant cells of sugarbeet and rice has been successfully reported. This method is convenient, simple and quick. However, electroporation cannot be applied to all the tissues, cell viability drops due to electric shock. Also, regeneration of plants from protoplasts is still difficult.
2) **Particle bombardment:** This is a relatively recent development but is widely used and is effective in introduction of DNA into plant cells. The technique involves coating 1μm diameter particles of tungsten or gold known as microprojectiles with DNA, which are then accelerated to high speed using a pulse of high pressure helium into an evacuated chamber containing the target tissues. These DNA coated particles penetrate through the cell wall releasing DNA from particles which can express transiently or get integrated into nuclear genome of that cell. With appropriate tissue culture and selection, transgenic plants can be regenerated. Particle bombardment has been used for transformation of monocotyledonous crop plants such as maize, rice, wheat etc.

(ii) Agrobacterium mediated Gene Transfer: form the most successful plant transformation system. This is vector based gene transfer method where the vectors are the Ti plasmids of *Agrobacterium tumefaciens* which is a soil inhabiting bacterium responsible for crown gall disease of many plants. The bacterium infects through a wound in the stem of the plant and a tumor develops at the junction of root and stem. The agent responsible for crown gall is Ti plasmid and not the bacterium itself. During infection, a small portion of Ti plasmid DNA, called T-DNA is transferred to the plant cell nucleus, where it becomes covalently inserted into the nuclear DNA, thus getting stably maintained in the genome of transformed cells. T-DNA

carries the genes responsible for tumor formation and for synthesis of unusual aminoacid derivatives known as opines (most common are octopine and nopaline).These opines are used as sole carbon/nitrogen source for inducing Agrobacterium strain. The genes responsible for transfer of T-DNA called virulence genes (*vir* genes) are also contained on the Ti plasmid. Agrobacterium infection requires wounded plant tissue because vir genes are induced by phenolic compounds released by the injured plant cells.

The regions of T-DNA absolutely required for its transfer and integration into the plant genome are border regions which are short repeat sequences of 25bp. Any DNA sequence inserted between the border repeats will be transferred to and integrated into the plant genome. Therefore, Ti-based plasmids are excellent vectors for introducing foreign genes into plants. In order to use them as vectors, the genes responsible for tumor formation must be removed. Ti-based plasmids lacking tumorigenic functions are known as disarmed vectors. These disarmed vectors are still too large to be conveniently used as vectors. Thus, smaller vectors described below have been constructed that are suitable for manipulation *in vitro.*

Cointegration and binary vector system: The **cointegration** technique is based on *in vivo* recombination of two plasmids. One plasmid carries desirable DNA sequence; the other plasmid contains *vir* genes and the border repeats of T-DNA. The recombination of these plasmids leads to large Ti plasmid which now can be used to transform plants.

The **binary** vector system uses two separate plasmids: mini-Ti plasmid to supply the disarmed T-DNA and second having *vir* genes. The mini-Ti plasmid bears the gene construct that will be inserted into the plant genome, along with a eukaryotic selectable marker between T-DNA border sequences, so that both genes will be inserted as a unit (Fig.7). This plasmid when placed into an Agrobacterium strain containing a plasmid with virulence functions, the *vir* gene products are able to drive the transfer of T-DNA into plant cells, even though T-DNA is located on a separate DNA molecule. This is the most frequently used approach as mini Ti plasmids are very easy to manipulate using standard recombinant DNA techniques.

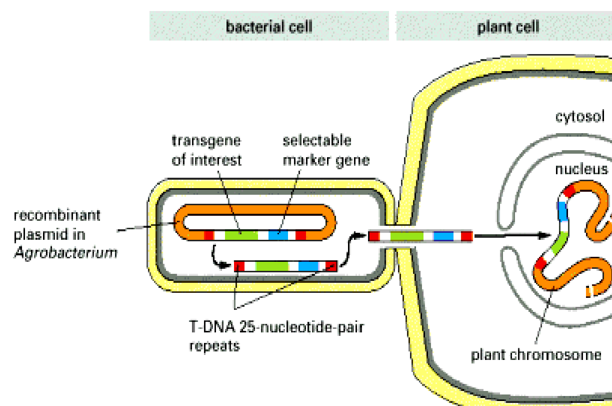

Fig. 7: Process of T DNA transfer from *Agrobacterium* to nucleus of plants

This method is preferred over all other techniques as Agrobacterium is capable of transferring large fragments (<50kb) of DNA very efficiently without much rearrangements. Also, the gene transferred is stably inherited.

Generation of transgenic plants and their identification

The advent of recombinant DNA technique and transformation methods for plants has given agricultural scientists a powerful new way of incorporating defined genetic changes into plants and thus generating transgenic plants. The continued development of Agrobacterium based transfer systems for improving its efficiency and applicability to more crops is rapididly replacing other methods for generation of transgenic plants.

Agrobacterium based generation of transgenic plants: There are few prerequisites for Agrobacterium mediated gene transfer which includes:

- In order to induce *vir* genes, plants must produce acetosyringone or Agrobacterium can be preinduced with synthetic acetosyringone.
- Following induction, the agrobacteria should have access to cells that are competent for transformation. Thus, wounded and dedifferentiated cells, fresh explants are used which have replicating DNA or are undergoing mitosis.
- Transformation competent cells should be able to regenerate in whole plants.

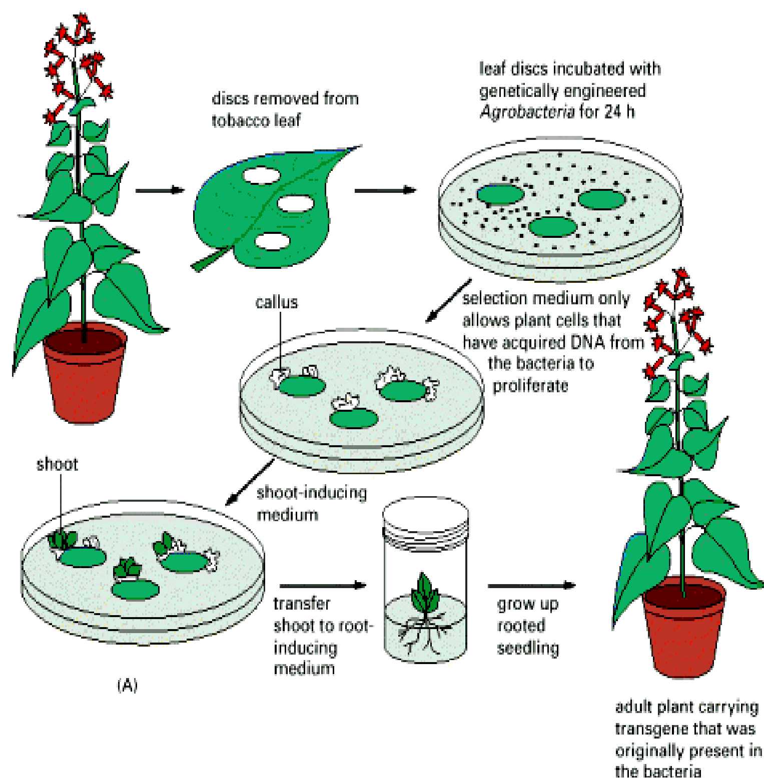

Fig. 8: *Agrobacterium* mediated development of transgenic plants in tobacco

Procedure: The procedure for Agrobacterium mediated gene transfer is summarized in fig.8. The explants used for inoculation or cocultivation with Agrobacterium carrying the vector include protoplasts, callus, tissue slices, sections of organs like leaf discs etc. In practice, the procedure can be performed with any tissue explant that provides a good source for initiation of

whole plant differentiation. So, a tissue segment like leaf disc is excised and is incubated in Agrobacterium suspension for few hours to 3-4 days followed by culturing on a media for bacterial growth to take place. Tissue explants are then transferred to media containing carbenicillin or cefotaxime which eliminate bacteria. After explants are inoculated with Agrobacterium carrying the vector with gene of interest, they are moved to media designed for selection of transformed plant cells. Selection is facilitated by selectable marker genes present in vector which is usually resistance to herbicide or antibiotics like kanamycin. Following selection, the transformed calli is put in regeneration medium for development of shoots and roots. The second level of selection for transformed tissue is done by expression of reporter gene or scorable marker gene like GUS. Different selectable marker genes and reporter genes with their substrate and assays are summarized in tables 2 and 3.These transgenic plants (T0) are then tested for stable integration and expression of genes by PCR or by Southern hybridization

Molecular Markers

A genetic marker is any character that can be measured in an organism which provides information on the genotype of that organism. A genetic marker may be a recognizable phenotypic trait (e.g. dwarfism, albinism, altered leaf or flower morphology), a biochemical trait (e.g. proteins like isozymes) or a molecular trait (molecular or DNA based). Use of phenotypic markers is limited by their dependence on expression of genes which are influenced by environmental or developmental conditions. Whereas molecular markers defined as readily detectable DNA sequences whose inheritance can be easily monitored, which are independent of developmental stage or environment and are numerous in number. Markers are used to study genetic diversity within species/strains or for marker assisted selection of desirable genes. The location of a desirable gene can be assigned by comparing the inheritance of a mutant gene with inheritance of marker whose chromosomal location is known. Coinheritance of gene of interest and markers suggests that they are physically close together on the chromosome. Therefore, the desirable features of a molecular marker are:

- Should be easy, fast and cheap to detect
- Should be reproducible
- Should be polymorphic
- Should have codominant inheritance to allow discrimination between homo and heterozygotes in diploids

There are a wide range of molecular markers available to detect polymorphism like RFLP, RAPD, AFLP, SSR etc.

1. **Restriction Fragment Length Polymorphism (RFLP)** was termed by Botstein in 1980. RFLPs rely on the combination of a probe and restriction enzyme (RE) to identify polymorphic DNA sequence using Southern blotting. RFLP is generated by the presence or absence of a recognition site for one restriction endonuclease in the same region of chromosome from different individuals of a species. In this method, DNA is digested with RE, electrophoresed, blotted on a membrane and probed with a labeled clone. Polymorphism in the hybridization pattern is a result of the same RE producing fragments of different lengths representing region of

same chromosome of different individuals. Single base pair changes, inversions, translocations, deletions etc may result in loss or gain of a recognition site which in turn leads to restriction fragments of different lengths between different genotypes. Since this approach requires large amount of highly pure DNA and radioactive or non radioactive detection method to identify polymorphic DNA bands, it is time consuming, laborious and expensive.

2. **Randomly Amplified Polymorphic DNA (RAPD)** is a PCR based molecular technique. This involves amplifying DNA segments randomly distributed throughout genome by PCR using single decamer primers at low stringency. Polymorphism occurs as a result of presence or absence of complementary sequence to the primer in the genome. The RAPDs may be of different types *viz.*Arbitarily Primed-PCR, DNA Amplification Fingerprinting (DAF) etc. These techniques differ in the length of primers used, the amplification conditions and the resolution of PCR products. RAPD needs small amount of DNA (15-30ng). Since, it is PCR based, it is quick and efficient technique. But since, it is not codominant marker and also not reproducible,it is used only as an initial approach to identify polymorphism.

3. **Amplified Fragment Length Polymorphism (AFLP)** is a combination of RFLP and RAPD techniques. It involves PCR amplification of genomic restriction fragments generated by specific RE and oligonucleotide adaptors of few nucleotide bases. Many potentially polymorphic fragments are generated by this approach which are separated on highly resolving sequencing gels and visualized using autoradiography or fluorescent dye. This is a highly sensitive, reproducible fingerprinting technique.

4. **Simple Sequence Repeats (SSR)** also called microsatellites are groups of repetitive DNA sequences that are present in a significant proportion of plant and animal genomes. They consist of tandemly repeated mono-, di-, tri-, tetra- and penta nucleotide units. The number of repeats at that locus varies in different individuals, thus displaying high levels of genetic variation. These SSR length polymorphisms are detected by PCR. Therefore, it is necessary to sequence the conserved flanking DNA to design PCR primers that will amplify the repeat sequences. Microsatellites provide reliable, reproducible molecular markers.

Table 2: Selectable marker genes used for gene transfer

	Selectable marker gene	Substrate used for selection
1.	Hygromycin phosphotransferase (*hpt*)	Hygromycin B
2.	Neomycin phophotransferase (*nptII*)	G418, kanamycin, neomycin
3.	Phophinothricin acetyltransferase (*bar*)	L-phophinothricin (PPT), bialaphos
4.	Bromoxynil nitrilase	Bromoxynil

Table3: Scorable marker genes used for gene transfer

S.No	Scorable marker gene	Substrate and assay	Identification
1.	β glucoronidase (GUS)	X-gluc	Fluorescence
2.	β galactosidase (lac Z)	X gal	Colony colour
3.	Neomycin phosphotransferase (NPT II)	Kanamycin+ P^{32}ATP	Radioactivity detection

Role of Biotechnology in Crop Improvement

The last decade has witnessed remarkable change which has taken plant biotechnology from study of basic science to large scale commercial applications. This is true for almost every aspect of plant biotechnology such as development of molecular markers to speed up plant breeding practices and using knowledge of genes and their expression to generate and commercialise transgenic crops. In general, the role of biotechnology in crop improvement can be divided into two categories: 1) those directed towards same goals as conventional plant breeding like improved yield, quality, resistance to pests and diseases, tolerance to abiotic stresses etc by molecular breeding or production of transgenic crops. 2) Novel applications such as use of plants as bioreactors to generate pharmaceuticals, vaccines or biodegradable plastics.

a) Molecular breeding: Molecular maps using markers RFLP, RAPD, SSR, ESTs for major crop species like rice, maize, tomato etc has been utilized very effectively in crop improvement programmes like marker assisted selection (MAS). Many markers have been identified which are closely linked to genes for agronomic traits of interest. These include markers for genes coding for:

i.) **Pest and disease resistance** (against viruses fungi, bacteria, nematodes, insects) for e.g. RFLP has been used to map Tm-2a locus in tomato resistant to tobacco mosaic virus (TMV), gene Mi in tomato for resistance against root knot nematode, two genes in rice for blast resistance and in many other crops. RAPD assisted selection has been done for *pto* gene conferring resistance to Pseudomonas in tomato. IARI has developed new improved Basmati by marker assisted transfer of X*a13 and Xa21* genes against bacterial leaf bight.

ii.) **Quality traits** For e.g. malting quality in barley, alkaloid levels etc.

iii.) **Abiotic stresses** e.g. tolerance to salinity or drought

iv.) **Developmental traits** for e.g. flowering time, vegetative period

v.) **Quantitative traits** e.g. high fibre strength in cotton

vi.) **Micronutrient uptake** e.g. high iron and zinc uptake in wheat

The role of molecular MAS for crop improvement is in increased speed and accuracy of selection, gene pyramiding, reduced cost of field based selection. Thus rather than growing breeding lines in the field and testing for important traits over the growing season, it is possible to extract DNA from 50ng of seedling leaflet and test for presence or absence of a range of traits in that DNA sample in one day. Plants lacking the required traits can then be removed early in the breeding programme. With the availability of more validated molecular marker, MAS therefore, becomes a highly cost effective and efficient process.

The same principle used in developing molecular markers can be applied for range of molecular diagnostics and DNA fingerprinting *viz.*identification of breeding lines and varieties, characterization of genetic resources and study of phylogenetic relationships.

b) Transgenic Plants: The development of transgenic plants is the result of integrated application of rDNA technology, gene transfer methods and tissue techniques. Transgenic plants have both basic and applied role in crop improvement.

i.) **Genes have been successfully transferred to many crops for resistance to various biotic stresses** such as the biopesticidal gene of bacterium *Bacillus thuringiensis* (Bt) has been

incorporated into cotton for resistance against bollworm, maize against European corn borer, tomato, tobacco against Bt cotton is already in markets and rest are in process. Also, plant derived insecticidal genes like protease inhibitors, lectins etc have also been integrated into many pulse crops which are under field trials.

Also, transgenic plants has been especially successful for control of viral diseases. The approach followed is to identify those viral genes or gene products which when present a wrong time or in improper amount, will intefere with the normal functions of the infection process and prevent disease development. Few examples of transgenic plants with viral resistance genes are in tobacco against TMV, rice against rice yellow mosaic virus, potato against potato virus Y and potato leaf roll virus etc.

ii.) **Genes resistant to abiotic stresses like herbicide resistance.** Herbicides are the method of choice to control weeds and hence maximize crop yields by reducing by reducing competition from weeds. This has necessitated development of safer, biodegradable herbicides and development of crop plants resistant to those herbicides. Also, because herbicide resistance genes are also effective selectable marker genes in culture, herbicide tolerant crop varieties were the first major transgenic trait to be produced and commercialized. Based on either expression of a herbicide insensitive gene, degradation of herbicide or overexpression of herbicide target gene product, engineered resistance is now available to a range of herbicides for e.g. transgenic petunia plants resistant to glyphosate of Roundup herbicide was developed by transfer of a gene for EPSPS (5-enol-pyruvyl-shikimate-3 phosphate synthase) that overproduces this enzyme. Transgenic tomato and maize plants using herbicide detoxifying gene GST (glutathione- S transferase) have been successfully used.

Also, extensive research is going on for producing transgenic crops against salt, drought, chilling stresses in rice, wheat, tomato etc. In most cases, advances to generate stress tolerant plants by traditional breeding are slow because of involvement of many genes and physiological processes. For cold and drought tolerance, recent research has shown that a series of functionally different cold and drought response genes show common promoter regulatory sequences. These results are significant because they show that introduction of a single regulatory gene confer tolerance to different stresses.

iii.) **Gene transfers to improve quality of food products**- Bruise resistant tomatoes were developed which expressed antisense RNA against polygalatouronase which attacks pectin in the cell walls of ripening fruit and thus softens the skin. This transgenic tomato was commercialized under the name of Flavr Savr. Also, tomatoes with delayed ripening were developed by using gene for ACC deaminase to degrade ACC which is immediate precursor to ethylene. This increases shelf life of tomato. Starch content in potatoes could be increased by 20-40% by using a bacterial ADP glucose pyrophosphorylase gene.

iv.) **Male sterlity and fertility restoration in transgenic plants** which is required for hybrid seed production. For many crops, it is difficult to generate commercial hybrid seed by conventional means. Hybrid seed is attractive to seed companies as farmers must purchase new seed from them each year, since hybrid varieties donot breed true. It is now possible to engineer male sterlity bt expression of a ribonuclease gene (*barnase*) specifically during development of

the tapetal layer that nourishes developing pollen grains. Developmental regulation of the ribonuclease by the TA29 tapetum specific promoter kills the tapetal cells leading to male sterility. Male sterile plants can be used as the female parent to produce hybrid seed. Fertility can be restored by expression of the *barstar* gene, which inactivates *barnase.* This technology can be used to produce hybrids of crops such as maize, sugarbeet or canola. It is not possible to produce hybrid canola conventionally but hybrid canola can exhibit hybrid vigor and increased yields.

c) Molecular Farming: A major role of biotechnology is the use of transgenic plants as factories for manufacturing speciality chemicals and pharmaceuticals such as transgenic tobacco plants carrying mannitol dehydrogenase gene from *E. coli* is used for increasing production of mannitol. Similarly production of Polyhydroxy butyrate (PHB) in plants provide attractive source of biodegradable plastics at low cost. Two genes which catalyse two steps in production of PHB, acetoacetyl CoA reductase (*phb B*) and polyhydroxy butyrate synthase (*phb C*) have been successfully transformed and expressed in *Arabidopsis thaliana.*.Efforts are on to create transgenic plants as source of edible vaccines, antibodies, pharmaceuticals.

d) To study regulated gene expression: Transgenic plants have proven to be particularly useful tools in studies on plant molecular biology, gene regulation, identification of regulatory sequences involved in differential expression of gene activity. T-DNA and transposable elements are used as molecular tags to produce mutations by becoming inserted within genes thus making it non functional. Using this approach regulatory sequences of many structural genes have been cloned like light inducible genes like ribulose biphosphate carboxylase *(rbcS*), chlorophyll a/b binding protein (Cab), heat shock protein genes etc. The genes for seed storage proteins provide an excellent example of a cell and tissue specific expression.

Although, biotechnology alone will not be able to generate sustainable food production, however, the “gene revolution’ will have immense role in crop improvement and agriculture.

Suggested Readings:

1. Adams, R.P. and Adams, J. E. (eds.) 1992, Conservation of Plant Genes, DNA Banking and *In Vitro* Technology. Academic Press, New York.
2. Bhojwani, S.S. (eds.) 1990, Plant Tissue Culture: Applications and Limitations. Elsevier, Amsterdam
3. Lewin, B. 2004 Genes VIII, Oxford University Press, New York.
4. Old, R.W. and Primrose, S.B. 1990. Principles of Gene Manipulation, An Introduction to Genetic Engineering. Blackwell Science Publishers, London.
5. Singer, M. and Berg, P. 1991. Genes and Genomes, University Science Books, Mill Valley, California and Blackwell Science Publishers.
6. Singh, B.D. 1998, Biotechnology, Kalyani publishers, New Delhi
7. Slater, A., Scott, N. and Fowler, M. 2003, Plant Biotechnology: The Genetic Manipulations of Plants, Oxford University Press.
8. Street, H. E. 1977, Plant Cell and Tissue Culture, Blackwell, London.
9. Vasil, I.K. and Thorpe, T. A. (eds.) 1994. Plant Cell and Tissue Culture. Kluwer Academic Publishers, London.