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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BITOECHNOLOGY

UNIT – I – PLANT BIOTECHNOLOGY– SBB3103

PLANT TISSUE CULTURE

It’s a collection of techniques used to maintain or growth of plant cell tissue or organ under sterile condition .

• A aseptic culture of plant cell, tissue under controlled condition to lead the cell multiplication of regeneration of organs or whole plants.

• The growth or maintenance of plant cells, tissues, organs or whole plants in vitro.

GENERAL TERM IN PTC

• Adventitious – developing from unusual points of origin such as shoot or root from callus or embryos from other than zygote.

• Asepsis: Without infection or contaminating microorganisms.

• Callus: An unorganized, proliferative mass of differentiated plant cells; a wound response.

• Cybrid: The viable cell resulting from the fusion of a cytoplast with a whole cell, thus creating a cytoplasmic hybrid.

• Explant: Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

• Gameto-clone: Plants regenerated from cell cultures derived from meiospores, gametes or gametophytes.

• Meristem culture: In vitro culture of a generally shiny, dome-like structure measuring less than 0.1 mm in length when excised, most often excised form the shoot apex.

• Somaclone: Plants derived from any form of cell culture involving the use of somatic plant cells.

• Totipotency: A cell characteristic in which the potential for forming all the cell types in the adult organism is retained.

HISTORY •

1902 - C.Haberlant-First attempt to culture isolated plant cells in vitro on artificial medium

• 1922 - WJ Robbins and W. Kotte-Culture of isolated roots (for short periods) (organ culture)

• 1934 - P R White-Demonstration of indefinite culture of tomato roots (long period)

• 1939 - R J Gautheret and P Nobecourt-First long term plant tissue culture of callus, involving explants of cambail tissues isolated from carrot.

• 1939 - P R White-Callus culture of tobacco tumor tissues from intersepcific hybird of Nicotina glaucum X N.longsdorffi.

• 1941 - J Van Overbeek-Discovery of nutritional value of liquid endosperm of coconut for culture of isolated carrot embryo.

• 1942 - P R White and A C Braun-Experiments on crownn-gall and tumor formation in plants, growth of bacteria free crown-gall tissues.

• 1948 - A Caplan and F C Stewart-Use of coconut milk plus 2, 4-D fro proliferation of cultured carrot and potato tissues.

1950 - G Morel-Culture of monocot tissues using coconut milk.

• 1953 - W H Muir-Inoculation of callus pieces in liquid medium can give a suspension of single cells amenable tosubculture. Development of technique for culture of single isolated cells.

• 1953 - W Tulecke-Haploid culture from pollen of gymnosperm (Ginkgo)

• 1955 - C O Miller, F Skoog and others-Discovery of cytokinins. E.g. Kinetin, or potent cell division factor.

• 1957 - F Skoog and C O Miller-Hypotheses that shoot and root initiation in cultured callus is regulated by the proportion of auxins and cytokinins in the culture medium.

• 1960 - E C Cocking-Enzymatic isolation and culture of protoplast.

• 1960 - G Morel-Development of shoot apex culture technique.

• 1966 - S G Guha and S C Maheshwari-Cultured anthers and pollen and produce haploid embryos.

• 1978 - G Melchers-Production of somatic hybrids from attached to plasmid vectors into naked plant protoplast.

• 1983 - M D Chilton-Production of transformed tobacco plants following single cell transformation or gene insertion.

Historical Developments and Applied Aspects (H R Dagla)

The success of plant biotechnology relies on the fundamental techniques of plant tissue culture. Understanding basic biology of plants is a prerequisite for proper utilization of the plant system or parts thereof. Plant tissue culture helps in providing a basic understanding of physical and chemical requirements of cell, tissue, organ culture, their growth and development. Establishment of cell, tissue and organ culture and regeneration of plantlets under in vitro conditions has opened up new avenues in the area of plant biotechnology.

Introduction

1. Plant tissue culture is a technique of culturing plant cells, tissues and organs on synthetic media under aseptic environment and controlled conditions of light, temperature, and humidity. The development of plant tissue culture as a fundamental science was closely linked with the discovery and characterization of plant hormones, and has facilitated our

understanding of plant growth and development. Furthermore, the ability to grow plant cells and tissues in culture and to control their development forms the basis of many practical applications in agriculture, horticulture industrial chemistry and is a prerequisite for plant genetic engineering

Plant Tissue Culture: Laboratory Requirements

Laboratory Requirements:

'Plant tissue culture' or in vitro cultivation of plant parts needs some basic requirements:

(a) Cultivation should be done under aseptic conditions.

(b) The isolated plant part should get an appropriate environment which will help to divide the cell and to get an expression of internal potential.

Basic facilities for plant tissue culture operations involving any type of in vitro procedures must include certain essential elements:

(a) Washing and storage facilities;

(b) Media preparation, sterilisation and storage room;

(c) Transfer area for aseptic manipulations;

(d) Culture rooms or incubators for maintenance of cultures under controlled conditions of temperature, light and humidity;

(e) Observation or data collection area;

(f) Transplantation area.

Washing and Storage Facilities:

An area with large sink (lead lined to resist acids and alkalis) and draining area is necessary with provision for running water, draining-boards or racks and ready access to a deionized, distilled and double-distilled apparatus.

Space should also be available to set up drying ovens, washing machines, plastic or steel buckets for soaking labware, acid or detergent baths, pipette washers, driers and cleaning brushes. For storage of washed and dried labware, the laboratory should be provided with dustproof cupboards or storage cabinets.

Media Preparation Room or Space:

This part is the central section of the laboratory where most of the activities are performed i.e., media preparation and sterilisation of media and glassware's needed for culture. There should be sufficient working bench as well as storage space.

The following items are essential in the room (Fig. 16.2A-D):

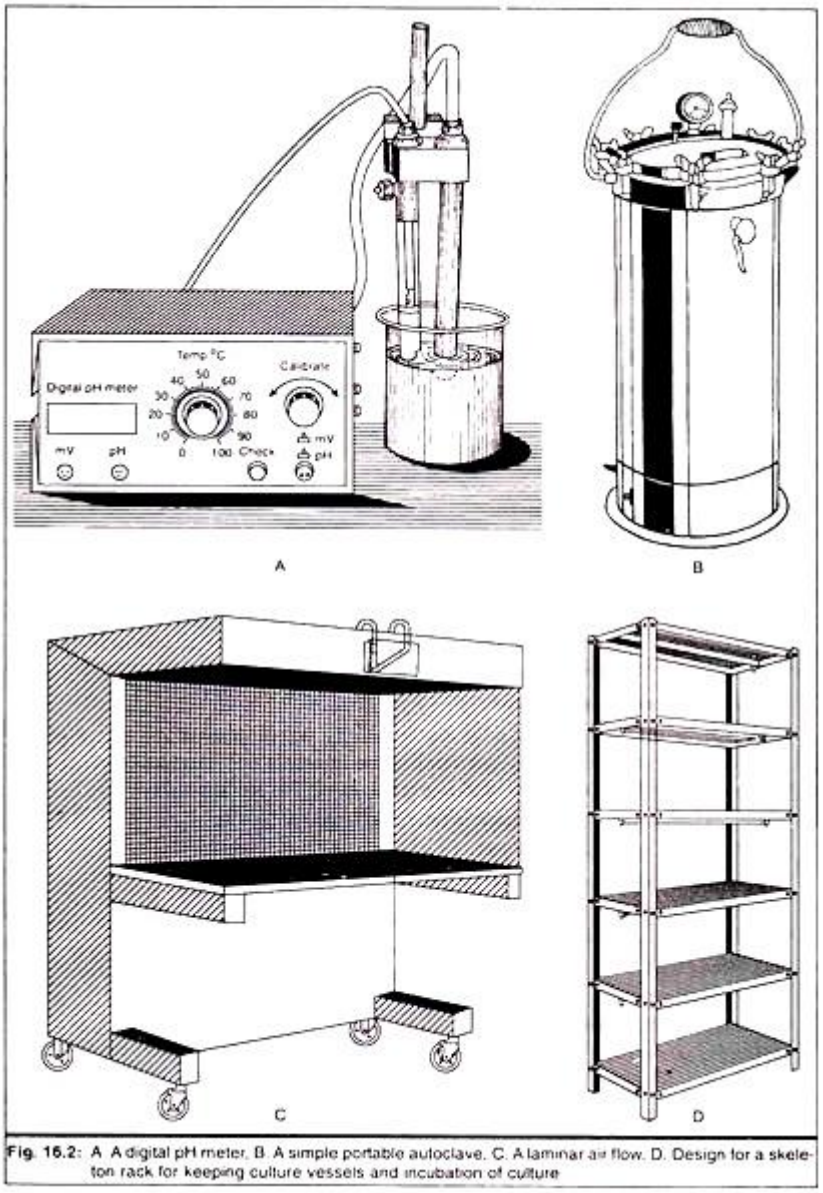


Fig. 16.2: A. A digital pH meter, B. A simple portable autoclave, C. A laminar air flow, D. Design for a skeleton rack for keeping culture vessels and incubation of culture

(i) Different types of glassware

(ii) Different kinds of balances

(iii) Required chemicals

(iv) Hot plates and Stirrer

(v) Water bath

vi) pH meter

(vii) Autoclave and Hot air oven

(viii) Microwave oven

(ix) Vortex, Shaker

(x) Centrifuge

(xi) Refrigerator and Freezer

(xii) Storage cabinet (Dust-free)

Transfer Area:
Tissue culture techniques can only be successfully carried out in a very clean laboratory having dry atmosphere with protection against air-borne microorganisms. For this purpose a sterile dust-free room/cabinet is needed for routine transfer and manipulation work.

The 'laminar air flow cabinet' (Fig. 16.2C) is the most common accessory used for aseptic manipulations now-a-days. The cabinet may be designed with horizontal air flow or vertical air flow where the air is forced into the cabinet through a bacterial HEPA (High Efficiency Particulate Air) filter. The air flows over the working bench at a constant rate which prevents the particles (microorganisms) from settling on the bench.

Before operation in the laminar air flow cabinet, the interior of the cabinet is sterilised with the ultraviolet (UV) germicidal light and wiping the floor of cabinet with 70% alcohol. Inoculation chamber, a specially designed air tight glass chamber fitted with UV light, may also be used as transfer area.

Culture Room:
Plant tissue cultures should be incubated under conditions of well-controlled temperature, illumination, photoperiod, humidity and air circulation. Incubation culture rooms, commercially available incubator cabinets, large plant growth chambers and walk-in-environmental rooms satisfy these requirements.

Culture rooms are constructed with proper air-conditioning; perforated shelves (Fig. 16.2D) to support the culture vessels, fitted with fluorescent tubes having a timing device to maintain the photoperiod, black curtains may be used to maintain total darkness.

For the suspension cultures, gyratory shakers are used. Air conditioners and heaters are used to maintain the temperature around 25 ± 2°C and humidity is maintained by uniform forced air-ventilation. The lighting is also done in a measured amount i.e., 40-200 fc (foot-candle).

Data Collection Area:

The growth and development of tissues cultured in vitro are generally monitored by observing cultures at regular intervals in the culture room or incubators where they have been maintained under controlled environmental conditions.

Arrangement should be there where the observations can be done under aseptic conditions using microscope. Special facilities are required for germplasm conservation i.e., cryopreservation accessories should be there.

Transplantation Area:

Plants regenerated from in vitro tissue culture are transplanted to soil in pots. The potted plants are ultimately transferred to greenhouse but prior to transfer the tissue culture grown plants are allowed for acclimatization under well humid condition and controlled temperature and under controlled entry of sunlight.

2. History

History of plant tissue culture is a record of systematic efforts by botanists to culture excised plant tissues and organs to understand their growth and development under controlled conditions.

COMPOSITION OF COMMONLY USED MEDIA

TABLE · Composition of commonly used plant tissue culture media

Components	*Amount (mg l^{-1})*				
	White's	*Murashige and Skoog (MS)*	*Gamborg (B5)*	*Chu(N6)*	*Nitsch's*
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	—	0.025
$CoCl_2.6H_2O$	—	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	0.8	—
$FeSO_4.7H_2O$	—	27.8	—	27.8	27.8
$Na_2EDTA.2H_2O$	—	37.3	—	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thiamine HCl	0.01	0.5	10	1	0.5
Pyridoxine (HCl)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	—	100	100	—	100
Others					
Glycine	3	2	—	—	2
Folic acid	—	—	—	—	0.5
Biotin	—	—	—	—	0.05
pH	5.8	5.8	5.5	5.8	5.8

TABLE [illegible] A selected list of elements and their functions in plants

Element	*Function(s)*
Nitrogen	Essential component of proteins, nucleic acids and some coenzymes. (Required in most abundant quantity)
Calcium	Synthesis of cell wall, membrane function, cell signalling.
Magnesium	Component of chlorophyll, cofactor for some enzymes.
Potassium	Major inorganic cation, regulates osmotic potential.
Phosphorus	Component of nucleic acids and various intermediates in respiration and photosynthesis, involved in energy transfer.
Sulfur	Component of certain amino acids (methionine, cysteine and cystine, and some cofactors).
Manganese	Cofactor for certain enzymes.
Iron	Component of cytochromes, involved in electron transfer.
Chlorine	Participates in photosynthesis.
Copper	Involved in electron transfer reactions, Cofactor for some enzymes.
Cobalt	Component of vitamin B_{12}.
Molybdenum	Component of certain enzymes (e.g., nitrate reductase), cofactor for some enzymes.
Zinc	Required for chlorophyll biosynthesis, cofactor for certain enzymes.

Totipotency is the potential or inherent capacity of a plant cell or tissue to develop into an entire plant if suitably stimulated. Totipotency implies that all the information necessary for growth and reproduction of the organism is contained in the cell. Although theoretically all plant cells are totipotent the meristematic cells are best able to express it.

• Dedifferentiation is the capacity of mature cells to return to meristematic condition and development of a new growing point

• Competency describes the endogenous potential of a given cell or tissue to develop in a particular way. For example, as embryogenically competent cells are capable of developing into fully functional embryos. The opposite is non-competent or morphogenetically incapable.

2.1 Cell Culture

The idea of experimenting with the tissues and organs of plants in isolation under controlled laboratory conditions arose during the later part of the nineteenth century. German botanist Gottlieb Haberlandt was the first person to culture isolated, fully differentiated cells in 1898 [2]. He selected single isolated cells from leaves and grew them on Knop's (1865) salt solution with sucrose. Haberlandt succeeded in maintaining isolated leaf cells alive for extended periods but the cells failed to divide because the simple nutrient media lacked the necessary plant hormones. Although he could not demonstrate

the ability of mature cells to divide, he was certain that in the intact plant body, the growth of a cell simply stops due to a stimulus released by the organism itself, after acquiring the features required to meet the need of the whole organism. Haberlandt's vision was to achieve continued cell division in explanted tissues on nutrient media (Box 1); that is, to establish true, potentially perpetual tissue culture. This goal was attained only after the discovery of auxins [3]. Although Haberlandt was unsuccessful in his attempts to culture cells, he foresaw that they could provide an elegant means of studying morphogenesis. And the result of such culture experiments should give some interesting insight into the properties and potentialities which the cell as an elementary unit of life possesses.

2.2 Organ Culture

In the early part of the 20th century, efforts in growing excised plant tissues in culture continued with the development of sterile working methods (Box 2) and discovery of the need for B vitamins and auxins for tissue growth. In 1922, Robbins (USA) and Kotte (Germany) reported some success with growing isolated root tips. The first successful experiment to maintain growth and cell division in plant cell culture was conducted by White (1934) who established cultures of isolated tomato roots under aseptic conditions. White's medium was simple, containing only sucrose, mineral salts and yeast extract, which supplied vitamins. The cultured roots maintained their morphological identity as roots with the same basic anatomy and physiology. This happened only because excised plant organs on nutrient media are capable of synthesizing hormones necessary to maintain cell division. Ball (1946) obtained whole plants from cultured shoot meristem. This heralded the present day method of in vitro vegetative multiplication. Ball is considered the father of socalled micropropagation [4]. Morel and Martin [5] cultured shoot meristem of virus-infected plants to raise healthy plants from Dahlia. The cells of the shoot tip of virus infected plants are free of virus or contain a negligible number of virus. Axillary bud proliferation has immense practical applications for large-scale clonal propagation of plants of importance in agriculture, horticulture and forestry.

2.3 Tissue or Callus Culture

A mass of unorganized protoplasmic (undifferentiated living) cells is known as 'callus' (Figure1a). White (1939) cultured tissue of plant tumors (galls) that were produced by a hybrid between Nicotiana glauca and N. langsdorffii on the same medium that was used for tomato roots. Proliferated cell masses from the original explants were divided and subcultured. Gautheret and Nobecourt in 1939 reported unlimited growth of cultures derived from carrot tap root tissue, using indole-3-acetic acid (IAA). The goal at that time was to establish unlimited growth of a culture by repeated subcultures. Much effort was devoted to determine the nutritional requirements for sustained growth. White and Braun (1942) initiated studies on crown gall and tumour formation in plants and Skoog (1944) initiated work on organogenesis in tobacco callus. Although continuously growing cultures could be established in 1939, the objective of Haberlandt to induce cell division in isolated vegetative cells, was not achieved, because the tissues used by them were not meristematic in nature. The most significant event that led to advancement in the field was the discovery of the nutritional properties of coconut milk. Van Overbeek and his coworkers (1941) cultured isolated embryo of Datura on a medium containing coconut milk. The combination of 2,4-D (2, 4-dichlorophenoxy acetic acid) and coconut milk had a remarkable effect on stimulating growth of cultured carrot and potato tissues [6–8]. In a

search for cell division factor, Skoog's group located such a factor in degraded DNA preparation. It was isolated, identified as 6- furfurylaminopurine, and named it Kinetin [9]. The related analogue, 6-benzylaminopurine, was then synthesized and that too stimulated cell division in cultured tissues. The generic term 'cytokinin' was given to this group of 6-substituted aminopurine compounds that stimulate cell division in cultured plant tissues. Later Zeatin was discovered as a natural plant hormone. Skoog and Miller [10] advanced the hypothesis that shoot and root initiation in cultured callus can be regulated by specific ratios of auxin and cytokinin. The availability of cytokinins made it possible to induce divisions in cells of mature and differentiated tissues. At this stage, the dream of Haberlandt was realized partially, for he foresaw the possibility of cultivating isolated single cells. Only small pieces of tissue could be grown in cultures. Further progress was made by Muir [11] who transferred callus of Tagetes erecta and Nicotiana tabacum to liquid medium (culture medium devoid of agar-agar) and agitated the cultures on a shaking machine, to break the tissue into single cells and small cell aggregates. Muir et al. (1954) succeeded in making single cells to divide by placing them individually on separate filter papers resting on top of a well-established callus culture. Callus tissue separated from the cells by thin filter paper, supplied the necessary factor(s) for cell division. Jones (1960) et al. designed a micro-chamber for growing single cells in hanging drops in a conditioned medium (medium in which callus has been grown previously). Using a micro-chamber and replacing the conditioned medium with a fresh medium containing coconut milk, Vasil and Hildbrandt (1965) raised whole plants starting from single cells of tobacco. They transferred single tobacco hybrid cells to a drop of culture medium on a slide, and observed separately under phase contrast microscope and photographed their observations. Cells were observed to divide and form callus which differentiated into roots and leafy shoots. However, they did not prove that the whole plants were the direct product of a single cell, rather than the product of a tissue mass within which somaclonal or other genetic changes might have taken place during growth.

Finally, Haberlandt's prediction, that one could successfully cultivate artificial embryos from vegetative cells, was proved by the research of Backs-Husemann and Reinert in Berlin. They mounted isolated single cells on microscope slides and photographed repeatedly. Isolated cells divided to form a mass of embryogenic and parenchyma cells which developed into heart shaped and torpedo-shaped embryos with recognizable cotyledons, hypocotyls and radicles [12]. Tuleke (1953) cultured pollen grains of Ginkgo biloba in a medium containing vitamins and amino acids and obtained cell clumps, some of which looked similar to embryos. Yamada et al. [13] reported that culture of Tradescantia reflexa anther produced haploid tissues. Guha and Maheshwari [14] reported that immature pollen grains produced embryos. Colchicine treatment can transform them into diploid fertile plants. Klercker (1892) and Kuster (1909) reported isolation and fusion of protoplasts, respectively. Cocking [15] developed enzymatic method of protoplast isolation. The method involved the enzymatic digestion of cell wall by cellulase and pectinase enzymes extracted from the fungus Myrothecium verrucaria. Cultured protoplasts regenerated new walls, developed colonies and eventually plantlets [16]. Protoplasts are now used for creation of somatic hybrids within and between species and genera. The first hybrid between N. Glauca and N. langsdorffii was produced by Carlson [17]. In 1978, Melchers et al. [18] produced a hybrid between potato and tomato, but the hybrid was sterile. Novel application of protoplast fusion is called cybrid production, where cytoplasm of two species or genera is fused with nuclear genome of only one cell (nuclear–cytoplasmic combination).

3. Applied Aspects of Plant Tissue Culture

Establishment of plant tissue culture techniques has enabled botanists to introduce this method in major areas of plant sciences such as plant breeding, industrial production of natural plant products, conservation of germplasm and genetic engineering.

3.1 Plant Breeding

Establishment of cellular totipotency , callus differentiation and vegetative multiplication under in vitro conditions has opened up new dimensions in the applied field of plant sciences. Rapid vegetative propagation or micropropagation of plants of elite characteristics is possible through axillary shoot induction (Figure 1b) and rooting them (Figure 1c) in vitro to raise complete plantlets. Somatic embryogenesis2 and organogenesis (callus differentiation) are other methods of micropropagation. Seedlings (Figure 1d) derived from mature seeds can also be used as a source for large-scale multiplication of rare and endangered plant species. Virus-free plants can be raised using apical meristems of virus-infected plants. Homozygous plants can be obtained in a single generation by diploidization3 of the haploid cells such as pollen grains. Protoplast technology has made it possible to develop somatic hybrids and cybrids of distantly related plant species and genus. Protoplasts are also a suitable material for genetic engineering of plants in a manner similar to gene transfer into bacteria. Cell culture may be an important source of induction and selection of cell variants for production of new varieties of economically important plants.

3.2 Industrial Production of Natural Plant Products

Plants produce a variety of natural compounds that are used as agricultural chemicals, pharmaceuticals and food additives. Cell culture technique is being used as an efficient system for production of high-value natural plant products at industrial level. In the 1950s and 1960s, great efforts were made by the Pfizer Company to culture plant cells in liquid medium (suspension culture4) similar to culture of microbes for production of natural plant products as an alternative to whole plants. Different kinds of bioreactors5 have been designed for large-scale cultivation of plant cells. Culture of hairy roots produced by transformation with Agrobacterium rhizogenes has been shown to be a more efficient system than cell culture for the production of compounds which are normally synthesized in roots of intact plants. The first tissue culture product to be commercialized by Mitsui Petrochemical Co. of Japan is shikonin, a natural colouring substance, from the cell cultures of Lithospermum erythrorhizon [19].

3.3 Conservation of Germplasm

Successful regenerating of whole plants from somatic and gametic cells and small shoot apices, and storage of germplasm is possible by using in vitro techniques. This is an efficient method by which small portions of plant parts in the form of cell, tissue and organ can be stored for longer periods in a limited space, free from contamination and infection.

3.4 Genetic Engineering of Plants

Protocol for cell culture and regeneration of plants from single cell is a basic requirement for development of genetically modified plants. Single cell culture and regeneration of plants from single cell is possible through plant tissue culture techniques. Agrobacterium tumefaciens-based vectors play an important role in genetic modification of plants. Smith and Townsend [20] had shown that A. tumefaciens, the Gram negative soil bacteria cause crown gall disease in some plants. They observed that crown gall tissue displayed the tumourigenic character of autonomous growth on salt–sugar medium, even in the absence of growth regulators. Braun [21] suggested that the bacterium introduces a tumourinducing property in plant genome. This was identified as Tiplasmid [22]. Phytohormone biosynthesizing genes from T-DNA6 of Ti-plasmid were removed to eliminate the aberrant cell proliferation ability of Ti-plasmid. A. tumefaciens can be used to transfer genes conferring desired traits into plant cells. This transformation system is species-specific and does not work in most monocotyledons including major cereals. Therefore, free DNA delivery techniques, such as electroporation, particle gun and microinjection are mostly used for genetic modification of cells, tissues and organs of monocots.

Types of Tissue Culture | Biotechnology

The following points highlight the top eight types of tissue culture. The types are: 1. Seed Culture 2. Embryo Culture 3. Meristem Culture 4. Bud Culture 5. Callus Culture 6. Cell Suspension Culture 7. Anther Culture 8. Protoplast Culture.

Type # 1. Seed Culture:

Seeds may be cultured in vitro to generate seedlings or plants. It is the best method for raising the sterile seedling. The seed culture is done to get the different kinds of explants from aseptically grown plants which help in better maintenance of aseptic tissue.

Type # 2. Embryo Culture:

Embryo culture is the sterile isolation and growth of an immature or mature embryo in vitro with the goal of obtaining a viable plant. In some plants seed dormancy may be due to chemical inhibitors or mechanical resistance, structures covering the embryo. Excision of embryos and culturing them in nutrient media help in developing viable seedlings.

Embryo developed from wide hybridisation between two different species may not mature fully due to embryo-endosperm incompatibility. So, the isolation and culture of hybrid embryos prior to abortion help in overcoming the post-zygotic barrier and production of interspecific or inter-generic hybrids.

Type # 3. Meristem Culture:

The apical meristem of shoots of angiosperms and gymnosperms can be cultured to get the disease free plants. Meristem tips, between 0.2-0.5 mm, most frequently produce virus-free plants and this method is referred to as meristem-tip culture.

This method is more successful in case of herbaceous plants than woody plants. In case of woody plants, the success is obtained when the explant is taken after the dormancy period is over. After the shoot tip proliferation, the rooting is done and then the rooted plantlet is potted.

Type # 4. Bud Culture:

Buds contain quiescent or active meristems in the leaf axils, which are capable of growing into a shoot. Single node culture, where each node of the stem is cut and allowed to grow on a nutrient media to develop the shoot tip from the axil which ultimately develops into new plantlet. In axillary bud method, where the axillary buds are isolated from the leaf axils and develop into shoot tip under little high cytokinin concentration.

Type # 5. Callus Culture:
Callus is basically more or less un-organised dedifferentiated mass of cells arising from any kind of explant under in vitro cultural conditions. The cells in callus are parenchymatous in nature, but may or may not be homogenous mass of cells. They are meristematic tissue, under special circumstances they may be again organised into shoot primordia or may develop into somatic embryos.

The callus tissue from different plant species may be different in structure and growth habit. The callus growth is also dependent on factors like the type of explant and the growth conditions. After callus induction it can be sub-cultured regularly with appropriate new medium for growth and maintenance.

Type # 6. Cell Suspension Culture:
The growing of individual cells that have been obtained from any kind of explant tissue or callus referred to as cell suspension culture. These are initiated by transferring pieces of tissue explant/callus into liquid medium (without agar) and then placed them on a gyratory shaker to provide both aeration and dispersion of cells. Like callus culture, the cells are also sub-cultured into new medium.

Cell suspension cultures may be done in batch culture or continuous culture system. In the later system, the culture is continuously supplied with nutrients by the inflow of fresh medium with subsequent draining out of used medium but the culture volume is constant. This culture method is mainly used for the synthesis of specific metabolite or for biomass production.

Type # 7. Anther Culture:
An important aspect of plant tissue culture is the haploid production by another culture or pollen culture which was first established by Guha and Maheswari (1964, 1966) in Datura.

During the last few decades, much progress has been made in different crops like rice, wheat, maize, mustard, pepper and others. The anthers bearing the uni-nucleate microspores are selected and allowed to grow in medium to produce callus from the pollen mass.

Then the triggering of these androgenic calli is directed to produce the embryos and haploid plants are developed from these androgenic embryos. The anther culture can be done with the isolated anthers on solid medium where anther wall will break open and the androgenic calli will be formed from the pollen.

In pollen culture, microspores of uni-nucleate stage are collected in liquid media and can be grown in suspension culture. In suspension, the uni-nucleate pollens may give rise to calli mass or the globular mass from which the plants can be raised either through embryogenic or organogenic pathway.

Type # 8. Protoplast Culture:

It is the culture of plant protoplasts i.e., culture of cells devoid of cell wall. Isolated protoplasts are usually cultured in either liquid or semisolid agar media plates. Protoplasts are isolated from soft parenchymatous tissue by enzymatic method and then viable protoplasts are purified and cultured.

The protoplast culture is aimed mainly to develop genetically transformed plant where the transgenic is put successfully within the plant protoplast and the transgenic plant is regenerated from that transformed protoplast. Another aspect of protoplast culture is somatic hybridization of two plant species through protoplast fusion.

UNIT I

Basic rDNA technology in relation to plant

Totipotency-Definition and Concept. Culture environment, Plant cell culture media, Plant hormones in agriculture and horticulture. Types of culture-solid and liquid, Factors affecting in vitro culture. Isolation of DNA, Vector, Transfer mechanism and expression. Agrobacterium mediated gene transfer vectors-Ti plasmid, Organization of T-DNA, Molecular biology of Agrobacterium infection, Plant virus vectors-CaMV, Gemini viruses, TMV, Brome mosaic virus. Agrobacterium vectors-cointegerate and binary vectors.

Cell Totipotency - Definition

A plant grows by increasing its cell population while the cells specialize their functions. Increasing cell population is done by cell division (also called mitosis). Before a mother cell divides into two daughter cells, it makes an exact copy of its genome first. As a result, the two daughter cells usually have exactly the same genetic makeup as their mother cell. Therefore, every living cell of a plant should contain all the genes the plant has and thus has the capacity to grow back to a full plant. This is called cell **totipotency**.

The process of specializing cells' functions is called cell **differentiation** . It is accompanied by **morphogenesis**, the change of the cells' morphology. Differentiation is done by turning on certain genes and turning off some others at a certain time. Therefore, for a highly differentiated cell to grow into a full plant, the differentiation process has to be reversed (called **de-differentiation**) and repeated again (called re-differentiation).

Theoretically, all living cells can revert to an undifferential status through this process. However, the more differentiated a cell has been, the more difficult it will be to induce its de-differentiation. Practically, the younger or the less differentiated a cell is, the easier to culture it into a full plant. The ease of fulfilling the cell totipotency also varies tissue by tissue, **genotype** by genotype and species by species. Genotype dependency is often the bottle-neck in plant tissue culture and also in plant **genetic engineering**.

GLOSSARY

Genome

All the genetic material in the haploid set of chromosomes for a particular organism.

Totipotency

The ability of a single plant cell to grow, divide, and differentiate into an entire plant. Mammalian cells do not have this ability.

Morphogenesis

The process of morphology development.

De-differentiation

The developmental process from a highly differentiated cell to an undifferentiated cell. It is a reverse to differentiation.

Genotype

The allelic composition of a cell or organism.

Tissue Culture

Plant cells are grown in culture which allows them to be manipulated and then induced to develop into whole plants.

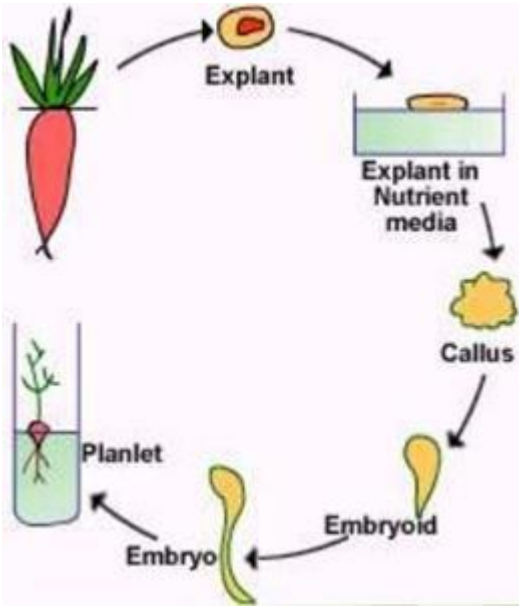
Genetic Engineering

The process of adding foreign DNA to the genome of an organism.

References

http://passel.unl.edu/pages/informationmodule.php?idinformationmodule=956783940&topicorder=2&maxto=10

Concept of totipotency



- As cell divide mitotically, they do so equationally to produce daughter cells.
- **G.Haberlandt's** claimed that one day, it could be possible to rear plants from isolated would be rarely surviving cellos flowering plants.
- He also stated that out of surviving somatic cells, artificial embryos would be reared asexually.
- Therefore, **every cell**

within the plant has a potential to rgenerate into a whole plant.

References

http://www.slideshare.net/swativasih/plant-biotechnology-introduction

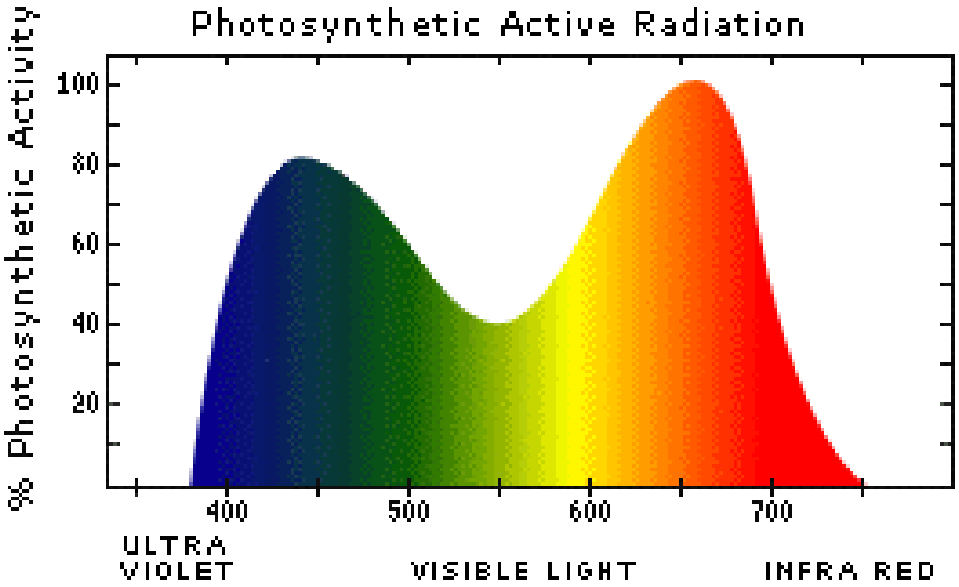
Culture environment

Most important environmental conditions in a tissue culture growth room are:

- Light
- Temperature
- Humidity
- Oxygen

LIGHT

For plants, it is measured in PAR (photosynthetically active radiation) - the number of photons per square meter per second for the spectral range from 400-700 nm.



- PAR is a more applicable measurement than other types such as lux because it measures the light spectrum that plant can use for photosynthesis.
- The amount of light needed for tissue culture explants is much lower compared to plants in grown in the field.
- Generally, in tissue culture growth rooms, 60-100 $\mu mol.m^{-2}s^{-1}$ PAR is used.

Light requirements of plants

The growth and development of plants is dependent on light for:

1. Photosynthesis

- The process whereby light energy is converted to chemical energy in the biosynthesis of chemicals from carbon dioxide and water.

2. Photomorphogenesis

- The light-induced development of structure or form.
- It does not necessarily involve the absorption of light energy, it uses receptor which act as switches to set in motion the morphogenetic processes of plants.

The influence of light on tissue culture

3 qualities of light which most clearly influence *in vitro* growth and morphogenesis.

1. Wavelength

Plants absorb blue and red lights, which have the greatest effect on plant growth.

Red light:

- Photosynthesis
- Seed germination, seedling growth
- Flowering, fruit development

Blue light:

- Photosynthesis
- Vegetative growth (leaf) growth

Growth rooms are mostly equipped with cool white fluorescent tubes.

Gro-lux tubes are specifically made to plants, but are more expensive.

Traditional incandescent light bulbs are not appropriate, since most of the light emitted is heat.

Limited reports on the effect of light on explant growth is available due to the complex relationship between light and plant.

The numerous factors that will affect plant response to light are:

- Plant species
- Type of explant (leaf, stem, root, etc)
- Type of development of the explant (embryo, callus, meristem, etc.)
- Often conflicting results are reported with different plants exposed to similar light conditions.

Blue light

Blue light (420nm) at a lower intensity stimulates callus and shoot development in tobacco explants.

While at higher intensities, it inhibits callus growth and cell division.

The intensity of blue light has the biggest effect on stimulation and inhibition of callus growth.

Red light

In general, red light (660 nm) promotes adventitious shoot formation in most

plants.

In addition, red light stimulates adventitious root growth in sunflowers and tobacco more than blue light.

While red light inhibits organogenesis in tobacco.

2. Photoperiod

Photoperiod is the length of time a plant receives light in 24 hours.

Photoperiod influences plants in 2 ways:

Growth of plant is proportional to the length of time that they are exposed to light.

High light (summer) = more growth

Low light (winter) = less growth

Plants are able to sense changes in the photoperiod and respond accordingly. In nature, photoperiod affects flowering and morphogenesis.

Photoperiod of tissue culture growth rooms is dependent on the type of explant cultured.

When uncertain, the photoperiod of plants in nature are used *in vitro.*

Most explants grow well with 14-16 hours of light.

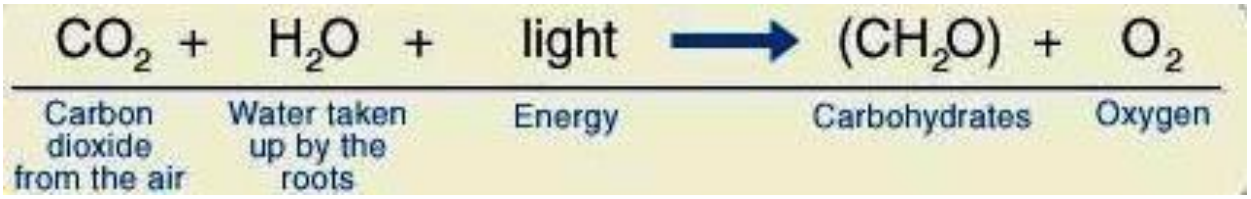
For specific purposes, complete darkness is used (eg., seed germination).

3. Light intensity

Temperature

In vivo photosynthesis:

- Increases with temperature up to a point.
- Although photosynthesis is low in tissue cultured explants, optimum temperature is still required for growth.



In vivo respiration:

Rapidly increases with temperature

Sugar, starch and oxygen is converted to CO_2 and energy.

The temperature of a growth room is usually kept constant at 24-28°C.

Sometimes in experiments, depending on the origins of the explants, lower temperature (18°C) for bulbous species, or higher temperature (28°C) for tropical species is chosen.

The temperature within the test tubes is 3-4°C higher than the growth room due to irradiance.

Sometimes alternating temperature conditions may be needed.

This is particularly evident in seed germination.

Common alternating temperature regimes include a 26°C daytime temperature, and 15°C night temperature.

For example, callus tissue of carrots grows best under a day temperature of 26°C and night temperature of 20°C.

Humidity

- Little is known about the influence of growth room humidity and *in vitro* growth.
- However, a growth room with high humidity increases the chances of contamination.
- Humidity is usually very high in the test tubes (90-100%).

This causes stomatal malfunction and hyperhydricity.

Oxygen

- Oxygen availability is important for *in vitro* root formation, as is the case *in vivo*.
- For woody plants, it is extremely difficult to regenerate roots when they are in solid medium.
- Root formation is much better in a liquid medium.
- In this case, the explant is supported on a paper-bridge or on rafts.

Culture room

Most commonly used conditions:

Temperature: 24ºC - 28ºC

Photoperiod: 16 – 24 hours

Lighting: 60 – 70 µmol m^{-2} s^{-1}

Plant cell culture media

Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues.

The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media.

Basically, the plant tissue culture media should contain the same nutrients as required by the whole plant. It may be noted that **plants in nature can synthesize their own food material.** However, **plants growing in vitro** are mainly **heterotrophic** i.e. they cannot synthesize their own food.

Composition of Media:

The composition of the culture media is primarily dependent on two parameters:

1. The particular species of the plant.

2. The type of material used for culture i.e. cells, tissues, organs, protoplasts.

Thus, the composition of a medium is formulated considering the specific requirements of a given culture system. The media used may be solid (solid medium) or liquid (liquid medium) in nature. The selection of solid or liquid medium is dependent on the better response of a culture.

TABLE 43.1 Composition of commonly used plant tissue culture media

Components	*Amount (mg l^{-1})*				
	White's	*Murashige and Skoog (MS)*	*Gamborg (B5)*	*Chu(N6)*	*Nitsch's*
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	—	0.025
$CoCl_2.6H_2O$	—	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	0.8	—
$FeSO_4.7H_2O$	—	27.8	—	27.8	27.8
$Na_2EDTA.2H_2O$	—	37.3	—	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thiamine HCl	0.01	0.5	10	1	0.5
Pyridoxine (HCl)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	—	100	100	—	100
Others					
Glycine	3	2	—	—	2
Folic acid	—	—	—	—	0.5
Biotin	—	—	—	—	0.05
pH	5.8	5.8	5.5	5.8	5.8

Major Types of Media:

The composition of the most commonly used tissue culture media is given in Table 43.1, and briefly described below.

White's medium:

This is one of the earliest plant tissue culture media developed for **root culture.**

MS medium:

Murashige and Skoog (MS) originally formulated a medium to **induce organogenesis,** and **regeneration** of plants in cultured tissues. These days, MS medium is widely used for many types of culture systems.

B5 medium:

Developed by Gamborg, B5 medium was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture.

N6 medium:

Chu formulated this medium and it is used for **cereal anther culture,** besides other tissue cultures.

Nitsch's medium:

This medium was developed by Nitsch and Nitsch and frequently used for **anther cultures**. Among the media referred above, MS medium is most frequently used in plant tissue culture work due to its success with several plant species and culture systems.

Synthetic and natural media:

When a medium is composed of **chemically defined components**, it is referred to as a **synthetic medium**. On the other hand, if a medium contains **chemically undefined compounds** (e.g., vegetable extract, fruit juice, plant extract), it is regarded as a **natural medium**. Synthetic media have almost replaced the natural media for tissue culture.

Expression of concentrations in media:

The concentrations of inorganic and organic constituents in culture media are usually expressed as mass values **(mg/l or ppm or mg/I)**. However, as per the

recommendations of the International Association of Plant Physiology, the concentrations of **macronutrients** should be expressed as **mmol/l** and **micronutrients** as **µmol/l**.

Constituents of Media:

Many elements are needed for plant nutrition and their physiological functions. Thus, these elements have to be supplied in the culture medium to support adequate growth of cultures in vitro. A selected list of the elements and their functions in plants is given in Table 43.2.

Table 43.2 A selected list of elements and their functions in plants

Element	*Function(s)*
Nitrogen	Essential component of proteins, nucleic acids and some coenzymes. (Required in most abundant quantity)
Calcium	Synthesis of cell wall, membrane function, cell signalling.
Magnesium	Component of chlorophyll, cofactor for some enzymes.
Potassium	Major inorganic cation, regulates osmotic potential.
Phosphorus	Component of nucleic acids and various intermediates in respiration and photosynthesis, involved in energy transfer.
Sulfur	Component of certain amino acids (methionine, cysteine and cystine, and some cofactors).
Manganese	Cofactor for certain enzymes.
Iron	Component of cytochromes, involved in electron transfer.
Chlorine	Participates in photosynthesis.
Copper	Involved in electron transfer reactions, Cofactor for some enzymes.
Cobalt	Component of vitamin B_{12}.
Molybdenum	Component of certain enzymes (e.g., nitrate reductase), cofactor for some enzymes.
Zinc	Required for chlorophyll biosynthesis, cofactor for certain enzymes.

The culture media usually contain the following constituents:

1. Inorganic nutrients
2. Carbon and energy sources
3. Organic supplements
4. Growth regulators
5. Solidifying agents
6. pH of medium

Inorganic Nutrients:

The inorganic nutrients consist of macronutrients (concentration >0.5 mmol/l) and micronutrients (concentration <0.5 mmol/l). A wide range of mineral salts (elements) supply the macro- and micronutrients. The inorganic salts in water undergo dissociation and ionization. Consequently, one type of ion may be contributed by more than one salt. For instance, in MS medium, K^+ ions are contributed by KNO_3 and KH_2PO_4 while NO_3^- ions come from KNO_3 and NH_4NO_3.

Macronutrient elements:

The six elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are the essential macronutrients for tissue culture. The ideal concentration of **nitrogen** and **potassium** is around **25 mmol/l** while for **calcium, phosphorus, sulfur** and **magnesium**, it is in the range of **1-3 mmol/l**. For the supply of nitrogen in the medium, **nitrates** and **ammonium** salts are together used.

Micronutrients:

Although their requirement is in minute quantities, micronutrients are essential for plant cells and tissues. These include **iron, manganese, zinc, boron, copper** and **molybdenum**. Among the microelements, iron requirement is very critical. Chelated forms of iron and copper are commonly used in culture media.

Carbon and Energy Sources:

Plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon for energy. Among the energy sources, **sucrose** is the most preferred. During the course of sterilization (by autoclaving) of the medium, sucrose gets hydrolysed to glucose and fructose.

The plant cells in culture first utilize glucose and then fructose. In fact, glucose or fructose can be directly used in the culture media. It may be noted that for energy supply, glucose is as efficient as sucrose while fructose is less efficient.

It is a common observation that cultures grow better on a medium with **autoclaved sucrose** than on a medium with filter-sterilized sucrose. This clearly indicates that the hydrolysed products of sucrose (particularly glucose) are efficient sources of energy. Direct use of fructose in the medium subjected to autoclaving, is found to be detrimental to the growth of plant cells. Besides sucrose and glucose, other carbohydrates such as **lactose, maltose, galactose, raffinose, trehalose** and **cellobiose** have been used in culture media but with a very limited success.

Organic Supplements:

The organic supplements include vitamins, amino acids, organic acids, organic extracts, activated charcoal and antibiotics.

Vitamins:

Plant cells and tissues in culture (like the natural plants) are capable of synthesizing vitamins but in suboptimal quantities, inadequate to support growth. Therefore the medium should be supplemented with vitamins to achieve good growth of cells. The vitamins added to the media include thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenic acid, biotin, ascorbic acid, myoinositol, Para amino benzoic acid and vitamin E.

Amino acids:

Although the cultured plant cells can synthesize amino acids to a certain extent, media supplemented with amino acids stimulate cell growth and help in establishment of cells lines. Further, organic nitrogen (in the form of amino acids such as L-glutamine, L-asparagine, L- arginine, L-cysteine) is more readily taken up than inorganic nitrogen by the plant cells.

Organic acids:

Addition of Krebs cycle intermediates such as citrate, malate, succinate or fumarate

allow the growth of plant cells. Pyruvate also enhances the growth of cultured cells.

Organic extracts:

It has been a practice to supplement culture media with organic extracts such as yeast, casein hydrolysate, coconut milk, orange juice, tomato juice and potato extract. It is however, preferable to avoid the use of natural extracts due to high variations in the quality and quantity of growth promoting factors in them. In recent years, natural extracts have been replaced by specific organic compounds e.g., replacement of yeast extract by L-asparagine; replacement of fruit extracts by L-glutamine.

Activated charcoal:

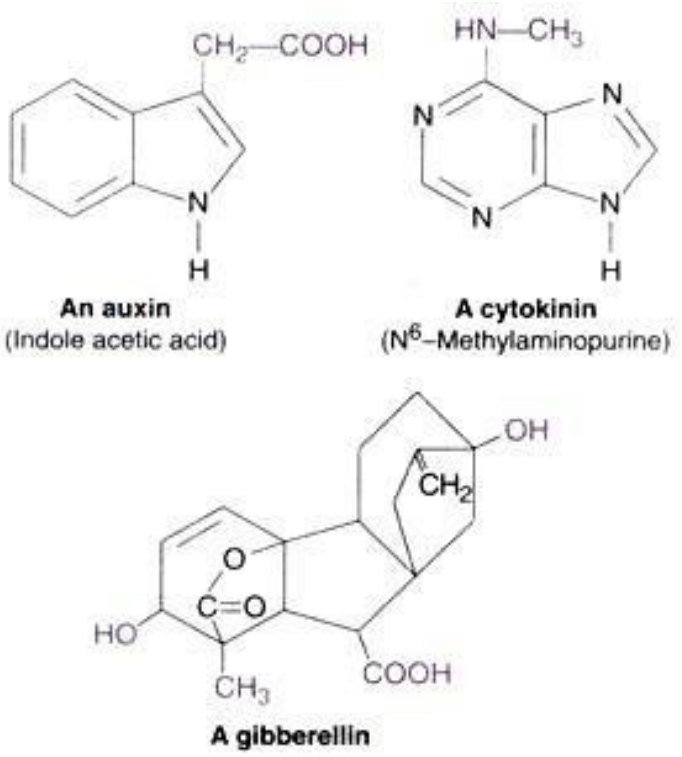
Supplementation of the medium with activated charcoal stimulates the growth and differentiation of certain plant cells (carrot, tomato, orchids). Some toxic/inhibitory compounds (e.g. phenols) produced by cultured plants are removed (by adsorption) by activated charcoal, and this facilitates efficient cell growth in cultures. Addition of activated charcoal to certain cultures (tobacco, soybean) is found to be inhibitory, probably due to adsorption of growth stimulants such as phytohormones.

Antibiotics:

It is sometimes necessary to add antibiotics to the medium to prevent the growth of microorganisms. For this purpose, low concentrations of streptomycin or kanamycin are used. As far as possible, addition of antibiotics to the medium is avoided as they have an inhibitory influence on the cell growth.

Growth Regulators:

Plant hormones or phytohormones are a group of natural organic compounds that promote growth, development and differentiation of plants. Four broad classes of growth regulators or hormones are used for culture of plant cells-auxins, cytokinins, gibberellins (Fig. 43.1) and abscisic acid. They promote growth, differentiation and



An auxin
(Indole acetic acid)

A cytokinin
(N^6–Methylaminopurine)

A gibberellin

Fig. 43.1 : Structures of selected plant growth regulators.

organogenesis of plant tissues in cultures.

Auxins:

Auxins induce cell division, cell elongation, and formation of callus in cultures. At a low concentration, auxins promote root formation while at a high concentration callus formation occurs. A selected list of auxins used in tissue cultures is given in Table 43.3.

Among the auxins, 2, 4-dichlorophenoxy acetic acid is most effective and is widely used in culture media.

TABLE 43.3 A selected list of plant growth regulators used in culture media

Growth regulator (abbreviation/name)	*Chemical name*
Auxins	
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
NAA	1-Naphthyl acetic acid
2, 4-D	2, 4-Dichlorophenoxy acetic acid
2, 4, 5-T	2, 4, 5-Trichlorophenoxy acetic acid
4–CPA	4-Chlorophenoxy acetic acid
NOA	2-Naphthyloxy acetic acid
MCPA	2-Methyl 4-chlorophenoxy acetic acid
Dicamba	2-Methoxy 3, 6-dichlorobenzoic acid
Picloram	4-Amino 2, 5, 6-trichloropicolinic acid
Cytokinins	
BAP	6-Benzyl aminopurine
BA	Benzyl adenine
2 iP (IPA)	N^6-(2-isopentyl) adenine
DPU	Diphenyl urea
Kinetin	6-Furfuryl aminopurine
Zeatin	4-Hydroxy 3-methyltrans 2-butenyl aminopurine
Thidiazuron	1-Phenyl 3-(1, 2, 3-thiadiazol-5 yl) urea

Cytokinins:

Chemically, cytokinins are derivatives of a purine namely adenine. These adenine derivatives are involved in cell division, shoot differentiation and somatic embryo formation. Cytokinins promote RNA synthesis and thus stimulate protein and enzyme activities in tissues. The most commonly used cytokinins are given in Table 43.3. Among the cytokinins, kinetin and benzyl-amino purine are frequently used in culture media.

Ratio of auxins and cytokinins:

The relative concentrations of the growth factors namely auxins and cytokinins are crucial for the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation and root initiation occur.

On the other hand, for axillary and shoot proliferation, the ratio of auxins to cytokinins is low. For all practical purposes, it is considered that the formation and maintenance of callus cultures require both auxin and cytokinin, while auxin is needed for root culture and cytokinin for shoot culture. The actual concentrations of the growth regulators in culture media are variable depending on the type of tissue explant and the plant species.

Gibberellins:

About 20 different gibberellins have been identified as growth regulators. Of these, gibberellin A_3 (GA_3) is the most commonly used for tissue culture. GA_3 promotes growth of cultured cells, enhances callus growth and induces dwarf plantlets to elongate. Gibberellins are capable of promoting or inhibiting tissue cultures, depending on the plant species. They usually inhibit adventitious root and shoot formation.

Abscisic acid (ABA):

The callus growth of cultures may be stimulated or inhibited by ABA. This largely depends on the nature of the plant species. Abscisic acid is an important growth regulation for induction of embryogenesis.

Solidifying Agents:

For the preparation of semisolid or solid tissue culture media, solidifying or gelling

agents are required. In fact, solidifying agents extend support to tissues growing in the static conditions.

Agar:

Agar, a polysaccharide obtained from seaweeds, is most commonly used as a gelling agent for the following reasons.

1. It does not react with media constituents.
2. It is not digested by plant enzymes and is stable at culture temperature.

Agar at a concentration of 0.5 to 1% in the medium can form a gel.

Gelatin:

It is used at a high concentration (10%) with a limited success. This is mainly because gelatin melts at low temperature (25°C), and consequently the gelling property is lost.

Other gelling agents:

Bio-gel (polyacrylamide pellets), phytagel, gelrite and purified agarose are other solidifying agents, although less frequently used. It is in fact advantageous to use synthetic gelling compounds, since they can form gels at a relatively low concentration (1.0 to 2.5 g/l).

pH of medium:

The optimal pH for most tissue cultures is in the range of 5.0-6.0. The pH generally falls by 0.3-0.5 units after autoclaving. Before sterilization, pH can be adjusted to the required optimal level while preparing the medium. It is usually not necessary to use buffers for the pH maintenance of culture media.

At a pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures. If the pH falls during the plant tissue culture, then fresh medium should be prepared. In general, pH above 6.0 gives the medium hard appearance, while pH below 5.0 does not allow gelling of the medium.

Preparation of Media:

The general methodology for a medium preparation involves preparation of stock solutions (in the range of 10x to 10Ox concentrations) using high purity chemicals and demineralized water. The stock solutions can be stored (in glass or plastic containers) frozen and used as and when required. Most of the growth regulators are not soluble in water.

They have to be dissolved in NaOH or alcohol.

Dry powders in Media Preparation:

The conventional procedure for media preparation is tedious and time consuming. Now a day, plant tissue culture media are commercially prepared, and are available in the market as dry powders. The requisite medium can be prepared by dissolving the powder in a glass distilled or demineralized water. Sugar, organic supplements and agar (melted) are added, pH adjusted and the medium diluted to a final volume (usually 1 litre).

Sterilization of Media:

The culture medium is usually sterilized in an autoclave at 121°C and 15 psi for 20 minutes. Hormones and other heat sensitive organic compounds are filter-sterilized, and added to the autoclaved medium.

Selection of a Suitable Medium:

In order to select a suitable medium for a particular plant culture system, it is customary to start with a known medium (e.g. MS medium, B5 medium) and then develop a new medium with the desired characteristics. Among the constituents of a medium, growth regulators (auxins, cytokinins) are highly variable depending on the culture system. In practice, 3-5 different concentrations of growth regulators in different combinations are used and the best among them are selected. For the selection of appropriate concentrations of minerals and organic constituents in the medium, similar approach referred above, can be employed.

Medium-utmost Important for Culture:

For tissue culture techniques, it is absolutely essential that the medium preparation and composition are carefully followed. Any mistake in the preparation of the medium is likely to do a great harm to the culture system as a whole.

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DNA isolation & extraction

CTAB technique / Protocol for DNA isolation / DNA extraction from plant leaf / leaf samples

Reagents needed

CTAB buffer
2% CTAB 20gm CTAB
20mM EDTA 40ml EDTA stock (0.5M)
100mM Tris-Cl pH 8.0 100ml Tris-Cl stock (1M)
1.4M NaCl 280ml NaCl stock (5M)
make up to 1 Litre with water, pH 7.5 - 8.0, and autoclave
+ 0.2% Mercaptoethanol
Wash Buffer
76% Ethanol
10mM NH4 Ac

DNA Extraction

1. Preheat 5ml CTAB (add 10µl mercaptoethanol to each 5ml CTAB) in a blue-topped 50ml centrifuge tube at 60-65^{o}C. Remove and discard midribs, and wrap laminae in aluminium foil and freeze in liquid nitrogen. 0.5 – 1.0 gm tissue/5ml CTAB
(Can store leaf material after liquid Nitrogen – 1-2 days at –20 or –80 for longer periods)
2. Gently crumble leaf tissue over cold pestle of liquid nitrogen. Grind frozen leaf with one spatula of fine sand adds 0.5 spatula of PVPP powder after grinding.
3. Scrape powder into dry tube and add pre-heated buffer and mix gently. Avoid leaving dry material around rim of tube. Adjust CTAB volume to give a slurry-like consistency, mix occasionally.
4. Incubate for 60 min at 60^{o}C
5. Add equal volume of chloroform/iso-amyl alcohol (24:1), Mix for about 3min, then transfer contents to narrow bore centrifuge tubes. Balance by adding extra chlor/iso. Spin 5,000rpm for 10min (ensure correct tubes used), brake off. (For extra pure DNA isolation - spin and retain supernatant before chloroform extraction).
6. Remove supernatant with wide-bore pastette (cut off blue tip) to clean tube, repeat chloroform extraction once. Supernatant should be clear, though may be coloured.
7. Precipitate DNA with 0.66 vol. of cold isopropanol - can leave overnight. Spool out or spin down DNA, 2min at 2,000rpm.

8. Transfer to 5ml wash buffer for 20min.

9. Dry briefly and resuspend in 1ml T.E. (can be left overnight)
10. Add 1µl 10mg/ml RNAse to each 1ml T.E./DNA mixture and incubate for 60min at 37 ^{o}C. (If RNase in the sample doesn't matter – stages 11 and 12 may be omitted)
11. Dilute with 2 volumes TE and add 0.3vol 3M Sodium acetate (pH 8) + 2.5 vol cold 100% ethanol,
12. Spool DNA out. Air dry and resuspend in 0.5 to 1ml TE or water (takes time) and freeze until required.

DNA Quantification

An approximate way to determine DNA concentration is to look at the viscosity of the solution: not accurate to 10% but, unlike spectrophotometry, you will not get results which are 10 or 100 times wrong!

In a microcentrifuge tube, DNA solutions stronger than 0.1 µg/µl will show a reluctance to pour when you tilt the tube. From about 0.5 µl/µg and above, you can tilt the tube - very gently - and the solution will stay at the end. If you dip a 10-200 µl (yellow) pipette tip into the solution and pull it away, a solution of 1µg/µl will form a distinct string from the surface to the tip which breaks when about 1 to 2 mm long.

Make a 0.8% agarose gel with 1x TAE and 0.1µl of Ethidium bromide (10mg/ml) per 10ml solution. Load samples undiluted and at a 1 in 10 (1+9) dilution., with 3µl loading buffer. Also include a Lamda ladder cut with HindIII and EcoRI. This contains 100ng of

DNA per microlitre and use as follows:

1µl ladder + 4µl water + 2µl loading buffer

2µl ladder + 3µl water + 2µl loading buffer

The different bands of the ladder are of known molecular weight and known DNA concentration. Match the brightness of your samples with those of the two dilutions of the ladder. Refer to the diagram to match the band with the concentration. Remember that although the ladder concentrations are absolute, you have loaded 5µl of sample and also diluted some of them. This must be taken into account when calculating the strength of the sample s in ng/µl.

Pestles and mortars washed for 20-30min in 0.25M HCl, rinsed in water and air- dried, all mess to be tidied up and tubes washed and left to drain.

References

http://www.le.ac.uk/bl/phh4/dnaiso.htm

Isolation of plasmid DNA

The various plasmid isolation techniques that are currently in use can be divided into three phases:

a. Growth of bacterial cells;
b. Harvesting and lysis of bacterial cells;
c. Purification of plasmid DNA.

One of the most common bacterial host strains used for the *in vivo* amplification of plasmids is *E. coli* XL1-Blue, a derivative of the *E. coli* K12 strain. XL1-Blue cells can be readily transformed with plasmids. They are suitable for α-complementation analysis, and can also be transduced with filamentous phages. Depending on the amount of plasmid DNA to be isolated, bacterial cells can be grown in various volumes of shaken culture in the presence of antibiotic(s) suitable for the desired selection. By applying the most commonly used, so-called "miniprep" plasmid isolation protocols, 1-10 μg of isolated plasmid can be prepared from 3-5 mL of cell culture volume.

Following bacterial growth, the cells can be pelleted by centrifugation in a microcentrifuge. After disposing of the supernatant, plasmid isolation from the pelleted cells can proceed principally in two different ways:

a. By applying a "classical" method involving phenol-chloroform extraction and subsequent precipitation of the plasmid by using ethanol (see below in details).
b. By using a plasmid isolation kit. In this case the isolation of the plasmid is performed using a miniaturised chromatographic column.

The "classical" method for plasmid isolation is composed of the steps described below.

a. Resuspension of the bacterial pellet in an isotonic solution. In this solution, the lysis of the cells does not yet take place. The ethylene diamine tetraacetate (EDTA) contained in the resuspension solution inhibits the nuclease activity of cellular enzymes via complexation of Mg^{2+} ions. Some earlier protocols also applied lysozyme to break down the bacterial cell wall. In addition to the above listed components, the resuspension solution can also contain RNase enzyme in order to break down ribonucleic acids that are later released into the lysate.
b. Alkaline lysis of the cells by applying an alkaline solution of sodium dodecyl sulfate (SDS) that disintegrates the lipid structure of the cell membrane. In addition, this

treatment will denature both proteins and DNA, and keep these molecules dissolved in their denatured form.

c. Precipitation of dissolved proteins, membrane debris and associated genomic DNA by applying a solution of acidic potassium acetate. The potassium salt of dodecyl sulfate is also insoluble. Thus, this component will also precipitate. Plasmid DNA will still remain dissolved during this step.
d. Sedimentation of the precipitated components in a microcentrifuge. The clear supernatant will contain the plasmid DNA.
e. Purification of plasmid DNA using a mixture of phenol and chloroform (care should be taken when working with phenol as it is corrosive to human tissues). To remove the protein content of nucleic acid preparations, a mixture is often used that contains phenol and chloroform in a volume ratio of 1:1, isoamyl alcohol in a volume ratio of 1:24 with regard to the rest of the components, and is saturated with TE pH 8.0 (tris-hydroxymethyl-aminomethane buffer containing 1 mM EDTA (ethylene diamine tetraacetate)). Phenol denatures proteins, and chloroform readily dissolves phenol, which has limited water solubility. When the nucleic acid preparation is shaken thoroughly with the described mixture and subsequently centrifuged, denatured proteins will be concentrated at the boundary of the upper aqueous and the lower phenol-chloroform phase of higher density. Isoamyl alcohol reduces the frothing associated with the separation procedure.
f. The addition of ethanol to the aqueous phase containing the plasmid will result in the precipitation of the plasmid DNA, which can thus be subsequently sedimented by centrifugation.
g. The precipitate containing the plasmid DNA is washed with 70 % ethanol in order to remove the salt content of the preparation.
h. The plasmid DNA is sedimented repeatedly by centrifugation, and then dissolved in TE solution (see point (e) above for composition). The preparation can be stored on ice or in a freezer. The TE solution may also contain DNase-free RNase enzyme in order to eliminate ribonucleic acids.

During plasmid isolation using commercially-available kits (Figure 10.6), the steps of resuspension, alkaline lysis, precipitation with acidic potassium acetate and subsequent centrifugation (steps (a)-(d) above) are performed similarly to those described for the “classical” method, which the difference that the reagents supplied by the kit manufacturer are used for the procedure. The supernatant resulting from this series of

steps will contain the plasmid DNA. This supernatant is loaded on top of a mini-column containing a silicate-based membrane. The mini-column is placed in an Eppendorf tube so that the flow-through can be collected upon centrifugation. Under the applied high ionic strength conditions, the column will bind DNA molecules in the size range of 100 base pairs to 10 kilo-base pairs. The column is subsequently washed with a wash buffer and a solution with high ethanol content. The ethanol is then removed via repeated centrifugation. The plasmid DNA is then dissolved in TE or a similar low ionic strength solution.

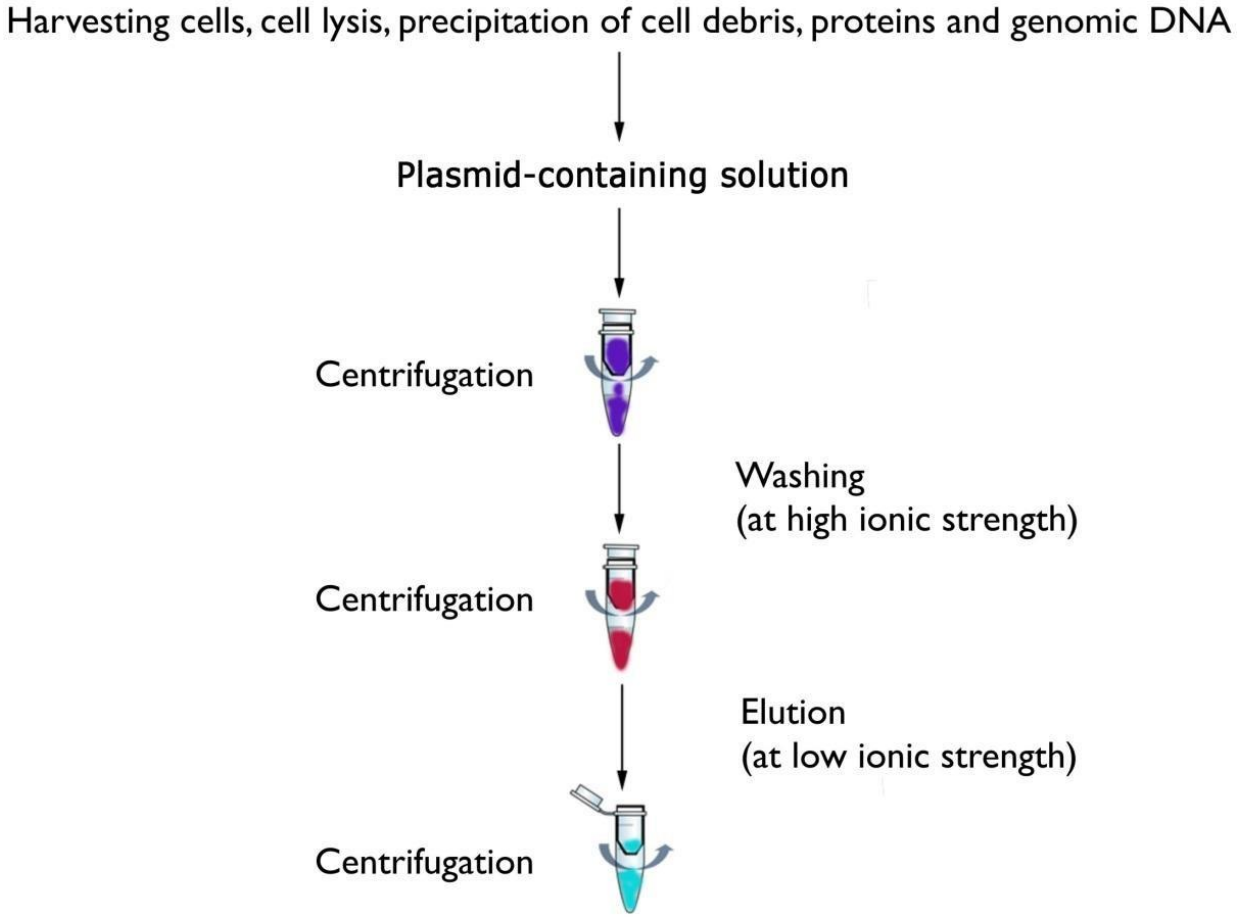

Figure 10.6. Isolation of plasmid DNA by using a mini-column containing a DNA-binding resin.

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Molecular biology of *Agrobacterium* infection

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DEPARTMENT OF BITOECHNOLOGY

UNITII- PLANT BIOTECHNOLOGY- SBB3103

PROCESS OF ANDROGENESIS (WITH DIAGRAM)

https://www.biologydiscussion.com/biotechnology/plant-biotechnology

A. In Vitro Pathways of Androgenesis:

The anther or pollen normally starts undergoing in the pathway of androgenesis within 2 weeks and it takes about 5-8 weeks to obtain complete plantlets.

Haploid plants are formed in two ways:

(i) Direct Androgenesis:

Embryos are directly formed from the pollen/microspore without callus.

(ii) Indirect Androgenesis:

Microspores undergoes division in unorganised fashion to give rise to callus and by embryogenic or organogenic induction the haploid plantlets may be obtained.

There are four different pathways to form the multicellular condition of pollen from the unicellular pollen (Fig. 21.4).

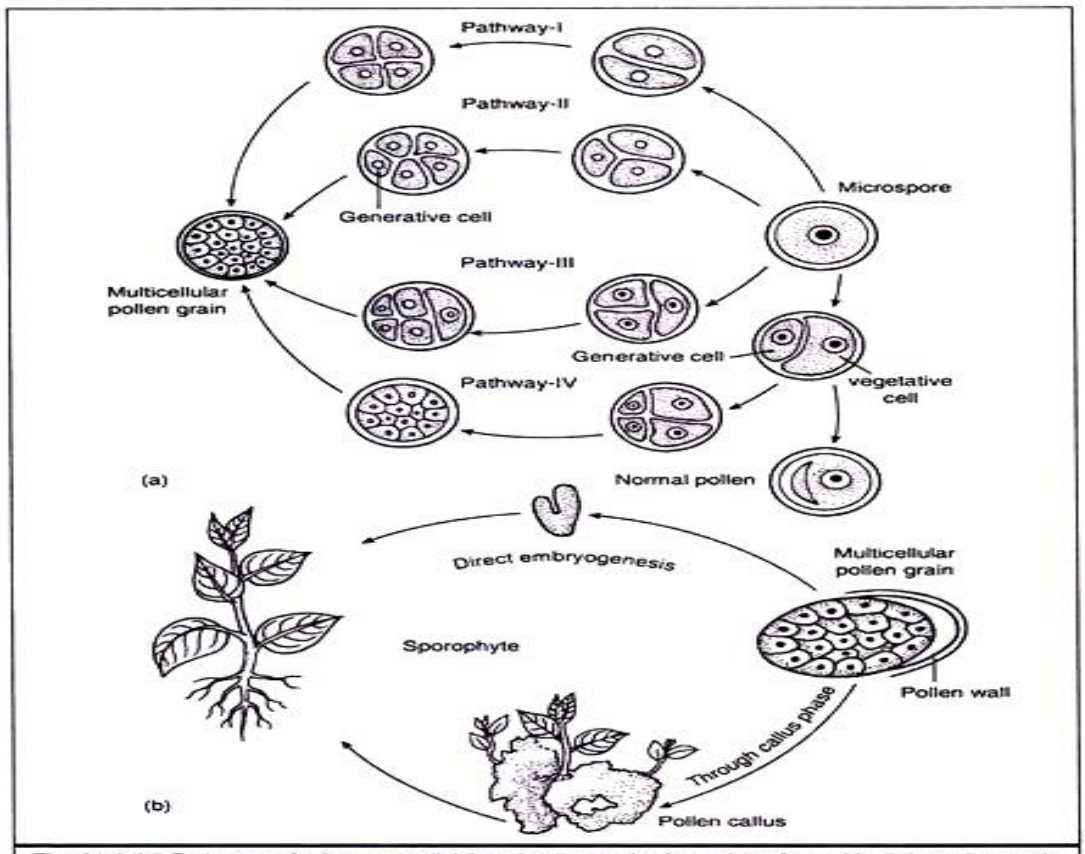


Fig. 21.4: (a) Pathways of microspore divisions leading to the formation of a multicellular pollen grain; (b) Multicellular pollen may directly form an embryo or produce sporophytes through a callus phase (after Bhojwani and Bhatnagar, 1990)

Pathway I:

The microspore divides by an equal division and two identical daughter cells contribute equally to the sporophyte development e.g., Datura innoxia.

Pathway II:
The uninucleate microspores divide unequally forming vegetative and generative cell. The sporophyte arises through divisions of vegetative cell, the generative cell gets degenerated e.g., Nicotiana tabacum.

Pathway III:
he uninucleate microspore undergoes a normal unequal division but the pollen embryos are formed from the generative cell alone. The vegetative cell does not divide, e.g., Hyocyamus niger.

Pathway IV:
The division of microspore is asymmetrical as in pathway II, but both the cells take part in embryo formation and sporophyte development e.g., Datura metal.

B. Factor Affecting Androgenesis:

(a) Stage of Pollen:
Anthers with microspores ranging from tetrad to the bi-nucleate stage are responsive to culture. As soon as the starch deposition starts within the microspore there is no further development towards sporophyte.

(b) Physiological Status of Donor Plant:
The anther should be collected from the flower buds of adult healthy plant and it is very important to use the healthy explant. The variation in response of anthers from plants grown under different environmental conditions may be due to the differences in endogenous level of growth regulators. Flowers from relatively younger plants, flowering at the beginning of flower-season are more responsive.

(c) Genotype of Plants:
Success of anther culture is highly dependent on the genotype of the plant. It has been observed that various species and cultivars exhibit different growth responses in culture.

(d) Pretreatment of Anthers:
The pathways towards androgenesis require to stop the development of pollen cell towards gamete formation and to force to develop the multicellular condition.

This induction can be given by different types of pre-treatments:
(i) Cold Shock:

In general cold shock or cold treatment between 3-6°C for 3-15 days may be applied. Cold treatment helps the weak and non-viable anthers/microspore to be killed, as a result the good material get more enrichment.

(ii) Hot Treatment:
In some species the hot water treatment (3'0-40°C for 24 hr-1 hr) helps more embryo development from the pollen. The temperature shock helps in dissolution of microtubules and causes abnormal division of microspores.

(iii) Chemical Treatment:
Chemicals like 2-chloroethyl phosphonic acid have a pronounced effect in increasing the haploid production in various species. It helps to develop the multinucleate condition with fewer starch grains.

(e) Culture Media:
Composition of medium is one of the most important factors determining the success of anther culture. But it is difficult to draw a conclusion about the suitable media composition for different genes/species or even genotypes.

Higher concentration of sucrose is essential for osmoticum and also androgenesis, chelated iron has been shown to induce embryogenesis. Minerals and growth regulators play important roles on embryogenesis but it totally depends on the endogenous level of hormones.

(f) Culture Conditions:
The physical environmental conditions in which the cultures to be placed can enhance the differentiation. The cultures are incubated generally at 24-28°C.

In the initial stages of induction of morphogenesis, darkness is normally more effective or cultures should be kept at low light intensity (500 lux). After formation of macroscopic structures, these can be transferred to a regeneration medium and kept at 14 hr. day light condition at 2000-4000 lux.

C. Diploidisation of Haploids:

The ploidy level of plants derived from anther or microspore culture is highly variable. This variation may be due to endomitosis or fusion of various nuclei during the developmental stages of anthers at the time of excision and culture.

For obtaining homozygous diploid lines, the plants derived through anther culture must be analysed for their ploidy level and then the following methods can be applied:

Colchicine Treatment:

As colchicine is a spindle inhibitor it is used to induce chromosome duplication in various ways:

(a) The regenerated plantlets can be treated with 0.5% colchicine solution.

(b) Repeated colchicine treatment to apical or axillary bud by cotton soaked in colchicine or by applying colchicine-lanolin paste.

(c) In case of cereals, the tillers can be put into colchicine solution cutting the crown and then again put back to soil.

(d) Anthers can be plated directly in colchicine supplemented media for a period and just after first division again the explants should be placed on colchicine-free media.

Endomitosis:

Haploids are in general unstable in culture and have a tendency to undergo endomitosis to form diploid cells. This property can be exploited in some cases to obtain the homozygous plant.

What is Gynogenesis?

The development of entire plants from unfertilized female gametes is termed, "gynogenesis." It serves as a valuable alternative to haploid production for species in which anther culture (Androgenesis) fails to give satisfactory results. It was first reported by a scientist named San Noeum in 1976 and, so far, it's been studied in 19 species belonging to 10 different families.

In nature, gynogenesis occurs through parthenogenesis, and in labs, the embryo formation by female gametophyte occurs via two pathways: direct embryogenesis (formation of an embryo without callus) and indirect embryogenesis (formation of an embryo with intermediary callus formation).

In this article, you will get an understanding of the history and the factors affecting gynogenesis.

HISTORY OF GYNOGENESIS

The research on haploid production by gynogenesis was initiated in 1965. However, the research didn't get much attention because of the breakthrough made by Guha and Maheshwari (1964) in the production of androgenic haploids.

In 1971, significant research on gynogenesis was initiated. A report published by Uchimiya et al. showed the division of haploid cells forming callus when unpollinated ovaries of *Zea mays* and ovules of *Solanum melongena* were cultured.

Successful reports on gynogenic haploids were first published in 1976 and 1979 by San Noeum and it has now been studied in several economically important plants. These include wheat, rice, barley, maize, sunflower, etc.

The production of a haploid by gynogenesis is influenced by several key factors that are discussed in the section below.

FACTORS AFFECTING GYNOGENESIS

1. EXPLANTS (THE PART OF THE PLANT TO START FROM)

Young flowers, ovary, ovule, and unfertilized parts of the embryo sac are considered suitable explants, depending on species and the application thereof. For example, only ovule culture has been successfully observed in *Gerbera jamesonii*. In rice and barley, culturing whole florets gives better results.

The stage of explant tissue required also differs significantly from case to case. When anthers are not mutated, the young flowers are used in most cases--except in the case of male-sterile plants.

2. Pre-treatments of the explant

Cold treatment, starvation, and heat shock of explants have been very useful in enhancing haploid production by gynogenesis. For example, treating sunflowers at 4℃ for 24-48 hours before culturing results in an increase of the overall embryo yield.

In rice, sugar beet, wheat, and *Salvia sclarea*, cold treatment at 8℃ for 14-16 days enhances the gynogenic response to multifold. In some species, like *Picea sitchensis* and cucumber spp., heat shock treatment (during the culture/induction phase) at 32-33℃ has been observed to promote the sporophytic development of the female gametophyte. However, in many other species like niger, *Cucurbita pepo,* and rice spp., any pretreatment causes detrimental effects.

3. Culture Medium

N6 and MS media with a high concentration of sucrose (different concentrations depending on the species) are the most suitable media to produce maternal haploids. In gramineous species, the production of haploid can be induced in a media containing hormone or growth regulators (like 2,4-D, NAA, and BAP). However, in some species like sunflower, the presence of hormones suppresses the embryo development from a female gametophyte.

It should also be noted that the process of gynogenesis is a multistep process. So, each step has different requirements for nutrients, light exposure, and hormone regulators.

4. Genotype

The haploid production also depends on the donor plants and the response varies from species to species. For example, in *Allium cepa*, the open-pollinated cultivars show low response to gynogenesis than inbred lines and F 1 hybrids.

In some species of plants (like sugar beet), the florets grown on the lateral branches have better embryogenic response compared to the florets grown on the main branch.

APPLICATION OF HAPLOID PRODUCTION BY GYNOGENESIS

Very few species have had their gynogenic response studied because it's less efficient, more tedious, and restricted in comparison to haploid production by androgenesis. However, this method has been observed as one of the best alternatives to haploid production in cases where androgenesis cannot be performed. Some examples are explained below.

1. Gynogenesis has been useful for haploid production in plants like sugar beet, onion, and melon.
2. It's been an essential technique for haploid production in male-sterile plants. Some successful cases have been observed in unfertilized ovary culture.
3. The technique has been used to produce green haploids where albinism was a problem. For example, in rice, 83% of green plants were observed in gynogenic cultures compared to 1% in androgenic plants.
4. In some species of plants (like rice), it has been found that gynogenic cultures are more efficient and yield more haploids than androgenic cultures.

Plant Protoplast Culture: Meaning, History and Principles

What is a Protoplast?

It is known that each and every plant cell possesses a definite cellulosic cell wall and the protoplast lies within the cell wall except some reproductive cells and the free floating cells in some fruit juices like coconut water.

Therefore, protoplast of plant cell consists of plasma-lemma and everything contained within it.

But those of importance to plant protoplast culture are produced experimentally by the removal of cell wall by either enzymatically or mechanical means from the artificially plasmolysed plant cells. Experimentally produced protoplasts are known as isolated protoplasts.

According to Torrey and Landgren (1977) “the isolated protoplasts are the cells with their walls stripped off and removed from the proximity of their neighbouring cells”. Vasil (1980) defines that “the protoplast is a part of plant cell which lies within the cell wall and can be plasmolysed and which can be isolated by removing the cell wall by mechanical or enzymatic procedure”. Therefore, isolated protoplast is only a naked plant cell surrounded by plasma membrane—which is potentially capable of cell wall regeneration, cell division, growth and plant regeneration in culture.

Brief Past History:

J. Klercker (1892):

First isolated protoplast mechanically from plasmolyzed cell of water warrior (Stratiotes aloides). No attempt was made to culture them.

E. Kiister (1927):

In the fruits of several plants like Solatium nigram, Lycopersicon esculenium etc. the cell wall are hydrolysed during fruits ripening process so that free protoplasts and protoplasmic units are left. Kuster preferred such physiological method for isolating protoplasts. No report of culture was available.

R. Chambers and K. Hofler (1931):

Were able to isolate few protoplasts by using thin slices of epidermis of onion bulb immersed in 1M sucrose until the protoplast shrunk away from their enclosing walls and then cutting sheets of epidermis with a sharp knife. Report of culture was not available.

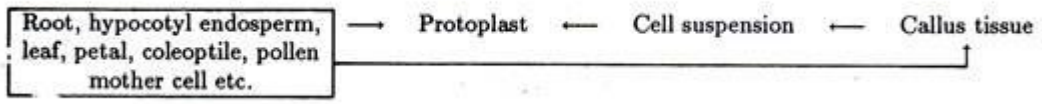
E. C. Cocking (I960):

First reported the enzymatic method for isolation of protoplast in a large number from root tip cells of Lycopersicon esculentum by using a concentrated solution of cellulase enzyme, prepared from cultures of the fungus Myrothecium verrucaria to degrade cell wall.

I. Takebe, Y. Otsuki and S. Aoki (1968):

First employed the commercial preparation of cellulase and macerozyme sequentially (in two steps) for the isolation of mesophyll protoplast of tobacco.

J. B. Power and E. C. Cocking (1968):
Demonstrated first that the mixture of such two enzymes (cellulase + macerozyme) can be used simultaneously (one step method) for the isolation of protoplasts.



I. Takebe, G. Labib, G. Melchers (1971):
First reported the plant regeneration from isolated protoplast in Nicotiana tabacum.

P. S. Carlson, H. H. Smith, R. D. Dearing (1972):
First reported a somatic hybrid in higher plants involving two different sexually compatible species of mesophyll protoplast (N. glauca x N. langsdorffi).

Different Sources of Plant Tissue and Their Condition for Protoplast Isolation:
Protoplast can be isolated either directly from the different parts of whole plant or indirectly from in vitro cultured tissue. Convenient and suitable materials are leaf, mesophyll and cells from liquid suspension cultures. Protoplast yield and viability are profoundly influenced by the growing conditions of plants serving leaf mesophyll sources.

The age of the plant and of the leaf and the prevailing conditions of light, photoperiod, humidity, temperature, nutrition and watering are contributing factors. Cell suspension cultures may provide a more reliable source for obtaining consistent quality protoplasts. It is necessary, however, to establish and maintain the cells at maximum growth rates and utilize the cell at the early log phase.

Principles of Protoplast Culture:
The basic principle of protoplast culture is the aseptic isolation of large number of intact living protoplasts removing their cell wall and cultures them on a suitable nutrient medium for their requisite growth and development. Protoplast can be isolated from varieties of plant tissues. Convenient and suitable materials are leaf mesophyll

and cells from liquid suspension culture. Protoplast yield and viability are greatly influenced by the growing condition of the plant as well as the cells.

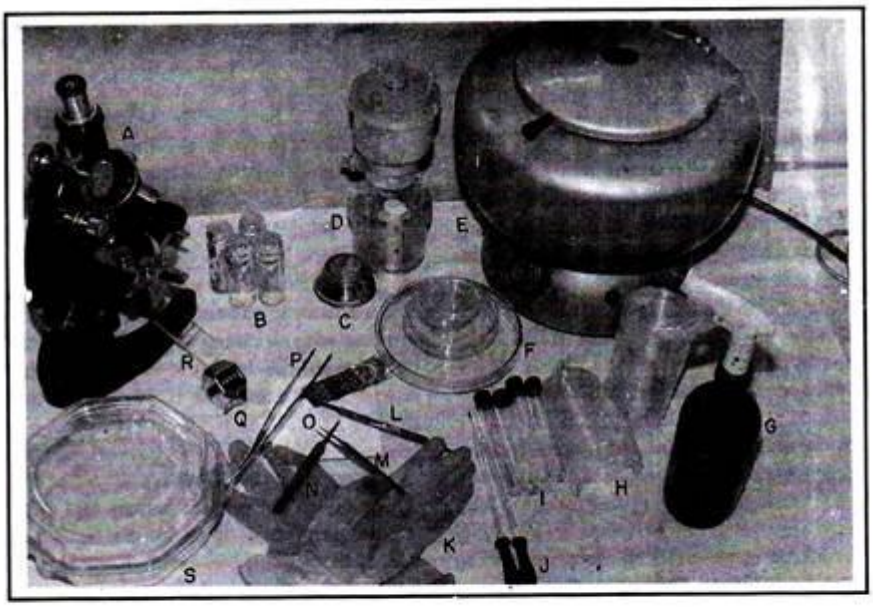


Fig 12.1

Instruments required for a plant protoplast culture. A. Compound microscope. B. Screw topped bottle. C. Nylon mesh. D. Bacterial filter. E. Centrifuge mechine. F. Petridishes. G. Alcohol sprayer. H. Disposable sterile petridishes. I. Screw capped centrifuge tube. J. Pasteur pipette. K. Hand gloves. L. Disposable sterile scalpel. M & N. Jewellery fine forceps. O. Tile. P. Long forceps. Q. Counter. R. Haemocytometer. S. Casserole. (Photograph taken by Mr. T. K. Bera)

The essential step of the isolation of protoplast is the removal of the cell wall without damaging the cell or protoplasts. The plant cell is an osmotic system. The cell wall exerts the inward pressure upon the enclosed protoplasts. Likewise, the protoplast also puts equal and opposite pressure upon the cell wall. Thus, both the pressures are balanced.

Now if the cell wall is removed, the balanced pressures will be disturbed. As a result, the outward pressure of protoplast will be greater and at the same time in absence of cell wall, irresistible expansion of protoplast takes place due to huge inflow of water from the external medium. Greater outward pressure and the expansion of protoplast cause it to burst.

So, the isolated protoplast is an osmotically fragile structure at its nascent stage. Therefore, if the cell wall is to be removed to isolate protoplast, the cell or tissue must be placed in a hypertonic solution of a metabolically inert sugar such as mannitol at higher

concentration (13%) to plasmolysis the cell away from the cell wall (Fig 12.2).

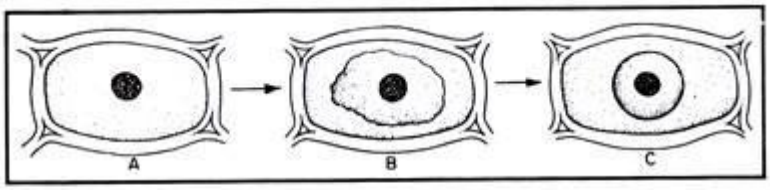


Fig 12.2

A – C. Showing the stages of plasmolysis. A. normal cell, B. Shrinking of protoplasm. C. complete plasmolysis

Mannitol, an alcoholic sugar, is easily transported across the plasmodesmata, provides a stable osmotic environment for the protoplasts and prevents the usual expansion and bursting of protoplast even after loss of cell wall. That is why, this hypertonic solution is known as osmotic stabilizer or plasmolyticum or osmolyticum.

Once the cells are stabilized in such a manner by plasmolysis the protoplasts are released from the containing cell wall either mechanically or enzymatically. Mechanical isolation (Fig 12.3) involves breaking open each cell compartment to liberate the protoplast. This operation can be done carefully on small pieces of tissue under a microscope using a micro-scalpel.

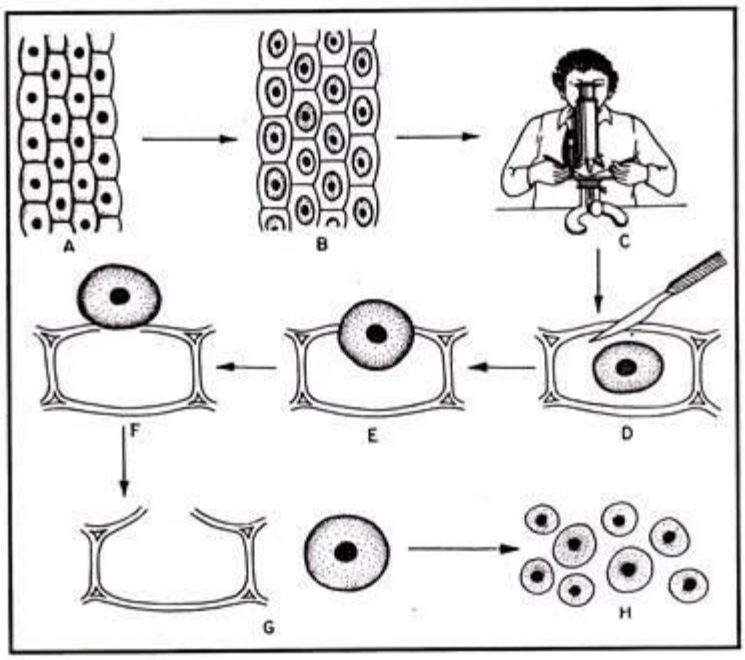


Fig 12.3

Method of mechanical isolation of protoplasts. A. A small piece of plant tissue. B. Plasmolysis of cells. C – D. Cutting of cell wall by microscalpel under microscope. E – F. Subsequent stages of liberation of protoplasts. G. Isolated protoplast and empty cell. H. Isolated protoplasts

But very few protoplasts are obtained for a lot of time and effort. Large-scale attempts at mechanical isolation involves the disrupting tissue with fine stainless steel-bristled brush. This process may liberate more protoplasts with less efforts, but the percentage of yield of intact protoplasts is still very low.

A considerably more efficient way of liberating the protoplasts is to digest the cell walls away around them, using cell wall degrading enzymes such as cellulase, hemicellulose, pectinase or macerozyme etc. These enzymes are isolated from fungi and available commercially (Table 12.1).

Table 12.1 Commercial enzymes, their commercial name and source

Enzyme	Source organism
A. Cellulose degrading enzymes	*Aspergillus niger*
Cellulysin (Onozuka R10)	*Trichoderma reessei (formally T. viride)*
Driselase	*Irpex lactes*
B. Hemicellulose degrading enzymes	
Hemicellulase	*Aspergillus niger*
Rhozyme HP150	*A. niger*
C. Pectin degrading enzymes	
Pectinase	*A. niger*
Macerase (Macerozyme)	*Rhizopus spp.*
Pectinol AC, Pectolyase Y23	*A. niger*
Pectic-acid acetyl transferase (PATE)	*A. japonicus*

Period of treatment and concentration of enzymes are the critical factors and both factors should be standardized for particular plant tissue. Intact tissue can be incubated with a pectinase or macerozyme solution which will dissolve the middle lamella between the cells and so separate them.

Subsequent treatment with cellulase will digest away the cellulosic layer of the cell wall. This process is known as sequential enzyme treatment or two step method as opposed to a mixed enzyme treatment (one step method) in which both cellulase and pectinase or macerozyme are mixed so that the entire wall is broken down in a single operation (Fig. 12.4).

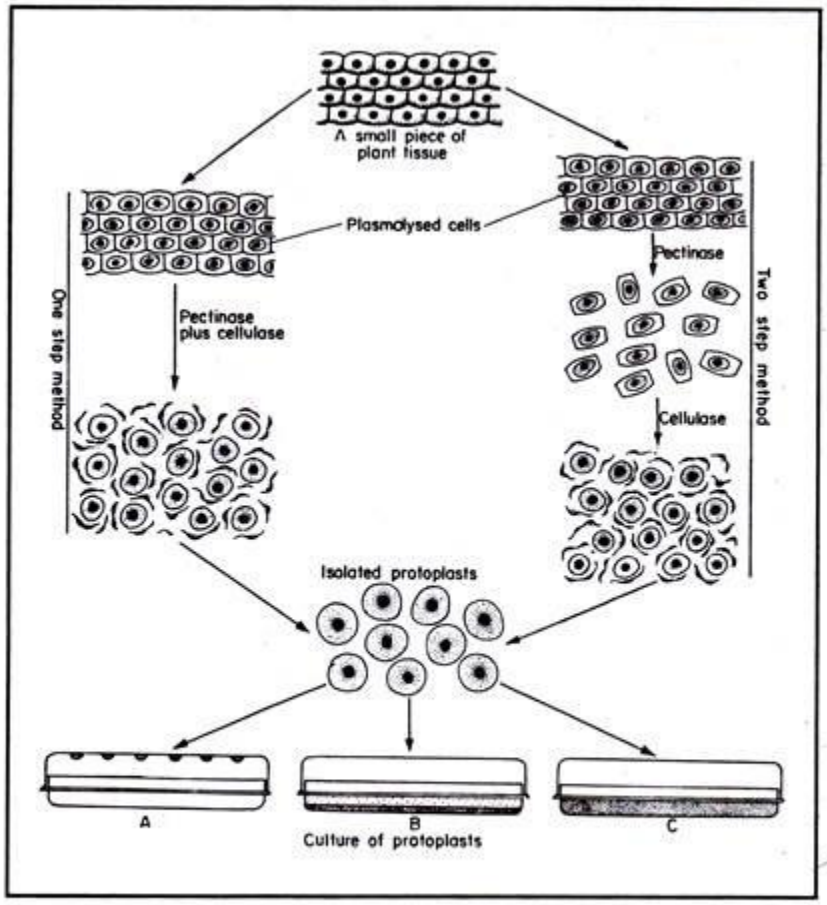


Fig 12.4

Methods of enzymatic isolation of a large number of protoplasts and their culture. A. Hanging-droplet method of culture. B. Co-culture. C. Plating of protoplasts

The isolated protoplasts can be cultured either static liquid or agarified medium. The protoplast media consist of mineral salts, vitamins, carbon sources and plant growth hormones as well as osmotic stabilizers and possibly organic nitrogen sources, coconut milk and organic acids.

In culture protoplast can reform a new cell wall around them. Once the wall is formed, the protoplast becomes a cell. The cells from protoplasts subsequently enter cell division which is followed by the formation of callus and cell cultures. Such callus also retain the capacity for morphogenesis and plant regeneration. A brief list of plant regeneration from plant protoplast culture is given below (Table 12.2).

Table 12.2 Species in which plant regeneration has been achieved from cultured protoplasts

Common name	Species	Family	Cell origin
Tobacco	*Nicotiana tabacum*	Solanaceae	Leaf, cell culture
Potato	*Solanum tuberosum*	Solanaceae	Leaf
Datura	*Datura innozia*	Solanaceae	Leaf
Petunia	*Petunia hybrida*	Solanaceae	Leaf
Carrot	*Daucus carota*	Umbelliferae	Cell culture
Rape seed	*Brassica napus*	Cruciferae	Leaf
Orange	*Citus sinensis*	Rutaceae	Nucellus callus
Asparagus	*Asparagus officinalis*	Liliaceae	Cladodes
Bromegrass	*Bromus inermis*	Poaceae	Cell culture

Germplasm Conservation and Cryopreservation (With Diagram)

Germplasm Conservation:

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells.

Germplasm provides the raw material for the breeder to develop various crops. Thus, conservation of germplasm assumes significance in all breeding programmes.

As the primitive man learnt about the utility of plants for food and shelter, he cultivated the habit of saving selected seeds or vegetative propagules from one season to the next one. In other words, this may be regarded as primitive but conventional germplasm preservation and management, which is highly valuable in breeding programme

The very objective of germplasm conservation (or storage) is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in future. In recent years, many new plant species with desired and improved characteristics have started replacing the primitive and conventionally used agricultural plants. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the primitive plants may be lost.

A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation. Its main objective is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

There are two approaches for germplasm conservation of plant genetic materials:

1. In-situ conservation

2. Ex-situ conservation

1. In-Situ Conservation:

The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries) is regarded as in-situ conservation. This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity. The in-situ conservation is considered as a high priority germplasm preservation programme.

The major limitations of in-situ conservation are listed below:

i. The risk of losing germplasm due to environmental hazards

ii. The cost of maintenance of a large number of genotypes is very high.

2. Ex-Situ Conservation:

Ex-situ conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions. For successful establishment of gene banks, adequate knowledge of genetic structure of plant populations, and the techniques involved in sampling, regeneration, maintenance of gene pools etc. are essential.

Germplasm conservation in the form of seeds:

Usually, seeds are the most common and convenient materials to conserve plant germplasm. This is because many plants are propagated through seeds, and seeds occupy relatively small space. Further, seeds can be easily transported to various places.

There are however, certain limitations in the conservation of seeds:

i. Viability of seeds is reduced or lost with passage of time.

ii. Seeds are susceptible to insect or pathogen attack, often leading to their destruction.

iii. This approach is exclusively confined to seed propagating plants, and therefore it is of no use for vegetatively propagated plants e.g. potato, Ipomoea, Dioscorea.

iv. It is difficult to maintain clones through seed conservation.

Certain seeds are heterogeneous and therefore, are not suitable for true genotype maintenance.

In vitro methods for germplasm conservation:
In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants. The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.

There are several advantages associated with in vitro germplasm conservation:
i. Large quantities of materials can be preserved in small space.

ii. The germplasm preserved can be maintained in an environment, free from pathogens.

iii. It can be protected against the nature's hazards.

iv. From the germplasm stock, large number of plants can be obtained whenever needed.

v. Obstacles for their transport through national and international borders are minimal (since the germplasm is maintained under aspectic conditions).

There are mainly three approaches for the in vitro conservation of germplasm:
1. Cryopreservation (freeze-preservation)

2. Cold storage

3. Low-pressure and low-oxygen storage

Cryopreservation:

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

Cryopreservation broadly means the storage of germplasm at very low temperatures:

i. Over solid carbon dioxide (at -79°C)

ii. Low temperature deep freezers (at -80°C)

iii. In vapour phase nitrogen (at -150°C)

iv. In liquid nitrogen (at -196°C)

Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods.

In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat, peanut, cassava, sugarcane, strawberry, coconut. Several plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

Mechanism of Cryopreservation:

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C). When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

Precautions/Limitations for Successful Cryopreservation:

Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.

Other precautions (the limitations that should be overcome) for successful cryopreservation are listed below:

i. Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.

ii. High intracellular concentration of solutes may also damage cells.

iii. Sometimes, certain solutes from the cell may leak out during freezing.

iv. Cryoprotectants also affect the viability of cells.

v. The physiological status of the plant material is also important.

Technique of Cryopreservation:

An outline of the protocol for cryopreservation of shoot tip is depicted in Fig. 48.1. The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages

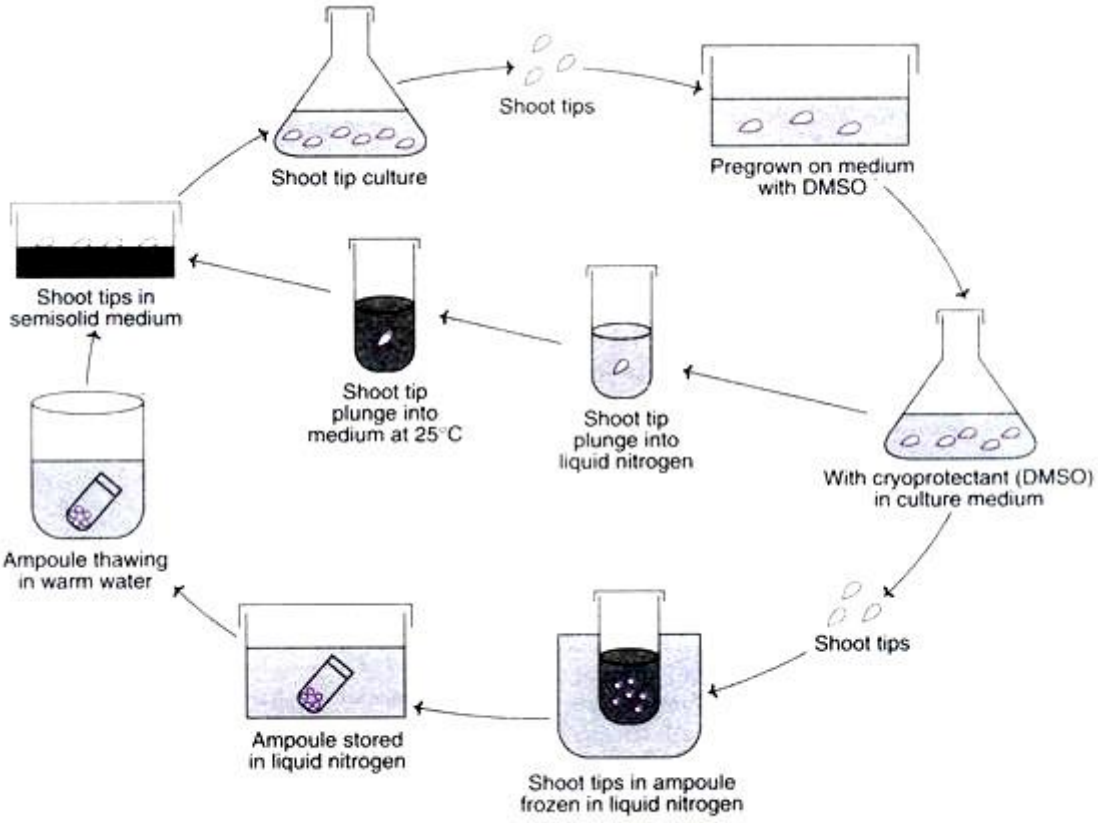


***Fig. 48.1** : An outline of the protocol for cryopreservation of shoot tip (DMSO–Dimethyl sulfoxide).*

1. Development of sterile tissue cultures

2. Addition of cryoprotectants and pretreatment

3. Freezing

4. Storage

5. Thawing

6. Re-culture

7. Measurement of survival/viability

8. Plant regeneration.

The salient features of the above stages are briefly described.

Development of sterile tissue culture:
The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these, meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

Addition of cryoprotectants and pretreatment:
Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation.

There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

Freezing:
The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

Four different types of freezing methods are used:

1. Slow-freezing method:
The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures.

2. Rapid freezing method:
This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

3. Stepwise freezing method:
This is a combination of slow and rapid freezing procedures (with the advantages of both), and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

4. Dry freezing method:
Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

Storage:
Maintenance of the frozen cultures at the specific temperature is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator — at 1 50°C in the vapour phase, or at -196°C in the liquid phase.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

The documented information must be comprehensive with the following particulars:
i. Taxonomic classification of the material

ii. History of culture

iii. Morphogenic potential

iv. Genetic manipulations done

v. Somaclonal variations

vi. Culture medium

vii. Growth kinetics

Thawing:
Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling. By this approach, rapid thawing (at the rate of 500- 750°C min^{-1}) occurs, and this protects the cells from the damaging effects ice crystal formation.

As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath. For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

Re-culture:

In general, thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a fresh medium following standard procedures. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

Measurement of survival/viability:

The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or re-culture.

The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA) are commonly used.

The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression.

$$\frac{No.of\ cells/organs\ growing}{No.of\ cells/organs\ thawed} \times 100$$

Plant regeneration:

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of certain growth promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

A selected list of plants (in various forms) that have been successfully used for cryopreservation is given in Table 48.1.

Table 48.1 A selected list of plants in various forms that are successfully cryopreserved

Plant material	*Plant species*
Cell suspensions	*Oryza sativa*
	Glycine max
	Zea mays
	Nicotiana tabacum
	Capsicum annum
Callus	*Oryza sativa*
	Capsicum annum
	Saccharum sp
Protoplast	*Zea mays*
	Nicotiana tabacum
Meristems	*Solanum tuberosum*
	Cicer arietinum
Zygotic embryos	*Zea mays*
	Hordeum vulgare
	Manihot esculenta
Somatic embryos	*Citrus sinensis*
	Daucus carota
	Coffea arabica
Pollen embryos	*Nicotiana tabacum*
	Citrus sp
	Atropa belladona

Cold Storage:

Cold storage basically involves germplasm conservation at a low and non-freezing temperatures (1-9°C) The growth of the plant material is slowed down in cold storage in contrast to complete stoppage in cryopreservation. Hence, cold storage is regarded as a slow growth germplasm conservation method. The major advantage of this approach is that the plant material (cells/tissues) is not subjected to cryogenic injuries.

Long-term cold storage is simple, cost-effective and yields germplasm with good survival rate. Many in vitro developed shoots/plants of fruit tree species have been successfully stored by this approach e.g. grape plants, strawberry plants.

Virus- free strawberry plants could be preserved at 10°C for about 6 years, with the addition of a few drops of medium periodically (once

in 2-3 months). Several grape plants have been stored for over 15 years by cold storage (at around 9°C) by transferring them yearly to a fresh medium.

Low-Pressure and Low-Oxygen Storage:

As alternatives to cryopreservation and cold storage, low-pressure storage (LPS) and low-oxygen storage (LOS) have been developed for germplasm conservation. A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure and low-oxygen is depicted in Fig. 48.2.

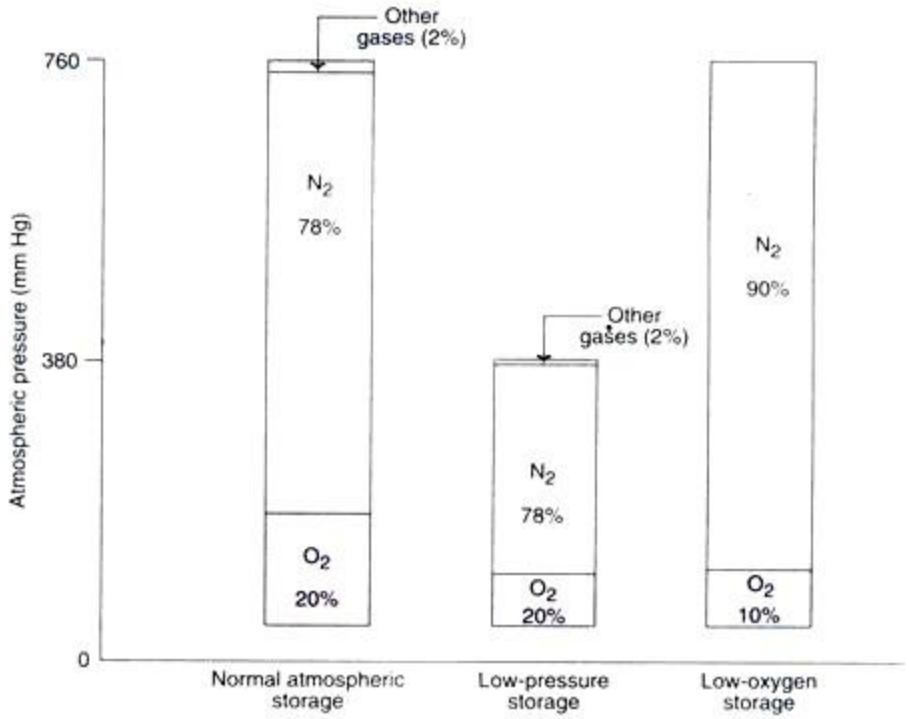


***Fig. 48.2** : A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure, and low-oxygen.*

Low-Pressure Storage (LPS):

In low-pressure storage, the atmospheric pressure surrounding the plant material is reduced. This results in a partial decrease of the pressure exerted by the gases around the germplasm. The lowered partial pressure reduces the in vitro growth of plants (of organized or unorganized tissues). Low-pressure storage systems are useful for short-term and long-term storage of plant materials.

The short-term storage is particularly useful to increase the shelf life of many plant materials e.g. fruits, vegetables, cut flowers, plant

cuttings. The germplasm grown in cultures can be stored for long term under low pressure. Besides germplasm preservation, LPS reduces the activity of pathogenic organisms and inhibits spore germination in the plant culture systems.

Low-Oxygen Storage (LOS):
In the low-oxygen storage, the oxygen concentration is reduced, but the atmospheric pressure (260 mm Hg) is maintained by the addition of inert gases (particularly nitrogen). The partial pressure of oxygen below 50 mm Hg reduces plant tissue growth (organized or unorganized tissue). This is due to the fact that with reduced availability of O_2, the production of CO_2 is low. As a consequence, the photosynthetic activity is reduced, thereby inhibiting the plant tissue growth and dimension.

Limitations of LOS:
The long-term conservation of plant materials by low-oxygen storage is likely to inhibit the plant growth after certain dimensions.

Applications of Germplasm Storage:

The germplasm storage has become a boon to plant breeders and biotechnologists.

Some of the applications are briefly described:

1. Maintenance of stock cultures: Plant materials (cell/tissue cultures) of several species can be cryopreserved and maintained for several years, and used as and when needed. This is in contrast to an in vitro cell line maintenance which has to be sub-cultured and transferred periodically to extend viability. Thus, germplasm storage is an ideal method to avoid sub-culturing, and maintain cells/ tissues in a viable state for many years.

2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolites (e.g. medicines).

3. Disease (pathogen)-free plant materials can be frozen, and propagated whenever required.

4. Recalcitrant seeds can be maintained for long.

5. Conservation of somaclonal and gametoclonal variations in cultures.

6. Plant materials from endangered species can be conserved.

7. Conservation of pollen for enhancing longevity.

8. Rare germplasms developed through somatic hybridization and other genetic manipulations can be stored.

9. Cryopreservation is a good method for the selection of cold resistant mutant cell lines which could develop into frost resistant plants.

10. Establishment of germplasm banks for exchange of information at the international level.

Limitations of Germplasm Storage:
The major limitations of germplasm storage are the expensive equipment and the trained personnel. It may, however, be possible in the near future to develop low cost technology for cryopreservation of plant materials.

Embryo Culture: Meaning, Categories, Principles and Protocols | Plant Tissue

What is Embryo Culture?

The embryo of different developmental stages, formed within the female gametophyte through sexual process, can be isolated aseptically from the bulk of maternal tissues of ovule, seed or capsule and cultured in vitro under aseptic and controlled physical conditions in glass vials containing nutrient solid or liquid medium to grow directly into plantlet.

Different Categories of Embryo-Culture and their Objectives:

Culture of embryo (Fig 10.1) may be divided into the following categories:

1. Culture of Mature and Intact Seed Embryo:

The aim of this study is to analyse the various parameters of embryonic growth and the metabolic and biochemical aspects of dormancy and germination.

2. Culture of Surgically Disected Embryo:

The mature seed embryo can be dissected surgically into a number of segments. Such embryo segments are cultured to analyse the relationship of different parts of the embryo to its final form in culture.

3. Culture of Immature Embryos or Pro-embryo's:

The term pro embryo means the early developmental stages of the embryo that precede cotyledon initiation. Globular and heart-shaped stages of embryo are appropriately called as pro embryos. The objective of such culture is to understand the control of differentiation and the nutritional requirements of such progressively developing embryos.

4. Culture of Intact Seed Containing Undifferentiated Embryo:

Each fruit of an orchid plant develops several thousand tiny seeds which contain morphologically undifferentiated embryos. These embryos are the spherical mass of tissue lacking both radicle and

plumule. There is even no storage tissue in the seeds and the seed coat is reduced to a membranous structure. For this reason the entire seed of orchid containing undifferentiated embryo is cultured and treated as embryo culture. In nature, these seeds germinate only in association with a proper fungus or else they perish. As a result, numerous seeds are lost. In vitro culture of orchid seeds is routinely employed for orchid propagation.

5. Culture of Adventives Embryos from Polyembryonic Seeds:

Besides the zygotic embryo produced from egg cell, some additional embryos are produced from nuclear tissue in polyembryonic seed like lemons and oranges. Such additional abortive embryos can be exploited in culture for clonal propagation.

6. Culture of Inviable or Abortive Embryos:

In many inter-specific or inter-generic breeding experiments, sometimes inviable or abortive embryos may develop due to unsuccessful crosses. As a result, the non-viable seeds do not germinate normally. But it is now possible to raise a hybrid plant by culturing the inviable embryos in vitro.

Principles of Embryo Culture:

The underlying principle of the method is the aseptic excision of the embryo and its transfer to a suitable nutrient medium for development under optimum culture conditions. In general, it is relatively easy to obtain pathogen-free embryos, since the embryo is lodged in the sterile environment of the ovule or seed or capsule or fruit. So, surface sterilization of the embryos as such is not necessary. Thus the entire seeds or fruits containing the ovule are surface sterilized and the embryos are then aseptically separated from the surrounding tissues.

Seeds with hard seed coats are generally surface sterilized and then soaked in sterile water for a few days aseptically so that seed can be cut easily to free the embryo. Although seeds are surface sterilized before soaking they may need to go through a second sterilization before embryo excision. Splitting open the seeds and transferring

embryos to the nutrient medium directly is the simplest technique that can be done with seeds.

In the isolation of comparatively smaller embryos, it is important that they are removed intact carefully from the ovule without any injury. This can be best achieved by carrying out the operation under a specially designed dissecting microscope. In case of orchid seeds, the entire seed or ovule is cultured because the seed contain morphologically undifferentiated spherical embryo having no functional storage tissues like endosperm and the seed coat is reduced to a membranous structure.

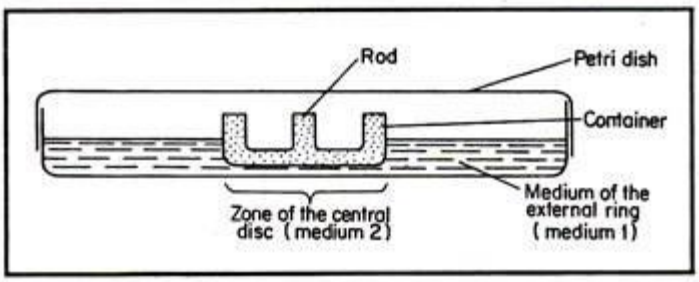
Although the entire ovule containing undifferentiated embryos is cultured, but it is referred to as embryo culture. Each fruit of an orchid contains thousands and thousands of tiny seeds, so a large quantity of sample can be cultivated easily just after excision of surface sterilized fruits. It is also important that the freed seeds containing the embryos not become desiccated during above operation.

The most important aspect of embryo culture work is the crucial selection of the medium necessary to sustain continued growth of the embryos. The formulation of nutrient media may vary depending upon the species used for study and many of them have not been rigorously determined.

Nevertheless it is possible to make certain generalization i.e. the younger the embryo the more complex is its nutrient requirements. Thus while the mature embryo can be grown in an inorganic salt medium supplemented with a carbon energy source such as sucrose, relatively young embryos require in addition, different combinations of vitamins, amino acids, growth hormones and in some cases natural endosperm extracts such as coconut milk. Since pro embryos are often submerged in the ovular sap under considerable osmotic pressure, culture of such embryo in presence of an osmoticum such as mannitol is often suggested.

The changing nutritional requirement for successful embryo culture has often meant transferring the embryo from one medium to

another for optimum growth. Monnier devised a culture method (Fig 10.2) which allowed for the uninterrupted growth of globular embryo to maturity. By this method, embryo can be grown in both solid and liquid medium at the same time.



❒ Fig 10.2

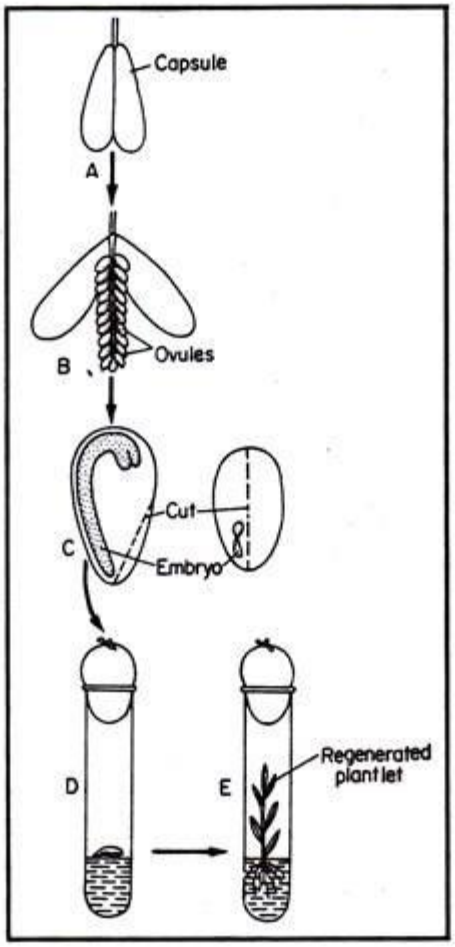
Device of Monnier for embryo culture

The composition of both media is different. Monnier also stressed the importance of obtaining uninjured embryos with their suspensor for successful culture. Suspensor is important for the growth of immature embryos. But embryo selected at later stages do not require the attached suspensor.

n culture, the embryos are not induced to form callus tissue but they are allowed to form a plantlet. After the embryos have grown into plantlets in vitro, they are generally transferred to sterile soil or vermiculite and grown to maturity in the green house.

Protocol for Embryo Culture:

The following protocol for embryo culture (Fig 10.3) is based on the method used for Capsella bursapastoris. With modification, this basic protocol should be applicable to embryo culture in general.



❒ Fig 10.3

Procedure of isolation of embryo of Capsella bursa-pastoris and its culture. A. a capsule. B. the capsule has been opened. C. incision of ovule to isolated embryo. D. Culture of isolated embryo. E. Development of plantlet from the cultured embryo

The steps are given below:

1. Capsules in the desired stages of development are surface sterilized for 5-10 minutes in 0.1% $HgCl_2$, either in a closed small room previously illuminated by UV lamps or in a Laminar air flow.
2. Wash repeatedly in sterile water.

3. Further operations are carried out under a specially designed dissecting microscope at a magnification of about 90X. The capsules are kept in a depression slide containing few drops of liquid medium.

4. The outer wall of capsule is removed by a cut in the region of the placenta; the halves are pushed apart with forceps to expose the ovules.

5. A small incision in the ovule followed by slight pressure with a blunt needle is enough to free the embryos.

6. The excised embryos are transferred by micro-pipettes or small spoon headed spatula to standard 10 cm petridishes containing 25 ml of solidified standard medium. Usually 6-8 embryos are cultured in a petridish.

7. The petridishes are sealed with cello-tape to prevent desiccation of the culture.

8. The cultures are kept in a culture room at 25 ± 1°C and given 16 hrs. illumination by cool white fluorescent tube.
9. Subcultures into fresh medium are made at approximately four weeks intervals.

In case of fresh seeds or dry and imbibed seeds, the schedule is slightly changed. Seeds are cleaned by 5% Teepol (a liquid detergent) for 10 minutes and dipped in 70% ethyl alcohol for 60 seconds. Surface sterilization in 0.1% $HgCl_2$ is followed by washing in sterile water, then the seeds are decotylated using a sharp scalpel and embryos are transferred to solid nutrient medium. In case of orchid seeds, after following the scheduled surface sterilization procedure, the fruits are excised and dusty seeds are gently spread over the medium.

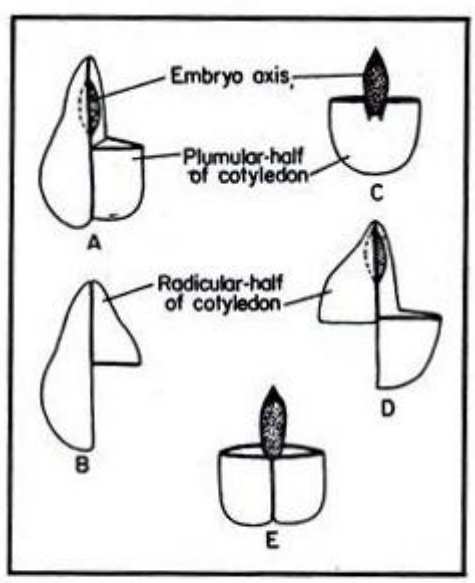
Precocious Germination Embryo in Culture:
A lot of cellular, physiological and biochemical changes take place during embryo development from the zygote to the fully formed embryo stage. After its full-term development, the embryo becomes dehydrated and enters a phase of metabolic quiescence and developmental arrest (dormancy) which may last from a few days to several months or even years.

During this stage, embryo is normally incapable of germination. Embryos of mangroves and some viviparous varieties of cultivated

plants (e.g. Sechium edule) germinate while still attached to the parent plant. They are able to bypass the stage of dormancy.

Similar phenomenon has been observed when excised immature plant embryos are grown in vitro. In culture, excised immature embryos do not proceed further then the embryo genic development for its maturation and start to germinate.

The immature embryos develop into weak seedling showing only those structures which are already present at the time of embryo excision. This phenomenon of seedling formation without completing normal embryo genic development is known as precocious germination. LaRue and Avery (1938) have demonstrated in vitro growth of Zizania aquaiica embryos excised from ovules at different stages of development (Fig10.4).



❒ Fig 10.4

Growth of embryos from ovule at different stages of development (after LaRue and Avery 1938)

Embryos 0.05 mm long show very little in vitro growth. Older, immature embryos (3.5 mm long) germinates precociously and form seedlings which are not as well developed as those formed by mature embryos in the same culture period. The main objective of culturing immature embryos is to stimulate normal embryological development in order to understand the factor(s) that regulate the orderly development of embryos in nature.

Secondary Metabolites in Plant Cultures: Applications and Production

The production process comprises of seven aspects.

The seven aspects are: (1) Selection of cell lines for high yield of secondary metabolites (2) Large scale cultivation of plant cells (3) Medium composition and effect of nutrients (4) Elicitor-induced production of secondary metabolites (5) Effect of environmental factors (6) Biotransformation using plant cell cultures and (7) Secondary metabolite release and analysis.

Secondary Metabolites:

The chemical compounds produced by plants are collectively referred to as phytochemicals. Biotechnologists have special interest in plant tissue culture for the large scale production of commercially important compounds. These include pharmaceuticals, flavours, fragrances, cosmetics, food additives, feed stocks and antimicrobials.

Most of these products are secondary metabolites— chemical compounds that do not participate in metabolism of plants. Thus, secondary metabolites are not directly needed by plants as they do not perform any physiological function (as is the case with primary metabolites such as amino acids, nucleic acids etc.). Although the native plants are capable of producing the secondary metabolites of commercial interest, tissue culture systems are preferred.

The advantages and limitations are listed:

Major Advantages:

1. Compounds can be produced under controlled conditions as per market demands.

Secondary Metabolites in Plant Cultures: Applications and Production

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Read this article to learn about the applications of secondary metabolites and the production process of secondary metabolites in plant cultures.

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The advantages and limitations are listed:

Major Advantages:

1. Compounds can be produced under controlled conditions as per market demands.

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2. Culture systems are independent of environmental factors, seasonal variations, pest and microbial diseases and geographical constraints.

3. Cell growth can be controlled to facilitate improved product formation.

4. The quality of the product will be consistent as it is produced by a specific cell line.

5. Recovery of the product will be easy.

6. Plant cultures are particularly useful in case of plants which are difficult or expensive to be grown in the fields.

7. Mutant cell lines can be developed for the production of novel compounds of commercial importance, which are not normally found in plants.

8. Biotransformation reactions (converting specific substrates to valuable products) can be carried out with certain cultured cells.

9. The production control is not at the mercy of political interference.

10. The production time is less and labour costs are minimal.

Considering the advantages listed above, about 25-30% of medicines for human use, and the various chemical materials for industrial purposes are obtained from plant tissue cultures. In general, tissue culture production of natural materials is cheaper compared to synthetic production. However, there are certain limitations associated with tissue cultures.

Limitations/Disadvantages:
1. In general, in vitro production of secondary metabolites is lower when compared to intact plants.

2. Many a times, secondary metabolites are formed in differentiated tissues/organs. In such a case, culture cells which are non-differentiated can produce little.

3. Cultured cells are genetically unstable and may undergo mutation. The production of secondary metabolite may be drastically reduced, as the culture ages.

4. Vigorous stirring is necessary to prevent aggregation of cultured cells. This may often damage the cells.

5. Strict aseptic conditions have to be maintained during culture technique: Any infection to the culture adversely affects product formation.

Why do Plants Produce Secondary Metabolites?
Based on the existing evidence, it is believed that the production of some secondary metabolites is linked to the induction of morphological differentiation.

Consider the following examples:
As and when available, the natural plant products are preferred to synthetic products, by man. According to a WHO survey, nearly 70-80% of the world population depends on herbal drugs. It is a fact that many chemicals with complex structures that cannot be chemically synthesized can be conveniently produced in plants.

The production of speciality chemicals by plants is a multibillion industry. The plant cell cultures provide laboratory managed sources for the supply of useful plant products. Although hundreds of new compounds are identified every year in plants, only a few of them are of commercial importance. Attempts are made to produce them in cell culture systems.

A selected list of plant products obtained from plant cell cultures along with their applications is given in Table 42.1.

Table 42.1 A selected list of secondary metabolites obtained from plant cell cultures along with their application(s)

Product	*Plant species*	*Uses*
Shikonine	*Lithospermum erythrorhizon*	Dye, pharmaceutical
Codeine, morphine	*Papaver somniferum*	Analgistic
Quinine	*Cinchona officinalis*	Antimalarial
Atropine	*Atropa belladonna*	Muscle relaxant
Digoxin	*Digitalis lanata*	Cardiovascular disorders
Reserpine	*Rauwolfia serpentina*	Hypotensive
Diosgenin	*Dioscorea deltoidea*	Antifertility
Vanillin	*Vanilla* sp	Vanilla
Jasmine	*Jasmium* sp	Perfume
Vinblastine, ajmalicine, vincristine	*Catharanthus roseus*	Anticancer
Taxol	*Taxus brevifolia*	Anticancer
Baccharine	*Baccharis megapotanica*	Anticancer
Cesaline	*Caesalpinia gillisesii*	Anticancer
Fagaronine	*Fagara zanthoxyloides*	Anticaner
Maytansine	*Maytenus bucchananii*	Anticancer
Harring tonine	*Cephalotaxus harringtonia*	Anticancer
Thalicarpine	*Thalictrum dasycarpum*	Anticancer
Ellipticine, 3-deoxycolchine	*Ochrosia moorei*	Anticancer
Pyrithrins	*Tagetus erecta* *Chrysanthemum cinceariefolium*	Insecticide
Rotenoids	*Derris elliptica* *Tephrosia* sp	Insecticide
Nicotine	*Nicotiana tabacum* *Nicotiana rustica*	Insecticide
Saffron	*Crocus sativus*	Food colour and flavouring agent
Stevioside	*Stevia rabaudiana*	Sweetener
Thaumatin	*Thaumatococcus damielli*	Sweetener
Capsaicin	*Capsicum frutesus*	Chilli
Rosamarinic acid	*Coleus blunei*	Spice, antioxidant
Anthraquinones	*Morinda citrifolia*	Laxative, dye
Berberine	*Coptis japonica*	Antibacterial
Sarcoplasmine (hyoscine)	*Datura stramonium*	Treatment of nausea

Shikonine is a dye produced by the cells Lithospermum erythrorhizon on a commercial scale. The other products successfully produced in plant cell cultures include analgistics (codeine) anti-malarial (quinine), muscle relaxants (atropine), drugs to control cardiovascular disorders (digoxin), hypotensives (reserpine), perfumes (jasmine), insecticides (pyrithrins), food sweeteners

(stevioside) and anticancer agents (vincristine). Sometimes, the cost of the plant products is unimaginably high. For instance, one kg of vincristine and vinblastine respectively cost $ 3, 500, 00 and $ 1,000,000!

Production of Secondary Metabolites:

The process of in vitro culture of cells for the large scale production of secondary metabolites is complex, and involves the following aspects:

1. Selection of cell lines for high yield of secondary metabolites.

2. Large scale cultivation of plant cells.

3. Medium composition and effect of nutrients.

4. Elicitor-induced production of secondary metabolites.

5. Effect of environmental factors.

6. Biotransformation using plant cell cultures.

7. Secondary metabolite release and analysis.

1. Selection of Cell Lines for High Yield of Secondary Metabolites:

The very purpose of tissue culture is to produce high amounts of secondary metabolites. However, in general, majority of callus and suspension cultures produce less quantities of secondary metabolites. This is mainly due to the lack of fully differentiated cells in the cultures.

Some special techniques have been devised to select cell lines that can produce higher amounts of desired metabolites. These methods are ultimately useful for the separation of producer cells from the non-producer cells. The techniques commonly employed for cell line selection are cell cloning, visual or chemical analysis and selection for resistance.

Cell Cloning:

This is a simple procedure and involves the growth of single cells (taken from a suspension cultures) in a suitable medium. Each cell

population is then screened for the secondary metabolite formation. And only those cells with high-yielding ability are selected and maintained by sub-cloning.

Single cell cloning:
There are certain practical difficulties in the isolation and culture of single cells.

Cell aggregate cloning:
Compared to single cell cloning, cell aggregate cloning is much easier, hence preferred by many workers. A schematic representation of cell aggregate cloning for the selection of cells yielding high quantities of secondary metabolites is given in Fig. 42.9. A high yielding plant of the desired metabolite is selected and its explants are first cultured on a solid medium. After establishing the callus cultures, high metabolite producing calluses are identified, and they are grown in suspension cultures.

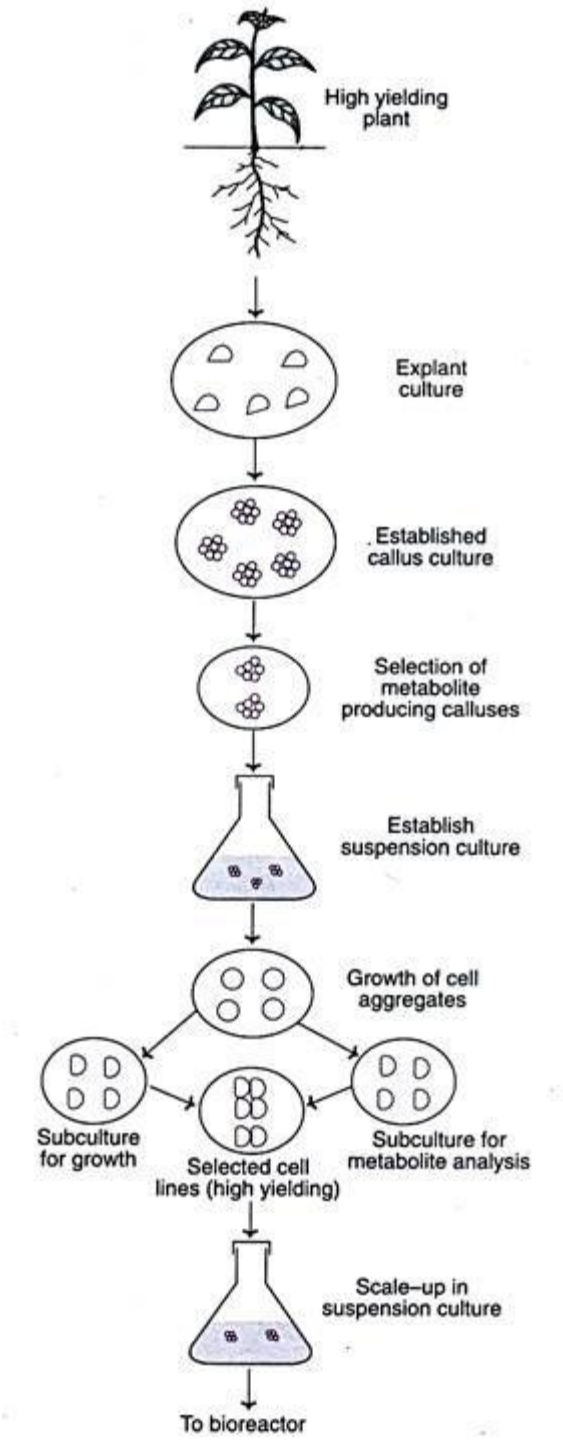

Fig. 42.9 : A schematic representation of cell aggregate cloning for the selection of high yielding cell lines.

Cell aggregates from these cultures are grown on solid medium. The freshly developed cell aggregates (calluses) are divided into two parts. One half is grown further, while the other half is used for the quantitative analysis of the desired metabolite produced. The cell lines with high yield of secondary metabolites are selected and used

for scale-up in suspension cultures. This is followed by large scale tissue culture in a bioreactor.

Visual or Chemical Analysis:
A direct measurement of some of the secondary metabolites produced by cell lines can be done either by visual or chemical analysis. Visual identification of cell lines producing coloured secondary metabolites (pigments e.g., β-carotene, shikonin) will help in the selection of high-yielding cells. This method is quite simple and non-destructive. The major limitation is that the desired metabolite should be coloured.

Certain secondary metabolites emit fluorescence under UV light, and the corresponding clones can be identified. Some workers use simple, sensitive and inexpensive chemical analytical methods for quantitative estimation of desired metabolites. Analysis is carried out in some colonies derived from single cell cultures. Radioimmunoassay is the most commonly used analytical method. Micro spectrophotometry and fluorescent antibody techniques are also in use.

Selection for Resistance:
Certain cells resistant to toxic compounds may lead to the formation of mutant cells which can overproduce a primary metabolite, and then a secondary metabolite. Such mutants can be selected and used to produce the desired metabolite in large quantities. One example is described.

Cell lines selected for resistance of 5-methyl- tryptophan (analogue of tryptophan) produce strains which can overproduce tryptophan. These tryptophan overproducing strains can synthesize 10-50 times higher levels of the natural auxin namely indole acetic acid (Note: The secondary metabolite indole acetic acid is derived from the primary metabolite tryptophan).

2. Large Scale (Mass) Cultivation of Plant Cells:
In order to achieve industrial production of the desired metabolite, large scale cultivation of plant cells is required. Plant cells (20-150 μm in diameter) are generally 10-100 times larger than bacterial or

fungal cell. When cultured, plant cells exhibit changes in volumes and thus variable shapes and sizes. Further, cultured cells have low growth rate and genetic instability. All these aspects have to be considered for mass cultivation of cells.

The following four different culture systems are widely used:

1. Free-cell suspension culture

2. Immobilized cell culture

3. Two-phase system culture

4. Hairy root culture.

Free-cell Suspension Culture:

Mass cultivation of plant cells is most frequently carried out by cell suspension cultures. Care should be taken to achieve good growth rate of cells and efficient formation of the desired secondary metabolite. Many specially designed bioreactors are in use for free-cell suspension cultures.

Some of these are listed below:

i. Batch bioreactors

ii. Continuous bioreactors

iii. Multistage bioreactors

iv. Airlift bioreactors

v. Stirred tank bioreactors.

Two important aspects have to be considered for good success of suspension cultures.

1. Adequate and continuous oxygen supply.

2. Minimal generation of hydrodynamic stresses due to aeration agitation.

Immobilized Cell Cultures:

Plant cells can be made immobile or immovable and used in culture systems. The cells are physically immobilized by entrapment. Besides individual cells, it is also possible to immobilize aggregate cells or even calluses. Homogenous suspensions of cells are most suitable for immobilization.

Surface immobilized plant cell (SIPC) technique efficiently retains the cells and allows them to grow at a higher rate. Further, through immobilization, there is better cell-to-cell contact, and the cells are protected from high liquid shear stresses. All this helps in the maximal production the secondary metabolite.

The common methods adopted for entrapment of cells are briefly described:

1. Entrapment of cells in gels:

The cells or the protoplasts can be entrapped in several gels e.g., alginate, agar, agarose, carrageenin. The gels may be used either individually or in combination. The techniques employed for the immobilization of plant cells are comparable to those used for immobilization of microorganisms or other cells.

2. Entrapment of cells in nets or foams:

Polyurethane foams or nets with various pore sizes are used. The actively growing plant cells in suspension can be immobilized on these foams. The cells divide within the compartments of foam and form aggregates.

3. Entrapment of cells in hollow-fibre membranes:

Tubular hollow fibres composed of cellulose acetate silicone polycarbonate and organized into parallel bundles are used for immobilization of cells. It is possible to entrap cells within and between the fibres. Membrane entrapment is mechanically stable. However, it is more expensive than gel or foam immobilization.

Bioreactors for Use of Immobilized Cells:

Fluidized bed or fixed bed bioreactors are employed to use immobilized cells for large scale cultivation. In the fluidized-bed reactors, the immobilized cells are agitated by a flow of air or by

pumping the medium. In contrast, in the fixed-bed bioreactor, the immobilized cells are held stationary (not agitated) and perfused at a slow rate with an aerated culture medium.

Biochemicals produced by using immobilized cells:

A selected list of the immobilized cells from selected plants and their utility to produce important bio-chemicals is given in Table 42.2.

TABLE 42.2 A selected list of plant species with immobilized cells employed for the production secondary metabolite(s)

Plant culture species	*Immobilization method*	*Substrate*	*Product*
Catharanthus roseus	Entrapment in agarose	Cathenamine	Ajmalicine
Digitaiis lanata	Entrapment in alginate	Digitoxin	Digoxin
Capsicum frutescens	Entrapment in polyurethane foam	Sucrose	Capsaicin
Catheranthus roseus	Entrapment in alginate, agarose, carrageenin	Sucrose	Ajmalicine
Petunia hybrida	Entrapment in hollow fibres	Sucrose	Phenolics
Morinda citrifolia	Entrapment in alginate	Sucrose	Anthraquinone
Solanum aviculare	Attachment polyphenylene beads	Sucrose	Steroid glycosides
Glycine max	Entrapment in hollow fibre	Sucrose	Phenolics

Two-phase System Culture:

Plant cells can be cultivated in an aqueous two phase system for the production of secondary metabolites. In this technique, the cells are kept apart from the product by separation in the bioreactor. This is advantageous since the product can be removed continuously. Certain polymers (e.g., dextran and polyethylene glycol for the separation of phenolic compounds) are used for the separation of phases.

Hairy Root Culture:

Hairy root cultures are used for the production of root-associated metabolites. In general, these cultures have high growth rate and genetic stability. For the production of hairy root cultures, the explant material (plant tissue) is inoculated with the cells of the pathogenic bacterium, Agrobacterium rhizogenes. This organism contains root-inducing (Ri) plasmid that causes genetic transformation of plant tissues, which finally results in hairy root cultures. Hairy roots produced by plant tissues have metabolite features similar to that of normal roots.

Hairy root cultures are most recent organ culture systems and are successfully used for the commercial production of secondary metabolites. A selected list of the plants employed in hairy root

cultures and the secondary metabolites produced is given in Table 42.3.

Table 42.3 A selected list of plant species used in hairy root cultures for the production of secondary metabolite(s)

Plant species	*Secondary metabolite(s)*
Nicotiana tabacum	Nicotine, anatabine
Atropa belladonna	Atropine
Datura stramonium	Hyoscyamine
Lithospermum erythrorhizon	Shikonin
Catharanthus roseus	Ajmalicine, serpentine
Cinchona ledgeriana	Quinine alkaloids
Mentha vulgaris	Monoterpenes
Solanum laciniatum	Steroid alkaloids

3. Medium Composition and Effect of Nutrients:

The in vitro growth of the plant cells occurs in a suitable medium containing all the requisite elements. The ingredients of the medium effect the growth and metabolism of cells. For optimal production of secondary metabolites, a two-medium approach is desirable.

The first medium is required for good growth of cells (biomass growth) while the second medium, referred to as production medium promotes secondary metabolite formation. The effect of nutrients (carbon and nitrogen sources, phosphate, growth regulators, precursors, vitamins, metal ions) on different species in relation to metabolite formation are variable, some of them are briefly described.

Effect of Carbon Source:

Carbohydrates influence the production of phytochemicals.

Some examples are given below:

1. Increase in sucrose concentration (in the range 4-10%) increases alkaloid production in Catharanthus roseus cultures.

2. Sucrose is a better carbon source than fructose or galactose for diosgenin production by Dioscorea deltoidea or Dalanites aegyptiaca cultures.

3. Low concentration of sucrose increases the production of ubiquinone-10 in tobacco cell cultures.

Effect of Nitrogen Source:
The standard culture media usually contain a mixture of nitrate and ammonia as nitrogen source. Majority of plant cells can tolerate high levels of ammonia. The cultured cells utilize nitrogen for the biosynthesis of amino acids, proteins (including enzymes) and nucleic acids. The nitrogen containing primary metabolites directly influence the secondary metabolites.

In general, high ammonium ion concentrations inhibit secondary metabolite formation while lowering of ammonium nitrogen increases. It is reported that addition of KNO_3 and NH_4NO_3 inhibited anthocyanin (by 90%) and alkaloid (by 80%) production.

Effect of Phosphate:
Inorganic phosphate is essential for photosynthesis and respiration (glycolysis). In addition, many secondary metabolites are produced through phosphorylated intermediates, which subsequently release the phosphate e.g., phenylpropanoids, terpenes, terpenoids. In general, high phosphate levels promote cell growth and primary metabolism while low phosphate concentrations are beneficial for secondary product formation. However, this is not always correct.

Increase in phosphate concentration in the medium may increase, decrease or may not affect product formation e.g.:
1. Increased phosphate concentration increases alkaloid (in Catharanthus roseus), anthraquinone (in Morinda citrifolia) and diosgenin (in Dioscorea deltoidea) production.

2. Decreased phosphate level in the medium increases the formation of anthocyanins and phenolics (in Catharanthus roseus), alkaloids (in Peganum harmala) and solasodine (in Solanum lanciatum).

3. Phosphate concentration (increase or decrease) has no effect on protoberberine (an alkaloid) production by Berberis sp.

Effect of Plant Growth Regulators:
Plant growth regulators (auxins, cytokinins) influence growth, metabolism and differentiation of cultured cells. There are a large number of reports on the influence of growth regulators for the

production of secondary metabolites in cultured cells. A few examples are given.

1. Addition of auxins (indole acetic acid, indole pyruvic acid, naphthalene acetic acid) enhanced the production of diosgenin in the cultures of Balanites aegyptiaca.

2. Auxins may inhibit the production of certain secondary metabolites e.g., naphthalene acetic acid and indole acetic acid inhibited the synthesis of anthocyanin in carrot cultures.

3. Another auxin, 2, 4-dichlorophenoxy acetate (2, 4-D) inhibits the production of alkaloids in the cultures of tobacco, and shikonin formation in the cultures of Lithospermum erythrorhizon.

4. Cytokinins promote the production of secondary metabolites in many tissue cultures e.g., ajmalicine in Catharanthus roseus; scopolin and scopoletin in tobacco; carotene in Ricinus sp.

5. In some tissue cultures, cytokinins inhibit product formation e.g., anthroquinones in Morinda citrifolia; shikonin in Lithospermum erythroshizon; nicotine in tobacco.

In actual practice, a combination of auxins and cytokinins is used to achieve maximum production of secondary metabolites in culture systems.

Effect of Precursors:
The substrate molecules that are incorporated into the secondary metabolites are referred to as precursors. In general, addition of precursors to the medium enhances product formation, although they usually inhibit the growth of the culture e.g., alkaloid synthesis in Datura cultures in increased while growth is inhibited by the addition of ornithine, phenylalanine, tyrosine and sodium phenyl pyruvate; precursors tryptamine and secologanin increase ajmalicine production in C. roseus cultures.

4. Elicitor-Induced Production of Secondary Metabolites:
The production of secondary metabolites in plant cultures is generally low and does not meet the commercial demands. There are

continuous efforts to understand the mechanism of product formation at the molecular level, and exploit for increased production. The synthesis of majority of secondary metabolites involves multistep reactions and many enzymes. It is possible to stimulate any step to increase product formation.

Elicitors are the compounds of biological origin which stimulate the production of secondary metabolites, and the phenomenon of such stimulation is referred to as elicitation. Elicitors produced within the plant cells are endogenous elicitors e.g., pectin, pectic acid, cellulose, other polysaccharides. When the elicitors are produced by the microorganisms, they are referred to as exogenous elicitors e.g., chitin, chitosan, glucans. All the elicitors of biological origin are biotic elicitors.

The term abiotic elicitors is used to represent the physical (cold, heat, UV light, osmotic pressure) and chemical agents (ethylene, fungicides, antibiotics, salts of heavy metals) that can also increase the product formation. However, the term abiotic stress is used for abiotic elicitors, while elicitors exclusively represent biological compounds.

Phytoalexins:
Plants are capable of defending themselves when attacked by microorganisms, by producing antimicrobial compounds collectively referred to as phytoalexins. Phytoalexins are the chemical weapons of defense against pathogenic microorganisms. Some of the phytoalexins that induce the production of secondary metabolites are regarded as elicitors. Some chemicals can also act as elicitors e.g., actinomycin-D, sodium salt of arachidonic acid, ribonuclease-A, chitosan, poly-L- lysine, nigeran. These compounds are regarded as chemically defined elicitors.

Interactions for Elicitor Formation:
Elicitors are compounds involved in plant- microbe interaction. Three different types of interactions between plants and microorganisms are known that lead to the formation of elicitors.

1. Direct release of elicitor by the microorganisms.

2. Microbial enzymes that can act as elicitors. e.g. endopolygalacturonic acid lyase from Erwinia carotovara.

3. Release of phytoalexins by the action of plant enzymes on cell walls of microorganisms which in turn stimulate formation elicitors from plant cell walls e.g., chitosan from Fusarium cell walls; α-1, 3-endoglucanase from Phytophthora cell walls.

Methodology of Elicitation:

Selection of microorganisms:

A wide range of microorganisms (viruses, bacteria, algae and fungi) that need not be pathogens have been tried in cultures for elicitor induced production of secondary metabolites. Based on the favourable elicitor response, an ideal microorganism is selected. The quantity of the microbial inoculum is important for the formation elicitor.

Co-culture:

Plant cultures (frequently suspension cultures) are inoculated with the selected microorganism to form co-cultures. The cultures are transferred to a fresh medium prior to the inoculation with microorganism. This helps to stimulate the secondary metabolism.

Co-cultures of plant cells with microorganisms may sometimes have inhibitory effect on the plant cells. In such a case, elicitor preparations can be obtained by culturing the selected microorganism on a tissue culture medium, followed by homogenization and autoclaving of the entire culture. This process releases elicitors. In case of heat labile elicitors, the culture homogenate has to be filter sterilized (instead of autoclaving).

In some co-culture systems, direct contact of plant cells and microorganisms can be prevented by immobilization (entrapment) of one of them. In these cultures, plant microbial interaction occurs by diffusion of the elicitor compounds through the medium.

Mechanism of Action of Elicitors:

Elicitors are found to activate genes and increase the synthesis of mRNAs encoding enzymes responsible for the ultimate biosynthesis

secondary metabolites. There are some recent reports suggesting the involvement of elicitor mediated calcium-based signal transduction systems that promotes the product formation. When the cells are pretreated with a calcium chelate (EDTA) prior to the addition of elicitor, there occurs a decrease in the production of secondary metabolite.

Elicitor-induced products in cultures:

In Table 42.4, a selected list of elicitor-induced secondary metabolites produced in culture systems are given.

TABLE 42.4 A selected list of elicitor-induced secondary metabolite production in plant cell cultures

Elicitor microorganism	*Plant cell culture(s)*	*Secondary metabolite(s)*
Aspergillus niger	*Cinchona ledgeriana, Rubia tinctoria*	Anthraquinones
Pythium aphanidermatum	*Catharanthus roseus*	Ajmalicine, Strictosidine Catharanthine
Botrytis sp	*Papaver somniferum*	Sanguinarine
Phytophthora megasperma	*Glycine max*	Isoflavonoids Gluceollin
Dendryphion sp	*Papaver somniferum*	Sanguinarine
Alternaria sp	*Phaseolus vulgaris*	Phaseollin
Fusarium sp	*Apium graveolens*	Furanocoumarins
Phythium aphanidermatum	*Daucus carota*	Anthocynins
Penicillium expansum	*Sanguinaria canadensis*	Benzophenan-thridine Alkaloids

5. Effect of Environmental Factors:

The physical factors namely light, incubation temperature, pH of the medium and aeration of cultures influence the production of secondary metabolites in cultures.

Effect of Light:

Light is absolutely essential for the carbon fixation (photosynthesis) of field-grown plants. Since the carbon fixation is almost absent or very low in plant tissue cultures, light has no effect on the primary metabolism.

However, the light- mediated enzymatic reactions indirectly influence the secondary metabolite formation. The quality of light is

also important. Some examples of light- stimulated product formations are given

1. Blue light enhances anthocyanin production in Haplopappus gracilis cell suspensions.

2. White light increases the formation of anthocyanin in the cultures of Catharanthus roseus, Daucus carota and Helianthus tuberosus.

3. White or blue light inhibits naphthoquinone biosynthesis in callus cultures of Lithospermum erythrorhizon.

Effect of Incubation Temperature:
The growth of cultured cells is increased with increase in temperature up to an optimal temperature (25-30°C). However, at least for the production some secondary metabolites lower temperature is advantageous. For instance, in C. roseus cultures, indole alkaloid production is increased by two fold when incubated at 16°C instead of 27°C. Increased temperature was also found to reduce the production of caffeine (by C. sineneis) and nicotine (by N. tabacum).

Effect of pH of the medium:
For good growth of cultures, the pH of the medium is in the range of 5 to 6. There are reports indicating that pH of the medium influences the formation of secondary metabolites. e.g., production of anthocyanin by cultures of Daucus carota was much less when incubated at pH 5.5 than at pH 4.5. This is attributed to the increased degradation of anthocyanin at higher pH.

Aeration of cultures:
Continuous aeration is needed for good growth of cultures, and also for the efficient production of secondary metabolites.

6. Biotransformation Using Plant Cell Cultures:
The conversion of one chemical into another (i.e., a substrate into a final product) by using biological systems (i.e. cell suspensions) as biocatalysts is regarded as biotransformation or bioconversion. The biocatalyst may be free or immobilized, and the process of biotransformation may involve one or more enzymes.

Biotransformation involving microorganisms and animal cells are described elsewhere.

The biotechnological application of plant cell cultures in biotransformation reactions involves the conversion of some less important substances to valuable medicinal or commercially important products. In biotransformation, it is necessary to select such cell lines that possess the enzymes for catalysing the desired reactions. Bioconversions may involve many types of reactions e.g., hydroxylation, reduction, glycosylation.

A good example of biotransformation by plant cell cultures is the large scale production of cardiovascular drug digoxin from digitoxin by Digitali lanata. Digoxin production is carried out by immobilized cells of D. lanata in airlift bioreactors. Cell cultures of Digitalis purpurea or Stevia rebaudiana can convert steviol into steviobiocide and steviocide which are 100 times sweeter than cane sugar.

A selected list of biotransformation's carried out in plant cell cultures is given in Table 42.5.

TABLE 42.4 A selected list of elicitor-induced secondary metabolite production in plant cell cultures

Elicitor microorganism	*Plant cell culture(s)*	*Secondary metabolite(s)*
Aspergillus niger	*Cinchona ledgeriana, Rubia tinctoria*	Anthraquinones
Pythium aphanidermatum	*Catharanthus roseus*	Ajmalicine, Strictosidine Catharanthine
Botrytis sp	*Papaver somniferum*	Sanguinarine
Phytophthora megasperma	*Glycine max*	Isoflavonoids Gluceollin
Dendryphion sp	*Papaver somniferum*	Sanguinarine
Alternaria sp	*Phaseolus vulgaris*	Phaseollin
Fusarium sp	*Apium graveolens*	Furanocoumarins
Phythium aphanidermatum	*Daucus carota*	Anthocynins
Penicillium expansum	*Sanguinaria canadensis*	Benzophenan-thridine Alkaloids

7. Secondary Metabolite Release and Analysis:
The methods employed for the separation and purification of secondary metabolites from cell cultures are the same as that used for plants.

Sometimes, the products formed within the cells are released into the medium, making the isolation and analysis easy. For the secondary metabolites stored within the vacuoles of cells, two membranes (plasma membrane and tonoplast) have to be disrupted. Permeabilizing agents such as dimethyl sulfoxide (DMSO) can be used for the release of products.

In general, separation and purification of products from plant cell cultures are expensive, therefore every effort is made to make them cost- effective. Two approaches are made in this direction:

1. Production of secondary metabolite should be as high as possible.

2. Formation of side product(s) which interfere with separation must be made minimal.

Once a good quantity of the product is released into the medium, separation and purification techniques (e.g. extraction) can be used for its recovery. These techniques largely depend on the nature of the secondary metabolite.

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DEPARTMENT OF BITOECHNOLOGY

UNIT – III– PLANT BIOTECHNOLOGY– SBB3103

UNIT - III

Methods to develop transgenic plants

Cross pollination, Direct gene transfer-Chemical methods, Particle Bombardment, Electroporation, Microinjection, Vacuum pressure, Pollen transformation, Lipofection, Macroinjection. Integration of transgenes, Inheritance of transgene analysis and Confirmation of transgene integration.

Gene Transfer Methods:

The gene transfer techniques in plant genetic transformation are broadly grouped into two categories:

I. Vector-mediated gene transfer

II. Direct or vector less DNA transfer

The salient features of the commonly used gene (DNA) transfer methods are given in Table 49.1.

Table 49.1 Gene transfer (DNA delivery) methods in plants

Method	*Salient features*
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

Direct or Vector-less DNA Transfer:

The term direct or vector less transfer of DNA is used when the foreign DNA is directly introduced into the plant genome. Direct DNA transfer methods rely on the delivery of naked

DNA into the plant cells. This is in contrast to the Agrobacterium or vector-mediated DNA transfer which may be regarded as indirect methods. Majority of the direct DNA transfer methods are simple and effective. And in fact, several transgenic plants have been developed by this approach.

Limitations of direct DNA transfer:

The major disadvantage of direct gene transfer is that the frequency of transgene rearrangements is high. This results in higher transgene copy number, and high frequencies of gene silencing.

Types of direct DNA transfer:

The direct DNA transfer can be broadly divided into three categories.

1. Physical gene transfer methods—electro- portion, particle bombardment, microinjection, liposome fusion, silicon carbide fibres.
2. Chemical gene transfer methods—Polyethylene glycol (PEG)-mediated, diethyl amino ethyl (DEAE) dextran-mediated, calcium phosphate precipitation.
3. DNA imbibition by cells / tissues / organs.

The salient features of the different methods for direct DNA transfer are given in Table 49.1 .

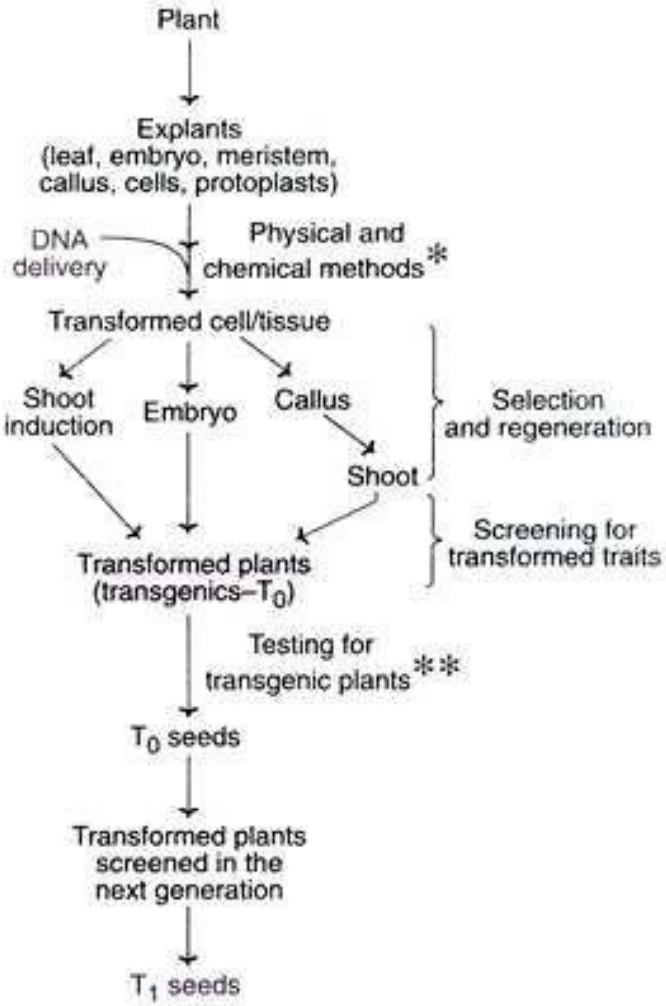


Fig. 49.9 : *An overview of the protocol for the production of transgenic plants using direct DNA delivery methods (*Electroporation, microinjection, macroinjection, bombardment, etc.; **Polymerase chain reaction, Southern hybridization)*

(A) Physical Gene Transfer Methods:

An overview of the general scheme for the production of transgenic plants by employing

physical transfer methods is

depicted in Fig. 49.9. Some details of the different techniques are described.

1. Electroporation:

Electroporation basically involves the use of high field strength electrical impulses to reversibly permeabilize the cell membranes for the uptake of DNA. This technique can be used for the delivery of DNA into intact plant cells and protoplasts.

The plant material is incubated in a buffer solution containing the desired foreign/target DNA, and subjected to high voltage electrical impulses. This results in the formation of pores in the plasma membrane through which DNA enters and gets integrated into the host cell genome.

In the early years, only protoplasts were used for gene transfer by electroporation. Now a days, intact cells, callus cultures and immature embryos can be used with suitable pre- and post-electroporation treatments. Electroporation has been successfully used for the production of transgenic plants of many cereals e.g. rice, wheat, maize.

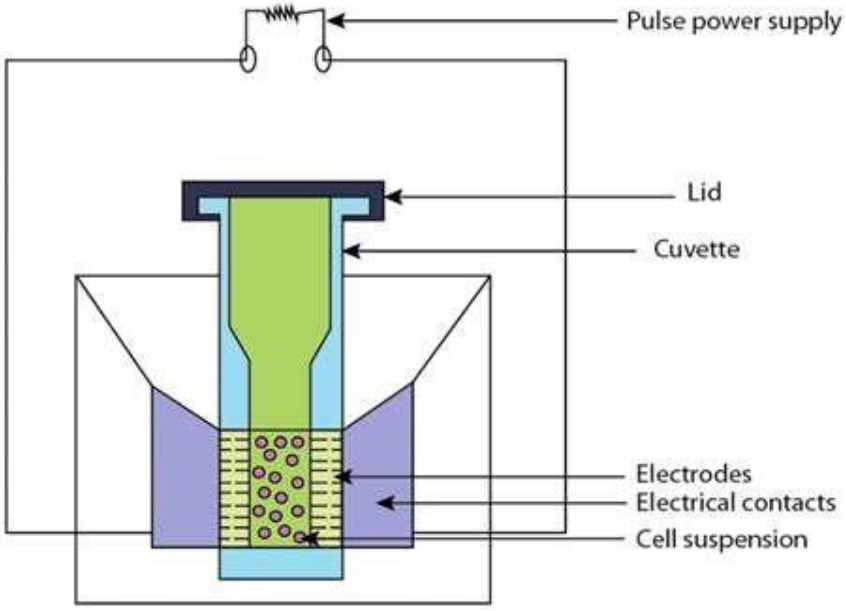
Electroporators

Benchtop electroporators are generally used as common lab equipment, residing atop a central bench or hood. Some units offer the possibility of electroporating multiple samples at the same time with special electrode assemblies that fit into multi-well cell culture dishes. Benchtop electroporators can also be set to different operating parameters, allowing researchers to optimize their field strengths depending on the cell type and whether or not the cell has a cell wall.

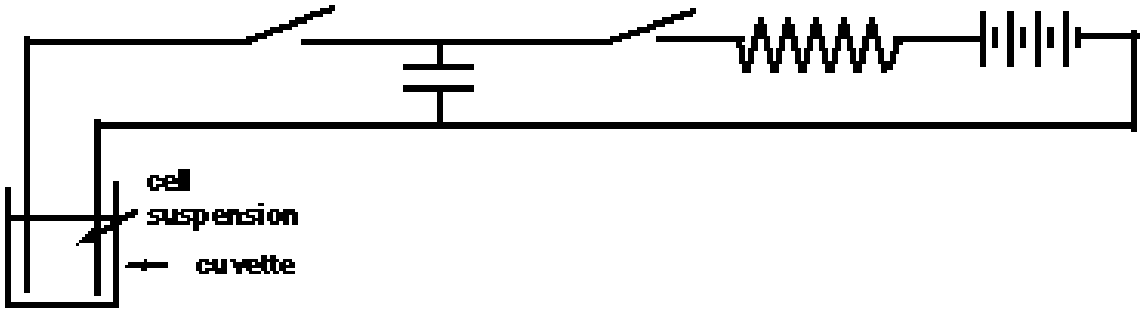
Electroporators have been used on a wide range of cells - including E. coli (for transformation) and mammalian cells such as neurons, astrocytes, neuroglia, lymphocytes, monocytes, fibroblasts, epithelial and endothelial cells from humans, mice, rats and monkeys (for transfection).

This is the method to direct gene transfer or transformation of plant cells. This technique is mostly used for monocotyledons plants. Introduction to DNA in to cells by exposing them for very brief period to high voltage electrical pulses, which is thought to induce transient pores in the plasma lemma, is called electroporation.

- The diagram shows an electrical circuit diagram for a simple electroporation device.



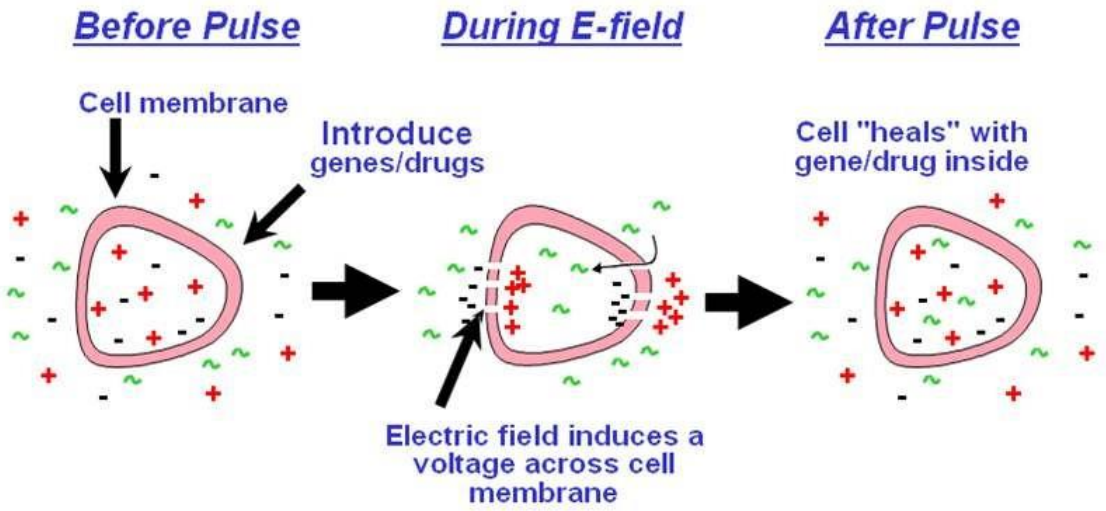
- A cell suspension, such as of plant protoplasts, is placed in the cuvette. A solution of DNA fragments containing the gene of interest is added. For example, the DNA could include a reporter gene such as that for chloramphenicol acetyltransferase (CAT).
- The capacitor is charged by closing the right-hand switch. When the capacitor has been charged, the direct current pulse is discharged in the cuvette suspension by closing the left-hand switch.
- The DC pulse is thought both to disrupt temporarily the membrane and to electrophorese DNA into cells.
- The cells are put in culture and assayed after various times (24 to 48 h) for the amount of CAT activity



Principle

In this approaches transfection mixture containing cells and DNA is exposed for a very brief period (few milliseconds) to a very high voltage gradient (eg. 4000 – 8000 V/cm). Which

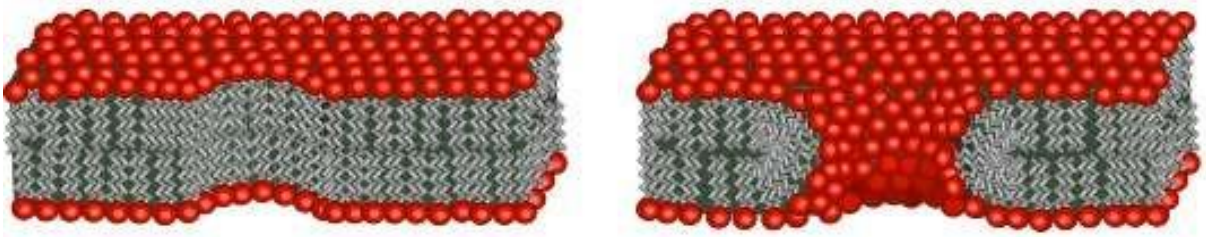
induces transient pores in the cell membrane through witch DNA seems to enter the cells. Treatment of cells before they are electroporated increases the frequency of transfection. This is most likely due to the cells arresting at the meta-phase, stage and the associated absence of nuclear envelope or to an unusual permeability of the plasma membrane.



Electroporation

Physical mechanism

Electroporation allows cellular introduction of large highly charged molecules such as DNA which would never passively diffuse across the hydrophobic bilayer core.[1] This phenomenon indicates that the mechanism is the creation of nm-scale water-filled holes in the membrane. Although electroporation and dielectric breakdown both result from application of an electric field, the mechanisms involved are fundamentally different. In dielectric breakdown the barrier material is ionized, creating a conductive pathway. The material alteration is thus chemical in nature. In contrast, during electroporation the lipid molecules are not chemically altered but simply shift position, opening up a pore which acts as the conductive pathway through the bilayer as it is filled with water.



Schematic showing the theoretical arrangement of lipids in a hydrophobic pore (top) and a hydrophilic pore (bottom).

Electroporation is a multi-step process with several distinct phases. First, a short

electrical pulse must be applied. Typical parameters would be 300-400 mV for < 1 ms across the membrane (note- the voltages used in cell experiments are typically much larger because they are being applied across large distances to the bulk solution so the resulting field across the actual membrane is only a small fraction of the applied bias). Upon application of this potential the membrane charges like a capacitor through the migration of ions from the surrounding solution. Once the critical field is achieved there is a rapid localized rearrangement in lipid morphology. The resulting structure is believed to be a "pre-pore" since it is not electrically conductive but leads rapidly to the creation of a conductive pore.

Evidence for the existence of such pre-pores comes mostly from the "flickering" of pores, which suggests a transition between conductive and insulating states.[16] It has been suggested that these pre-pores are small (~3 Å) hydrophobic defects. If this theory is correct, then the transition to a conductive state could be explained by a rearrangement at the pore edge, in which the lipid heads fold over to create a hydrophilic interface. Finally, these conductive pores can either heal, resealing the bilayer or expand, eventually rupturing it. The resultant fate depends on whether the critical defect size was exceeded which in turn depends on the applied field, local mechanical stress and bilayer edge energy.

Method

There are two systems to electroporation

1. Low voltage and long pulses method (300 -400 v cm-1 for 10 -50 milli- seconds)
2. High voltage short pulses approach (1000-1500 v cm-1- for 10 micro seconds).

Generally low voltage and high pulses produces high rates of transient transformation. While high voltage short pulses give high rates of stable transformation. electroporation has also been used to deliver DNA into intact plant cells. The cell wall presents and effective barrier to DNA therefore it has to be weakened by mild enzymatic treatments so as allow the entry of DNA in to the cell cytoplasm. Electroporation of intact maize and sugerbeet cells resulted in low levels of transient expression. Stable integration has not been reported.

Advantages of electroporation:

i. This technique is simple, convenient and rapid, besides being cost-effective.
ii. The transformed cells are at the same physiological state after electroporation.

iii. Efficiency of transformation can be improved by optimising the electrical field strength, and addition of spermidine.

Limitations of electroporation:

i. Under normal conditions, the amount of DNA delivered into plant cells is very low.

ii. Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.

iii. Regeneration of plants is not very easy, particularly when protoplasts are used.

2. Particle Bombardment (Biolistics):

Particle (or micro projectile) bombardment is the most effective method for gene transfer, and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms.

The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistics. There are other names for this technique- particle gun, gene gun, bio blaster. A diagrammatic representation of micro projectile bombardment system for the transfer of genes in plants is depicted in Fig. 49.10, and briefly described below.

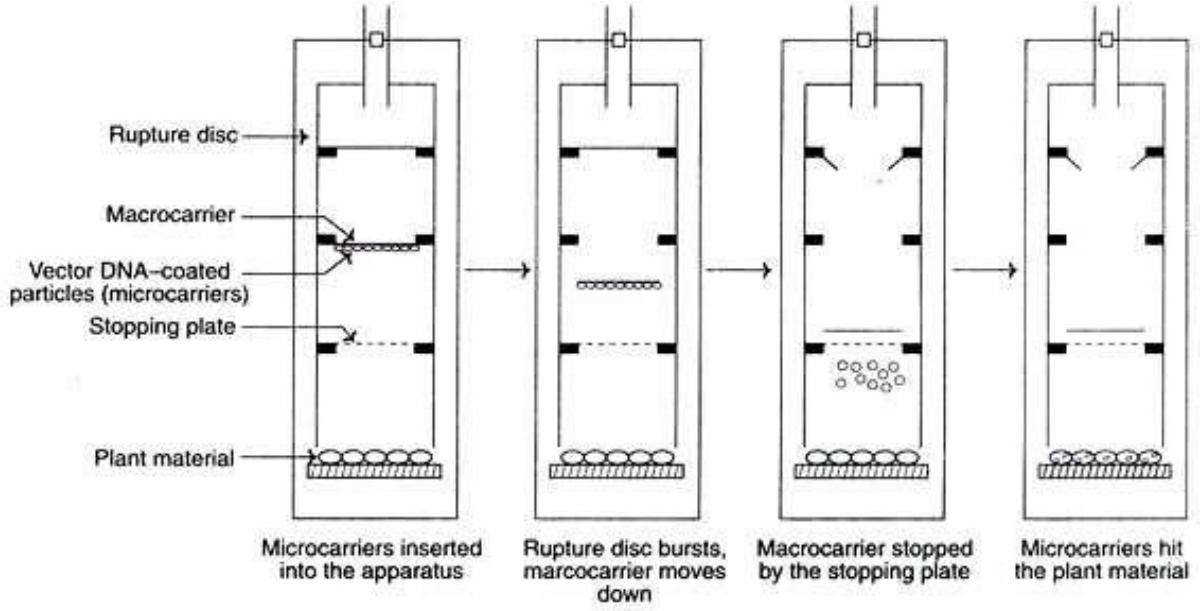


Fig. 49.10 : A diagrammatic representation of particle bombardment (biolistics) system for gene transfer in plants.

Micro carriers (micro projectiles), the tungsten or gold particles coated with DNA, are carried by macro carriers (macro projectiles). These macro-carriers are inserted into the apparatus and pushed downward by rupturing the disc.

The stopping plate does not permit the movement of macro carrier while the micro carriers (with DNA) are propelled at a high speed into the plant material. Here the DNA segments are released which enter the plant cells and integrate with the genome.

Plant material used in bombardment:

Two types of plant tissue are commonly used for particle bombardment:

1. Primary explants which can be subjected to bombardments that are subsequently induced to become embryogenic and regenerate.
2. Proliferating embryonic tissues that can be bombarded in cultures and then allowed to proliferate and regenerate.

In order to protect plant tissues from being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.

Transgene integration in bombardment:

It is believed (based on the gene transfer in rice by biolistics) that the gene transfer in particle bombardment is a two stage process.

1. In the pre-integration phase, the vector DNA molecules are spliced together. This results in fragments carrying multiple gene copies.
2. Integrative phase is characterized by the insertion of gene copies into the host plant genome.

The integrative phase facilitates further transgene integration which may occur at the same point or a point close to it. The net result is that particle bombardment is frequently associated with high copy number at a single locus. This type of single locus may be beneficial for regeneration of plants.

The success of bombardment:

The particle bombardment technique was first introduced in 1987. It has been successfully used for the transformation of many cereals, e.g. rice, wheat, maize. In fact, the first commercial genetically modified (CM) crops such as maize containing Bt-toxin gene were developed by this approach.

A selected list of the transgenic plants (developed by biolistics) along with the sources of the plant materials used is given in Table 49.2.

TABLE 49.2 A selected list of transgenic plants (along with cell sources) developed by microprojectile bombardment

Plant	*Cell source(s)*
Rice	Embryonic callus, immature zygotic embryos
Wheat	Immature zygotic embryos
Sorghum	Immature zygotic embryos
Corn	Embryonic cell suspension, immature zygotic embryos
Barley	Cell suspension, immature zygotic embryos
Banana	Embryonic cell suspension
Sweet potato	Callus cells
Cotton	Zygotic embryos
Grape	Embryonic callus
Peas	Zygotic embryos
Peanut	Embryonic callus
Tobacco	Pollen
Alfalfa	Embryonic callus

Factors affecting bombardment:

Several attempts are made to study the various factors, and optimize the system of particle bombardment for its most efficient use. Some of the important parameters are described.

Nature of micro particles:

Inert metals such as tungsten, gold and platinum are used as micro particles to carry DNA. These particles with relatively higher mass will have a better chance to move fast when bombarded and penetrate the tissues.

Nature of tissues/cells:

The target cells that are capable of undergoing division are suitable for transformation. Some more details on the choice of plant material used in bombardment are already given.

Amount of DNA:

The transformation may be low when too little DNA is used. On the other hand, too much DNA may result is high copy number and rearrangement of transgenes. Therefore, the quantity of DNA used should be balanced. Recently, some workers have started using the chemical aminosiloxane to coat the micro particles with low quantities of DNA adequate enough to achieve high efficiency of transformation.

Environmental parameters:

Many environmental variables are known to influence particle bombardment. These factors (temperature, humidity, photoperiod etc.) influence the physiology of the plant material, and consequently the gene transfer. It is also observed that some explants, after bombardment may require special regimes of light, humidity, temperature etc.

The technology of particle bombardment has been improved in recent years, particularly with regard to the use of equipment. A commercially produced particle bombardment apparatus namely PDS-1000/HC is widely used these days.

Advantages of particle bombardment:

i. Gene transfer can be efficiently done in organized tissues.
ii. Different species of plants can be used to develop transgenic plants.

Limitations of particle bombardment:

i. The major complication is the production of high transgene copy number. This may result in instability of transgene expression due to gene silencing.
ii. The target tissue may often get damaged due to lack of control of bombardment velocity.
iii. Sometimes, undesirable chimeric plants may be regenerated.

3. Microinjection:

Microinjection is a direct physical method involving the mechanical insertion of the desirable DNA into a target cell. The target cell may be the one identified from intact cells, protoplasts, callus, embryos, meristems etc. Microinjection is used for the transfer of cellular organelles and for the manipulation of chromosomes.

The technique of microinjection involves the transfer of the gene through a micropipette (0.5-10.0 pm tip) into the cytoplasm/nucleus of a plant cell or protoplast.

While the gene transfer is done, the recipient cells are kept immobilized in agarose embedding, and held by a suction holding pipette (Fig. 49.11).

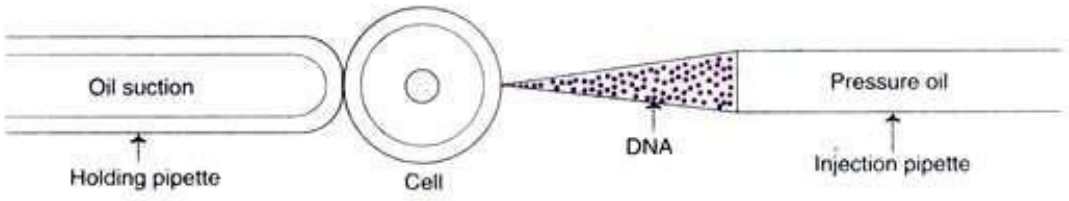


Fig. 49.11 : Microinjection of DNA by holding pipette method.

As the process of microinjection is complete, the transformed cell is cultured and grown to develop into a transgenic plant. In fact, transgenic tobacco and Brassica napus have been developed by this approach. The major limitations of microinjection are that it is slow, expensive, and has to be performed by trained and skilled personnel.

4. Liposome-Mediated Transformation:

A liposome is an artificially-prepared spherical vesicle composed of a lamellar phase lipid bilayer. The liposome can be used as a vehicle for administration of nutrients and pharmaceutical drugs. Liposomes can be prepared by disrupting biological membranes (such as by sonication).

They are successfully used in mammalian cells for the delivery of proteins, drugs etc. Liposomes carrying genes can be employed to fuse with protoplasts and transfer the genes.

Discovery

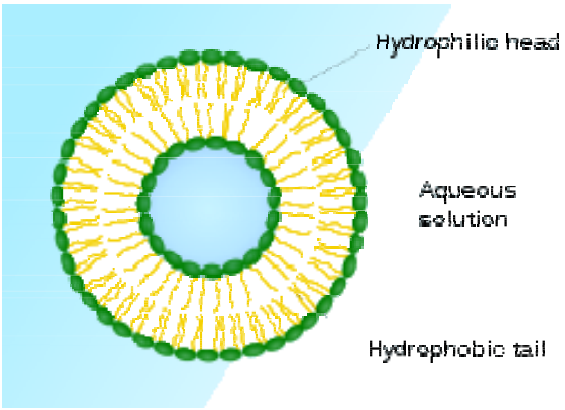
Liposomes were first described by British haematologist, Alec D Bangham in 1961 (published 1964), at the Babraham Institute, in Cambridge.

Liposomes are often composed of phosphatidylcholine-enriched phospholipids and may also contain mixed lipid chains with surfactant properties such as egg phosphatidylethanolamine. A liposome design may employ surface ligands for attaching to unhealthy tissue.

The major types of liposomes are the multilamellar vesicle (MLV), the small unilamellar liposome vesicle (SUV), the large unilamellar vesicle (LUV), and the cochleate vesicle.

Liposomes should not be confused with micelles and reverse micelles composed of monolayers.

Lipofection is the transformation of cells using liposomes. Liposomes are artificial phospholipid vesicles used to deliver a variety of molecules including DNA into the cells. Liposomes can be preloaded with DNA and are then fused with the protoplasts to release the content into the cell. Animal cells, yeast protoplasts, plant cells, bacteria are susceptible to liposome transformation.



Membrane – membrane fusion and endocytosis are two common methods by which the liposomes fuse with the cells. DNA in solution spontaneously complexes with liposomes. Liposomes are positively charged vesicles produced from acidic lipids such as phosphatidyl glycerol and phosphatidyl serine. The liposomes may be multi lamellar vesicles having a size of 0.1 to 10 micrometer or uni lamellar types having 20-25 nano meters size. The liposome containing DNA is prepared as water- in -oil emulsion through sonication of the phospholipids and aqueous buffer in organic solvent. The organic solvent is the evaporated to leave the emulsion of liposomes.

The efficiency of transformation increases when the process is carried out in conjunction with polyethylene glycol (PEG). Liposome-mediated transformation involves adhesion of liposomes to the protoplast surface, its fusion at the site of attachment and release of plasmids inside the cell (Fig. 49.12).

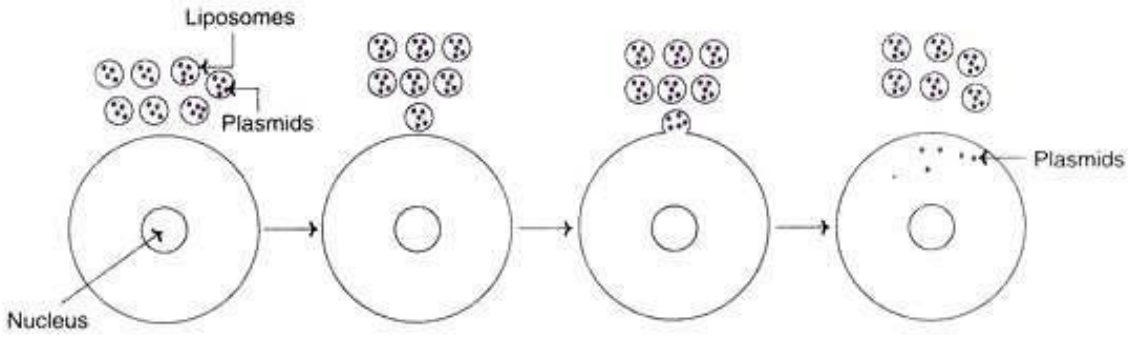


Fig. 49.12 : A diagrammatic representation of fusion of plasmid–filled liposomes with protoplasts.

Example

Liposome-mediated plant cell transformation offers several unique features, the most important of which is the protection of the nucleic acids against nucleases present in the plant cell media. Conditions for efficient fusion of liposomes and plant cell membranes, gene delivery and gene expression, were investigated using plasmids containing the chloramphenicol acetyl transferase (CAT) gene, and a cloned dimer of the tomato yellow leaf curl virus (TYLCV) genome, a geminivirus. Negatively charged large unilamellar vesicles were used to encapsulate the DNA and to introduce it into tobacco or tomato protoplasts and calli.

These results indicate that liposome-mediated gene transfer can be used to study transient gene expression and virus replication in plant cells.

Advantages of liposome fusion:

i. Being present in an encapsulated form of liposomes, DNA is protected from environmental insults and damage.
ii. DNA is stable and can be stored for some time in liposomes prior to transfer.
iii. Applicable to a wide range of plant cells.
iv. There is good reproducibility in the technique.

Limitations of liposome fusion:

The major problem with liposome-mediated transformation is the difficulty associated with the regeneration of plants from transformed protoplasts.

5. Silicon Carbide Fibre-Mediated Transformation:

The silicon carbide fibres (SCF) are about 0.3-0.6 pm in diameter and 10-100 pm in length. These fibres are capable of penetrating the cell wall and plasma membrane, and thus can deliver DNA into the cells. The DNA coated silicon carbide fibres are vortexed with ‘plant material (suspension culture, calluses). During the mixing, DNA adhering to the fibres enters the cells and gets stably integrated with the host genome. The silicon carbide fibres with the trade name Whiskers are available in the market.

Advantages of SCF-mediated transformation:

i. Direct delivery of DNA into intact walled cells. This avoids the protoplast isolation.
ii. Procedure is simple and does not involve costly equipment.

Disadvantages of SCF-mediated transformation:

i. Silicon carbide fibres are carcinogenic and therefore have to be carefully handled.

ii. The embryonic plant cells are hard and compact and are resistant to SCF penetration.

In recent years, some improvements have been made in SCF-mediated transformation. This has helped in the transformation of rice, wheat, maize and barley by using this technique.

(B) Chemical Gene Transfer Methods:

1. Polyethylene glycol (PEG)-mediated transfer:

Polyethylene glycol (PEG), in the presence of divalent cations (using Ca^{2+}), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. In this way, the DNA enters nucleus of the protoplasts and gets integrated with the genome.

The procedure involves the isolation of protoplasts and their suspension, addition of plasmid DNA, followed by a slow addition of 40% PEG-4000 (w/v) dissolved in mannitol and calcium nitrate solution. As this mixture is incubated, protoplasts get transformed.

Advantages of PEG-mediated transformation:

i. A large number of protoplasts can be simultaneously transformed.

ii. This technique can be successfully used for a wide range of plant species.

Limitations of PEG-mediated transformation:

i. The DNA is susceptible for degradation and rearrangement.

ii. Random integration of foreign DNA into genome may result in undesirable traits.

iii. Regeneration of plants from transformed protoplasts is a difficult task.

2. Deae Dextran-Mediated transfer:

The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The major limitation of this approach is that it does not yield stable trans-formants.

Calcium Phosphate Co- Precipitation-Mediated Transfer:

The DNA is allowed to mix with calcium chloride solution and isotonic phosphate buffer to form DNA-calcium phosphate precipitate. When the actively dividing cells in culture are exposed to this precipitate for several hours, the cells get transformed. The

success of this method is dependent on the high concentration of DNA and the protection of the complex precipitate. Addition of dimethyl sulfoxide (DMSO) increases the efficiency of transformation.

Dna Imbibition By Cells/Tissues:

Some workers have seriously tried to transform cells by incubating cell suspensions, tissues, embryos and even seeds with DNA. The belief is that the DNA gets imbibed, and the cells get transformed. DNA imbibition approach has met with little or no success.

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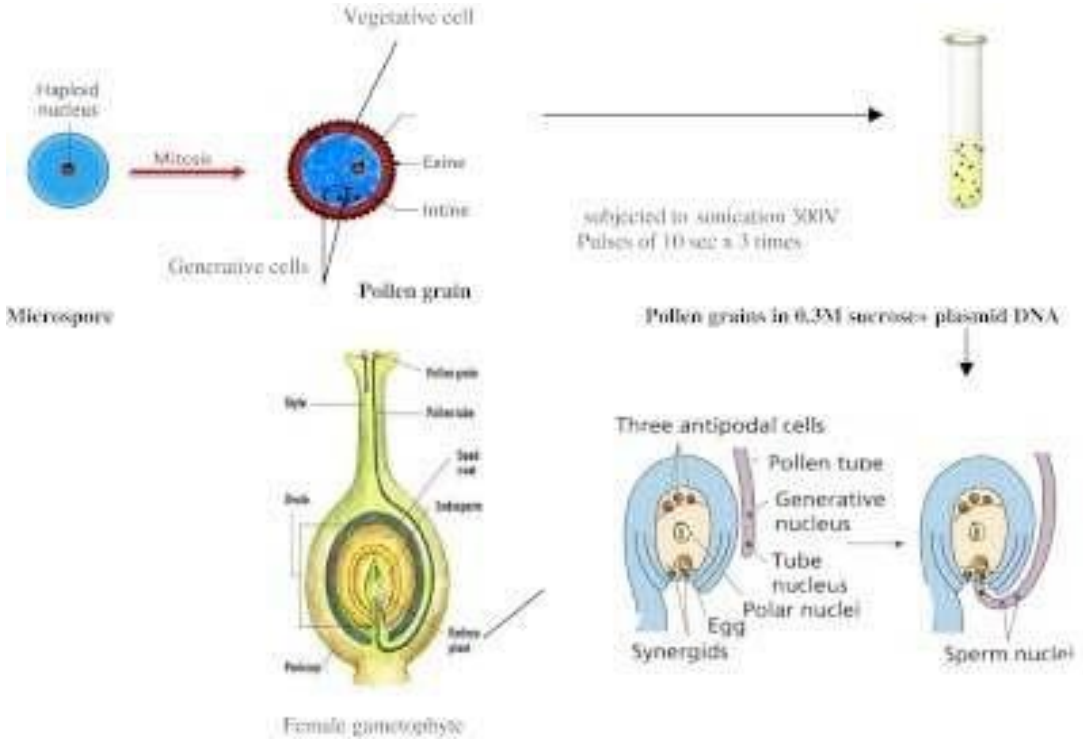
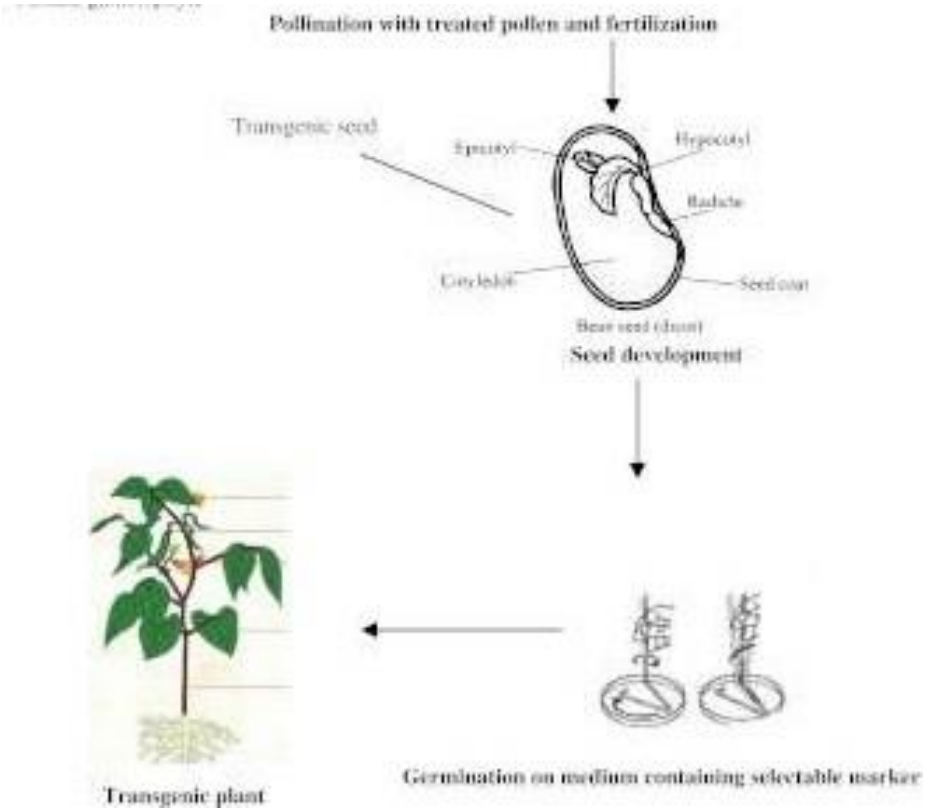
Pollen transformation





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Pollen grains—a target for gene transfer

Formation of gametes is a key step in the life-cycle of sexually reproducing plants. The whole concept has centered on the assumption that at fertilization, the egg cell accepts the genome of the sperm cell and consequently introduction of foreign gene into the pollen grain prior to fertilization will give rise to transgenic zygote and plants. The treated pollen grains can be used for pollinating the stigma of male sterile or emasculated flowers, develop seeds and transgenic plants selected on medium supplemented with selectable marker chemicals.

Pollen grain is an ideal target for transformation, since they occur in large numbers, can be easily isolated from anthers and transgenes can be introduced directly in the form of naked DNA or through mediation of *Agrobacterium*. The mature pollen grain harbours a vegetative cell and a generative cell, the latter giving rise to two male gametes. Sporogenous initial cells also called as pollen mother cells, undergo meiosis and a mitosis resulting in a tetrad of microspores. Each tetrad is enclosed in a thick callose wall and individual microspores are released from the tetrad by the action of callase—an enzyme produced by the surrounding tapetal cells. The microspore enlarges and undergoes an

asymmetric division resulting in a large vegetative cell and a small generative cell. The generative cell is engulfed in the cytoplasm of the vegetative cell. The generative cell undergoes mitosis (second pollen mitosis) to form two sperm cells. The timing of second mitosis varies in different plant species and in some cases, as in case of gramineae and crucifers, it takes place when the microspores are inside the anthers, while in others, it may occur during pollen tube growth on stigma.

Pollen grain transformation

Desired foreign gene should be introduced into the pollen grain and they can subsequently be used for pollination and fertilization. At fertilization, the egg cell fuses with one of the sperm cells of the pollen grain and zygote is produced. Hence, it is appropriate to integrate transgenes into the particular generative cell of the pollen, which will fuse with the egg cell and subsequently give rise to the zygote. Often, it is difficult to introduce genes into mature pollen grains with conventional methods of transformation because of the thick cell walls of pollen grains. Some of the initial experiments on treatment of pollen with exogenous DNA did not yield transgenic plants after pollination and fertilization. Particle gun bombardment of pollen grains and vacuum infiltration can help in introduction of transgenes into pollen grains. Presence of thick cell walls of pollen grains coupled with the release of strong nucleases by germinating pollen grains have been hindrances for successful entry and integration of transgenes.

Co-cultivation of pollen grains with *Agrobacterium* and application of *Agrobacterium* prior to or after pollination have been reported to give rise to transgenic zygotes and plants. However, these results could not be reproduced in all laboratories and contradictory evidence has slowed down further research in this area. Some researchers showed that transformation of cereals by pollen pathway was difficult and they suggested that transformation observed in earlier studies was probably an artifact. However, since the successful reports on pollen-mediated gene transfer using ultrasonication leading to transgenic plant development in some of the crop plants like *Zea mays*, *Sorghum bicolor* and *Brassica juncea*, there is renewed interest in pollen-mediated transformation.

Pollen transformation is conducted on the basis of the assumption that DNA uptake by pollen will lead to integration of transgene into the germline with subsequent transmission to the progeny. Mature pollen grain contains a vegetative cell and two sperm cells. Since only one of the sperm cells fuse with the egg cell giving rise to the zygote and

the second sperm cell fuses with the central cell to produce the endosperm, the introduction of DNA into the sperm cell, destined to fuse with the egg cell is important in determining the development of transgenic seed, while DNA transferred to vegetative cell and to the second sperm cell involved in triple fusion to produce endosperm will not produce transgenic plants, with subsequent transmission of the trait to the progeny. Hence, it will be ideal to introduce the transgene into the microspore when it is uninucleate, so that the transgene is passed on to both the generative cells.

Electroporation of pollen grains have been used to introduce DNA into pollen grains and development of transgenic plants containing genes encoding B-glucuronidase and chloramphenicol acetyl transferase has also been demonstrated.

Advantages and disadvantages of pollen mediated transformation Advantages

1 Fast and regeneration independent method
2 Not prone to chimerism
3 Avoids somaclonal variation
4 Genotype independent
5 Cheap method
6 No need for skilled workers
7 No need of *in vitro* conditions
8 Can be routinely employed
9 Availability of large number of pollen grains
10 Can be used by plant breeders

Disadvantages

1 Not demonstrated in many species
2 Genes have to be introduced into pollen at correct stage
3 Frequency of transformation is low

Macroinjection.

Injection of DNA solution (5-10 µl) by micropipettes into the developing floral side shoot (tillers) of plant is known as macroinjection. Within the floral tillers are archesporial

cells that give rise to pollen in the developing sac by two meiotic cell divisions. Such cells might also be able to take up large molecules such as DNA.

Macroinjection is an approach to the delivery of exogenous DNA that could circumvent the expense, time and genome stress associated with the transformation of cells in culture and subsequent plant regeneration. Also, plant regeneration from transformed protoplasts, still remains a problem. Therefore, cultured tissues, which facilitate the development of immature structures, provides an alternate cellular target for transformation. These immature structures may include immature embryos, meristems, immature pollen, germinating pollen, etc.. The main disadvantage of this technique is the likelihood for the production of chimeric plants with only a part of the plant transformed. However, from this chimeric plant, transformed plants of single cell origin can be subsequently obtained. To escape these problems Pollen-Tube Pathway (PTP) utilizing the normal fertilization cycle was developed to eliminate the regeneration phase. PTP based transformation is an injection/delivery of naked DNA into ovaries to produce transformed progeny. In this approach the stigma is cut off and a drop of DNA solution is applied to the cut end of the style of recently pollinated plant florets. This procedure was used for the first time in rice. Afterwards, other species such as wheat, soybean, *Petunia hybrida* and watermelon were subjected. In rice, the transformation efficiency was higher after excising the palea than after cutting top floret. A variation is the injection of a bacterial inoculum or plasmid DNA into inflorescences with pollen mother cells in the premeiotic stage without removing the stigma. Such an approach has been employed for rye. Although the results of the described experiments could be encouraging, the final transformation efficiency was about 10-fold lower than with biolistics. However, this approach has recently been introduced as a potential method for stable plant transformation.

Integration of transgenes,

Two different ways for alteration of covalent linkage between DNA molecules is possible: either a region of sequence identity between the partners is needed (homologous recombination) or no sequence-specific requirements have to be fulfilled (illegitimate recombination also referred to as non-homologous end joining). But stable integration and expression of introduced gene is essential to realize transgene advantage in the genetically-engineered crops. However, it has been clear for sometime that the introduced genes are not expressed uniformly in independent transgenic plants and that

transgenic expression can change in successive generations. In addition, introduced genes can suppress the expression of related endogenous genes and/or transgenes already present in the genome[1]. The ability to recognize self, from non-self, a characteristic of the immune system, and the existence of self-DNA protection systems might modify the foreign DNA to make it nonfunctional or eliminate it altogether.

Although several factors contribute to inactivation and elimination of intrusive nucleic acids, methylation of intruder DNA and homology-dependent ectopic pairing are probably the major factors that contribute to transgene inactivation and gene silencing. The integration intermediates may be the prime targets for DNA methyltransferases that enforce repression of transgenes. Also, evidence is rapidly accumulating that silencing of single copy foreign genes, multicopy transgenes integrated either at the same locus or at unlinked loci frequently cause silencing of themselves and of homologous host sequences. The frequency of silencing encountered in multicopy transformants has led to the speculation that enhanced DNA : DNA pairing of the repetitive elements in such complex inserts might act as a signal for detection, resulting in highly efficient silencing. Therefore, suitable transformation constructs need to be designed to avoid host surveillance processes and facilitate predictable integration.

The intrinsic ability of viral sequences or mobile genetic elements to excise and reinsert several times in search of a compatible genomic environment to attain stable integration is probably a mechanism for escaping the self-protection system of the host. The investigations into the sequence complexity of a genomic organization suggest that genomes are made up of 'isochores', i.e. very long stretches of DNA with high compositional homogeneity. Carels *et al*. found that almost all genes in maize are present in isochores covering an extremely low GC-range (1–2%) that represent only 10–20% of the genome. From this discovery, they developed the concept of 'gene space', in which genomic regions are represented by a single family of isochores, and noted that the gene space in maize corresponded to the only genome compartment in which certain mobile sequences can be transposed. The implication is that exogenous DNA arriving within the GC-rich gene space is likely to be actively transcribed, whereas DNA inserted into other regions is unlikely to be transcribed. Axiomatically, the GC-rich transgenes inserted into transcriptionally-active DNA remain functional, whereas AT-rich sequences in transgenes may mark them for inspection as invasive DNA. A precedent for this generalization has been observed in mammals: mobile sequences that have undergone numerous

amplification and translocation events during the evolution of mammals, and several integrated viral sequences, were predominantly found in isochores of matching GC- composition. Thus, it can be inferred that a matching genomic environment is essential for stable integration and expression of inserted sequences, and that excision and reinsertions may continue until the candidate transgene finds a compatible gene space in the host genome, else it would be eliminated/inactivated through the genome scanning mechanism. The observations recorded from isochore studies suggest that genomic systems exist that facilitate identification and inactivation (or elimination) of the foreign sequences that are compositionally different or incompatible from the genomic sites into which the insertions take place.

Iglesias *et al.* have made an important discovery in relation to the stable integration and expression of transgene from the physical mapping of introduced DNA with respect to the spatial sites and sequence composition of chromosomal segments. Using fluorescence *in situ* hybridization to probe the physical location of transgene insertion, they demonstrated in tobacco that the stably-expressed inserts were present in the vicinity of telomeres, whereas the unstably-expressed inserts occupied intercalary and paracentromeric locations. Also, the stably-expressed loci comprised relatively simple T- DNA arrangements that were flanked on at least one side by plant DNA containing AT-rich regions that bind to nuclear matrices *in vitro*. It may be important to mention here that the matrix attachment regions (MARs) that serve as a link between nuclear attachment sites and expressed sequence tags (ESTs), and facilitate gene expression, remain associated mainly with such telomeric regions

In view of the foregoing information, the following may be considered for efficient transgene integration:

1. Identification of isochores in the genome map of the target host is of value to design gene constructs amenable for stable integration. The isochore sizes and borders can be physically determined by compositional mapping, i.e. hybridizing probes for landmark genes or single copy anonymous sequences, both at the DNA and chromosome level.
2. Since MARs reduce variation in and increase transgene expression level, it is of utmost topographic value that the isochores that are predominant in sub- telomeric regions may be targeted to serve as ideal sites for stable transgene integration.
3. The flanking regions of transgenes should preferably contain sequences homologous to compatible gene space of isochores so as to facilitate improved integration of transgenes

by homologous recombination, and also attain a possible knock out of redundant endogenous sequences/allele replacement by double cross over.

4. The haploid cells in higher plants may possibly serve as a better experimental system to improve the efficiency of targeted transformation.

Inheritance of transgene analysis

To date, various transgenes have been successfully introduced into nuclear genomes of many plants species with verified methods. Under standing of the inheritance and stability of the newly introduced transgenes is of great importance in determining the value and application of genetically engineered organisms (GMOs) in agriculture. Characterization of the transgene locus/loci on the molecular level (transmission of the transgene) as well as segregation analysis of the transgene-encoded phenotype (expression of the transgene) in the subsequent progenies allowed insight into the nature of transgene inheritance. Integration of transgenes at a single Mendelian locus, regardless of copy number, is typically observed in transformants produced both by direct DNA delivery and by Agrobacterium-mediated transformation. Multiple complete and/or partial transgene copies inherited as digenic or multigenic Mendelian traits have also been documented. However, the non-Mendelian segregation occurred at a frequency between 10% and 50% of lines due to either unstable transmission of the transgene or poor expression.

The regular transgene transmission as well as its expression is a main prerequisite for the production of new cultivars in generatively propagated plants. Therefore, the knowledge of distortion frequency and the sources of this phenomenon have a substantial importance for breeding of transgenic varieties.

Agrobacterium-mediated transformation usually produces transgenic plants with a low copy number and the transgenes are transmitted to progeny according to Mendelian and in some cases non-Mendelian inheritance. The characteristic features of the transgene integration pattern resulting from DNA delivery through particle bombardment often include integration of the full-length transgene as well as rearranged copies of the introduced DNA. Copy numbers of both the transgene and rearranged fragments are often highly variable. Multiple transgene copies most frequently are inherited as a single locus. A variable proportion of the transgenic events exhibited a Mendelian ratio vs events exhibiting segregation distortion. In some cases, the transgenic locus has not been stably

inherited. Both deletion of a transgene locus and rearrangement of inserted T-DNA with either retention or loss of expression have been reported. Duplication or amplification of transgenes, and the epistatic interaction between different loci and/or allelic interaction within a single locus also exist. Furthermore, mitotic/meiotic recombination has been observed for transgenic loci in various plant species

The introduction of a transgene into a recipient genome is a complex event depending on the transgene itself and the host genome. The transgene expression level may vary extremely, depending on a number of factors in which the 'positional effects' play a major role. The same is true for transgene inheritance, where the site of transgene integration determines its stability. If integration occurs in a transcriptionally active area, the resulting expression may be influenced by proximal regulatory sequences. In situations where integration occurs in the repeat-sequence regions of heterochromatin, inactivation of transgenes may. Several other factors are also known. Generally, they are known genetic mechanisms, including transgene deletion, duplication, rearrangement, repeated sequence recombination, and gene interactions. The frequency of distortion in transgene inheritance varied between 10% and 50% of lines (independent transformants). However, these data are most frequently based on a very low number of lines analysed. The consequence of non-Mendelian inheritance for transgenic breeding is that an increasing number of lines should be produced after transformation

Confirmation of transgene integration.

Commonly used methods to confirm the putative transgenic plants are polymerase chain reaction, Southern blotting, Western blotting, Northern blotting, functional assay (testing the presence of selectable marker and the target gene), *in situ* hybridization, and progeny analysis (segregation of the target gene). Not all transgenic plants produce the same amount of protein from the target gene and selection based on the Western blot is necessary. This is because a positive correlation usually exists between the effectiveness of the gene in the bioassay and the amount of protein it produces. For example, the level of rice chitinase accumulating in transgenic sorghum plants with the *chi11* gene was positively correlated with resistance to sorghum stalk rot.

PCR, a simple and rapid procedure, is utilized to confirm whether a putatively transgenic plant that has survived selection is indeed transgenic. Usually, two primers (one forward and one reverse) specific for the selectable marker (bar gene, for example) are

used in a PCR reaction with genomic DNA extracted from the transgenic plants. A thermostable DNA polymerase amplifies the region between the two primers during the multiple amplification cycles of the PCR, which yields a DNA fragment of predicted size (the length equal to the number of base pairs between the two primers in the transgene). This fragment is easily detected on an agarose gel by staining with ethidium bromide. PCR is a very sensitive and rapid method for identification of transgenic plants in the seedling stage and requires only a small amount of plant tissue.

In Southern blotting, DNA fragments from transgenic plants generated by digestion with restriction enzyme(s) are first separated according to fragment size by electrophoresis through an agarose gel. The DNA fragments then are transferred to a solid support, such as a nylon membrane or a nitrocellulose sheet. The transfer is affected by simple capillary action, sometimes assisted by suction or electric current. The DNA binds to the solid support, usually because the support has been treated to carry a net positive charge, or some other means of binding such as inducing covalent binding of the DNA to the support. DNA fragments maintain their original positions in the gel after transfer to the membrane. Hence, larger fragments will be localized toward the top of the membrane and smaller fragments toward the bottom. The positions of specific fragments can then be determined by "probing" the membrane. The probe consists of the DNA fragments of interest, such as a cloned gene, which has been labeled with a radioactive isotope or some other compound that allows its visual detection. Under the proper set of conditions, the denatured single-stranded probe will hybridize to its complementary single strands of genomic DNA affixed to the membrane. In this way, the size of the fragment on which the probe resides in the genomic DNA can be determined. In transgenic plant experiments, the Southern blot often is used to determine whether an introduced gene is indeed present in the plant DNA and whether multiple transgenic plants carry the introduced gene on the same size of DNA fragment (suggesting independent transformation events). The results of Southern blots also indicate whether a single copy of the gene has been inserted or if multiple copies are likely to be present.

Northern blotting – the name was derived as a play on words from the Southern blot – is very similar to the Southern blot, except that instead of restriction enzyme-digested DNA, native RNA is separated according to size by electrophoresis through an agarose gel and then transferred to a solid support. The rest of the Northern blot procedure is very similar

to that of the Southern blot and it is used to determine whether the introduced gene has been transcribed into messenger RNA and accumulates in the transgenic plant.

The Western blotting procedure detects the protein of the transgene in an extract of proteins prepared from various parts of the transgenic plants and is, therefore, an assay for a functional transgene. In this technique the proteins are first electrophoresed in an SDS-polyacrylamide gel and the proteins are then transferred to nitrocellulose membrane by electrophoretic transfer. The membrane is then treated with an antibody specific for the protein encoded by the transgene followed by a second antibody coupled to an enzyme, which can act on a chromogenic (or fluorogenic) substrate leading to visualization of the transgene protein with increased sensitivity. The expression level of the protein can be quantified using known amounts of the transgenic-encoded protein.

Functional assay-When the selectable markers used are antibiotic-resistant or herbicide-resistant genes, a functional assay can be made by spraying antibiotics or smearing herbicide on the leaves of those putative transgenic seedlings or plants in later segregating populations. Sensitive plants typically will turn brown and shrivel up whereas resistant (transgenic) plants will stay healthy and green. Such an assay provides the initial screening of large number of putative transgenic plants and reduces the work load by eliminating escapes during selection.

Progeny test-With stable transformed genes, progeny testing should show the presence and activity of the selectable marker and target genes, such as the gene *gfp* encoding green fluorescent protein, or bar and disease resistance. However, segregation does not always follow the typical Mendelian fashion. For example, among the progeny in the *Agrobacterium*-mediated wheat transformation experiments reported, segregation in the T_1 generation had ratios of 32:0, 1:34, 0:40 and 74:0 in addition to the expected 1:1, 3:1 or 15:1 ratios. This variability indicates aberrant segregation. However, in other cases, segregation follows the normal Mendelian pattern. For example, among six sorghum T_0 transgenic plants produced by biolistic bombardment, all showed typical 3:1 segregation ratios in the T_1 generation.

Advantages and disadvantages of different methods of transformation

No.	Method	Advantage	Disadvantage
1	*Agrobacterium*-mediated transformation of protoplasts	No chimeras	Protoplast regeneration is essential. Tedious procedure
2	*Agrobacterium*-mediated transformation of explants	Easy	Chimeras, somaclonal variation, *in vitro* culture necessary
3	Floral dip method with *Agrobacterium*	Easy, No need of tissue culture	Demonstrated only in a few species
4	Pistil transformation	Easy, No tissue culture	Demonstrated only in a few species
5	Particle gun bombardment using explants	Genotype independent	Costly, somaclonal variation, chimeras, complex DNA integration pattern
6	Seedling transformation	Easy, tissue No culture	Not demonstrated in all species
7	Microinfection into protoplasts	No chimeras	Tedious, Time consuming, requirement for skilled personnel
8	Macroinfection		Tedious, Time consuming, requirement for skilled personnel, Chimeras
9	Liposome mediated DNA transfer into protoplasts	No chimeras	Tedious, requirement for regeneration from protoplasts, chimeras, somaclonal variation
10	PEG mediated DNA transfer to protoplasts	No chimeras	Tedious, requirement for regeneration from protoplasts, chimeras, somaclonal variation
11	Electroporation of protoplasts	No chimeras	Tedious, requirement for regeneration from protoplasts, chimeras, somaclonal variation

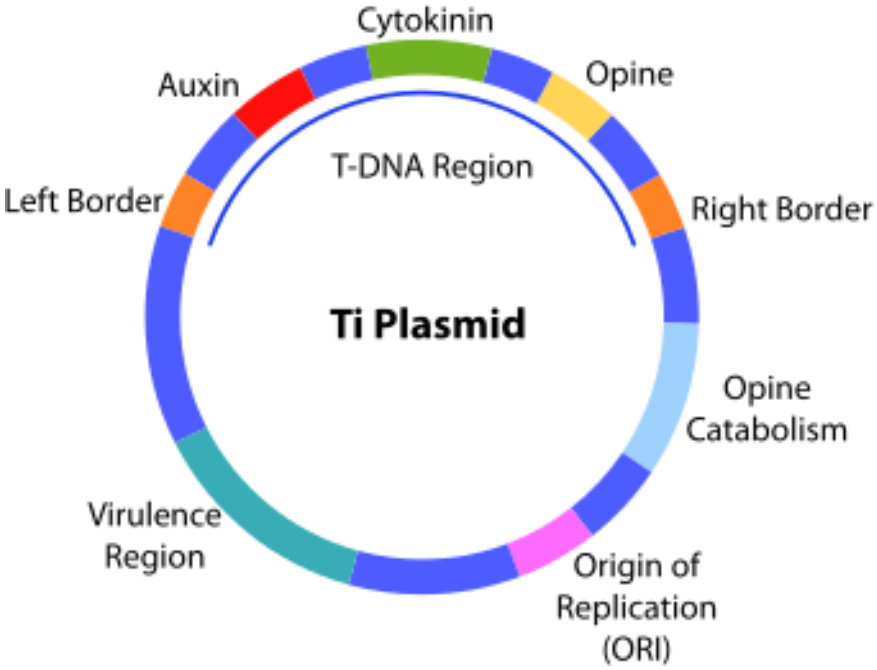
12	Laser-mediated DNA transfer		Needs highly skilled personnel, Not demonstrated in different plants, costly
13	Ca- phosphate for DNA transfer to protoplasts	No chimeras	Tedious, protoplast regeneration needed
14	Silicon fibre mediated	Easy	Toxic to cells
15	Electrophoretic DNA transfer		Not demonstrated in different plants

The Ti plasmid

The Ti plasmid contains genes for auxin and cytokinin formation, and for synthesis or utilization of opines. Ti plasmids range in size from 200 to 800 kbp. Of this, about 30% is common to nopaline and octopine plasmids (Genes VIII, Benjamin Lewin, pp. 525).

The genes of common region are required for the interaction between *Agrobacterium* and plant hosts. The part of Ti plasmid which is transferred to the plant nucleus is called T-DNA. It gets incorporated into the plant genome. The T-DNA is referred to as the T-region.

T-region is approximately 10 to 30 kbp in size and represents less than 10% of the Ti plasmid. Ti plasmids generally contain one T-region, but some Ti plasmids have multiple T- regions on them. T-region is flanked by T-DNA border repeats. These are 25 bp long and highly homologous in sequence. The T-DNA border sequences flank the T-region in directly repeated orientation. The T-DNA border sequences delimit the T-DNA , and are the target of the border-specific endonucleases.



The plasmid has 196 genes that code for 195 proteins. There is one structural RNA. The plasmid is 206,479 nucleotides long; the GC content is 56% and 81% of the material is coding genes. There are no pseudogenes.

The Ti plasmids are classified into different types based on the type of opine produced by their genes. The different opines specified by pTi are octopine, nopaline, succinamopine and leucinopine.

The modification of this plasmid is very important in the creation of transgenic plants.

A **Ti** or **tumour inducing** plasmid is a plasmid that often, but not always, is a part of the genetic equipment that *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* use to transducer its genetic material to plants. The Ti plasmid is lost when *Agrobacterium* is grown above 28°C. Such cured bacteria do not induce crown galls,
i.e. they become avirulent. pTi and pRi share little sequence homology but are functionally rather similar.

Characteristics features

- *Agrobacterium* is called the natural genetic engineer.
- Size of the plasmid: ~250 kbp (200 – 800 Kbp).
- Contains one or more T-DNA region.
- Contains a region enabling conjugative transfer.
- Contains regions for opine synthesis and catabolism.
- Responsible for crown gall disease in plants.

Virulence region

Genes in the virulence region are grouped into the operons *virABCDEFG*, which code for the enzymes responsible for mediating conjugative transfer of T-DNA to plant cells.

- *virA* codes for a receptor which reacts to the presence of phenolic compounds such as acetosyringone, syringealdehyde or acetovanillone which leak out of damaged plant tissues.
- *virB* encodes proteins which produce a pore / pilus-like structure.
- *virC* binds the overdrive sequence.
- *virD1* and *virD2* produce endonucleases which target the direct repeat borders of the T-DNA segment; virD4 is the coupling protein.

- *virE* binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes, beginning with the right border.
- *virG* activates vir-gene expression after binding to a consensus sequence, once it has been phosphorylated by *virA*.

References

https://en.wikipedia.org/wiki/Ti_plasmid
https://molbioandbiotech.wordpress.com/2007/08/31/ti-plasmid-contd/

Molecular biology of *Agrobacterium* infection

Mechanism of agrobacterium mediated gene transfer Agrobacterial virulence system – The Vir genes

The T-DNA transfer is mediated by products encoded by the 30-40 kb vir region of the Ti plasmid. They are involved in activation and transfer of the T-DNA from the bacterial cell into plant cell. This region is composed by at least six essential operons (Vir A, Vir B, Vir C, Vir D, Vir E, Vir g) and two non-essential (Vir F, Vir H). Each of the operons is inducible. The number of genes per operon differs, virA, virG and virF have only one gene; Vir E, Vir C, Vir H have two genes, while Vir D and Vir B have four and eleven genes respectively. Vir A and Vir G are constutively expressed. Others are expressed by the activity of Vir G.

Size of the Vir gene operons

Vir-A (2kbp, 1 gene), Vir-B (9.5kbp, 11 genes), Vir-G (1kbp, 1 gene), Vir-C (2kbp, 2 genes), Vir-D (4.5kbp, 4genes), Vir-E (2kbp, 2genes) and Vir-F (? 1 gene)

Molecular biology of *Agrobacterium* infection

Functions of Virulence genes in Ti plasmid of Agrobacterium

A wounded plant cell releases phenolic compounds such as **acetosyringone, hydroxyacetosyringone, syringealdehyde or acetovanillone** at the injured site. The agrobacterium gets attracted towards these compounds. **Chv-E, a sugar binding protein** recognizes and binds to these phenolic compounds.

Vir A

Vir A is localized as a receptor on the plasma membrane of the bacterial cell and is constitutively expressed at low level. Vir A acts in conjunction with Chv-E. Vir A gets

activated with the binding of signals and **autophosphorylates** (autokinase activity – phosphorylation mostly at tyrosine amino acid and sometimes at serine amino acid) and in turn **phosphorylates Vir G**.

Vir G – DNA binding protein

Its location is cytosol. Before activation the gene products are at low level, but once they are activated, they in turn activate its own gene and also activate all other Vir genes, i.e., the activated, phosphorylated Vir-G produced in large numbers then starts to assemble themselves into dimers. By acting as a **transcriptional activator**, this **dimeric Vir- G** binds the regulatory regions of other **Vir operons** and enhances the **expression** of other Vir proteins: **Vir C, Vir D, Vir E, Vir B, Vir F, Vir H**.

Vir C

This consists of two genes called Vir C1 and Vir C2. Location of these proteins is cytosol and they bind to over drive region at the right end of the right border of T-DNA, acts as a helicase and helps in unwinding the dsDNA. It also helps in activating cleavage of DNA at the right border. They help Vir-D products in this process. It also forms a complex with Vir C2, interacts with Vir D1 / D2 / D4.

Vir D

It consists of four genes namely, Vir D1, Vir D2, Vir D3, and Vir D4. Vir D1 is considered as Topoisomerase and it binds to the right border of the T-DNA. It helps Vir D2 to recognise and cleave within the 25bp Right border sequence

The Vir D2 is considered as an endonuclease. They recognize the RB sequence ACC and cuts at that sequence to generate – ACC 5'. The 5' of the nicked T-DNA gets covalently bound to Vir D2, thus forming the T-DNA-Vir D2 Complex. Binding to the 5' end, it alsoprevents 5'exonuclease activity on T-DNA. Also guides the T-DNA complex through the nuclear pores. It has one NLS sequence. Perhaps this protein is also involved in recombination events in the host cells.

Vir D4 is the ATP-dependent linkage of protein complex necessary for T-DNA translocation.

Vir E

It consists of two genes called Vir E1 and Vir E2. They act as single strand binding

protein (ssBPs), which in turn bind to the peeled of single strand of T-DNA region from right border to the left border. This protects from the exonuclease or endonuclease digestive activity. They may also help in transporting the DNA across the channel produced by Vir B products. This protein is also believed to be involved in recombination in the host.

Vir E1 acts as a chaperone which stabilizes Vir E2 in the Agrobacterium. It is essential for the export of E2 into the plant cell and into the nucleus.

Vir E2 binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes. It has two NL sequences.

Vir F

It consists of few sub groups of F genes. The Vir-F genes help in **DNA transfer**. It is believed that some Vir-F-pin genes **activate several host cell genes**.

Vir H

Not much is known about this gene. Vir H operon consist of two genes that code for Vir H1 & Vir H2 protein. These Vir proteins are not essential but could **enhance the transfer efficiency, detoxifying contains plant compounds** that can effect bacterial growth.

Vir B

It consists of 11 genes called **Vir B1 to Vir B11**. Their location is plasma membrane.

They perhaps organize into **channel like structure** and involved in the **transport of T-DNA** in **single strand form**.

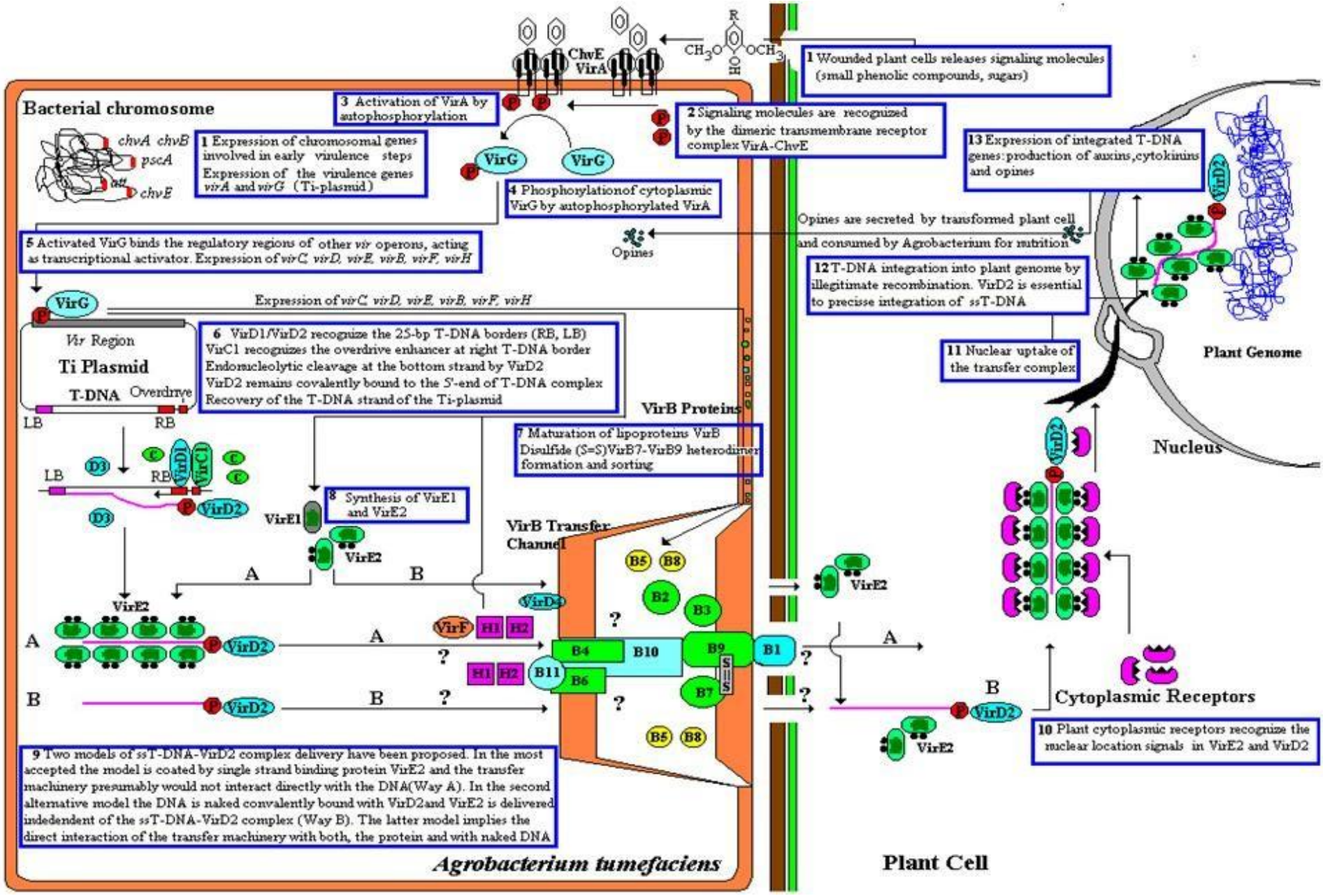
The B-11 has **ATPase activity**.

Vir B7, Vir B8, Vir B9 and VirB10 are the primary constituents of the T-DNA transport pore.

Vir B7, a lipoprotein, is anchored to the outer membrane, while Vir B8 and Vir B10 are inner membrane proteins, and VirB10 participate in the formation of oligomeric complexes.

Vir B4 and Vir B11 are homo- and heterodimmers (Dang and Christie, 1997).

The Vir B7 – Vir B9 heterodimmer is assumed to stabilises other Vir proteins during assembly of functional transmembrane channel.


Bacterial chromosome
chvA chvB
pscA
att
chvE
1 Expression of chromosomal genes involved in early virulence steps
Expression of the virulence genes virA and virG (Ti-plasmid)
3 Activation of VirA by autophosphorylation
ChvE
VirA
2 Signaling molecules are recognized by the dimeric transmembrane receptor complex VirA-ChvE
1 Wounded plant cells releases signaling molecules (small phenolic compounds, sugars)
VirG
VirG
4 Phosphorylationof cytoplasmic VirG by autophosphorylated VirA
5 Activated VirG binds the regulatory regions of other vir operons, acting as transcriptional activator. Expression of virC, virD, virE, virB, virF, virH
Opines
Opines are secreted by transformed plant cell and consumed by Agrobacterium for nutrition
13 Expression of integrated T-DNA genes: production of auxins, cytokinins and opines
12 T-DNA integration into plant genome by illegitimate recombination. VirD2 is essential to precisse integration of ssT-DNA
11 Nuclear uptake of the transfer complex
Plant Genome
Nucleus
Expression of virC, virD, virE, virB, virF, virH
Vir Region
Ti Plasmid
T-DNA
Overdrive
LB
RB
6 VirD1/VirD2 recognize the 25-bp T-DNA borders (RB, LB)
VirC1 recognizes the overdrive enhancer at right T-DNA border
Endonucleolytic cleavage at the bottom strand by VirD2
VirD2 remains covalently bound to the 5'-end of T-DNA complex
Recovery of the T-DNA strand of the Ti-plasmid
VirB Proteins
7 Maturation of lipoproteins VirB
Disulfide (S=S)VirB7-VirB9 heterodimer formation and sorting
8 Synthesis of VirE1 and VirE2
VirE1
VirE2
VirB Transfer Channel
A
B
VirD4
VirF
H1
H2
B5
B8
B2
B3
B4
B10
B9
B1
B11
B6
B7
S
S
VirE2
VirD2
Cytoplasmic Receptors
10 Plant cytoplasmic receptors recognize the nuclear location signals in VirE2 and VirD2
9 Two models of ssT-DNA-VirD2 complex delivery have been proposed. In the most accepted the model is coated by single strand binding protein VirE2 and the transfer machinery presumably would not interact directly with the DNA(Way A). In the second alternative model the DNA is naked convalently bound with VirD2and VirE2 is delivered indedendent of the ssT-DNA-VirD2 complex (Way B). The latter model implies the direct interaction of the transfer machinery with both, the protein and with naked DNA
Agrobacterium tumefaciens
Plant Cell

Vir B & Vir D4

Assemble into a secretion system which spans the inner and outer bacterial membranes. Required for Export of the T-complex and Vir E2 into the plant cell.

Plant cytoplasmic receptors recognize the nuclear location signals in Vir E2 and Vir D2. They then drag the entire ssT-DNA complex into nucleus via nuclear pore complex. Vir D2 helps in integrating the T-DNA into plant nuclear chromosome through illegitimate recombination. The T-DNA now undergoes replication, transcription and translation along with the host chromosome. Their gene products results in the expression of auxin, cytokinins and opines. Opines are transported from plant cytoplasm to cytoplasm of agrobacterium. The bacteria utilizes these opines as their carbon and energy source for their metabolic activities. The T-DNA generated auxin and cytokinin along with the endogenous auxin and cytokinin induces abnormal proliferation of root and stem cells respectively at the injured site, which results in the formation of tumor cells, **the crown gall (crown gall disease).**

Genes in Agrobacterial chromosome

CHV Genes: They are located on bacterial chromosomes. There are several loci and their functions are varied. The loci chvA and chvB, involved in the synthesis and excretion of the β -1,2 glucan (Cangelosi et al., 1989); the chvE required for the sugar enhancement of vir genes induction and bacterial chemotaxis (Ankenbauer et al., 1990, Cangelosi et al., 1990, 1991); the cel locus, responsible for the synthesis of cellulose fibrils (Matthysse 1983); the pscA (exoC) locus, playing its role in the synthesis of both cyclic glucan and acid succinoglycan (Cangelosi et at., 1987, 1991); and the attlocus, which is involved in the cell surface proteins (Matthysse, 1987).

Role of Chv genes :

Chv-A : Codes for inner membrane protein essential for the transport of β -1, 2 glucan from bacterial cytoplasm into periplasm
Chv-B : An inner membrane protein likely to be involved in the synthesis of β -1, 2 glucan

Chv-D : Required for expression of Vir genes Chv-E : Required for the expression of Vir genes

Exo-locus

Exo-C - Exo-biosynthesis of attachment polysaccharides

Exo-C - is involved required for the synthesis of β-1, 2 glucan

Cel - Cellulose fibril synthesis to enable bacterial cells to be firmly adhere to plant cell walls Psc-I

Modification of Ti plasmid in Agrobacterium

Agrobacterium Ti plasmids are used for reconstructing the plasmid for dual purposes. One for using T DNA to transfer the required a foreign gene into plant cells. Another, as Ti plasmid contains Vir genes, Ori and a marker gene, can be used as helper plasmid.

The T DNA containing part of the Ti plasmid has been cloned into suitable plasmids found in E.coli. These plasmids are reconstructed in such way they contain T DNA borders with over drive at the RB. They have required Ori for replication in E.coli and also in Agrobacterium. They have antibiotic markers one for T DNA and another for E.coli. Auxin and Cytokinin synthesizing genes have been removed. In their place a strong antibiotic gene is placed at the left border of the T DNA under strong opine promoters. Another construct contains a strong and universal promoter such as 35s CAMV with few cloning sites. This plasmid also contains its own Ori and marker gene. Such vectors are called Binary vectors; originally they were used as Bac vectors.

Ti Plasmid-Derived Vector Systems:

The ability of Ti plasmid of Agrobacterium to genetically transform plants has been described. It is possible to insert a desired DNA sequence (gene) into the T-DNA region (of Ti plasmid), and then use A. tumefaciens to deliver this gene(s) into the genome of plant cell.

In this process, Ti plasmids serve as natural vectors. However, there are several limitations to use Ti plasmids directly as cloning vectors:

i. Ti plasmids are large in size (200-800 kb). Smaller vectors are preferred for recombinant experiments. For this reason, large segments of DNA of Ti plasmid, not essential for cloning, must be removed.

ii. Absence of unique restriction enzyme sites on Ti plasmids.

iii. The phytohormones (auxin, cytokinin) produced prevent the plant cells being regenerated into plants. Therefore auxin and cytokinin genes must be removed.

iv. Opine production in transformed plant cells lowers the plant yield. Therefore opine synthesizing genes which are of no use to plants should be removed.

v. Ti plasmids cannot replicate in E. coli. This limits their utility as E. coli is widely used in recombinant experiments. An alternate arrangement is to add an origin of replication to Ti plasmid that allows the plasmid to replicate in E. coli.

Considering the above limitations, Ti plasmid- based vectors with suitable modifications have been constructed.

These vectors are mainly composed of the following components:

1. The right border sequence of T-DNA which is absolutely required for T-DNA integration into plant cell DNA.
2. A multiple cloning site (poly-linker DNA) that promotes the insertion of cloned gene into the region between T-DNA borders.
3. An origin of DNA replication that allows the plasmids to multiply in E. coli.
4. A selectable marker gene (e.g. neomycin phosphotransferase) for appropriate selection of the transformed cells.

Two types of Ti plasmid-derived vectors are used for genetic transformation of plants— co-integrate vectors and binary vectors.

Co-integrate vector:

In the co-integrate vector system, the disarmed and modified Ti plasmid combines with an intermediate cloning vector to produce a recombinant Ti plasmid (Fig. 49.5).

Production of disarmed Ti plasmid:

The T-DNA genes for hormone biosynthesis are removed (disarmed). In place of the deleted DNA, a bacterial plasmid (pBR322) DNA sequence is incorporated. This disarmed plasmid, also referred to as receptor plasmid, has the basic structure of T-DNA (right and left borders, virulence genes etc.) necessary to transfer the plant cells.

Construction of intermediate vector:

The intermediate vector is constructed with the following components:

i. A pBR322 sequence DNA homologous to that found in the receptor Ti plasmid.

ii. A plant transformation marker (PTM) e.g. a gene coding for neomycin phosphotransferase II (npt II). This gene confers resistance to kanamycin in the plant

cells and thus permits their isolation.

iii. A bacterial resistance marker e.g. a gene coding for spectinomycin resistance. This gene confers spectinomycin resistance to recipient bacterial cells and thus permits their selective isolation.

iv. A multiple cloning site (MCS) where foreign genes can be inserted.

v. A Co/E_1 origin of replication which allows the replication of plasmid in E. coli but not in Agrobacterium.

vi. An oriT sequence with basis of mobilization (bom) site for the transfer of intermediate vector from E. coli to Agrobacterium.

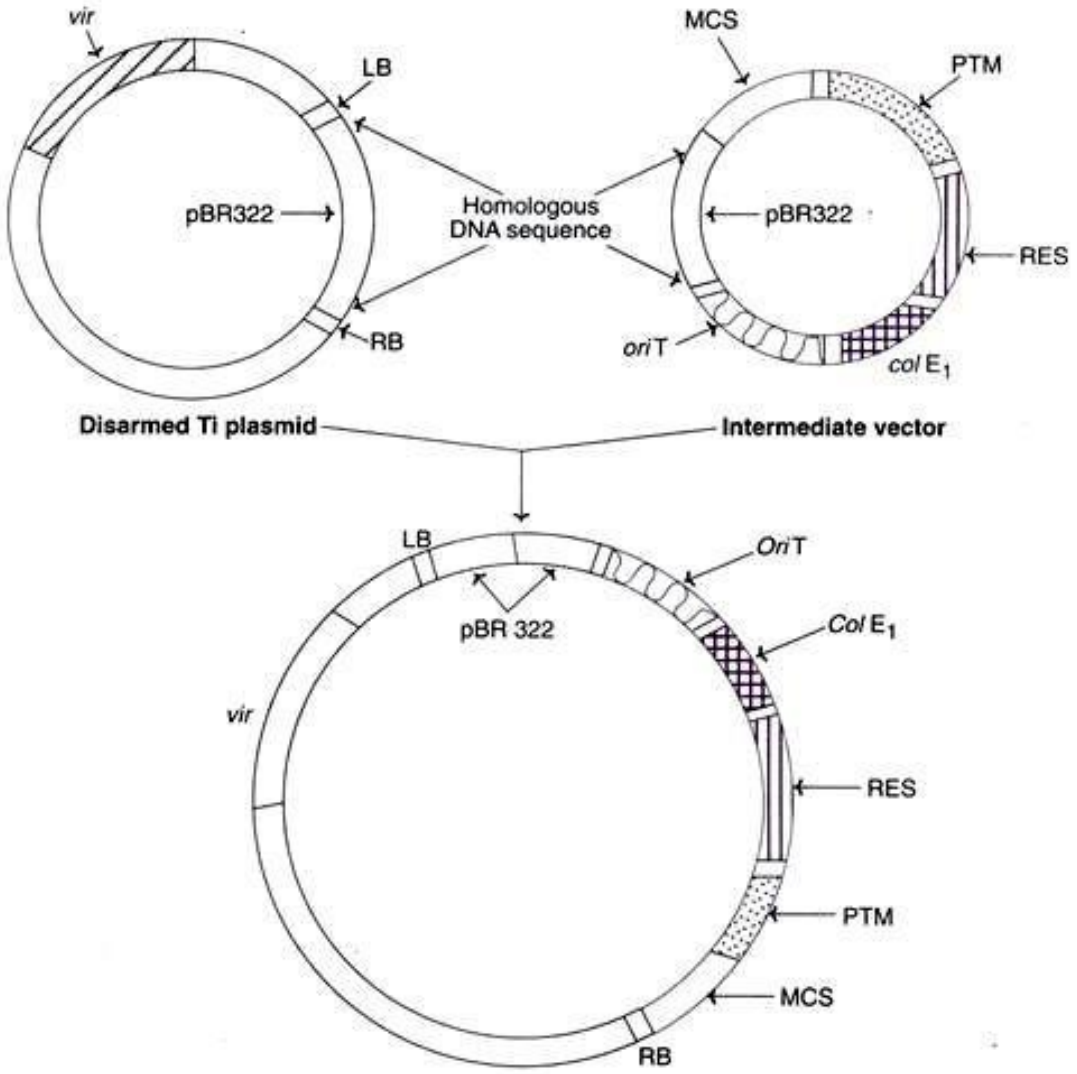


Fig. 49.5 : Cointegrate vector system (vir-Ti plasmid virulence region; pBR322-Bacterial plasmid 322; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; col E_1-Origin of a replication from col E_1 plasmid; ori T-Origin of transfer site for conjugative plasmid mobilization).

Production and use of co-integrate vectors:

The desired foreign gene (target-gene) is first cloned in the multiple cloning site of the intermediate vector. The cloning process is carried out in E. coli, the bacterium where the cloning is most efficient. The intermediate vector is mated with Agrobacterium so that the foreign gene is mobilised into the latter.

The transformed Agrobacterium cells with receptor Ti plasmid and intermediate vector are selectively isolated when grown on a minimal medium containing spectinomycin. The selection process becomes easy since E. coli does not grow on a minimal medium in which Agrobacterium grows.

Within the Agrobacterium cells, intermediate plasmid gets integrated into the receptor Ti plasmid to produce co-integrate plasmid. This plasmid containing plant transformation marker (e.g. npt II) gene and cloned target gene between T-DNA borders is transferred to plant cells. The transformed plant cells can be selected on a medium containing kanamycin when the plant and Agrobacterium cells are incubated together.

Advantages of co-integrate vector:

i. Target genes can be easily cloned
ii. The plasmid is relatively small with a number of restriction sites.
iii. Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

Binary vector:

The binary vector system consists of an Agrobacterium strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in E. coli and Agrobacterium.

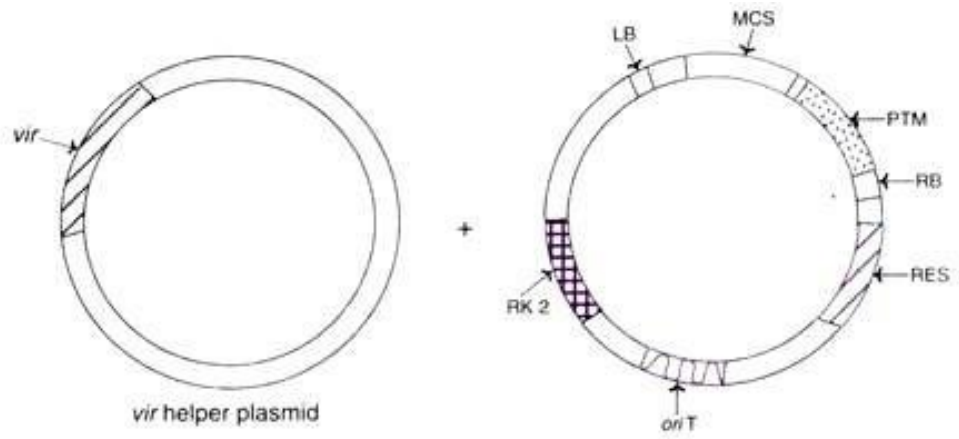


Fig. 49.6 : Binary vector system (vir-Ti plasmid virulence region; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; oriT-Origin of transfer site for conjugative plasmid mobilization; RK_2-Origin of replication from plasmid).

A diagrammatic representation of a typical binary vector system is depicted in Fig. 49.6. The binary vector has the following components.

1. Left and right borders that delimit the T-DNA region.
2. A plant transformation marker (PTM) e.g. npt II that confers kanamycin resistance in plant transformed cells.
3. A multiple cloning site (MCS) for introducing target/foreign genes.
4. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E. coli and Agrobacterium.
5. oriT sequence for conjugal mobilization of the binary vector from E. coli to Agrobacterium.
6. A broad host-range origin of replication such as RK_2 that allows the replication of binary vector in Agrobacterium.

Production and use of binary vector:

The target (foreign) gene of interest is inserted into the multiple cloning site of the binary vector. In this way, the- target gene is placed between the right and left border repeats and cloned in E. coli. By a mating process, the binary vector is mobilised from E. coli to Agrobacterium. Now, the virulence gene proteins of T-DNA facilitate the transfer of T-DNA of the vector into plant cells.

Advantages of binary vectors:

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co-integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti

plasmid.

ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

Plant Transformation Technique Using Agrobacterium:

Agrobacterium-mediated technique is the most widely used for the transformation of plants and generation of transgenic plants. The important requirements for gene transfer in higher plants through Agrobacterium mediation are listed.

i. The explants of the plant must produce phenolic compounds (e.g. autosyringone) for activation of virulence genes.

ii. Transformed cells/tissues should be capable to regenerate into whole plants.

In general, most of the Agrobacterium-mediated plant transformations have the following basic protocol (Fig. 49.7).

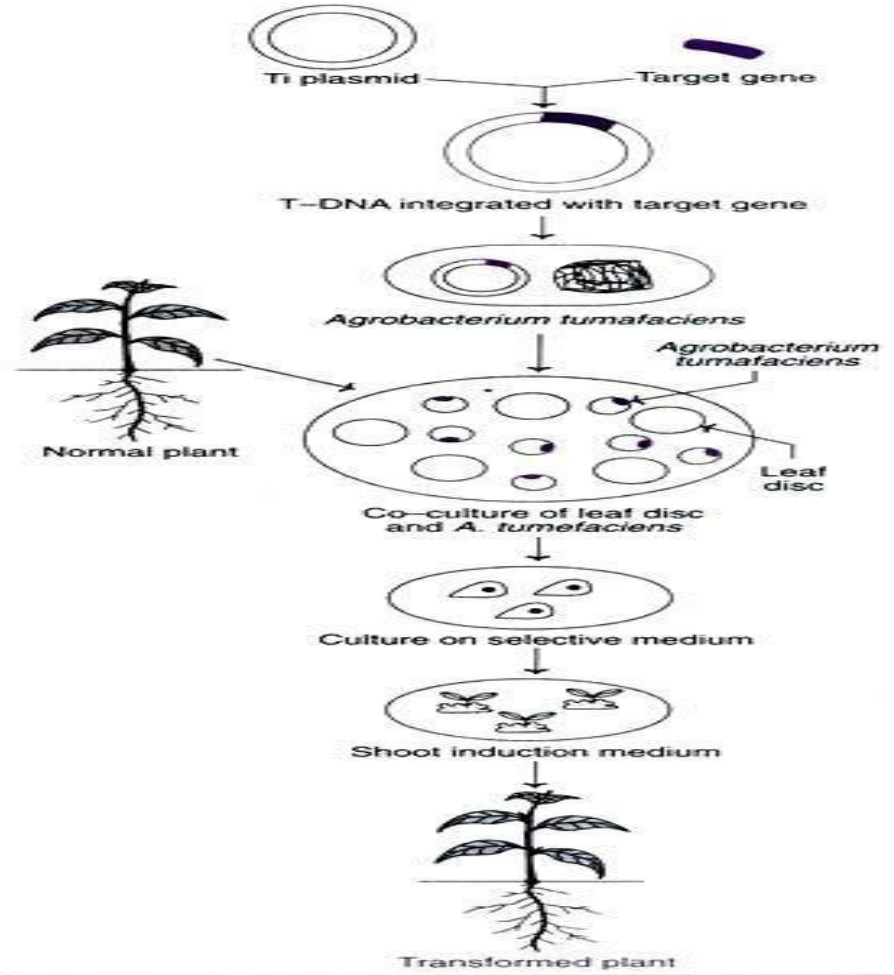


Fig. 49.7 : Transformation technique using Agrobacterium-mediated gene transfer.

1. Development of Agrobacterium carrying the co-integrate or binary vector with the desired gene.
2. Identification of a suitable explant e.g. cells, protoplasts, tissues, calluses, organs.
3. Co-culture of explants with Agrobacterium.
4. Killing of Agrobacterium with a suitable antibiotic without harming the plant tissue.
5. Selection of transformed plant cells.
6. Regeneration of whole plants.

Advantages of Agrobacterium- mediated transformation:

i. This is a natural method of gene transfer.
ii. Agrobacterium can conveniently infect any explant (cells/tissues/organs).
iii. Even large fragments of DNA can be efficiently transferred.
iv. Stability of transferred DNA is reasonably good.
v. Transformed plants can be regenerated effectively.

Limitations of Agrobacterium- mediated transformation:

i. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g. cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.
ii. The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

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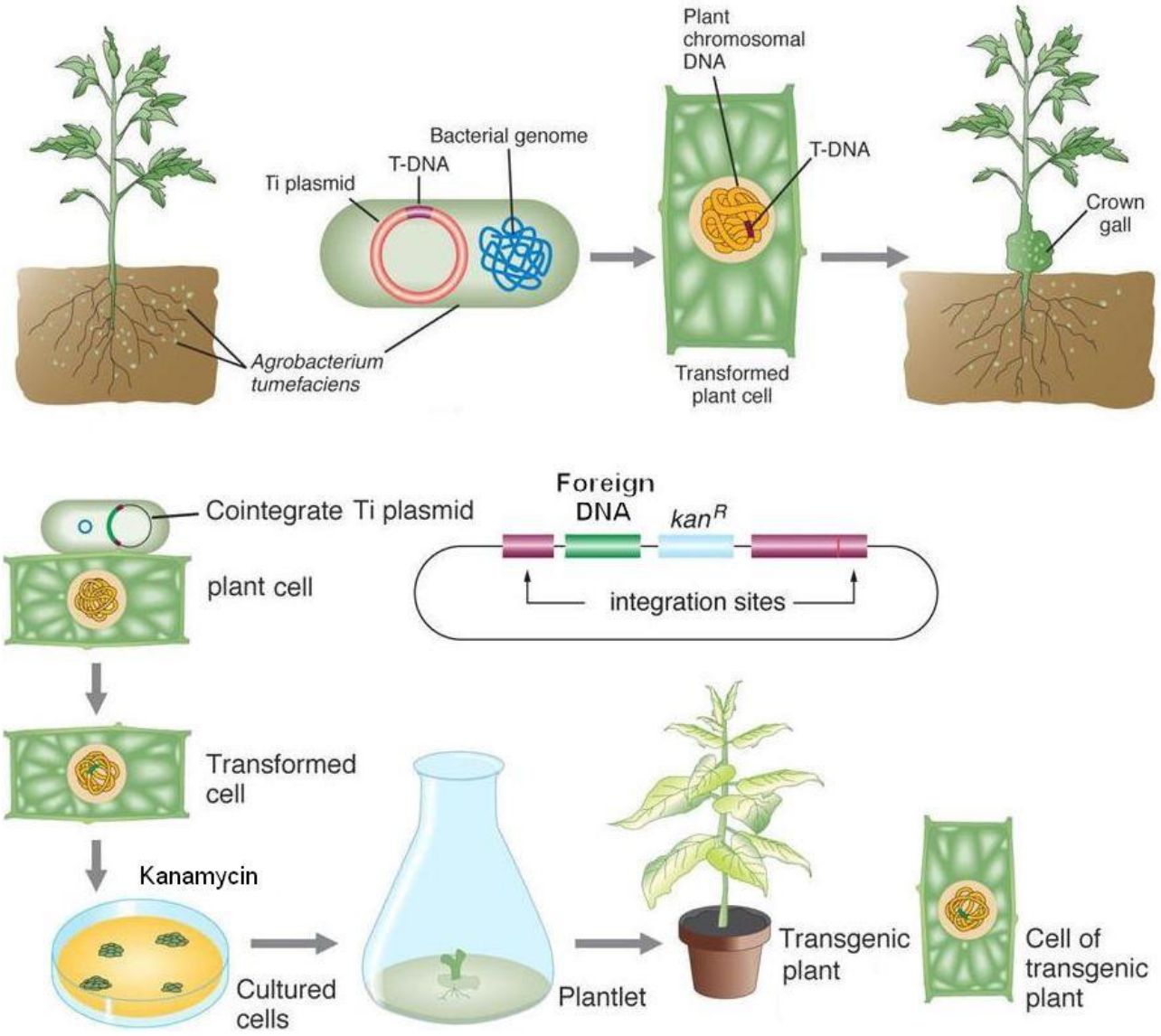
Production of transgenic plants by use of co-integrated *Ti* plasmids

The soil bacterium **Agrobacterium tumefaciens** causes **crown gall disease** in plants by transfering the **T-DNA** region of a **tumor-inducing** (**Ti**) **plasmid** into host cells (Top). The **T-DNA** region of the **Ti** plasmid can be genetically engineered to contain an **antiobiotic resistance gene** (**kan^{R}**) as well as a **foreign gene** of interest (inset diagram). Infection of plant cells in culture with bacteria containing this **co-integrated Ti plasmid** allows the **foreign DNA** to be transfered into the host cell.

Integration of the foreign **DNA** disrupts tumor formation, and only those plant cells with the **kan^{R}** gene will grow in culture

containing antibiotic. Plants are easily regenerated from cultured cells (**calluses**): the adult transgenic plant expresses the foreign gene.



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Cauliflower mosaic virus

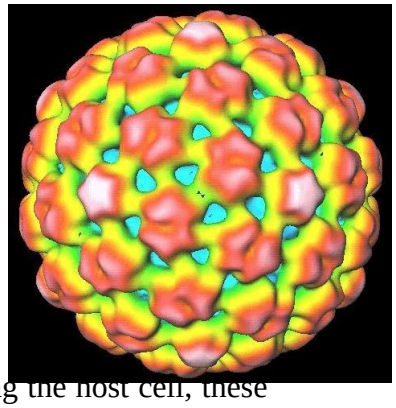
Introduction

Cauliflower mosaic virus (CaMV) is the type species of the family ***Caulimoviridae***. This family is grouped together with *Hepadnaviruses* into the *Pararetrovirus* group due to its mode of replication via reverse transcription of a pre-genomic RNA intermediate.

CaMV **infects** mostly plants of the ***Brassicaceae*** **family** (such as caulifower and turnip) but some CaMV strains (D4 and W260) are also able to infect ***Solanaceae*** **species** of the genera *Datura* and *Nicotiana*. CaMV induces a variety of systemic symptoms such as **mosaic, necrotic lesions on leaf surfaces, stunted growth, and deformation of the overall plant structure.** The symptoms exhibited vary depending on the viral strain, host ecotype, and environmental conditions.

CaMV is transmitted in a non-circulatory manner by **aphid species** such as ***Myzus persicae***. Once introduced within a plant host cell, virions migrate to the nuclear envelope of the plant cell.

Structure of CaMV



The CaMV particle is an **icosahedron** with a diameter of **52 nm** built from **420 capsid protein** (CP) subunits arranged with a triangulation T = 7, which surrounds a solvent-filled central cavity.

CaMV contains a **circular, double-stranded DNA** molecule of about **8.0 kilobases**, interrupted by **Site specific discontinuities or single strand site specific breaks** that result from its replication by reverse transcription. After entering the host cell, these single stranded "nicks" in the viral DNA are repaired, forming a supercoiled molecule that binds to histones resulting in the generation of **minichromosomes**.

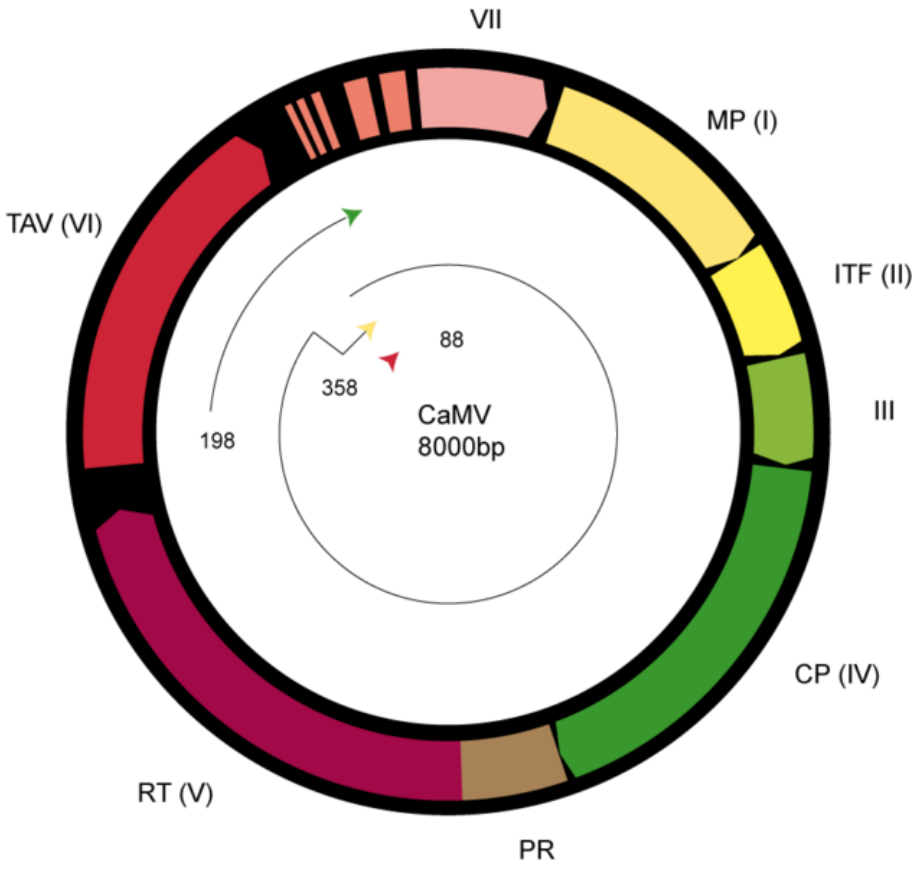
Genome of CaMV

The promoter was named CaMV 35S promoter ("35S promoter") because the coefficient of sedimentation of the viral transcript, whose expression is naturally driven by this promoter, is 35S. It is one of the most widely used, general-purpose constitutive promoters. It was discovered at the beginning of the 1980s, by Chua and collaborators at The Rockefeller University.

The 35S RNA is particularly complex, containing a highly structured 600 nucleotide long leader sequence with six to eight short open reading frames (ORFs).

This leader is followed by **seven tightly arranged, longer ORFs** that encode all the **viral proteins**. The mechanism of expression of these proteins is unique, in that the ORF VI protein (encoded by the 19S RNA) controls translation reinitiation of major open reading frames on the polycistronic 35S RNA, a process that normally only happens on bacterial mRNAs. TAV function depends on its association with polysomes and eukaryotic initiation factor eIF3.

- ORF I - Movement Protein, P1
- ORF II - Insect Transmission Factor, P2
- ORF III - Structural Protein, DNA-Binding Capabilities, P3
- ORF IV - Capsid Protein, P4
- ORF V - Protease, Reverse Transcriptase and RNaseH, P5
- ORF VI - Translational Activator, Inclusion Body Formation/Trafficking; Possibly more functions (See Below), P6
- ORF VII - Unknown (Appears to not be required for infection), P7



Brief history about Cauliflower mosaic virus

The first of what are now known to be caulimoviruses – family***Caulimoviridae*** – was described in **1933** as **dahlia mosaic virus**.**Cauliflower mosaic virus** (CaMV) was described in **1937**, and shown to have particles containing a DNA genome in **1968**. This was visualised by electron microscopy by two groups in **1971** as relaxed open circles **or also as** linear forms **– unlike the supercoiled DNAs of papilloma- or papovaviruses**. By **1977,** it was known that the "nicked circular form" was infectious, and fragments of the genome had been cloned in *E coli*. Physical and biochemical characterisation of the genome in **1978** showed that it consisted of **three discrete lengths of single-stranded DNA** – alpha, beta and gamma, with alpha being the full genome-length – that annealed to one another to give a **circular double-stranded form** about 8000 nucleotides in length. By **1979** it was known that only the alpha strand was transcribed to give mRNA.

The complete sequence of the viral genome was published in **1980,** and was **predicted to encode six proteins**:

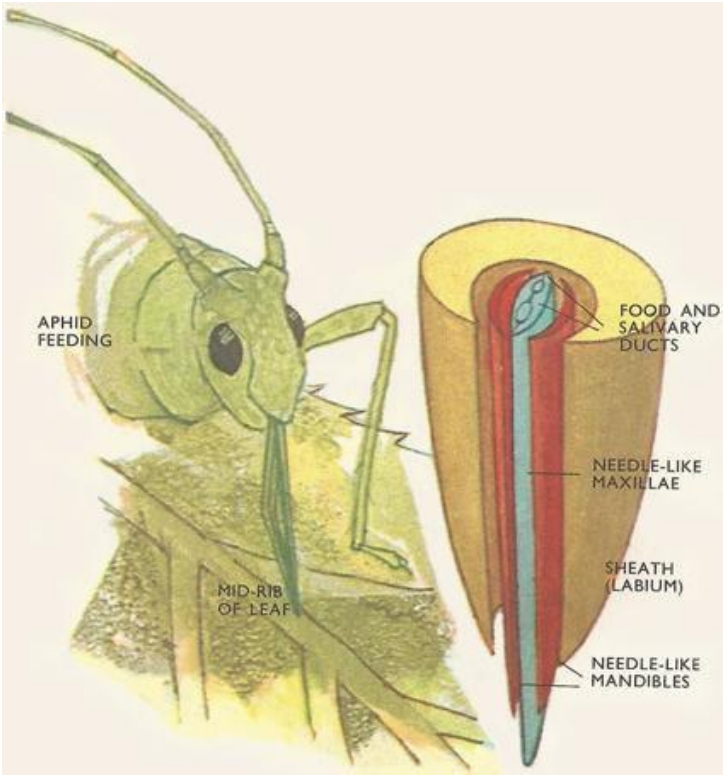
In nature, CaMV is spread through aphid transmission and usually targets Brassica crops such as cabbage, broccoli and cauliflower.

Anatomy of the aphid

Like all insects, the body of the aphid can be divided into three sections: head, thorax, and abdomen. The overall appearance is usually pear-shaped, but the color varies from species to species – greens, browns, reds, blacks, grays. The length of most aphids is only about 2 or 3 millimeters (less than ½ inch). The three pairs of legs, one to each thoracic segment, are long in comparison with the body. They are not, however, adapted for leaping; aphids are very sluggish walkers. Some individuals may have two pairs of transparent wings and are capable of flight.

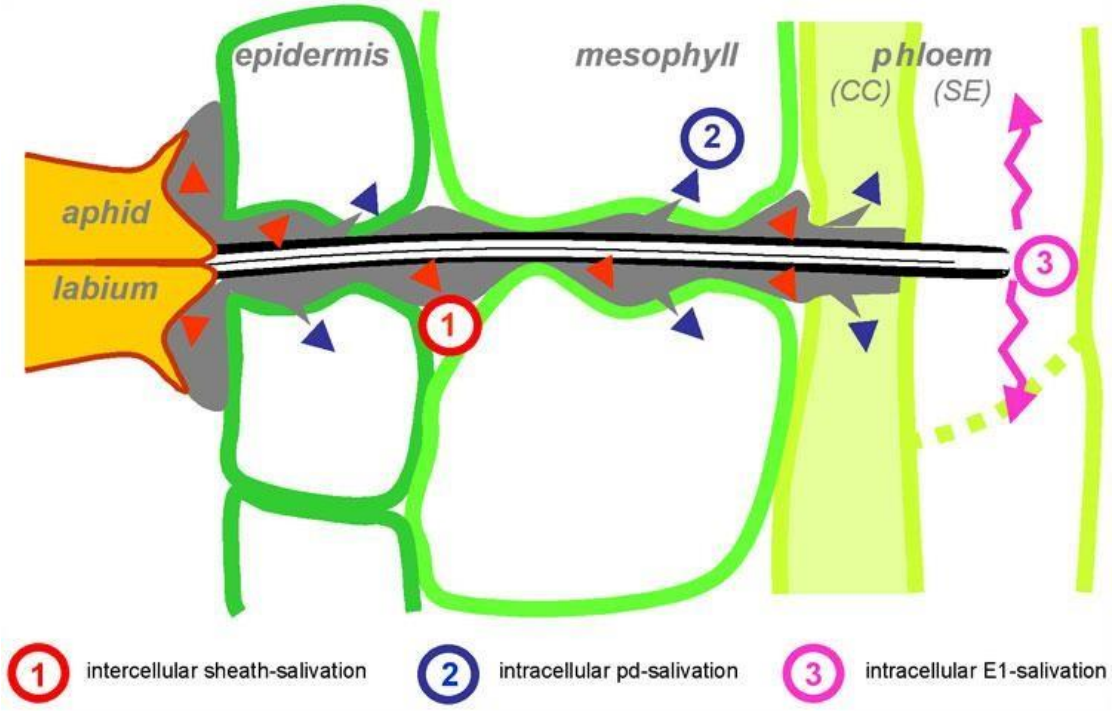
Mandibles and maxillae in aphids

The mouth parts on the head are adapted to piercing plant surfaces and sucking liquid foods from the tissues inside. The two jaws (mandibles) are needle-like. They come together to form a piercing outer stylet. The two secondary jaws (maxillae) form an inner stylet surrounding two canals. At rest, the needles are drawn up under the head and protected by an outer sheath formed from the lower lip (labium). When feeding begins the sheath is withdrawn and the needles are plunged into the food-conducting tissues of the plant. Saliva passes down one of the canals inside the needles and partially digests the plant fluids. Muscular action then pumps the sap up the other canal into the mouth.

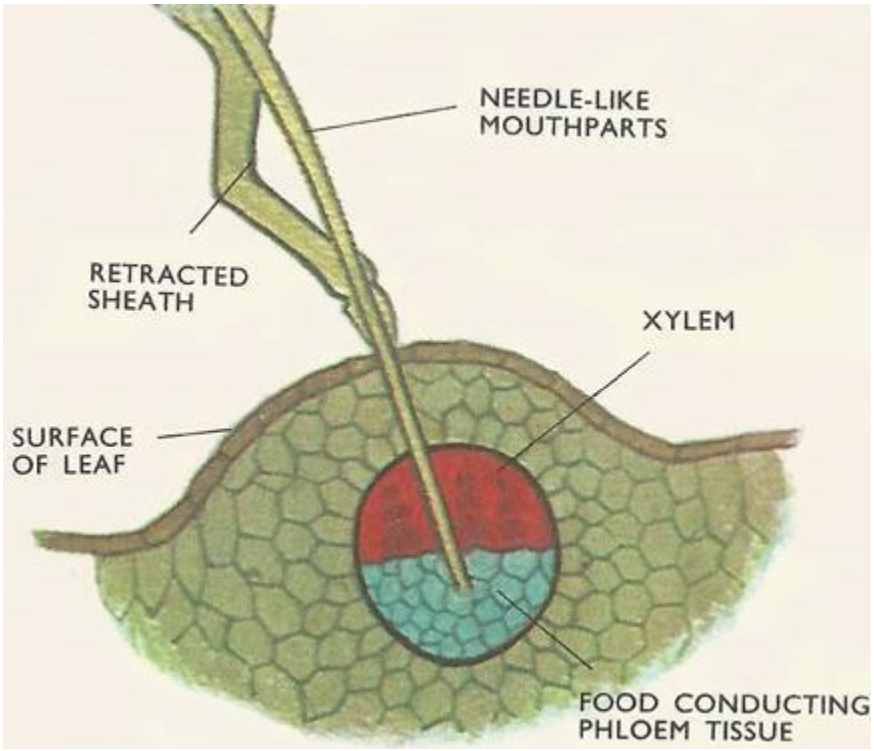


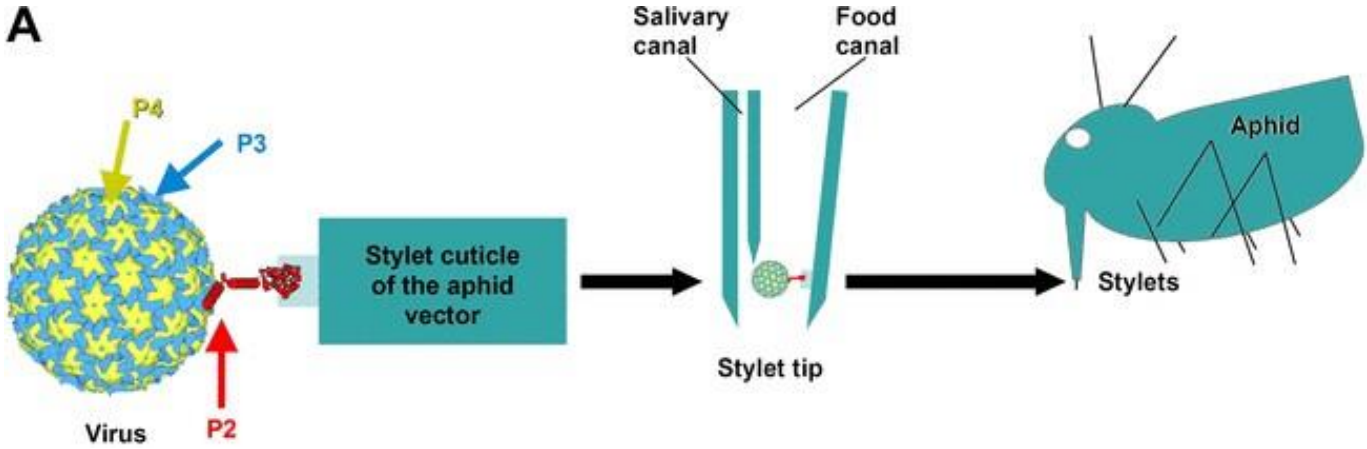
The mouth parts of aphids are adapted for piercing plant surfaces and sucking sap. The two maxillae come together as shown to form canals – one for injecting saliva, one for absorbing food.

Types of salivary secretion in aphid



Three identified salivary secretion phases into the plant during stylet penetration by aphids. (1) Gelling saliva (red arrows), forming the salivary sheath, (2) watery saliva (blue arrows), intracellularly secreted during brief stylet punctures (pd waveform), (3) watery saliva (purple arrows), into phloem sieve elements (E1 waveform), preceding phloem feeding. Salivation (3) might be mainly responsible for the induced resistance that is systemically spread, whereas salivation (1) and (2) may have more local effects.





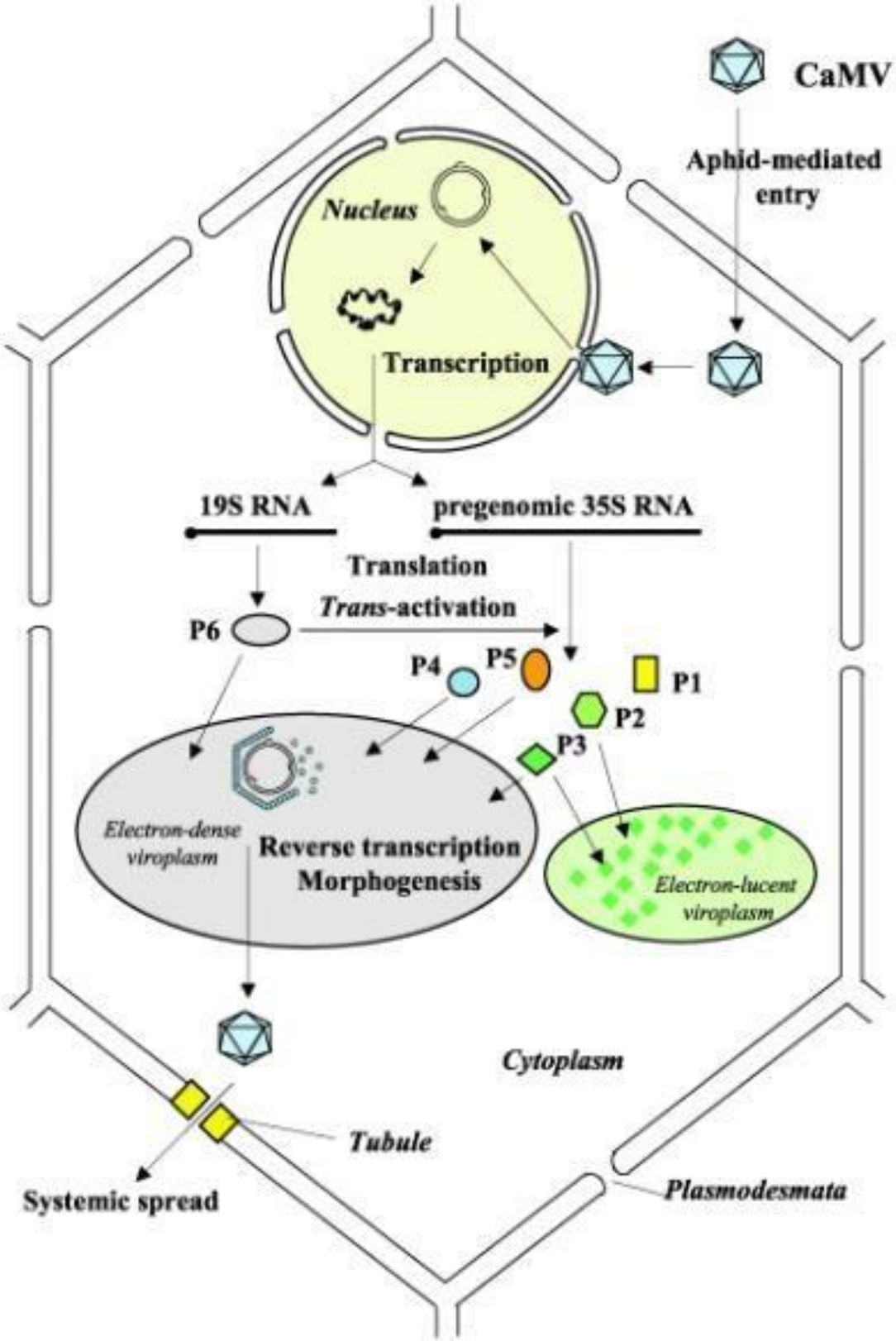
The stylets formed by the mandibles and maxillae plunge deep into plant stems and leaf mid-ribs. They locate the phloem – special tissue used for conducting plant food material.

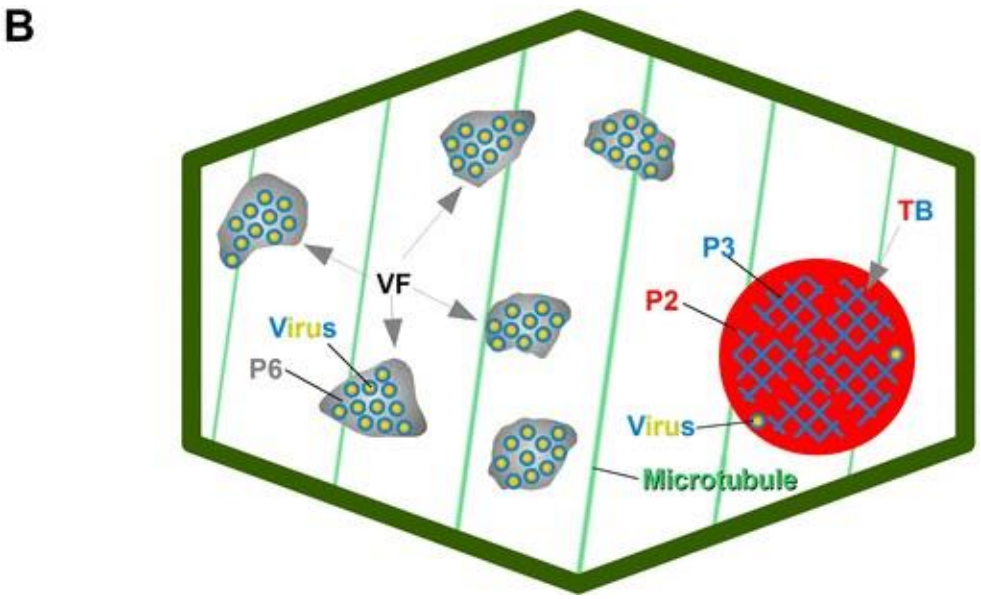
(A) Left: the CaMV transmissible complex comprises the virus particle, composed of capsid protein P4 (yellow), virus-associated protein P3 (blue) and the helper component P2 (red). P2 binds via its C-terminus to P3 and via its N-terminus to a protein receptor localized in the stylet tips of the aphid vector (middle and right).

CaMV Life Cycle

Each step in the CaMV life cycle is outlined below, referencing Khelifa et al. 2010 and Yasaka et al. 2014:

1. The virus enters the host cell via an aphid vector. Once introduced within a host cell, virions migrate to the nuclear envelope, where they presumably decapsidate (Karsies et al., 2002).
2. The virion(s) is transported via NLS to the nuclear pore and imported into the nucleus.
3. DNA sequence discontinuities are repaired and the genome is formed into minichromosomes by the histones in the cell.
4. These mini chromosomes are then translated by RNA Polymerase II into two mRNAs, the polycistronic 35S pregenomic RNA comprising the entire genome encoding 6 proteins and the subgenomic 19S RNA encoding a single protein, P6.
5. After translation in the cytoplasm, P6 condenses and aggregates as small inclusion bodies to form **viroplasms** or a cytoplasmic **Viral Factories** (VF) or **electron dense inclusion bodies (ediB)** (Khelifa et al. 2010). P6 then transactivates translation (i.e. TAV
   - translational transactivation) of all other viral proteins, P1 through P5 from the 35S RNA. Among these, P5 replicates the genome by reverse transcription using 35S RNA as a template to produce viral genomic DNA.
6. Parallely, certain amount of P2, P3 and limited virions are packed together to form **Transmission body** or **electron lucent inclusion bodies (eliB)**. The viral capsid proteins are then formed by P3 from eliB and P4 proteins, which cover the viral genomic DNA as an envelope.
7. In addition to its functions regarding translational activation and formation of inclusion bodies, P6 has been shown to interact with a number of other CaMV proteins, such as P2 and P3, suggesting that it may also contribute in some degree to viral assembly and aphid-mediated transmission. In addition, P6 has been shown to bind to P7; investigating interactions between the two may help to elucidate the as yet unknown function of P7.
8. The newly transcribed viral DNA is encapsidated, resulting in generation of virus particles and disperses from the electron dense inclusion bodies, in turn finally comes out of the plant cell thro microtubule channels or plasmodesmata. The virus then enters the next cell thro plasmodesmata and systemically infects the entire plant body.


CaMV
Aphid-mediated entry
Nucleus
Transcription
19S RNA
pregenomic 35S RNA
Translation
Trans-activation
P6
P4
P5
P1
P2
P3
Electron-dense viroplasm
Reverse transcription
Morphogenesis
Electron-lucent viroplasm
Cytoplasm
Tubule
Systemic spread
Plasmodesmata



The CaMV transmissible complex and the transmission body.

(B) Infected cells contain many cytoplasmic virus factories (VF), where most virus particles (blue-yellow circles) accumulate in a matrix composed of virus protein P6 (grey), and a single transmission body (TB). The TB (also cytoplasmic) is composed of P2 (red) and P3 (blue) as well as scattered virus particles. P3 in TBs is most likely in a conformation that differs from virus-associated P3. The spatial separation of the components of the transmissible complex (P2 in the TB and most virus particles in VFs) lead us to propose that they unite only at the moment of vector acquisition (Drucker et al., 2002). Cortical microtubules are designated in green and the cell wall in dark green. Cell organelles are not shown, for clarity. The CaMV model is from Plisson et al. (2005).

CaMV can be used as a vector for gene manipulation

1. Two genomic regions, ORF II (codes for ITF) and ORF VII (unknown function) can be replaced with foreign gene of interest.
2. Naked DNA is infective, being able to enter the plant cells directly if rubbed onto a leaf with mild abrasives.
3. As a DNA virus, whose genome is known to be packed with histones to form nucleosomes (minichromosomes) and transcribed by RNA polymerase II, it is more suited for exploitation as an experimental tool than any other plant virus.

Disadvantages of CaMV vector

1. The genome is so tightly packed with the coding regions that there is little room

for insertion of foreign DNA. Most deletions of any significant size destroy virus infectivity. The theoretical packaging capacity is of 1000 nucleotides.

2. CaMV-derived vectors are restricted to members of crucifera that can be infected by viral DNA.
3. CaMV DNA has multiple cleavage sites for most of the commonly used restriction endonucleases. This would limit the usefulness of wild isolates of CaMV.
4. To allow the movement of CaMV throughout the vasculature of the plant, the DNA must be encapsidated, and this would impose serious constraints on the size of the foreign DNA that can be inserted into the viral genome.

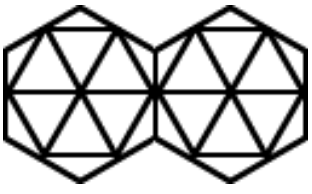
Gemini virus

Introduction

Geminiviridae is a family of plant viruses. There are currently 325 species in this family, divided among 7 genera. Diseases associated with this family include: bright yellow mosaic, yellow mosaic, yellow mottle, leaf curling, stunting, streaks, reduced yields. They have single-stranded circular DNA genomes encoding genes that diverge in both directions from a virion strand origin of replication (i.e. geminivirus genomes are ambisense). According to the Baltimore classification they are considered class II viruses. It is the largest known family of single stranded DNA viruses.

These viruses are responsible for a significant amount of crop damage worldwide. Epidemics of geminivirus diseases have arisen due to a number of factors, including the recombination of different geminiviruses coinfecting a plant, which enables novel, possibly virulent viruses to be developed. Other contributing factors include the transport of infected plant material to new locations, expansion of agriculture into new growing areas, and the expansion and migration of vectors that can spread the virus from one plant to another.

Structure of Gemini virus



The genome can either be a single component between 2500-3100 nucleotides, or, in the case of some begomoviruses, two similar-sized components each between 2600 and 2800 nucleotides. They have elongated, geminate capsids with two incomplete T=1 icosahedra joined at the missing vertex. The capsids range in size from 18-20 nm in diameter with a length of

about 30 nm. Begomoviruses with two component (i.e. bipartite) genomes have these components separated into two different particles both of which must usually be transmitted together to initiate a new infection within a suitable host cell.

Replication of genome in Gemini virus

- Geminivirus genomes encode only a few proteins; thus, they are dependent on host cell factors for replication: these include factors such as DNA polymerase - and probably repair polymerases - in order to amplify their genomes, as well as transcription factors.
- Geminiviruses replicate via a rolling circle mechanism like bacteriophages such as M13, and many plasmids. Replication occurs within the nucleus of an infected plant cell. First the single-stranded circular DNA is converted to a double-stranded circular intermediate. This step involves the use of cellular DNA repair enzymes to produce a complementary negative-sense strand, using the viral genomic or plus-sense DNA strand as a template.
- The next step is the rolling circle phase, where the viral strand is cleaved at a specific site situated within the origin of replication by the viral Rep protein in order to initiate replication. This process in a eukaryotic nucleus can give rise to concatemeric double- stranded forms of replicative intermediate genomes, although double-stranded unit circles can be isolated from infected plants and cells.
- New single-stranded DNA forms of the virus genome (plus-sense) are probably formed by interaction of the coat protein with replicating DNA intermediates, as genomes lacking a CP gene do not form ssDNA. The ssDNA is packaged into germinate particles in the nucleus. It is not clear if these particles can then leave the nucleus and be transmitted to surrounding cells as virions, or whether ssDNA associated with coat protein and a movement protein is the form of the genome that gets trafficked from cell to cell via the plasmodesmata.
- These viruses tend to be introduced into and initially infect differentiated plant cells, via the piercing mouthparts of the vector insect: however, these cells generally lack the host enzymes necessary for DNA replication, making it difficult for the virus to replicate. To overcome this block geminiviruses can induce plant cells to reenter the cell cycle from a quiescent state so that viral replication can occur.

Gemini viruses can be used as a cloning vector

→ These viruses contain ssDNA that appears to replicate via double stranded intermediate and thus makes invivo manipulation in bacterial plasmids in a more convenient way.

→ An attractive feature is the ability of bipartite geminiviruses to contain a deletion or a replacement of viral coat protein sequences by foreign genes without interfering with the replication of the viral genome.

Disadvantages of Gemini virus

→ These are not readily transferred by mechanical means from plant to plant, but transmitted in nature by insects in a persistent manner.

→ The small particle size may present packaging problems for modified DNA molecules and any useful genetic modifications will have to solve the problems of a vector, which in its natural state causes severe disease in susceptible plants.

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Brome mosaic virus

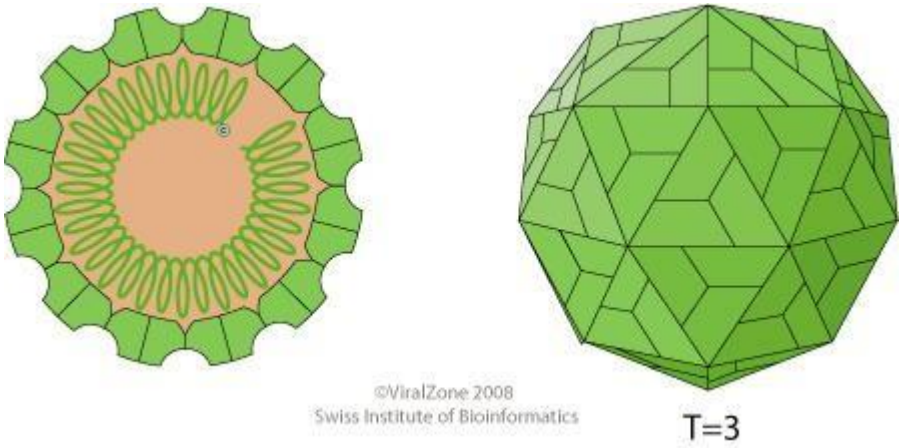
Brome mosaic virus (**BMV**) is a small (28 nm, 86S), positive-stranded, icosahedral RNA plant virus belonging to the genus *Bromovirus*, family *Bromoviridae*, in the alphavirus- like superfamily.

BMV infecting plants

BMV commonly infects *Bromus inermis* and other grasses, can be found almost anywhere wheat is grown, and thrives in areas with heavy foot or machinery traffic. It is also one of the few grass viruses that infects dicotyledonous plants; however, it primarily infects monocotyledonous plants, such as barley and others in the family Gramineae.

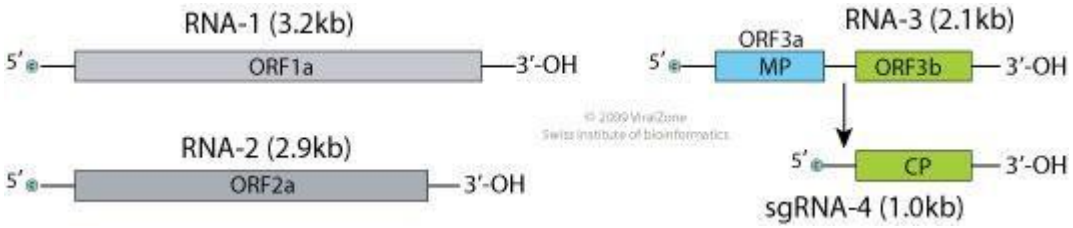
Genomic sequence studies in BMV

BMV was first isolated in 1942 from bromegrass (*Bromus inermis*),[2] had its genomic organization determined by the 1970s, and was completely sequenced with commercially available clones by the 1980s. The sequence similarities of RNA replication genes and strategies fo r BMV have been shown to extend to a wide range of plant and animal viruses beyond the alphaviruses, including many other positive-strand RNA viruses from other families. Understanding how these viruses replicate and targeting key points in their life cycle can help advance antiviral treatments worldwide.



Virion

Non-enveloped, virion about 26 nm in diameter with T=3 icosahedral symmetry, composed of 180 capsid proteins: 12 pentamers and 20 hexamers. The genomic RNA does not fill completely the interior of the particle, there is a central cavity of about 8 nm.



Genome

Segmented, tripartite linear ssRNA(+) genome composed of RNA1, RNA2, RNA3. Each genomic segment has a 3' tRNA-like structure and a 5'cap.

Gene expression

Genomic RNA serves as messenger RNAs. RNA1 and RNA2 encode respectively proteins 1a and 2a, both involved in genome replication and internal transcription of sgRNA4 from the minus-strand copy of RNA3. RNA3 and sgRNA4 are translated respectively into movement and capsid proteins.

BMV has a genome that is divided into three 5' capped RNAs. RNA1 (3.2 kb) encodes a protein called 1a (109 kDa), which contains both an N-proximal methyltransferase domain and a C-proximal helicase-like domain. RNA2 (2.9 kb) encodes the 2a protein (94 kDa), the RNA-dependent RNA polymerase, responsible for replication of the viral genome. The dicistronic RNA3 (2.1 kb) encodes for two proteins, the 3a protein (involved in cell-to-cell migration during infection) and the coat protein (for RNA encapsidation and vascular spread), which is expressed from a subgenomic replication intermediate mRNA, called RNA4 (0.9 kb). 3a and coat protein are essential for systemic infection in plants but not RNA replication

Tobacco mosaic virus has a rod-like appearance. Its capsid is made from 2130 molecules of coat protein and one molecule of genomic single strand RNA, 6400 bases long. The coat protein self-assembles into the rod-like helical structure (16.3 proteins per helix turn) around the RNA which forms a hairpin loop structure. The protein monomer consists of 158 amino acids which are assembled into four main alpha-helices, which are joined by a prominent loop proximal to the axis of the virion. Virions are ~300 nm in length and ~18 nm in diameter. Negatively stained electron microphotographs show a distinct inner channel of ~4 nm. The RNA is located at a radius of ~6 nm and is protected from the action of cellular enzymes by the coat protein. There are three RNA nucleotides per protein monomer. X-ray fiber diffraction structure of the intact virus was studied based on an electron density map at 3.6 Å resolution.

Genome

The TMV genome consists of a 6.3-6.5 kb single-stranded (ss) RNA. The 3'-terminus has a tRNA-like structure. The 5' terminus has a methylated nucleotide cap (m7G5'pppG). The genome encodes 4 open reading frames (ORFs), two of which produce a single protein due to ribosomal readthrough of a leaky UAG stop codon. The 4

genes encode a **replicase** (with methyltransferase [MT] and RNA helicase [Hel] domains), an RNA-dependent **RNA polymerase**, a so-called **movement protein** (MP) and a **capsid protein** (CP).

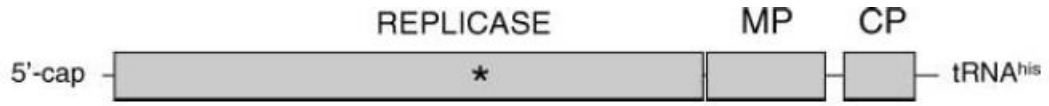


Figure 4 Genetic map of *Tobacco mosaic virus* (TMV). The ~6,400 nucleotide TMV RNA has a 5′-cap and 3′-tRNA-like structure. The open reading frames are indicated by boxes. The replicase consists of a 126-kDa protein and, by readthrough of an amber stop codon (asterisk), the 183-kDa protein. The replicase proteins are translated from the genomic RNA. The 30-kDa movement protein (MP) and the 17.5-kDa capsid protein (CP) are expressed from separate subgenomic RNAs (not shown).

Replication

Following entry into its host via mechanical inoculation, TMV uncoats itself to release its viral [+]RNA strand. As uncoating occurs, the MetHel:Pol gene is translated to make the capping enzyme MetHel and the RNA Polymerase. Then the viral genome will further replicate to produce multiple mRNAs via a [-]RNA intermediate primed by the $tRNA_{HIS}$ at the [+]RNA 3' end. The resulting mRNAs encode several proteins, including the

coat protein and an RNA-dependent RNA polymerase (RdRp), as well as the movement protein. Thus TMV can replicate its own genome. After the coat protein and RNA genome of TMV have been synthesized, they spontaneously assemble into complete TMV virions in a highly organized process. The protomers come together to form disks or 'lockwashers' composed of two layers of protomers arranged in a helix. The helical capsid grows by the addition of protomers to the end of the rod. As the rod lengthens, the RNA passes through a channel in its center and forms a loop at the growing end. In this way the RNA can easily fit as a spiral into the interior of the helical capsid.

TMV as a vector

Tobacco mosaic virus (TMV) is one of the most well characterized plant viruses and was the first plant virus examined for vector development. While original TMV-based

viral vectors depended on usage of the entire viral genome, second generation vectors were “deconstructed” and consisted of the only sections of genomes that were important for replication; these were subcloned into an assortment of plasmid constructs. The first of these new series of vectors, the MagnICON vector system of ICON Genetics, is a core technology of Dr. Gleba and coworkers. A technique for transfecting plants with these recombinant virus vector modules, known as “magnifection,” involved the infiltration of a suspension of *A. tumefaciens* using a vacuum into all mature leaves of a tobacco plant, thus infecting the whole plant in its entirety. These constructs are part of the T-DNA of a binary vector, and researchers need to merely mix different *Agrobacterium* strains which harbour these constructs prior to agroinfecting plants. As an example, yields of recombinant human growth hormone protein (hGH) reaching up to 10% of total soluble protein or 1 mg/g of fresh weight leaf biomass have been achieved using this system of expression. Further modifications, including alteration of transcript splicing sites, modification of codon usage patterns, and the introduction of introns into TMV coding sequences, have improved protein expression further.

The technology has been built upon so that it could be incorporated into the Gateway cloning system, a series of plasmids that are used for a wide variety of expression systems. In this case, the need for traditional cloning has been circumvented by a system that involves site-specific recombination. In this way, foreign genes and other DNA fragments alike can be transferred between plasmids to enable the desired recombination event to take place. Protein expression can also be enhanced 10- to 25-fold by the coexpression of the RNA silencing suppressor gene of Tomato bushy stunt virus known as

P19. For example, Lindbo coinoculated plants with a TMV-based vector and a viral suppressor of RNA silencing; this culminated in the production of tremendous levels of recombinant protein (between 600 and 1200 micrograms of GFP per gram of infiltrated tissue) after one week postinfection.

Other researchers have found that gene expression can be increased several times by placing the foreign gene open reading frame (ORF) closer to the 3′ end of the TMV RNA. This TRBO (TMV RNA-based overexpression) vector lacks the coat protein coding sequence and can produce 100-fold more recombinant protein in plants than the P19-enhanced transient expression system. Lindbo’s group found that this vector could generate 100 times more of a variety of recombinant proteins than their P19 silencer system.

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SCHOOL OF BIO

AND CHEMICAL ENGINEERING
DEPARTMENT OF BITOECHNOLOGY

UNIT – IV – PLANT BIOTECHNOLOGY– SBB3103

TRANSGENIC PLANTS

Production of transgenic plants for herbicide resistance

Weed Control Practices

The tandem technique of soil-tilling and herbicide application is an example of how farmers control weeds in their farms.

Generally, they till their soil before planting to reduce the number of weeds present in the field. Then they apply broad-spectrum or non-selective herbicides (one that can kill all plants) to further reduce weed growth just before their crop germinates. This is to prevent their crops from being killed together with the weeds. Weeds that emerge during the growing season are controlled using narrow-spectrum or selective herbicides. Unfortunately, weeds of different types emerge in the field, and therefore, farmers have to use several types of narrow-spectrum herbicides to control them. This weed control method can be very costly and can harm the environment.

Researchers postulated that weed management could be simplified by spraying a single broad-spectrum herbicide over the field anytime during the growing season.

Development of Glyphosate and Glufosinate Herbicide Tolerant Plants

Herbicide-tolerant (HT) crops offer farmers a vital tool in fighting weeds and are compatible with no-till methods, which help preserve topsoil. They give farmers the flexibility to apply herbicides only when needed, to control total input of herbicides and to use herbicides with preferred environmental characteristics.

Technology Background

How do these herbicides work?

These herbicides target key enzymes in the plant metabolic pathway, which disrupt plant food production and eventually kill it. So how do plants elicit tolerance to herbicides? Some may have acquired the trait through selection or mutation; or more recently, plants may be modified through genetic engineering.

Why develop HT crops?

What is new is the ability to create a degree of tolerance to broad-spectrum herbicides - in particular glyphosate and glufosinate - which will control most other green plants. These two herbicides are useful for weed control and have minimal direct impact on animal life, and are not persistent. They are highly effective and among the safest of agrochemicals to use. Unfortunately, they are equally effective against crop plants.

How do Glyphosate and Glufosinate HT crops work?

1. **Glyphosate-tolerant crops**

   Glyphosate herbicide kills plants by blocking the EPSPS enzyme, an enzyme involved in the biosynthesis of aromatic amino acids, vitamins and many secondary plant metabolites. There are several ways by which crops can be modified to be glyphosate-tolerant. One strategy is to incorporate a soil bacterium gene that produces a glyphosate-tolerant form of EPSPS. Another way is to incorporate a different soil bacterium gene that produces a glyphosate degrading enzyme.

2. **Glufosinate-tolerant crops**

   Glufosinate herbicides contain the active ingredient phosphinothricin, which kills plants by blocking the enzyme responsible for nitrogen metabolism and for detoxifying ammonia, a by-product of plant metabolism. Crops modified to tolerate glufosinate contain a bacterial gene that produces an enzyme that detoxifies phosphonothricin and prevents it from doing damage.

Other methods by which crops are genetically modified to survive exposure to herbicides including: 1) producing a new protein that detoxifies the herbicide; 2) modifying the herbicide's target protein so that it will not be affected by the herbicide; or 3) producing physical or physiological barriers preventing the entry of the herbicide into the plant. The first two approaches are the most common ways scientists develop herbicide tolerant crops.

Safety Aspects of Herbicide Tolerance Technology

Toxicity and Allergenicity

Government regulatory agencies in several countries have ruled that crops possessing herbicide-tolerant conferring proteins do not pose any other environmental and health risks as compared to their non-GM counterparts.

Introduced proteins are assessed for potential toxic and allergenic activity in accordance with guidelines developed by relevant international organizations. They are from sources with no history of allergenicity or toxicity; they do not resemble known toxins or allergens; and they have functions, which are well understood.

Effects on the Plants

The expression of these proteins does not damage the plant's growth nor result in poorer agronomic performance compared to parental crops. Except for expression of an additional enzyme for herbicide tolerance or the alteration of an already existing enzyme, no other metabolic changes occur in the plant.

Persistence or invasiveness of crops

A major environmental concern associated with herbicide-tolerant crops is their potential to create new weeds through outcrossing with wild relatives or simply by persisting in the wild themselves. This potential, however, is assessed prior to introduction and is also monitored after the crop is planted. The current scientific evidence indicates that, in the absence of herbicide applications, GM herbicide-tolerant crops are no more likely to be invasive in agricultural fields or in natural habitats than their non-GM counterparts (Dale et al., 2002).

The herbicide-tolerant crops currently in the market show little evidence of enhanced persistence or invasiveness.

Advantage of Herbicide Tolerant Crops

- Excellent weed control and hence higher crop yields;
- Flexibility – possible to control weeds later in the plant's growth;
- Reduced numbers of sprays in a season;
- Reduced fuel use (because of less spraying);
- Reduced soil compaction (because of less need to go on the land to spray);
- Use of low toxicity compounds which do not remain active in the soil; and
- The ability to use no-till or conservation-till systems, with consequent benefits to soil structure and organisms (Felsot, 2000).

A study conducted by the American Soybean Association (ASA) on tillage frequency on soybean farms showed that significant numbers of farmers adopted the "no-tillage" or "reduced tillage" practice after planting herbicide-tolerant soybean varieties. This simple weed management approach saved over 234 million gallons of fuel and left 247 million tons of irreplaceable topsoil undisturbed.

Current Status of Herbicide Tolerance

From 1996 to 2013, herbicide- tolerant crops consistently occupied the largest planting area of biotech crops. In 2013 alone, herbicide tolerant crops occupied 99.4 million hectares or 57% of the 175.2 million hectares of biotech crops planted globally. The most common are the

glyphosate and glufosinate tolerant varieties. The following table shows countries that have approved major HT crops for food, feed and/or cultivation.

Crop	**Countries**
Alfalfa	Australia, Canada, Japan, Mexico, New Zealand, Philippines, Singapore, South Korea, United States of America (USA)
Argentine Canola	Australia, Canada, Chile, China, European Union (EU), Japan, Mexico, New Zealand, Philippines, Singapore, South Africa, South Korea, USA
Carnation	Malaysia
Chicory	USA
Cotton	Argentina, Australia, Brazil, Canada, China, Colombia, Costa Rica, EU, Japan, Mexico, New Zealand, Paraguay, Philippines, Singapore, South Africa, South Korea, USA
Flax, Linseed	Canada; Colombia; USA
Maize	Argentina, Australia, Brazil, Canada, China, Colombia, EU, Honduras, Indonesia, Japan, Malaysia, Mexico, New Zealand, Panama, Paraguay, Philippines, Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, USA, Uruguay
Polish Canola	Canada
Potato	Australia, Canada, Japan, Mexico, New Zealand, Philippines, South Korea, USA
Rice	Australia, Canada, Colombia, Honduras, Mexico, New Zealand, Philippines, Russian Federation, South Africa, USA
Soybean	Argentina, Australia, Bolivia, Brazil, Canada, Chile, China, Colombia, Costa Rica, EU, India, Indonesia, Japan, Malaysia, Mexico, New Zealand, Paraguay, Philippines, Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, USA, Uruguay
Sugarbeet	Australia, Canada, China, Colombia, EU, Japan, Mexico, New Zealand, Philippines, Russian Federation, Singapore, South Korea, USA
Wheat	Australia, Colombia, New Zealand, USA

Source: ISAAA's GM Approval Database. http://www.isaaa.org/gmapprovaldatabase/.

A literature review conducted by the Council for Agricultural Science and Technology concluded that the environment benefits from the use of HT crops. In the US, for example, no-till soybean acreage has increased by 35% since the introduction of HT soybean. A similar trend is observed in Argentina where soybean fields are 98% planted with HT varieties. The CAST paper entitled “Comparative Environmental Impacts of Biotechnology-derived and Traditional Soybean, Corn and Cotton Crops” is available at http://www.cast-science.org.

For the first 17 years of commercialization (1996-2012), benefits from herbicide tolerant crops are valued at US$ 47.7 billion, 41% of global biotech crop value of US$ 116.9 billion, and for 2012 alone at US$ 6.6 billion or 35% of global value of US$ 18.7 billion.

References

http://isaaa.org/resources/publications/pocketk/10/default.asp

Plant derived vaccines

Vaccine

- A vaccine is a biological preparation that improves immunity to a particular disease.
- A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins.
- The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.
- Vaccination is the most effective and cost-efficient method for fighting human and animal diseases, preventing the spread of infectious diseases, completely eradicating some diseases and even helping to prevent cancer and autoimmune disorders.
- There are now more then 25 vaccines licensed for use in humans with many more in the development pipeline.
- An effective vaccine should be inexpensive, confer protective immunity to the vaccine with few side effects and safe, stable and easy to administer.

Different methods of vaccine production

- **Traditional vaccines:**

Traditional vaccines are live, killed or otherwise attenuated/modified pathogens (e.g., influenza vaccines produced in specific pathogen free-eggs). However, they have been increasingly supplanted by recombinant subunit vaccines produced in genetically modified cells because they offer increased safety, less antigenic competition, the ability to target vaccines to specific sites and the ability to differentiate between infected and vaccinated animals.

- **Production systems for recombinant subunit vaccines:**

Recombinant subunit vaccines currently on the market are produced in bacteria, yeast or insect cells and mammalian cells.

- Bacterial systems for vaccine production cannot produce more complex proteins that folds correctly and undergo forms of post-translational modifications (e.g. N-glycosylation). Furthermore, the presence of endotoxins and other pyrogens has limited their use.

- Since yeast cells are eukaryotes, they can fold and assemble complex recombinant proteins and carry out N-glycosylation however, the glycan structures often differ to those found in mammals.
- Insect cells culture medium is much less expensive and tend not to harbor mammalian pathogens. However, insect cells have limited scalability and major differences in glycan structures could raise challenges for the production of some recombinant proteins.
- In case of mammalian cells factors such as the cost of infrastructure and consumables, and the need for extensive product validation to prove that the final product is pathogen-free and does not contain oncogenic agents make this platform commercially unfeasible for vaccines required on a large scale.

Plants as a platform for the production of cost-effective proteins

- Plants have been used to produce over 200 medically relevant proteins and their many benefits now make them a serious competitor to mammalian cells for biopharmaceutical production.
- In terms of vaccine production, plants share the advantages of other eukaryotic systems but lack their principle disadvantages in terms of cost, safety and scalability.
- Plant-derived vaccines can be produced either stably by nuclear and chloroplast transformation or transiently by tobacco mosaic virus (TMV) based expression.

Advantages of plant-derived vaccines

- With plants, there is no need to build and run expensive fermenters, hire skilled workers and pay for expensive culture media.
- Very high yields of recombinant proteins can be achieved by chloroplast transformation. In two recent reports, approximately 70% and 72% of total soluble protein (TSP) has been obtained by plastid transformation, respectively.
- Growth of plants can be scaled up according to the required amount of protein.
- Plants tend not to harbor human pathogens and any colonizing bacteria or animal-derived material can be removed using appropriate sanitary measures before processing.
- Transgenic plants can be grown at the site where the vaccine is needed. This advantage can save the costs related to transportation and cold storage.
- Plant-derived vaccines have the potential to be used as oral vaccine, thus evading the costs related to sterile needles and trained medical staff.
- Plants-derived vaccines are likely to be more stable. Proteins expressed in certain plant tissues (e.g., cereal seeds) remain stable for years at ambient temperatures without loss of activity.

- Plant-derived vaccines survive in the stomach through bioencapsulation, which allows gradual release and, in some, cases this makes the vaccine more efficacious than the same subunit delivered through the parenteral route.

Vector	Cost to produce	Length of time to produce	Glycosylation	Risk of pathogen contamination	Cost to store
Bacteria	Low	Short	No	Medium	Moderate
Mammalian cells	High	Long	Yes	High	Expensive
Transgenic plants	Low-medium	Long	Yes(some differences)	Low	Low

Plant-derived vaccines: brief history

- The first demonstration of expression of a vaccine antigen within plants occurred in 1990 when Curtiss and Cardineau expressed the *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco.
- This demonstration was closely followed by plant expression of the hepatitis B surface antigen (HbsAg), the *E. coli* heat–labile enterotoxin responsible for diarrhoea, the Norwalk virus capsid protein and the rabies virus glycoprotein.
- Proteins produced in these plants induced synthesis of antigen specific mucosal IgA and serum IgG when delivered orally to mice and humans.
- The latest landmark in the development of pharmaceutical-producing plants sees a tomato-derived vaccine against cholera and hepatitis C
- Researchers have combined genetic sequences of these two pathogens and introduced them into plants. The tomato plants then produce key proteins of both pathogens.
- One of the advantages of the tomato-derived vaccine is that it is easily stored in the seed of the tomatoes themselves, according to lead researcher.
- Various vaccine antigens against different human diseases have been successfully expressed in chloroplast. However, none of the chloroplast-derived vaccine candidate has yet entered into clinical trials.
- So far, few plant-derived vaccines against human diseases, expressed either by nuclear transformation or TMV based expression, have shown promising results in phase I and II clinical trials upon oral delivery.

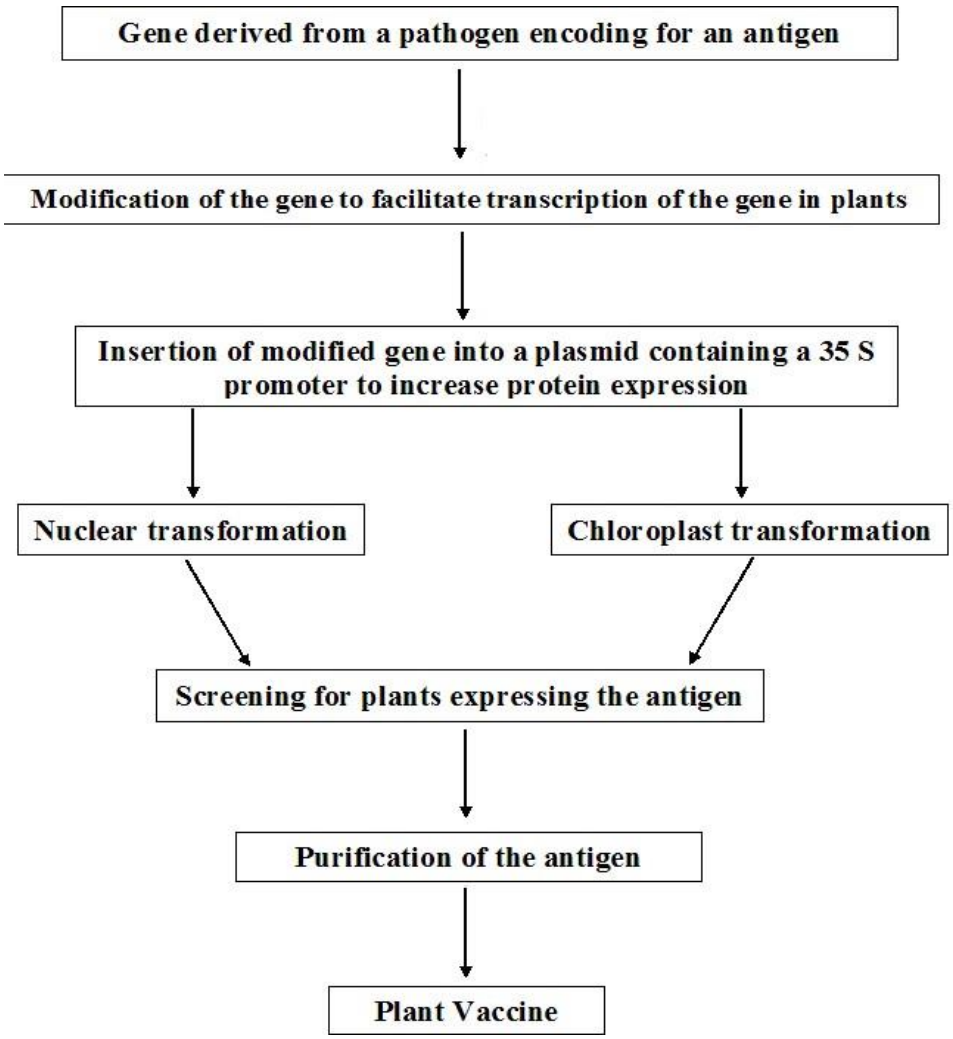
Diversity of plant-based systems

- To date many plant species have been used for vaccine production.

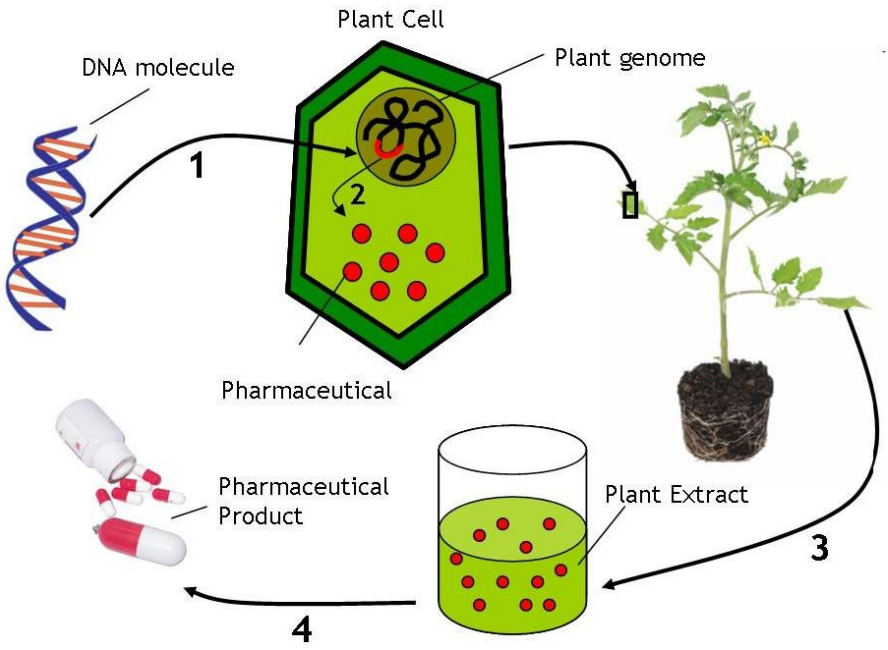
- Early studies used tobacco and potato but now tomato, banana, corn, carrot, lettuce and others are being used for this purpose.
- The choice of the plant species (and tissue in which the protein accumulates) is important and is usually determined through how the vaccine is to be applied in the future.

Procedure of plant-derived vaccines

- A DNA molecule carrying the genetic information for a pharmaceutical substance is introduced into the plant genome.
- This process is called transformation. The genes can be incorporated permanently (stable transformation) or for a short period of time (transient transformation). The transformed plant acts as a bioreactor producing large quantities of the pharmaceutical using its protein making machinery
- Through industrial processing, the pharmaceutically active substance is extracted from the plant and made into in a formulated product, for example a pill.



Flow diagram of the general procedures used to produce plant made vaccines



Strategies for vaccine production in plants

- **Transgenic plants:**

The most widely used strategy for vaccine production in plants is the nuclear transgenic system, in which the antigen transgenes are transferred to the plant nuclear genome.

- **Transplastomic plants:**

Instead of introducing transgenes into the nuclear genome, they can be targeted to the chloroplast genome using particle bombardment or other physical DNA delivery techniques, ensuring that the transgene is embedded in a chloroplast DNA homology region.

- **Plant viral expression system:**

This expression system is based on infection of a plant by a plant virus, which is competent to independently replicate, transcribe, and translate so as to produce many copies of a recombinant protein introduced into the plant viral genome.

Antigens produced in transgenic plants

Recombinant protein (vaccine)	Transgenic plant	Protection against
Rabies glycoprotein	Tomato	Rabies virus
Foot and mouth virus (VPI)	Arabidopsis	Foot and mouth virus
Herpes virus B surface antigen	Tobacco	Herpes simplex virus
Cholera toxin B subunit	Potato	*Vibrio cholerae*
Human cytomegalovirus glycoprotein B	Tobacco	Human cytomegalovirus

Oral delivery, mucosal and systemic antibody responses

- Plant-derived vaccines have demonstrated the ability to induce both systemic and mucosal immune responses.
- Most infectious agents enter the body through mucosal membranes. Induction of mucosal immunity is best achieved by direct vaccine delivery to mucosal surfaces.
- While effective inducers of systemic immunity, vaccines delivered by injection are not efficient at inducing mucosal responses.
- The major obstacle to oral vaccination is the digestion of the antigenic protein in the stomach.
- Vaccines derived and delivered by plant cells have been shown to overcome this problem through the protective effect of the plant cell wall.
- Like liposomes and microcapsules, the plant cell wall allows gradual release of the antigen onto the vast surface area of the lower digestive tract.
- Further problems may be associated with poor immunogenicity or the induction of tolerance. Binding to a targeting molecule or carrier peptide, has been shown to overcome poor immunogenicity of orally delivered subunit vaccines.
- In specific circumstances, for example cancer therapy, injection of the drugs, after purification from the producing plant, may be preferred.

Targets for plant-derived proteins

Vaccines against infectious diseases:

- There is a large and fast growing list of protective antigens from microbial and viral pathogens that have been expressed by plants.
- The initial focus was upon human pathogens. However, today attention has also spread to animal pathogens (e.g. Newcastle and foot and mouth disease).

Vaccines against autoimmune diseases:

- Transgenic plants expressing autoantigens are being produced in attempt to cure diseases in which the immune system recognises the body's own proteins as foreign. The diseases include arthritis, multiple sclerosis, myasthenia gravis, and type I diabetes.
- The rational is that an appropriate oral dose of a plant-derived autoantigen will inhibit the development of the autoimmune disease.

Table 2. Transient production of antigens in plants after infection with plant viruses expressing a recombinant gene

Protein	Plant	Carrier	Refs
Influenza antigen	Tobacco	TMV	28
Murine zona pellucida antigen	Tobacco	TMV	28
Rabies antigen	Spinach	AIMV	29
HIV-1 antigen	Tobacco	AIMV	30
Mink enteritis virus antigen	Black eyed bean	CPMV	31
Colon cancer antigen	Tobacco	TMV	32

AIMV, alfalfa mosaic virus; TMV, tobacco mosaic virus; CPMV, cowpea mosaic virus.

Vaccines against human tumours:

- Particular proteins have been shown to over-express on the cell surface of many tumours, including melanom and breast cancer. Naturally acquired, actively induced or passively administered antibodies against these antigens have been able, in some cases, to eliminate circulating tumour cells and micrometastasis. However, cancer vaccine development is complicated due to the tumour antigens also being auto-antigens.

Advantages of plant-derived vaccines

- Cost effective.
- Easy to administer.
- Easy to store.
- Acceptable to poor developing country.
- Safe
- Activate both mucosal and systemic immunity.
- Heat stable.
- Do not required cold chain maintenance.
- No fear of contamination.

Disadvantages

- One of the fears is that GM-pollen may outcross with sexually compatible plants (related crops or weeds) and affect biodiversity.
- Producing vaccines in plants also has a drawback because of the associated contamination risk for food crop production.
- A soya field was found to be contaminated with transgenic maize producing the pharmaceutically-active substance trypsin causing the complete harvest of 13,500 tons of soya beans to be destroyed. Hence, research is moving towards non-foods such as tobacco.
- There is also the risk that the pharma plants could be eaten by animals.
- The active substances could enter the groundwater and lead to harmful effects.
- Antibiotic resistance marker genes can spread from GM food to pathogenic bacteria.
- If a vaccine was consumed inadvertently, it could lead to desensitisation so that vaccinations would eventually cease to work.

References

http://www.bioethics.iastate.edu/retreat_2003/powerpoint/wang.ppt

Edible vaccine

- Edible Vaccine involves introduction of selected desired genes into plant and then inducing these altered plants to manufacture the altered protein.

How it acts?

- Antigen in transgenic plant
  > Ingestion
  >Delivered by bioencapsulation
  >Taken up by Mcell
  > Pass on to the Macrophage
  >IgG, IgE responses
  > Local IgA response & Memory cells
  > Neutralize the attack by the real infectious agent.

Advantage and Disadvantage of different plants

Plant	Advantages	Disadvantages
Potato	• Easily transformed. • Easily propagated. • Stored for long periods without refrigeration.	• Need cooking which denature antigen.
Banana	• Do not need cooking. • Protein not destroyed even after cooking. • Inexpensive. • Grown widely in developing countries.	• Trees take 2-3 to mature years. • Spoils rapidly after ripening.
Rice	• Commonly used in baby food. • High expression of antigen.	• Grows slowly. • Requires glasshouse condition.
Tomato	• Grow quickly. • Cultivate broadly. • High content Vitamin-A may boost immune response.	• Spoils readily.

Clinical trials on:

- **ETEC**

11 Volunteers were feed raw transgenic Potatoes expressing LT-B.

10 (95%) of these individuals developed neutralizing antibodies and 6 (55%) develop mucosal response.

- **Norwalk Virus**

20 people fed with transgenic potato .

19 (95%)of them expressing Norwalk virus antigen showed sero conversion.

- **Hepatitis B**

First human trials of potato-based vaccine against Hepatitis B have reported encouraging results.

The amount of HBsAg needed for one dose could be achieved in a single potato.

Levels of specific antibodies significantly exceeded the protective level of 10mIU/mL in human.

Advantages of Edible Vaccine

- Cost effective.
- Easy to administer.
- Easy to store.
- Acceptable to poor developing country.
- Fail safe
- Activate both mucosal and systemic immunity.
- Heat stable.
- Do not required cold chain maintenance.
- No fear of contamination.

Future of Edible Vaccine

- Resistance to GM foods may affect future of Edible Vaccine.

Limitations

- Transgenic contamination can occur.
- Antibiotic resistance marker genes can spread from GM food to pathogenic Bacteria.
- Difficulty in dose maintenance.

Edible plant derived vaccine may lead to a future of safer and more effective immunization.

Plantibodies

A **plantibody** is an antibody that is produced by plants that have been genetically engineered with animal DNA. An antibody (also known as an immunoglobulin) is a complex protein within the body that recognizes antigens on viruses and other dangerous compounds in order to alert the immune system that there are pathogens within the body.[1] The transgenic plants become transformed with the DNA and produce antibodies that are similar to those inserted. The term plantibody and the concept are trademarked by the company Biolex.

A plantibody is produced by insertion of antibodies into a transgenic plant. The plantibodies are then modified by intrinsic plant mechanisms (N-glycosylation). Plantibodies are purified through processes such as filtration, immunofluorescence, chromatography, and diafiltration. It is more cost effective to produce antibodies in transgenic plants than in transgenic animals.

Antibodies produced from plants (Smith & Glick, 2000)

- Functional antibodies can be produced from plants
- Targeted to intercellular space, chloroplast, seeds and tubers
- Benefit for topical immunotherapy
- Can be produced from plants in large quantities
- Plantibodies provides increased stability. e.g: Secretory Ig A
- Plants can assemble complex secretory antibodies
  - e.g: Construction of tobacco plants expressing 4 transgenes
- Quadruple transgenics efficiently assembled secretory immunoglobulins

Full-size monoclonal antibodies recently produced in transgenic plants

Plant	Antibody type (target)	Purpose
Tobacco	IgG (low molecular weight phosphonate ester)	Catalytic antibodies
Tobacco	IgG (nematode)	Plant pathogen resistance
Tobacco	sIgA/G (*Streptococcus mutans*)	Therapeutic (topical)
Soybean, rice	IgG (herpes simplex virus)	Therapeutic (topical)
Tobacco	IgG (colon cancer)	Therapeutic (systemic injection)
Alfalfa	IgG (human IgG)	Diagnostic
Tobacco	IgG (rabies virus)	Therapeutic: first IgG expressed in plant showing therapeutic activity (systemic injection)
Tobacco	IgG (hepatitis B virus)	Immunopurification of hepatitis B surface antigen
Tobacco	IgG (hepatitis B virus)	Therapeutic

Anti-rabies virus mAb

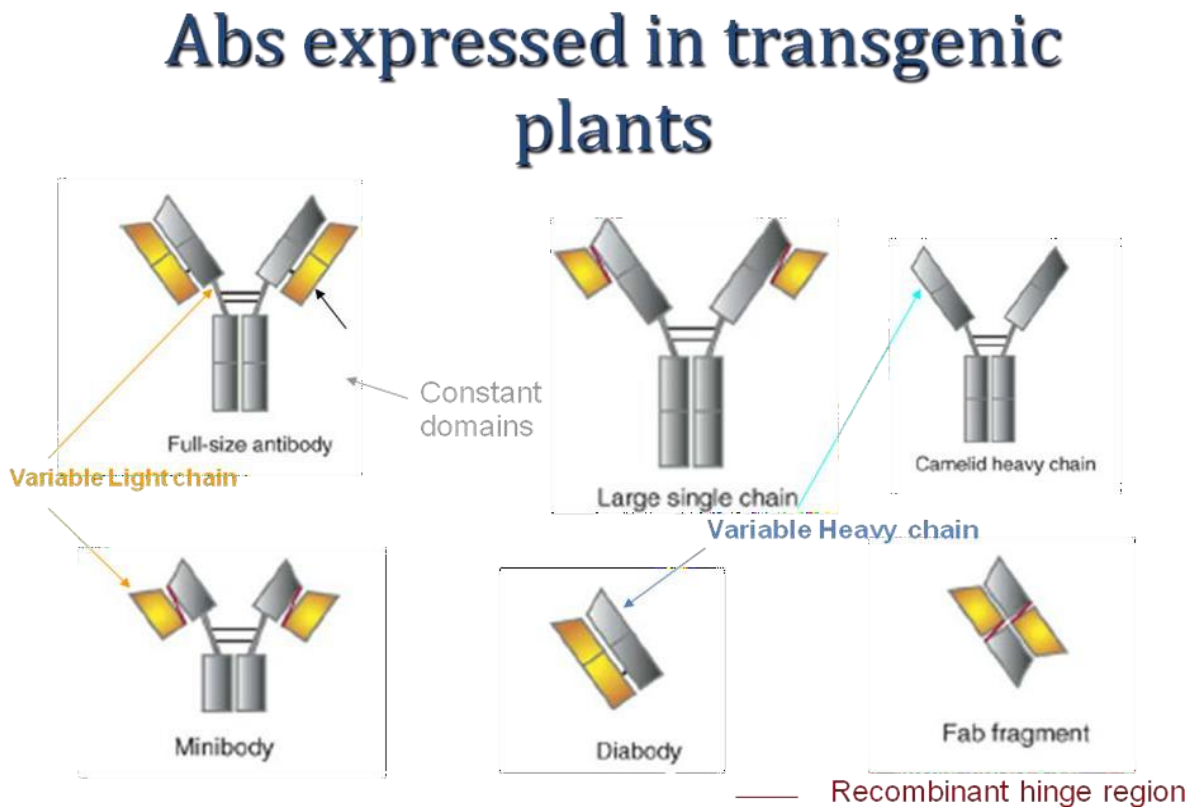
- After exposure treated with Ab

- Used to be made in horses
- First mAb made in transgenic plants
- 4 genes – 2 H, 2 L
- Transgenic plant for each one and crossing plants
- Later used single binary vector with two promoters

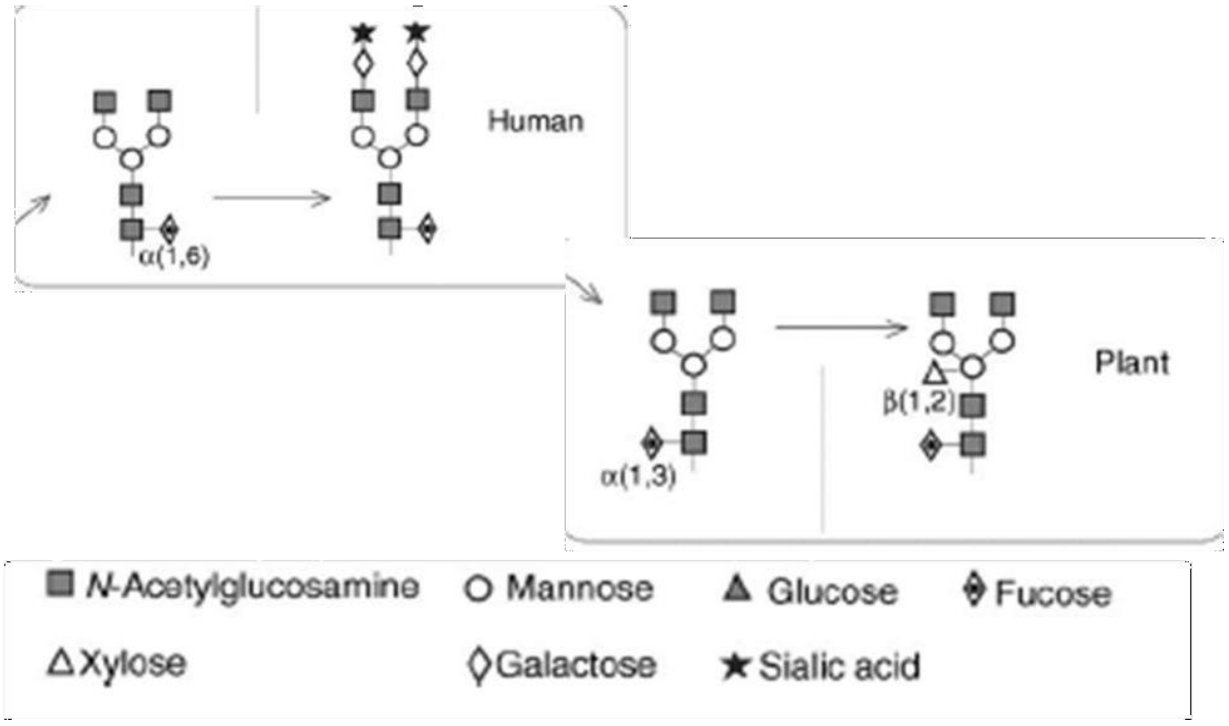
Functional Abs

- Need to be properly folded and assembled
- Need disulfide bond formation and glycosylation
- Down stream processing:
    - Purification of Ab mostly with Protein A or G
- Glycosylation is different in plants
    - β 1,2 Xylose and α 1,3 fucose
- Retain in ER, only mannose is attached

  Shorter half-life of Ab



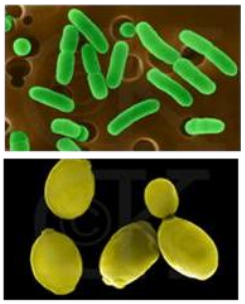
Glycosylation in Golgi



Production Costs for Antibodies

Production costs	cost in $ / gram
hybridomas	1000
transgenic animals	100
transgenic plants	10

Source: Daniell et al. (2001) TIPS 6, 219-226



E. coli & yeast

Tr. animals and animal cells

Transgenic plants

Antibodies: a compelling success story

- high specificity: *in vitro* and *in vivo* diagnostics
- low toxicity: therapeutic applications
- high drug approval rates (24 approved mAbs)
- major products in biotechnology (~240 in clinical trials)
- inherently stable human proteins
- injectable, topical and oral applications
- applicable for chronic conditions
- potential long-lasting benefits

Comparison of Mammalian and Plant-produced Antibodies

- peptide sequence: identical
- correct cleavage of Ig-derived signal peptides
- kinetics & affinity: identical
- stability in seeds > 30 months
- antibody types: plant system more versatile (sIgA)
- post-translational processing: different
- core glycan identical, terminal sugar different plus xylose & fucose
- antigenicity & clearance: apparently identical (shorter half-life)

Advantages of Plantibodies

- Produced in containment
- Controlled conditions
- Correctly folded, soluble proteins
- High levels of expression
- Cost effective for large scale production
- Safety issues

Disadvantages of Plantibodies

- Sporadic transgene silencing
- Glycolsylation patterns
- Inefficient expression
- Environmental contamination

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Insect resistance plants

Definitions of an insect-resistant plant are many and varied. In the broadest sense, plant resistance is defined as "the consequence of heritable plant qualities that result in a plant being relatively less damaged than a plant without the qualities." In practical agricultural terms, an insect-resistant crop cultivar is one that yields more than a susceptible cultivar when confronted with insect pest invasion. Resistance of plants is relative and is based on comparison with plants lacking the resistance characters, i.e., susceptible plants.

Tools of molecular biology and genetic engineering have provided humankind with unprecedented power to manipulate and develop novel crop genotypes towards a safe and sustainable agriculture in the 21st century. Technologies and chemical inputs that have proven harmful to human health and environment need to be replaced with safer alternatives to manage insect pests in agricultural ecosystems.

Many insecticidal proteins and molecules are available in nature which are effective against agriculturally important pests but are innocuous to mammals, beneficial insects and other organisms. Insecticidal proteins present in Bacillus thuringiensis (Bt), which have shown efficacy as spray formulations in agriculture over the past five decades, have been expressed in many crop species with positive results. Large scale cultivation of Bt-crops raises concerns about the possible development of resistant insects. Many strategies have been formulated to prevent/delay the development of resistance.These strategies have to be given serious consideration in India where the first Bt-crop containing resistance to insect pests, particularly Helicoverpa armigera, has been released for commercial cultivation in the farmers' fields.

In addition to Bt, proteinase inhibitors present in several plant species offer a good source of resistance to insect pests. A combination of proteinase inhibitors has been suggested as a viable alternative to Bt to manage insects such as H.armigera. In recent years, several novel insecticidal proteins have been discovered in bacteria such as Photorhabdus luminescens. The judicious expression of multiple insecticidal proteins that differ in their mechanisms of toxicity will provide formidable barriers for insects to develop resistance. Finally, deployment of integrated pest management (IPM) strategies during the cultivation of transgenic crops will ensure durable insect resistance.

Expression of Insecticidal proteins of Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a Gram-positive, aerobic, sporulating bacterium which synthesizes crystalline proteins during sporulation. These crystalline proteins are highly insecticidal at very low concentrations

Expression of Vegetative insecticidal proteins of Bt

Research efforts in the past five years have led to the discovery of novel insecticidal proteins which are produced by certain isolates of B. thuringiensis. These proteins unlike well characterized crystal proteins are produced during vegetative growth of cells and are secreted into the growth medium.

Expression of other insecticidal proteins from bacteria, plants and animals

Proteinase inhibitors
Plant lectins
α -amylase inhibitors
Insect chitinases
Plant metabolic enzymes
Insecticidal viruses
Genes from bacteria other than Bt
Novel genes of plant origin

Stress tolerance plants

Abiotic stress is one of the primary causes of crop losses worldwide but crop plants are affected by a variety of abiotic stresses. Abiotic stresses includes drought, salinity, heat, cold, flooding, and ultraviolet radiation etc. Currently, there are no economically viable technological means to facilitate crop production under stress conditions using breeding methods. However, the development of crop plants tolerant to drought stress is considered a promising approach, which may help satisfy growing food demands from both developing and under-developed countries. By contrast, improvement of stress tolerance by genetic engineering overcomes the bottlenecks of plant breeding methods. Transgenic approaches can be used in combination with conventional breeding strategies to create crops with enhanced drought tolerance. The current genetic engineering strategies rely on the transfer to the targetplant of one or several genes that are either involved in signaling and/or regulatory pathways, or that encode enzymes present in

pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or pathways that encode stress tolerance-conferring proteins.

Plants have evolved various mechanisms to cope with stress conditions and these include the shifts in the physiology of the plant and the expression of stress-associated genes, leading to the formation of a wide variety of low molecular mass metabolites collectively known as compatible solutes such as proline, glycine betaine, sugars such as sucrose, trehalose and fructans and sugar alcohols like sorbitol, mannitol, ononitol, pinitol and polyols These osmolytes are uniformly neutral with respect to the perturbation of cellular functions even when present at high concentrations. Accumulation of these molecules either actively or passively helps plants to retain water within cells and protects cellular compartments from injury caused by dehydration or maintains turgor pressure during water stress. Moreover, these molecules stabilize the structure and function of certain macromolecules, signaling functions or induction of adaptive pathways and scavenge reactive oxygen species. However, the molecular and cellular interactions of these solutes are not completely understood.

Identifying the mechanisms developed and deployed by plants to counteract abiotic stresses and maintain their growth and survival under harsh conditions thus holds great significance. Recent investigations have shown that phytohormones, including the classical auxins, cytokinins, ethylene, and gibberellins, and newer members including brassinosteroids, jasmonates, and strigolactones may prove to be important metabolic engineering targets for producing abiotic stress-tolerant crop plants.

Strategies for Improving Crops Against Stresses

- Use of Naturally Stress Tolerant Plants
- Selection and Breeding for Drought Tolerance
- Molecular Breeding
- Transgenic Approach

Plant as bioreactors

Plant as bioreactors refer to the use of transgenic plants and cell cultures of plants to make unlimited quantities of commercially important substances like recombinant proteins including antibodies and vaccines using biotechnology oriented techniques.

Most of the research has been directed towards using plant bioreactors to make the following

- Therapeutic proteins
- Edible vaccines
- Antibodies for immunotherapy

Using genetic engineering, cereal plants, fruits plants, legumes and vegetable plants have the capacity to become low cost bioreactors to make molecules that in the normal scheme of things would not have been available from plants. Human growth hormone was the first drug that was produced using plant bioreactors, in this case from the transgenic tobacco.

Here we discuss the different plant bioreactor expression systems especially with reference to where the protein compartmentalizes in these bioreactors.

Seed-based plant bioreactors

Plant seeds accumulate large amount of proteins during their development stage and therefore plant bioreactors based on seed platforms are reckoned as suitable for storing recombinant proteins.

An example is the successful expression of the human lysosomal enzyme alpha-L-iduronidase in Arabidopsis thaliana seeds. In seed bioreactors the expression of recombinant proteins is controlled using seed specific promoters like for example in maize globulin-1, and in rice glutelin promoter Gt-1. The advantage of these systems is that, proteins do not degrade at ambient temperature and are stable for long term storage.

However factors such as specificity of expression and subcellular storage environment would decide how specifically seeds could be used for producing desired molecules.

Seed Protein Storage Vacuole Bioreactors

In seed bioreactors, protein storage vacuoles comprising the sub compartments namely, matrix, globoid and crystalloid are dominant compartments for storing recombinant proteins. The matrix is suitable for soluble storage proteins, globoid for hydrolytic enzyme, and crystalloid for BP-80 TMD and the CT of alpha-tonoplast intrinsic protein sequences.

Seed Oil Body Bioreactors

Seed oil body is encircled by the protein oleosin which are ideal carriers for heterologous protein production and also provide a recognition signal for lipase binding during oil mobilization in seedlings. Seed oil body is bioreactors that can store large amount of macromolecules.

An example is the expression of fusion protein containing oleosin and the β-glucuronidase in seed oil body. Another example is the manufacture of the anticoagulant hirudin in the oil body of seeds Brassica napus and Brassica carinata.

Plant Suspension Cultures

They are used to express recombinant proteins, secondary metabolites and antibodies transported to subcellular organelles. For example, is the expression of 80-kDa human lysosomal protein (controlled by 35S CaMV and a signal peptide) in transgenic tobacco BY-2 cells culture media.

Hairy Root System Bioreactor

The Hairy Root System with its rhizosecretion is due to infection of the soil bacterium-Agrobaterium rhizogenes. It offers extreme biosynthetic stability and is suitable for making biopharmaceuticals as for example scopolamine in Hyoscyamus muticus L. hairy root culture

Chloroplast bioreactor

Insulin, interferons and other biopharmaceutical proteins can be made using Chloroplast bioreactor. One method is foreign genes are inserted into nuclear chromosomes and with peptides target expressed proteins into chloroplast. An example is the high yield in the expression of human serum albumin protein in chloroplast.

Comparative study of plant bioreactor systems

Cost wise, seed, hairy root, cultured cell suspension, oil body and chloroplast bioreactor systems are on the cheaper side to set-up and in terms of product stability seed and oil body systems offer the best prospects. In terms of scale-up capacity seed, oil body and chloroplast bioreactor systems are most suitable.

Advantages and Disadvantages

Generally the practice has been to use cloned animal cells to make antibodies for use as drugs. But there is always the remote chance of unwanted allergic reactions due to

antibodies of animal origin, and that apart, contamination of the antibody product due to proteins and viruses of animal origin is a distinct possibility. Such problems do not arise when plants are used to make antibodies because plants do not generally serve as hosts for human and animal pathogens.

Making recombinant proteins in transgenic plants is relatively cheap as compared to the cost of running fermentors although extraction and purification processes have to be efficient. For example these proteins are expressed in the plant seeds which all look similar and the recombinant protein has to be cleaved enzymatically or by other means. But these seeds can be stored and processed when required unlike animal cell production methods which require immediate purification.

Conclusion

More and more uses of plant bioreactors are coming up these days. For example, plant bioreactors are being investigated for making enzymes suitable for use in feed additives and in food. Another use of plants is to make genetically engineered plants that can produce seeds which can function as a delivery mechanism for various industrial enzymes.

As you can see these processes go far beyond the application of biotechnology in traditional agriculture, and so today, transgenic plants can produce on a mass scale proteins for agricultural, veterinary and pharmaceutical use. These processes are mainly carried out by cost conscious biopharma and enzyme companies.

Recombinant & subunit vaccine

Plant based vaccines are subunit vaccines in which the antigen of interest is expressed in plant tissues.

Molecular basis of photosynthesis

What is photosynthesis?

Photosynthesis is a biological process whereby the Sun’s energy is captured and stored by a series of events that convert the pure energy of light into the biochemical energy needed to power life. This remarkable process provides the foundation for essentially all life and has over geologic time profoundly altered the Earth itself. It provides all our food and most of our energy resources.

Perhaps the best way to appreciate the importance of photosynthesis is to examine the consequences of its absence. The catastrophic event that caused the extinction of the

dinosaurs and most other species 65 million years ago almost certainly exerted its major effect not from the force of the comet or asteroid impact itself, but from the massive quantities of dust ejected into the atmosphere. This dust blocked out the Sun and effectively shut down photosynthesis all over the Earth for a period of months or years. Even this relatively short interruption of photosynthesis, miniscule on the geological time scale, had catastrophic effects on the biosphere. Photosynthesis means literally “synthesis with light.” As such, it might be construed to include any process that involved synthesis of a new species under the action of light. However, that very broad definition might include a number of unrelated processes that we do not wish to include, so we will adopt a somewhat narrower definition of photosynthesis:

Photosynthesis is divided into two sets of reactions: the light-dependent (light) reactions and the light-independent (dark) reactions. As their names imply, the first set depends directly on light, whereas the second set does not. Nevertheless, even the dark reactions will cease if the plants are deprived of light for too long because they rely on the products of the light reactions.

The light reactions, which convert the energy in light into chemical energy, take place within the thylakoid membranes of the chloroplasts, whereas the dark reactions, which use that chemical energy to fix CO_2 into organic molecules, take place in the stroma of the chloroplast. In the light reactions, the energy of light is used to "split water," stripping a pair of electrons from it (and causing the two hydrogens to be lost), thus generating molecular oxygen. The energy in light is transferred to these electrons, and is then used to generate adenosine triphosphate (ATP) and the electron carrier NADPH. These two products carry the energy and electrons generated in the light reactions to the stroma, where they are used by the dark reactions to synthesize sugars from CO_2 .

Within the chloroplasts of higher plants and algae, photosynthesis converts light into biological energy, fueling the assimilation of atmospheric carbon dioxide into biologically useful molecules. Two major steps, photosynthetic electron transport and the Calvin-Benson cycle, require many gene products encoded from chloroplast as well as nuclear genomes. The expression of genes in both cellular compartments is highly dynamic and influenced by a diverse range of factors. Light is the primary environmental determinant of photosynthetic gene expression. Working through photoreceptors such as phytochrome, light regulates photosynthetic genes at transcriptional and posttranscriptional levels. Other processes that affect photosynthetic gene expression include photosynthetic activity, development, and biotic and abiotic stress. Anterograde (from nucleus to chloroplast) and retrograde (from chloroplast to nucleus) signaling

insures the highly coordinated expression of the many photosynthetic genes between these different compartments. Anterograde signaling incorporates nuclear-encoded transcriptional and posttranscriptional regulators, such as sigma factors and RNA-binding proteins, respectively. Retrograde signaling utilizes photosynthetic processes such as photosynthetic electron transport and redox signaling to influence the expression of photosynthetic genes in the nucleus. The basic C3 photosynthetic pathway serves as the default form used by most of the plant species on earth. High temperature and water stress associated with arid environments have led to the development of specialized C4 and CAM photosynthesis, which evolved as modifications of the basic default expression program. The goal of this article is to explain and summarize the many gene expression and regulatory processes that work together to support photosynthetic function in plants.

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Gene expression in the developing seed

The seed develops from the ovule and contains the embryo and endosperm, surrounded by the maternally derived seed coat. The function of the seed is to protect the embryo, to sense environmental conditions favorable to germination and to nourish the germinating seedling.

Fruits develop from organs of the flower and thus involve differentiation or redifferentiation of preexisting organs. Evolutionarily, floral organs represent modified leaves and so the fruit is also a modified leaf. Fruits serve 2 functions: to protect the seeds during development, and then to disperse the seeds following maturation.

Seed Development

All mature seeds contain an embryo and a protective covering called a seed coat (testa). In early development all angiosperm seeds also contain an endosperm, but in many seeds the endosperm is completely absorbed by the developing embryo. The embryo and endosperm are products of fertilization while the seed coat develops from the integuments of the ovule.

The seed coat contains a variety of adaptations related to protection and dispersal mechanisms. The seed coat usually forms a dry tissue. It may contain waxes for water impermeability, mucilage to make seeds sticky, compounds resistant to digestion by

animals, etc. In pomegranate, the seed coat forms the fleshy tissue that is consumed by humans. The seed coat often contains multiple layers with different characteristics.

Maternal tissues appear to have an important influence on seed development. An arabidopsis mutant called *aberrant testa shape (ats)* that lacks one of the 2 integuments also lacks several cell layers in the testa (3 layers vs. 5 normally). The seed are abnormally shaped in this mutant and seed shape shows maternal effect (ie. the genotype of the maternal parent determines the shape of the seed). Therefore, the seed coat and not the embryo determine the shape of the seed, and the embryo just grows to fill in the shape determined by the testa.

Another maternal gene called *FBP7* is specifically expressed in the ovule and seed coat and is required for normal ovule development. Downregulation of this gene in transgenic plants resulted in degeneration of the endosperm that was dependent on maternal genotype. This demonstrates the interaction between maternal tissues and those produced by fertilization.

The importance of global gene regulation

Several genes have been identified that negatively regulate seed development until fertilization has occurred. A mutant screen on a sterile line identified 3 genes that regulate seed development. Seeds develop on these mutants in the absence of fertilization. They are called *fis* for *fertilization independent seeds*. The genes appear important for control of seed development by fertilization. Several similar genes have been identified and cloned. They include:

FIE = fertilization independent endosperm, encodes a WD type POLYCOMB protein

MEDEA encodes a SET domain type POLYCOMB protein

FIS2 = fertilization independent seed2, encodes a zinc finger protein

POLYCOMB proteins are involved in chromatin structure and regulate (repress) the expression of genes in big portions of the genome. Therefore, the repression of large groups of genes is necessary to inhibit seed development until fertilization has occurred.

All three genes show parent-of-origin effects (imprinting). The maternally inherited gene is expressed and required but the paternally inherited gene is not expressed or required for seed development. (I.e. heterozygous mutants show 50% seed abortion, even when fertilized by wild type pollen [Luo, 2000 #167].

MEDEA and FIE proteins have been shown to interact by yeast 2-hybrid [Yadegari, 2000 #166].

Preparation for developmental arrest (seed/embryo maturation).

Most cell division is complete by the beginning of the maturation phase of embryo

development, but the embryo can increase in size up to 100 fold. This is by cell expansion and accompanies a massive accumulation of storage compounds. The major storage compounds are proteins, starch and lipids. These storage compounds are what give nutritional value to important crops such as cereals and beans. They are also valuable for other uses such as production of vegetable oil and starch which are used in a wide variety of ways ranging from cooking to industrial lubricants and plastics. Therefore there is a huge economic interest in seed storage compounds.

Accumulation of storage products

Storage proteins represent an important source of amino acids, nitrogen and carbon for the germinating seedling. Storage protein mRNAs represent up to 20% of the total mRNA found in a maturation phase embryo. They are synthesized on the RER and accumulate in the vacuole or as membrane bound vesicles called protein bodies. The storage proteins are encoded by several multigene families with up to 55 different genes coding for a given storage protein. Synthesis is controlled at the transcriptional level, with a few regulatory genes each controlling particular classes of storage proteins. An example is the *opaque2* gene of maize which codes for a transcription factor.

The regulation of starch and lipid accumulation, although no less important, is less well understood. These compounds are produced by complex enzymatic pathways. Each class of compound is a mixture of molecules with different chain lengths, chain branching characteristics, levels of saturation and other chemical modifications. Thus the synthesis of these compounds is much less straight forward than storage proteins.

Acquisition of dessication tolerance

At the end of embryonic development, most seeds dehydrate to about 5% moisture content. Such severe dehydration is lethal to most plant tissues and embryos express a developmental program that allows them to survive. Acquisition of dessication tolerance is part of the seed maturation program. Two problems faced by desiccated cells are high ionic concentrations and membrane stresses. At such low moisture levels, solutes would tend to crystallize and precipitate. Hydrophobic interactions with the aqueous solution are important for maintaining the integrity of the lipid bilayer. With no aqueous phase, the membrane becomes unstable and leaky.

A group of proteins called dehydrins are expressed in late maturation. The role for these proteins in desiccation tolerance is supported by their induction by drought stress in vegetative tissues and during desiccation of the resurrection plant, one of the few plants that can tolerate desiccation of postembryonic tissues. They are hypothesized to function in ion sequestration and in forming a protective layer for stabilizing membranes.

Coupling of morphogenetic and maturation programs

Morphogenesis and maturation appear to be controlled by independent developmental programs. Viviparous mutants fail to undergo the maturation program leading to seed dormancy but instead germinate directly. Morphogenesis in viviparous mutants is normal whereas other mutants arrested at various stages of morphogenesis undergo normal maturation as evidenced by the absence of necrosis following desiccation and the accumulation of storage proteins.

Integration of these programs involves both hormonal mechanisms and genetic programs. ABA is necessary to induce the expression of genes involved in maturation and desiccation tolerance. Viviparous mutants are either ABA deficient or insensitive. An ABA independent genetic program is also necessary to confer ABA sensitivity to the embryo and mutants in this program show ABA insensitive vivipary. The *LEC* gene, in which mutants both display seedling instead of embyro morphological characteristics and bypass embryo maturation are likely candidates for coordinating the two different programs.

Fruit Development

Contributions of different flower parts to the fruit

Most fruit develops from the ovary. In fact some schemes classify fruit derived from a single ovary as “true fruits” while “false fruits” are composed of tissues derived from flower parts other than the ovary or from more than one ovary.

In “true fruits” the outside of the fruit is called the pericarp and develops from the ovary wall. The pericarp can be dry and papery, like in maple or dandelions, woody like in nuts or fleshy as in berries (grapes and tomatoes) and stone fruits (cherries and peaches). These pericarp differences reflect adaptations to different dispersal mechanisms (eg. wind for papery pericarps, animal consumption for fleshy fruits). The fruit can contain a single seed as in corn, or many seeds like a pea pod or pumpkin. The pericarp of some fruits is further differentiated into specialized layers called exocarp, meso- and endocarp. For

example in citrus the rind is the exocarp, the white covering is the mesocarp and the juice sacs are the endocarp.

Many fruits we consider berries, such as raspberries and strawberries, are botanically not classified as berries. Raspberries are examples of aggregate fruits. Each juicy little sphere is actually an individual fruit of the same class as cherries, and what we consider as the fruit is really an aggregation of fruits.

Strawberries and apples are examples of accessory fruits, where some of the fleshy tissue is derived from flower parts other than the ovary. Strawberry fruits are actually what we consider the seeds. They are called achenes, which are dry fruits in the same category as dandelions. The fleshy part that we eat develops from the receptacle. Most of the fleshy tissue in apples develops from the hypanthium which is a region of the flower where sepals, petals and stamens are all fused to the ovary. Thus all floral organs contribute to the fleshy portion of apples.

Phases of fruit development

Fruit development can generally be considered to occur in four phases: fruit set, a period of rapid cell division, a cell expansion phase, and ripening/maturation.

Fruit set involves the decision whether to abort the ovary or proceed with fruit development. Fruit set is normally dependent on pollination. Pollen triggers fruit development indicating that positive signals are generated during pollination. In the absence of these signals, the flowers abscise. Growing pollen produces GA and application of GA can induce parthenocarpic fruit, therefore it is believed that GA is a triggering signal. Lagging slightly behind the growing pollen tube is a wave of increased auxin production by the style and then the ovary. Auxin application can also induce parthenocarpy and so it is thought that GA acts by inducing auxin production. However, most GA deficient mutants are able to produce fruit indicating that this is not the sole mechanism to induce fruit development and in an auxin insensitive tomato mutant, fruit growth is normal.

Continued fruit development usually relies on the continued presence of developing seeds. Seed abortion or removal causes fruit abortion, which can be reversed with auxin application. For example. removal of strawberry “seeds” prevents the development of the receptacle as a “fruit” but if auxin is applied following seed removal, fruit development continues. Commercial crops that produce parthenocarpic (seedless)

fruits, such as bananna, often show quantitaive or qualitative differences in GA or auxin content in the ovary when compared to nonparthenocarpic varieties.

The **phase of rapid cell division** involves all growing parts of the fruit. This is thought to be controlled by the developing seeds. The number of fertilized ovules in a fruit is correlated with both the initial cell division rate and the final size of the fruit. Also, fruits with an uneven distribution of seeds are often lopsided. There is a correlation between cytokinin levels in developing embryos and cell division in surrounding tissues but there is no direct evidence that embryo cytokinin in fact regulates fruit cell division. It is difficult to reconcile the complete development of parthenocarpic fruit with the requirement of embryos for cell division except to say that parthenocarpy represents an abnormal situation.

The cell division phase gradually shifts into the **cell expansion phase**. The rate and duration of cell division varies among fruits and also among tissues within a fruit. Tissues made up of many small cells at maturity continue dividing while tissues composed of large cells have begun expanding. In tomato the cell division phase lasts approximately 7-10 days while cell expansion lasts 6-7 weeks. Cell expansion accounts for the largest increase in fruit volume, often contributing in excess of a 100 fold size increase. Gibberellins are also associated with fruit expansion and removal of the seeds from pea pods inhibited GA biosynthesis in the pericarp. Many believe that auxins from seeds regulate cell expansion of the pericarp, but auxin application does not always compensate for seed removal, and in an auxin insensitive tomato mutant, fruit growth is normal.

Fruit ripening

Ripening represents the shift from the protective function to dispersal function of the fruit. Ripening occurs synchronously with seed and embryo maturation, as described in the lecture on embryo development. In dry fruits (cereals, nuts, dandelions) ripening consists of desiccation and is considered maturation. Ripening in fleshy fruits is designed to make the fruit appealing to animals that eat the fruit as a means for seed dispersal. Ripening involves the softening, increased juiciness and sweetness, and color changes of the fruit. Fleshy fruits are either climacteric or non-climacteric. Climacteric fruits produce a respirative burst with a concomitant burst in ethylene synthesis, as the fruits ripen. These include fruits with high degrees of flesh softening, like tomato, banana, avacado, peach etc.

Ripening has been most intensively studied in tomato. Ethylene is a major regulator of the ripening process. Inhibitioin of ethylene with inhibitors, transgenic approaches or

mutants blocks ripening. Exogenous ethylene accelerates ripening. There are also developmental factors involved because fruit does not attain competence to respond to ethylene until near the end of the cell expansion phase (the mature green stage). Several genes associated with ripening are ethylene inducible. This occurs transcriptionally in most genes but at least one is known where mRNA accumulation is regulated post-transcriptionally. None of these genes are induced until competence for ethylene response is attained.

The tomato *never-ripe* mutation blocks fruit ripening and is insensitive to ethylene. The mutated gene is similar to the ethylene receptor isolated from arabidopsis, suggesting that *never-ripe* is an ethylene receptor mutant. *NR* mRNA is not expressed until the mature green stage, suggesting that lack of this ethylene receptor might be related to the lack of competence to respond to ethylene at earlier stages.

Ethylene production is autocatalytic. That is, exposure to ethylene stimulates the synthesis of more ethylene. This occurs because the genes for the biosynthetic enzymes (e.g. ACC SYNTHASE) are ethylene inducible. The result is a positive feedback loop. Furthermore, the *Never-ripe* gene is ethylene inducible, resulting in a positive feedback loop for ethylene sensitivity as well. Both these factors contribute to the dramatic burst of ethylene production during ripening.

Fruit softening involves a partial breakdown of cell walls. Several enzymes are known to be involved in this process. Polygalacturonase hydrolyzes bonds in pectins. The gene for this enzyme is ethylene inducible.

Changes in fruit color involve changes in the expression of pigment biosynthetic genes. The major pigment in tomato is a carotenoid. The first committed step in carotenoid biosynthesis is catalyzed by phytoene synthase, and the gene for this enzyme is induced by ethylene.

Germination

Seeds have mechanisms to ensure germination occurs only under favorable environmental conditions for seedling growth. The primary factors are water availability and season. All seeds must imbibe water to germinate and for some this is the only requirement. Some also contain growth inhibitors that must be leached out of the seed. Some have impervious seed coats that must be fractured by freezing or passage through the digestive tract of an animal. Yet others have light or photoperiod requirements. All these mechanisms ensure the seeds germinate in the correct season and when moisture is available.

Arabidopsis seeds have certain requirements for germination, including a period of dormancy (which can be substituted for by cold treatment) and light (a phytochrome response). Mutations in a gene called *DAG1* (*Dof Affecting Germination1*) cause seeds that germinate in the dark without a dormancy period. Dof proteins are zinc finger transcription factors. The gene is expressed in the maternal tissues and all seeds of a mutant show this phenotype even if they result in pollination by a wild type (i.e. the embryo is wild type). Therefore, the maternal tissues during seed development control the dormancy behavior of the seed after being shed from the plant.

Upon imbibition, active metabolism resumes. Imbibed seeds contain high levels of GA. It is produced by the germinating embryo and stimulates the synthesis of hydrolytic enzymes by inducing the transcription of their genes. These enzymes appear after radicle elongation and are therefore postgerminative. The hydrolytic enzymes include proteases, amylases and lipases that break down storage compounds making building blocks available to the growing seedling. One enzyme of particular importance is a- amylase which cleaves starch into glucose and maltose molecules. This reaction is of economic importance to the malting industry and so the regulation of a-amylase gene expression has been carefully studied. It is transcriptionally induced by GA. Plants also contain a unique metabolic pathway called the glyoxylate cycle. This enables plants to convert fatty acids of the stored lipids into carbohydrates, specifically glucose and sucrose. In contrast, animals are unable to convert fatty acids to glucose.

GA and ABA act antagonistically to regulate the germination vs. maturation programs. ABA promotes maturation while GA promotes germination. As mentioned, ABA is necessary for seed maturation because ABA deficient mutants are viviparous and desiccation intolerant. Therefore, without ABA, seeds directly enter the germination program. Exogenous ABA can inhibit germination following dormancy. Conversely, promotes germination. GA is required for germination because GA deficient mutants are unable to germinate. Exogenous GA application to developing seeds can block maturation and induce vivipary. The VP1/ABI3 protein is a central regulator in these functions. This protein is a transcription factor that promotes the expression of maturation genes and inhibits the expression of germination genes. Mutants in this gene are ABA insensitive.

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Safety regulations on transgenic plants

Harmonisation of regulatory oversight of transgenic plants does not mean that all regulatory agencies must have identical legislation, information requirements, assessment processes and resulting decisions. Rather, it means that they seek to build consensus on the science that they use to assess the safety of the release of transgenic plants. Even though there may be differences in the outcomes of biosafety assessments because of the different environments into which transgenic plants may be released, there is recognition that much information (data) already available are relevant to transgenic plant biosafety assessment. The Organisation of Economic Cooperation Member countries, through the development of Consensus Documents, present this knowledge in a format that can expedite the safety assessment of transgenic plants. These documents can be considered as "mutual recognition of data," and are a means of moving towards harmonised regulatory decision making. To date the OECD Member countries are working on three types of Consensus Document: plant species biologies which focus specifically on centres of origin and diversity, and on related plant species with which that species can hybridise; general trait documents which focus on scientific issues arising from the development of such general traits as coat protein mediated virus resistance; and, specific trait documents which focus on the characteristics of specific genes and the resulting gene products that confer the novel trait to the transgenic plant.

The two main agencies identified for implementation of the rules on transgenic plants in India are the Ministry of Environment & Forests and the Department of Biotechnology, Government of India. The rules have also defined competent authorities and the composition of such authorities for handling of various aspects of the rules. There are six competent authorities as per the rules.

i. Recombinant DNA Advisory Committee (RDAC)
ii. Review Committee on Genetic Manipulation (RCGM)
iii. Genetic Engineering Approval Committee (GEAC)
iv. Institutional Biosafety Committees (IBSC)
v. State Biosafety Coordination Committees (SBCC)
vi. District Level Committees (DLC).

Out of these, the three agencies that are involved in approval of new transgenic crops are:

1. IBSC set-up at each institution for monitoring institute level research in genetically modified organisms.
2. RCGM set-up at DBT to monitor ongoing research activities in GMOs and small scale field trials.
3. GEAC in the Ministry of Environment and Forests set-up to authorize large-scale trials and environmental release of genetically modified organisms.

The Recombinant DNA Advisory Committee (RDAC) constituted by DBT takes note of developments in biotechnology at national and international level and prepares suitable recommendations. The State Biotechnology Coordination

Committees (SBCCs) set up in each state where research and application of GMOs are contemplated, coordinate the activities related to GMOs in the state with the central ministry. SBCCs have monitoring functions and therefore have got powers to inspect, investigate and to take punitive action in case of violations. Similarly, District Level Committees (DLCs) are constituted at district level to monitor the safety regulations in installations engaged in the use of GMOs in research and application.

The approvals and prohibitions under Rules 1989 are summarized below:

•No person shall import, export, transport, manufacture, process, use or sell any GMOs, substances or cells except with the approval of the GEAC.
•Use of pathogenic organisms or GMOs or cells for research purpose shall be allowed under the Notification, 1989 of the EPA, 1986.
•Any person operating or using GMOs for scale up or pilot operations shall have to obtain permission from GEAC.
•For purpose of education, experiments on GMOs IBSC can look after, as per the guidelines of the Government of India.
•Deliberate or unintentional release of GMOs not allowed.
•Production in which GMOs are generated or used shall not be commenced except with the approval of GEAC
•GEAC supervises the implementation of rules and guidelines.
•GEAC carries out supervision through SBCC, DLC or any authorized person.
•If orders are not complied, SBCC/DLC may take suitable measures at the expenses of the person who is responsible.
•In case of immediate interventions to prevent any damage, SBCC and DLC can take suitable measures and the expenses incurred will be recovered from the person responsible.
•All approvals shall be for a period of 4 years at first instance renewable for 2 years at a time.

GEAC shall have powers to revoke approvals in case of:

i. Any new information on harmful effects of GMOs.
ii. GMOs cause such damage to the environment as could not be envisaged when approval was given.
iii. Non-compliance of any conditions stipulated by GEAC.

Recombinant DNA guidelines, 1990:

With the advancement of research in biotechnology initiated by various Indian institutions and industry, Department of Biotechnology had formulated Recombinant DNA Guidelines in 1990. These guidelines were further revised in 1994 to cover R&D activities on GMOs, transgenic crops, large-scale production and deliberate release of GMOs, plants, animals and products into the environment, shipment and importation of GMOs for laboratory research.

For research, the guidelines have been classified into three categories, based on the level of the associated risk and requirement for the approval of competent authority.

•Category I activities include those experiments involving self cloning using strains and also inter-species cloning belonging to organism in the same exchanger group which are exempt for the purpose of intimation and approval of competent authority.
•Category II activities which require prior intimation of competent authority and include experiments falling under containment levels II, III and IV (details of each containment level provided separately in the guidelines).
•Category III activities that require review and approval of competent authority before commencement include experiments involving toxin gene cloning, cloning of genes for vaccine production, and other experiments as mentioned in the guidelines. The levels of risk and classification of the organisms within these categories have been defined in these guidelines. Appropriate practices, equipment and facilities necessary for safeguards in handling organisms, plants and animals in various risk groups have been recommended. The guidelines employ the concept of physical and biological containment and the principle of good laboratory practices. For containment facilities and biosafety practices, recommendations in the WHO laboratory safety

manual on genetic engineering techniques involving microorganisms of different risk groups have been incorporated therein.

The guidelines categorize experiments beyond 20 liters capacity for research and industrial purposes as large-scale. In case of cultivation of plants, this limits is 20 acres area. The guideline gives principles of occupational safety and hygiene for large-scale practice and containment. Safety criteria have also been defined in the guidelines. Physical containment conditions that should be ensured for large-scale experiments and production have been specified in the guidelines.

For release to the environment the guidelines specify appropriate containment facilities depending on the type of organisms handled and potential risks involved. The guidelines require the interested party to evaluate rDNA modified organism for potential risk prior to application in agriculture and environment like properties of the organism, possible interaction with other disease causing agents and the infected wild plant species. An independent review of potential risks should be conducted on a case-to-case basis. A copy of the guidelines can be accessed at http://www.dbtindia.nic.in

Guidelines for research in transgenic plants, 1998:

In 1998, DBT brought out separate guidelines for carrying out research in transgenic plants called the Revised Guidelines for Research in Transgenic Plants. These also include the guidelines fortoxicity and allergenicity of transgenic seeds, plants and plant parts.

These guidelines cover areas of recombinant DNA research on plants including the development of transgenic plants and their growth in soil for molecular and field evaluation. The guidelines also deal with import and shipment of genetically modified plants of research purposes. Genetic engineering experiments on plants have been grouped under three categories.

•Category I includes routine cloning of defined genes, defined non-coding stretches of DNA and open reading frames in defined genes in E. coli or other bacterial/fungal hosts which are generally considered as safe to human, animals and plants.

•Category II experiments include experiments carried out in lab and green house/net house using defined DNA fragments non-pathogenic to human and animals for genetic transformation of plants, both model species and crop species.

•Category III includes experiments having high risk where the escape of transgenic traits into the open environment could cause significant alterations in the biosphere, the ecosystem, plants and animals by dispersing new genetic traits the effects of which cannot be judged precisely. This also includes experiments having risks mentioned above conducted in green houses and open field conditions. To monitor the impact of transgenic plants on the environment over a period of time, a special Monitoring cum Evaluation Committee (MEC) has been set up by the RCGM. The committee undertakes field visits at the experimental sites and suggests remedial measures to adjust the trial design, if required, based on the on-the-spot situation. This committee also collects and reviews information on the comparative agronomic advantages of the transgenic plants and advises the RCGM on the risks and benefits from the use of transgenic plants under evaluation. The guidelines include complete design of a contained green house suitable for conducting research with transgenic plants. Besides, it provides the basis for generating food safety information on transgenic plants and plant parts. A copy of the guidelines can be accessed at http://www.dbtindia.nic.in

Seed policy 2002:

The Seed Policy 2002 contains a separate section (No. 6) on transgenic plant varieties. It has been stated that all genetically engineered crops/varieties will be tested for environment and

biosafety before their commercial release as per the regulations on guidelines of the EPA, 1986. Seeds of transgenic plant varieties for research purposes will be imported only through the National Bureau of Plant Genetic Resources (NBPGR) as per the EPA, 1986. Transgenic crops/varieties will be tested to determine their agronomic value for at least two seasons under the All India Coordinated Project Trials of ICAR, in coordination with the tests for environment and bio-safety clearance as per the EPA before any variety is commercially released in the market. After the transgenic plant variety is commercially released, its seed will be registered and marketed in the country as per the provisions of the Seeds Act.

After commercial release of a transgenic plant variety, its performance in the field, will be monitored for at least 3 to 5 years by the Ministry of Agriculture and State Departments of Agriculture.

It has also been mentioned that transgenic varieties can be protected under the PVP legislation in the same manner as non-transgenic varieties after their release for commercial cultivation. A copy of seed policy is placed in Annex-2. In addition the above, Ministry of Agriculture has issued a notification on November 12, 2003 nominating the Central Institute of Cotton Research (CICR) to act as a referral laboratory for Bt cotton seeds.

Safety assessment of transgenic crops

Commercial production of a transgenic crop is the culmination of a four step process. The first step begins in government or private research laboratories and greenhouses, where scientists investigate potential beneficial traits, identify genes and carry out genetic transformations. If these lab results are successful, the plant may advance to the second step i.e. open field trials, where breeding and testing continue in a real life environment. The third step is securing regulatory approval in the country where the plant will be grown, and/or its products consumed by humans or animals. The fourth and final step is market acceptance and large scale production.

Safety assessment of a transgenic crop is the most important step in this development process. Extensive testing and a long approval process precede every transgenic crop introduction. The approval process includes comprehensive analysis of the risks and their scientific management to ensure food, feed and environmental safety before introduction into the market.

Safety assessment of any transgenic crop starts with determining whether the product is substantially equivalent (except for defined differences) to conventional varieties. Further analysis then focuses on the evaluation of the defined differences by assessing potential safety risks of the host plan, gene donor(s) and the protein introduced.

Experiments are designed to systematically identify the hazards, to assess risks and to take steps to manage the risk by applying logical strategies. Information on the following aspects is required to be generated on a case-to-case basis:

i. Characteristics of the donor organisms providing the target gene such as identification, pathogenicity, toxicity and allergenicity, the geographical origin, distribution pattern and survival mechanisms and the method of transfer of its genetic material to other organisms.

ii. Characteristics of the vectors used such as the origin, identity and habitat, sequence, frequency of mobilization and the ability to get established in other hosts.

iii. Characteristics of the transgenic inserts such as the specific functions including the marker gene inserts, the expression levels and the toxicity of the expressed product on the host plant, humans or animals.

iv. Characteristics of the transgenic plants including methods of detection of the transgenic plant as well as the escaped transgenic traits in the environment, toxicity and pathogenicity of the transgenic plants and their seeds to other plants, human and animals, possibility of and the extent of transgenic pollen escape and pollen transfer to wild near relatives, and the impact on the environment
While information on some of these aspects may be available but many others need to be investigated using appropriately designed experiments.
Toxicity and allergenicity data are generated using standard protocols devised by national and international agencies. For minimizing the risk arising from the limited release of transgenic plants, the following may be taken into consideration:
i. Special separation for isolation, for preventing reproduction/ fertilisation and seed setting.
ii. Biological prevention of flowering by making use of sterility properties
iii. Human intervention for removal of reproductive structures of flowers.
iv. Controlling the reproductive structures of transgenic plants like the seeds and the plant propagules from unaccounted spread.
v. Controlling and destroying volunteer plants from experimental field.
vi. To take into account the proximity to human activity in case the transgenic plants have allergenic properties to human and animals.
vii. Appropriate training of field personnel handling the transgenic plants.
viii. Plans for handling unexpected events.
ix. Documentation of previous published information, if any, including any documented evidence of effects of the release to the ecosystem.

All the data generated by the developing organizations is then submitted in detailed formats to the government for seeking permission for commercial release of the transgenic crop. The initial risk assessment in India begins at the institutional level itself. The Institutional Biosafety Committee evaluates the proposal for research or commercialization following which it is passed on to Review Committee on Genetic Manipulation and then Genetic Engineering Approval Committee. At the commercialization phase, another round of assessment with respect to agronomic benefits is undertaken under the ICAR system. In fact, even after the release of the crop there is continuous monitoring by Monitoring and Evaluation Committees at the center and the state levels.

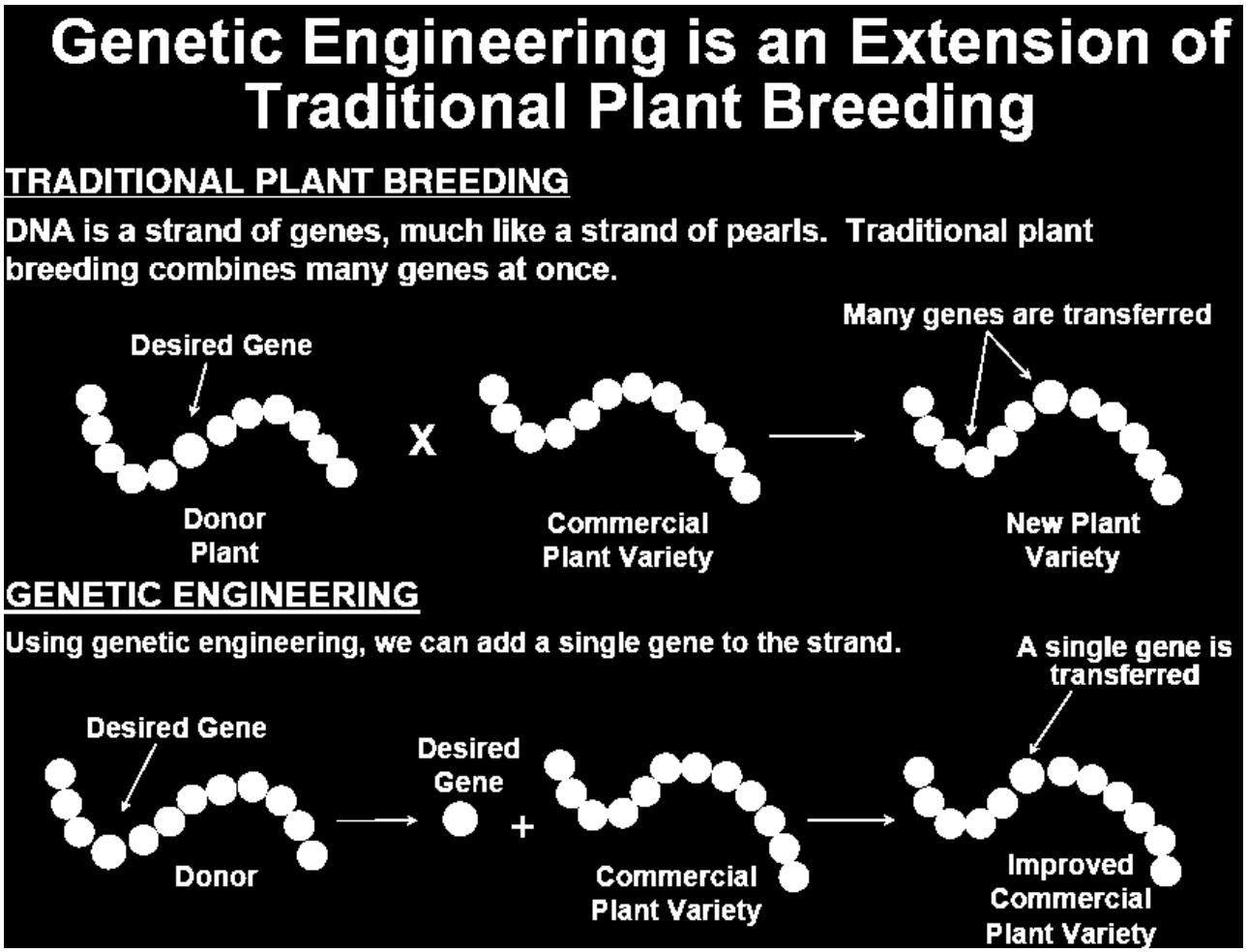
Biosafety

- Protecting human & animal health and environment from the possible adverse effects of the products of modern biotechnology
- Precautionary Approach is adopted for assessment of Biosafety

Why BiosafetyConcerns

- Classical plant breeding is limited to the introduction of required characters into plant by genetic crossing during sexual reproduction

- Due to limitations of conventional breeding for attaining the desirable traits use of modern biotechnology particularly genetic engineering/ recombinant DNA technology has been taken advantage of and development of transgenics started
- A transgenic crop is a crop which contains a gene or genes of a different species artificially inserted in its genome, which may come from an unrelated plant or from a completed different species.



Comparison of breeding and transgenic technology

Breeding	**Transgenic Technology**
Exchange of genes within a species	No barrier
Gene of interest with flanking sequence transferred	Only Gene of Interest
Simple Technology History of Safety	Intensive Technology New Technology Time needed for Safety History

Development of GM Crop/TransgenicsTransgenics

- Identify gene(s)-giving a desired trait
- Make copies of the gene
- Transfer to plant tissue
- Regenerate plants
- Lab analysis and safety testing
- Development of a variety
- Field tests
- Approval by Government agencies

- Commercialization
- Monitoring of efficacy and safety

GLOBAL SCENARIO

- First crop introduced was Flavr Savr tomato in USA in 1995
- So far 19 crops approved for commercial cultivation
- Include canola, carnation, chicory, cotton, linseed, green pepper, maize, melon, potato, rice, soybean, squash, sugarbeet, sunflower, tobacco and tomato
- Major characteristics are insect resistance, herbicide tolerance, virus resistance and improved product quality
- Major countries include USA, Canada, Japan, China, EU, Argentina, South Africa
- Only four crops being marketed commercially I.e., corn, cotton, soybean and canola
- Commercial production initiated for papaya, squash and tobacco in USA
- Others are approved but not yet being marketed
- Area under cultivation: 90 million hectares
- No. of countries: 21
- No. of farmers: 8.25 million
- Crops: Corn, soybean, cotton and canola
- Transgenic traits: insect resistance & herbicide tolerance

BIOSAFETY CONCERNS

- Environmental safety
- Food safety to human and animal health
- Risk management

Although the development of transgenic crops using recombinant DNA techniques is relatively recent, their applications are increasing rapidly because of advantages over the conventional crops. However, as more and more transgenic crops are released for field-testing and commercialization, concerns have been expressed regarding potential risks to both human health and environment. These apprehensions arise because transgenic technology crosses the species barrier as compared to classical selection techniques, thereby permitting the gene transfer among microorganisms, plants and animals. There is no evidence that any unique hazards exist in the development of transgenic crops, because of novel combinations of genes.

Transgenic crops are not toxic nor are likely to proliferate in the environment. However, specific crops may be harmful by virtue of novel combinations of traits they possess. This means that the concerns associated with use of GMOs can differ greatly depending on the particular gene- organism combination and therefore a case-by-case approach is required for risk assessment and management. Potential risks from the use of transgeniccrops broadly fall under two categories.

i. Human health

ii. Environment

Risk to human health:

Risks to human health are related mainly to toxicity, allergenicity and antibiotic resistance. The risk of toxicity may be directly related to the nature of the product whose synthesis is controlled by the transgene or the changes in the metabolism and the composition of the organisms

resulting from gene transfer. Most of the toxicity risks can be assessed using scientific methods both qualitatively and quantitatively.

The introduction of newer proteins in transgenic crops from the organisms, which have not been consumed as foods, sometimes has the risk of these proteins becoming allergens. However, it may be noted that there is no evidence that transgenic crops pose more risks than conventional products in triggering allergies. Further, the new transgenic crops can be tested for allergens prior to their commercial release. For example, when it was found that the consumption of transgenic soybean with a methionine-producing gene from brazil nut could trigger an allergic response in those allergic to brazil nut, the product was not released for sale. The use of genes for antibiotic resistance as selectable markers has also raised concerns regarding the transfer of such genes to microorganisms and thereby aggravate the health problems due to antibiotic resistance in the disease causing organisms. Although, the probability of such transfer is extremely rare, steps are being taken to reduce this risk by phasing out their use.

Allergenicity

The possibility that we might see an increase in the number of allergic reactions to food as a result of genetic engineering has a powerful emotional appeal because many of us experienced this problem before the advent of transgenic crops, or know of someone who did.

However, there is no evidence so far that genetically engineered foods are more likely to cause allergic reactions than are conventional foods. Tests of several dozen transgenic foods for allergenicity have uncovered only a soybean that was never marketed and the now-famous StarLink corn. Although the preliminary finding is that StarLink corn is probably not allergenic, the scientific debate continues. Every year some people discover that they have developed an allergy to a common food such as wheat or eggs, and some people may develop allergies to transgenic foods in the future, but there is no evidence that transgenic foods pose more of a risk than conventional foods do.

Horizontal transfer and antibiotic resistance

The use of antibiotic resistance markers in the development of transgenic crops has raised concerns about whether transgenic foods will play a part in our loss of ability to treat illnesses with antibiotic drugs. At several stages of the laboratory process, developers of transgenic crops use DNA that codes for resistance to certain antibiotics, and this DNA becomes a permanent feature of the final product although it serves no purpose beyond the laboratory stage. Will transgenic foods contribute to the existing problems with antibiotic resistance?

One aspect of this topic is the risk of horizontal gene transfer, that is, transfer of DNA from one organism to another outside of the parent-to-offspring channel. Transfer of a resistance gene from transgenic food to micro-organisms that normally inhabit our stomach and intestines, or to bacteria that we ingest along with food, could help those micro-organisms to survive an oral dose of antibiotic medicine. Although horizontal transfer of DNA does occur under natural circumstances and under laboratory conditions, it is probably quite rare in the acid environment of the human stomach.

Another concern is that the enzyme product of the DNA might be produced at low levels in transgenic plant cells. While high processing temperatures would inactivate the enzyme in processed foods, ingestion of fresh or raw transgenic foods could result in the stomach containing a small amount of an enzyme that inactivates an orally administered dose of the antibiotic. This issue was raised during the approval processes for Calgene's FlavrSavr tomato and Ciba-Geigy's Bt corn 176. In both cases, tests showed that orally administered antibiotics would remain effective. While

the risks from antibiotic resistance genes in transgenic plants appear to be low, steps are being taken to reduce the risk and to phase out their use.

Eating foreign DNA

When scientists make a transgenic plant, they insert pieces of DNA that did not originally occur in that plant. Often these pieces of DNA come from entirely different species, such as viruses and bacteria. Is there any danger from eating this "foreign" DNA?

We eat DNA every time we eat a meal. DNA is the blueprint for life and all living things contain DNA in many of their cells. What happens to this DNA? Most of it is broken down into more basic molecules when we digest a meal. A small amount is not broken down and is either absorbed into the blood stream or excreted in the feces. We suspect that the body's normal defense system eventually destroys this DNA. Further research in this area would help to determine exactly how humans have managed to eat DNA for thousands of years without noticing any effects from the tiny bits that sneak into the bloodstream.

So far there is no evidence that DNA from transgenic crops is more dangerous to us than DNA from the conventional crops, animals, and their attendant micro-organisms that we have been eating all our lives.

Changed nutrient levels

How do genetically engineered foods compare with conventional foods in nutritional quality? This is an important issue, and one for which there will probably be much research in the future, as crops that are engineered specifically for improved nutritional quality are marketed. However, there have been only a few studies to date comparing the nutritional quality of genetically modified foods to their unmodified counterparts.

The central question for GE crops that are currently available is whether plant breeders have accidentally changed the nutritional components that we associate with conventional cultivars of a crop. Because isoflavones are thought to play a role in preventing heart disease, breast cancer, and osteoporosis, the isoflavone content of RoundupReady soybeans has been investigated by several researchers.

The studies completed so far do not resolve the issue of whether RoundupReady soybeans have isoflavone levels comparable to conventional varieties, but the differences found in experiments appear to be small or moderate in comparison with natural variation in isoflavone levels. Additional evidence may clarify the arguments for and against Roundup applications as a risk factor in soybean cultivation.

Industry studies submitted in support of applications for permission to sell transgenic crops indicate that the nutritional components that are commonly tested are similar in transgenic foods and conventional foods.

Risk to environment:

Crop-to-weed gene flow

Hybridization of crops with nearby weeds may enable weeds to acquire traits we wish they didn't have, such as resistance to herbicides. Research results indicate that crop traits may escape from cultivation and persist for many years in wild populations. Genes that provide a competitive edge, such as resistance to viral disease, could benefit weed populations around a crop field.

Many cultivated crops have sexually compatible wild relatives with which they hybridize under favorable circumstances. The likelihood that transgenes will spread can be different for each crop in each area of the world.

For example, there are no wild relatives of corn in the United States or in Europe for transgenic corn to pollinate, but such wild relatives exist in Mexico.

Soybeans and wheat are self-pollinating crops, so the risk of transgenic pollen moving to nearby weeds is small. However, that small risk must balanced against the fact that there are wild relatives of wheat in the United States.

There are no wild relatives of soybean in the United States, but such wild relatives exist in China. Thus each crop must be evaluated individually for the risk of gene flow in the area where it will be grown.

Antibiotic resistance

There is also concern that transgenic plants growing in the field will transfer their antibiotic resistance genes to soil micro-organisms, thus causing a general increase in the level of antibiotic resistance in the environment. However, many soil organisms have naturally occurring resistance as a defense against other organisms that generate antibiotics, so genes contributed occasionally by transgenic plants are unlikely to cause a change in the existing level of antibiotic resistance in the environment.

Leakage of GM proteins into soil

Many plants leak chemical compounds into the soil through their roots. There are concerns that transgenic plants may leak different compounds than conventional plants do, as an unintended consequence of their changed DNA.

Speculation that this may be happening leads to concern about whether the communities of micro-organisms living near transgenic plants may be affected. The interaction between plants and soil micro-organisms is very complex, with the micro-organisms that live around plant roots also leaking chemical compounds into the soil. Much more research must be done before we understand the relationships that occur between micro-organisms and conventional crops. Attempts to discover whether transgenic plants are changing the soil environment, and whether they are changing it in good ways or bad ways, are hindered by our lack of basic scientific knowledge.

Reductions in pesticide spraying: are they real?

One of the most appealing arguments in favor of transgenic plants is the potential for reducing the damage we do to our environment with conventional methods of farming. Pest- resistant crops such as Bt corn and Bt cotton have been promoted as a means to reduce the spraying of pesticides, while herbicide-tolerant crops such as RoundupReady soybeans are said to reduce the application of herbicides. Large reductions in chemical spraying have been claimed to result from the introduction of these transgenic varieties. Are the claims true?

Bt cotton is the only crop for which claims of reduced spraying are clear. Analysts paint a mixed picture on the results of planting RoundupReady soybeans. Bt corn and herbicide-tolerant cotton and corn have not resulted in clear reductions in the spraying of chemicals.

Concerns about damage to current farming practices

Crop-to-crop gene flow

Hybridization of transgenic crops with nearby conventional crops raises concerns about separation distances to ensure purity of crops and about who must pay if unwanted genes move into a neighbor's crop. As "Identity Preservation" and segregation of GM from non-GM crops become factors in marketing products, it will be important to ensure that hybridization is not occurring in the field.

If GM pollen pollinates plants in a neighboring field, then the issue of genetic trespass may arise. What level of GM presence, if any, should be allowed in products that are sold as organic or conventional? Should GM farmers and companies bear responsibility for preventing gene flow, or should conventional and organic farmers pay to protect their products from gene flow? Should GM versions of outcrossing plants be banned as too risky, while GM versions of self-pollinating plants

are permitted? These issues have already prompted several lawsuits and they will continue to be a factor in the development and use of trangenic plants for years to come.

Global status of transgenic crops

Crops approved for commercial use:

The first commercial transgenic crop was "Flavr Savr" tomato with delayed ripening characteristics introduced in USA in 1995. Seventeen crops have so far been approved in various countries for planting in various countries across the world incorporating one or more of the basic phenotypical characteristics such as herbicide tolerance, insect resistance, male sterility, modified colour, delayed ripening and virus resistance. The following table lists these products along with the genetically improved trait and countries where they have been approved.

S. No.	Crop	Uses	Countries where approved
5.	**Flax, Linseed**	Herbicide tolerance, antibiotic resistance and improved weed protection	Canada, US
6.	**Green pepper**	Virus resistance	China
7.	**Maize**	Herbicide tolerance, improved weed protection, resistance against insects and restored fertility of seeds	Canada, Japan, US, Argentina, European Union, South Africa, Philippines,
8.	**Melon**	Delayed ripening	
9.	**Polish Canola**	Herbicide tolerance and improved weed control	Canada
10.	**Potato**	Improved protection from insect and leaf roll virus	US, Canada,
11.	**Rice**	Herbicide resistance	US
12.	**Soybean**	Improved weed control and herbicide tolerance, increased cooking quality	US, Argentina, Japan, Canada, Uruguay, Mexico, Brazil and South Africa
13.	**Squash**	Resistance against watermelon mosaic virus and zucchini yellow mosaic virus	US
14.	**Sugar beet**	Herbicide tolerance	US, Canada
15.	**Sunflower**	Herbicide tolerance	Canada
16.	**Tobacco**	Herbicide tolerance	US
19.	**Tomato**	Improved shelf life, taste, color and texture, improved insect resistance, virus resistance	US, Mexico, Japan, China

Source: http://www.agbios.com/

IPR and Bioethics

What is bioethics?

Ethics is the discipline concerned with what is good or bad, right or wrong. It has theoretical and practical aspects. Ethics seeks to establish norms or standards of conduct (normative ethics), and to analyze the basis of judgments about what is right and wrong (descriptive ethics). Applied or practical ethics is the application of theoretical ethical tools and ethical norms to address actual

moral choices. Bioethics deals with the ethical implications of biological research, and the biological and medical applications of research. Specific bioethics issues arise in debates over the dignity of the human being, beginning-of-life and end-of-life issues, consent to medical treatment, freedom of research, the consent of the donor of human genetic material, access to health care and distribution of health resources, and equitable access to the outcomes of biological research, as well as animal protection and environmental ethics.

Ethics versus morality

'Ethics' and 'morality' are often used interchangeably, but they do have different aspects. For instance, practical ethics aims to guide right behavior; 'morality' refers to the underlying moral values that are used to assess what is right and wrong. In the field of IP, some patent laws refer to inventions the exploitation of which would be contrary to order public or morality, and some trademark laws refer to trademarks that are contrary to morality. In this sense, ' morality' could refer to the shared values of a community, values that might differ from one community to another.

Law versus ethics

Law and ethics are closely interrelated, but they are not the same thing. Some acts that are legal might be considered unethical. As a simple example, it is normally unethical to tell a lie, but only in some circumstances is it a true crime. There can be strong commonality and consistency between the law of human rights, and ethical norms and expectations, but it would actually reduce the legal effect and status of human rights law to regard it as giving ethical guidance only. Sometimes legislators choose not to pass laws on certain issues, as a conscious choice to allow communities' ethical considerations to govern behavior, instead of legal rules. Certain forms of stem cell research may not actually break the law of a particular country, but some might still argue that it is unethical.

What is intellectual property protection?

Intellectual property refers to legal rights resulting from intellectual activity in the industrial, scientific, literary and artistic fields. IP systems protect certain well-defined subject matter by giving limited entitlements to eligible right holders to exclude others from certain uses of the protected material. But an
IP right does not give the holder the entitlement to use or market a product.

IP rights are normally created, administered and exercised separately under the national jurisdiction of each country. Their legal effect is restricted to the territory of the states where they are granted. Several international treaties lay down general legal and administrative standards. But these international standards need to be implemented through national laws and may be applied in diverse ways.

Some questions of potential interest to the bioethics community are barely dealt with at international level at all, but are left to national or regional authorities to determine. These include the definition of the core concept of 'invention', and the notions of 'morality' and 'order public' that should apply in the interpretation and administration of patent law.

Patents protect eligible inventions, including some forms of biotechnological invention (the exact scope of protectable inventions varies from one national system to another). The patent is the form of IP most pertinent to biotechnology, and most often discussed in the context of bioethics. But a number of other forms of intellectual property may also be considered relevant, for instance:

- Plant breeders rights or plant variety rights systems give IP rights over new plant varieties, generally with an exception for further breeding.
- Copyright and sui generis database rights may have ethical implications for access to genetic information.

- Trademarks may help ensure honest commercial practices, for instance with regard to counterfeit medicines.
- Law of confidentiality and the protection of undisclosed information may have ethical implications, for instance concerning obligations to protect individual genetic information.
- Bioethics issues concerning clinical trials and informed consent questions may be relevant to the protection of test data concerning the safety and efficacy of chemical entities, because of the public interest role of this information, and concerns about duplication of trials involving human or mammal subjects.
- Within the domain of unfair competition, international IP law includes a general requirement to suppress behavior 'contrary to honest commercial practices.'

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