Studies on Genetic Transformation in *Arachis hypogea*

A dissertation submitted in
Partial fulfillment of the requirements for the degree of
Master of Science in
Biotechnology

By

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CERTIFICATE

This is to certify that the dissertation entitled "**Studies on Genetic Transformation in *Arachis hypogea***" submitted in partial fulfillment for the award of Master of Science in Biotechnology to Swami Ramanand Teerth Marthawada University , is a bonafide work carried by **Ms.B.Sandhya** under our guidance and supervision.

The results embodied in this dissertation have not been submitted to any other university or institution for the award of any degree or diploma.

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Studies On

Genetic Transformation

In

Arachis Hypogea

Glossary of Abbreviations

ABA.....(±)-cis, trans-Abscisic Acid

DNA.....Deoxyribonucleic Acid

EDTA.....Ethylene diamine tetracetic acid

IAA.....Indole-3-Acetic Acid

MPB.....Microprojectile Particle Bombardment

MS.....Murashige and Skoog

NAA.....α -Naphthaleneacetic acid

PIG.....Particle Inflow Gun

Tween 20.....Polyoxyethylenesorbitan Monolaurate

X-GLUC.....5-Bromo-4-Chloro-3-Indoyl-β-D-Glucuronide

2,4-D.....2,4-Dichlorophenoxyacetic Acid

EXECUTIVE SUMMARY

Crop improvement through conventional breeding is limited to the genetic variability available through outcrossing within species and among related, sexually compatible species. Because of these biological limitations, increases in yield and productivity can not be sustained indefinitely without new sources of genetic variability. To meet the needs for future generations, emerging technologies such as tissue culture and plant genetic engineering are beginning to overcome the limitations faced by conventional plant breeding.

Genetic engineering is the manipulation of the host organism by introducing foreign DNA. Plant genetic engineering has progressed at a rapid rate in the past decade, with transformation systems having been developed for many different plant species. Several techniques are available for transferring foreign DNA to plant cells. Some of these techniques are species and genotype independent.

The main barrier to obtaining transgenic plants is the lack of reliable regeneration methods. Control of gene expression once integration has occurred is another important issue. Apart from the standardised in-vitro regeneration system, comparative studies of the available transformation techniques contribute lot in refining protocols for transgenic plant production. The primary aim of this dissertation work is understanding the basic principles, methodologies of tissue culture and genetic transformation to carry out *Agrobacterium* mediated and particle bombardment based gene transfer to compare the efficiency of these systems.

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Introduction

INTRODUCTION

The cultivated groundnut (*Arachis hypogea* L.) is an important food legume known for its high protein and oil content (Cobb and Johnson; 1973), high energy value (Pattee et al, 1974) and adaptability to wide range of ecological conditions. It is a native of Brazil and is cultivated in the tropical and subtropical as well as warm temperate zones. Groundnut is self pollinating, relatively photoinsensitive and is an excellent source of some essential nutrients such as carbohydrates, trace elements, and vitamins. Its cultivation improves soil fertility contributing to the ecological sustainability since it fixes atmospheric nitrogen with the help of bacteria in root nodules. In addition, it is grown for forage and to control soil erosion in hilly areas. The cultivated peanut however, is susceptible to several diseases and insect pests which cause tremendous loses in crop yields. Efforts aimed at improving the quality of groundnut by employing traditional plant breeding technique have met with some initial success. Further genetic manipulation is required to incorporate desirable characters like higher oil content and quality, resistance to drought, insect pests, viral diseases and fungal pathogens found in some wild species into commercially grown varieties. However, all these traits are not adequately available in the wild and cultivated germplasm. The development of gene transfer technology has significantly increased the accessible gene pool for crop improvement. The ability to introduce foreign DNA into plant cells has been one of the most significant advances in the plant biotechnology. Various biotechnological tools including marker assisted selection and gene transfer across the species barrier has opened up novel opportunities for enhancing the germplasm base of high yielding varieties.

The application of genetic engineering principles to improve groundnut depends upon 1) an efficient system for plant regeneration from groundnut cells and tissues 2) a method to deliver genes with high frequencies into groundnut and 3) genetic constructs with promoters and reporter genes allowing detection of transient expression and stable transformation in peanut cells. Our current ability to insert foreign genes into plant cells and tissue to regenerate viable and fertile novel plants has provided unique opportunity to plant breeders. This allows for explosive expansion of our understanding in the field of plant biology and provides us with the technology to modify and improve crop plants in many ways.

The most widely used technique for the transfer of desired genes into crop plants is genetic transformation using *Agrobacterium tumefaciens*, a host pathogenic soil bacterium. Many plant species especially dicots have been successfully transformed using this method. However many monocots remain recalcitrant to "*Agrobacterium*" technique. Recently developed technique of plant transformation such as "Particle gun or Biolistic" have solved the major problem of incompatibility between the technique used and the crop plant transformed. This process is based on accelerating high velocity microprojectiles coated with desired genes (foreign DNA) into the target plant tissue. The transformed plant tissue is later selected for the presence of foreign DNA and regenerated using tissue culture techniques. The Biolistic technique has demonstrated broad utility and appears to be applicable for most plant species, which are difficult to transform using "*Agrobacterium*" technique. Several other methods like chemically stimulated DNA uptake by protoplasts, microinjection and macroinjection, liposome mediated gene transfer, calcium phosphate precipitation method, transformation using pollen or pollen tube and transformation by ultrasonication are also used.

In the present work, major emphasis was on the developmental aspects of tissue culture and genetic transformation techniques such as *Agrobacterium*-mediated transformation and biolistics. Young leaves and embryonic explants of groundnut (cultivar JL24) were transformed with *Agrobacterium* strain C58 containing the binary plasmid. Biolistic-based transformation using young leaves and somatic embryos by isolated plasmid DNA containing *NPTII* (Neomycin phospho transferaseII) and *GUS* (β-glucoronidase) as a reporter genes.

Objectives

OBJECTIVES

(1) To develop the best regeneration system via organogenesis and somatic embryogenesis for carrying out genetic transformation.

(2) To carry out *Agrobacterium* tumefaciens-mediated and Biolistic based transformation in the groundnut variety J1.24.

(3) To compare the efficiency of the above two transformation systems

Review Of Literature

REVIEW OF LITERATURE

Techniques for selecting and upgrading plant products have been refined over centuries of human history. The genetic manipulation of crop species involves the creation of new allelic combinations, whether by recombination of existing allelic variation within the species, or by introduction of novel alleles of 'new' genes from outside of the species. After the rediscovery of Mendel's laws of genetics in the first decade of twentieth century (Borlaug, 1983) plant breeding became a science based endeavour. In essence, the technologies to achieve this are already developed or are presently, in an advanced state of development. First, the technologies for intra and inter specific, sexual hybridisation are used to generate new allelic combinations, and hence phenotypic variability, by exploiting the process of random reassortment of chromosomes and genetic recombination within chromosomes. Such techniques are in practical terms, operationally simple and have been the 'standard fare' of crop improvement programs for many years. Various forms of mutagenesis can also be used to generate additional variability. But there are certain limitations of this technique due to barrier to sexual compatibility and availability of limited genetic pool for crop improvement. Now "Gene revolution" has emerged that holds the promise of introducing crop improvement at the molecular level producing plants with far greater range of genetic variability.

This present era is biotechnology-based agricultural science where in the 'foreign' genes are introduced to produce highly variable transgenic plants of high quality. The manipulation of plant genes includes isolation, identification and analysis of structure and regulation of beneficial genes, followed by their transfer into plants. The discovery of DNA structure (1953), plasmid (1959), restriction enzymes (1970), plant regeneration (1970), recombinant DNA technology (1971), and Ti plasmid (1974)

collectively made the genetic transformation and the engineering of first transgenic plant (1983) possible.

Tissue culture

In tissue culture systems for plant transformation, accessibility of regenerable cells that are amenable to the gene transfer techniques, and that retain the capacity for regeneration into whole plant makes it an ideal system. Without appropriate plant regeneration systems, the generation of genetic variable plants, commercial cloning for the rapid multiplication of desirable or difficult to propagate species would be impossible (Tissue culture technology; George Morel *et al)*

3.0.1 Cellular totipotency

Unlike animal cells, in plants even highly mature and differentiated cells retain the ability to regress to the meristematic state as long as they have an intact membrane system and viable nucleus. This inherent capacity of the plant cell to give rise to a whole plant is called cellular totipotency.

3.1 Modes of plant regeneration

The term plant tissue culture has generated into an all encompassing, convenient term to describe all types of sterile plant culture procedures pertaining to the growth of plant protoplasts, cells, tissues, organs, embryos and platelets. Plant regeneration through tissue culture is used here to describe the production of plants in sterile culture. Plant material is taken from its normal growing (in vivo) situation, and cultured invitro (meaning literally, 'in glass'). When plant material comes out of culture it is moved into ex vitro ('out side' glass) environment and it once again become adapted to normal invivo growth. The invitro multiplication of plants is called micropropogation.

Plant regeneration through tissue culture can be accomplished using one of the following three methods:

(1) Embryo culture

(2) Somatic embryogenesis

(3) Organogenesis

3.1.1 Embryo culture

Embryo culture is the aseptic culture of zygotic embryo. The embryo is excised from the seed or the ovule and planted on a substitute endosperm environment (*i.e* nutrient medium). Subsequent embryo development and germination occurs as it would from the seed.

3.1.2 Somatic embryogenesis

Somatic or asexual embryogenesis is the production of embryo-like structures from the somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin. Such embryos can further develop and germinate into plants through the events that correspond with zygotic stages. Production of somatic embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct mode of somatic embryogenesis involves the formation of an asexual embryo from a single cell and group of cells on a part of the explant tissue without an intervening callus phase. The indirect mode of embryogenesis consists of establishing an explant in culture, subsequent proliferation of callus and initiation of proembroys on medium containing high concentration of auxins and transfer of callus to nutrient medium devoid of growth regulators in order to induce bipolar embryo formation from pro-embryo initials. When conditions are suitable these embryos germinate to produce plants. Generally, only a small percentage of the cells of the explant contribute to the formation of the callus. These cells are usually located on the surface layers or are in physical contact with the nutrient medium. The

callus is heterogeneous in nature and may be composed of numerous cell types including pro-embryo initials, single cells or multicellular groups. When the callus is transferred to medium containing low auxins levels, further embryogenesis occurs to give rise to more pro-embryos, pro-embryo initials develop into bipolar embryos, in a non-synchronised fashion.

3.1.3 Organogenesis

Plant development through organogenesis is the formation and outgrowths of shoots from callus or initiation and outgrowth of axillary buds generated from cultured tips, followed by adventitious rooting. The shoot is a unipolar structure and is physically connected to the tissue of origin. Occasionally, roots may give rise to shoots (Dixon *et al)*. The production of adventitious shoots in vitro is more common and easier to control. The production through organogenesis can be achieved through one of the following three modes:

-Production of adventitious organs directly from explant without an intervening callus phase

-Emergence of adventitious organs from a callus derived from the explant

-Production of plantlets from outgrowth of axillary buds

Direct Organogenesis

Adventitious shoots, which arise directly from the tissues of the explant, can provide a reliable method for micropropogation. Induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived, and is highly dependent on plant genotype. In this process the explant is established on the nutrient medium containing moderate levels of auxins and cytokinins (to avoid callus production) and subsequently initiates shoot buds. Multiplication is achieved through subculture of the shooting clump and planting out in separate vessels.

Indirect Organogenesis

Propagation by indirect organogenesis carries a risk that the regenerated plants will differ genetically from each other and from the stock plant. Only a small percentage of the explant's cells divide in-vitro to give rise to the organogenic callus. Again, the majority of these cells are usually located on the surface of the explant or those in contact with the nutrient medium. A high concentration of auxin and low concentration of cytokinin promotes callusing. Callus production on an agar medium can also be induced by the inclusion of an auxin alone. However, addition of cytokinins to the medium invariably enhances callus proliferation. Typically the cellular composition of the callus has been found to be quite heterogeneous consisting of wide variety of cell sizes, types and groups. Often, tannin bearing, secretory and lignified cells develop as the callus ages. The occurrences of meristematically active individual or group of cells in the callus is common. These cells are the precursors of root and shoot organ. The ploidy levels of cells in callus may vary tremendously (Vasil *et al).*

3.2 Initiating tissue cultures

3.2.1 Explants

The term explant is used to describe the initial piece of the plant introduced in vitro. Explant selection plays a major role in successful plant regeneration studies. The part of the plant from which explants are obtained depends on:

- The kind of the culture to be initiated
- The purpose of the proposed culture
- The plant species to be used.

For optimum success, explants must be obtained from healthy vigorous plants. Practically any part of the plant can be successfully cultured invitro and can regenerate plantlets provided the explant is obtained at proper

physiological stage of development of donar plant. Immature tissues and organs are invariably more morphogenetically plastic in-vitro than mature tissues and organs. Furthermore, meristematic tissues or organs should be selected in preference to other tissue sources because of their clonal properties, culture survival, and growth rates and totipotentiality invitro.

Choice of explant

Following explant selection disinfection of tissues is necessary in order to eradicate surface microorganisms. The presence of any contaminant will interfere with the growth of explants or cultures. Fungal and bacterial explant contaminations in plant cultures are usually detectable 1-14 days after planting. Contaminated cultures should be discarded immediately since their presence only leads to further air-borne contamination of the culture room. Prior washing of the explant with soap and water or dipping in ethanol is recommended to induce adequate wetting and initial cleaning. Most commonly a dilute solution of sodium hypochlorite (0.25-2.63%) is used as a disinfectant. An emulsifier such as Tween-20 (polyoxyethylene sorbitan monolaurate) is added at the rate of 1 drop per 100 ml of solution. Mechanical agitation using a stirrer and/or application of vacuum is sometimes helpful to dislodge air bubbles and facilitate an even distribution of the disinfectant over the explant.

3.2.2 MEDIA

For successful aseptic culture of isolated plant cells and tissues on artificial media be it liquid or agar in sterile containers like test tubes or flasks, the nutritional and hormonal requirements have almost been perfected over the last two to three decades, based on the nutritional needs of whole plants. The composition of the medium is adapted to the cultural needs and the objective of the experimental studies of regeneration, micropropogation,

cytodifferentiation, experimental androgenesis, biosynthesis of secondary metabolites or biotransformation of cells.

Several media have been formulated and modified from time to time to suit nutrition of particular tissues whether it be isolated cells or somatic embryos.

A complete medium necessarily should contain following components:

1.Macro / major inorganic nutrients
2.Micro / trace elements
3.Iron source
4.Sugar / carbon source
5.Organic supplement (vitamins)
6.Amino acid supplement

The most commonly used media are Murashige and Skoog, Whites, Schenk and Hildebrandt, Hellers and Lismaier media (George et al)

Choice of media

A large number of different media formulations are available in the literature, and before deciding on the formulation of a medium suitable for a specific purpose, it may be helpful to consult one of the larger texts which lists culture requirements for a variety of different species. If no information is available in the literature, start with a widely used standard medium such as MS, SH or Gamborg's B5, and then experiment by varying the levels of plant growth regulators. In the majority of cases, this involves manipulation of the concentration of an auxin and a cytokinin (Morel *et al*). Bhojwani and Razdan suggest addition of five different auxin concentrations and five different cytokinin concentrations to the selected basal medium, to give a total of 25 different combinations and then select the concentration range giving the best growth.

3.2.3 Mineral Nutrition

Nutrition of cells and tissues in culture is based on the mineral nutrition of green plants growing on soil. A soil lacking in essential elements does not support healthy and wholesome growth, and the plant exhibits deficiency symptoms, and so also explanted tissues in culture.

In regard to mineral nutrition, deficiency symptoms as reflected in callus cultures are: occurrence of pigmentation, absence of vessel elements, presence of narrow cambial zone, cellular hypertrophy (due to non availability of N. P. K); symptoms of chlorosis in the absence of Fe and S and inhibition of cell division due to B-vitamin deficiency. The cell walls are composed exclusively of C, H and O. While the proteins of the cytoplasm and the organic constituents of the nucleic acids of the nucleus are made up of C, H, O, N and P. Relatively small quantities of sulphur form a part of the sulphur containing aminoacids like cystine and methionine. Sulphur is also present in such substances as butathione believed to be concerned with the oxidation reduction reactions in plants.

Calcium is important in that it is a constituent of the structure of the middle lamella of the cell wall and Mg is an essential constituent of the chlorophyll molecule. In the absence of Mg, leaves become chlorotic. Phosphorus is a structural component of the nucleic acids, DNA and RNA As a part of fatty substances, the phospholipids are an essential structural component of the cell membrane. Phosphorus is also involved in all energy-transfer processes in the cell. The remaining eight elements take part in catalytic processes going on in the cell. Copper is a part of certain oxidative enzymes such as tyrosinase and ascorbic oxidase, which serve to oxidise phenolic substances. Iron functions as a respiratory electron carrier through such compounds as cytochromes and the oxidative enzymes, peroxidase and catalase.

Potassium is supplied at a concentration 20 mM or higher as the nitrate or chloride, and chloride ions are important in that they stimulate the production of necessary enzymes. The exact role of boron in cell metabolism is rather obscure, though implicated in sugar transport in speeding up the rate of sugar movement in the plant. Optimum concentrations of P, Mg, Ca and S for cell growth vary from 1-3 mM.

3.2.4 Phytohormones

Endogenous hormone levels have to be supplemented with auxins and cytokinins in an otherwise complete basal medium of inorganic salts. Sucrose, nitrogen source and vitamins in order to trigger cell division in a tissue explant. The hormonal content of a culture medium is crucial to any sustained growth of the cultures.

The growth and development of higher plant tissues in vitro is controlled by gradients of endogenous plant growth substances. There are five known classes of growth substances, namely indolyl auxins, cytokinins, gibberellins, unsaturated hydrocarbon gases and inhibitors. The two latter groups are represented by ethylene and abscisic acid as dominant members of their respective classes.

Auxins

All auxins are used in the concentration range 10^{-7} to 10^{-5} M or 0.01-5.0 mg/l. Stock solutions of phytohormones are prepared in double distilled water and stored in amber bottles in a refrigerator. Aliquots of 1-2 ml quantities are used. All auxins are dissolved in 0.1 N KOH or in 5-ml ethanol, and made up to the required volume. For 2, 4-D, dissolve 50 mg of the substance in 2-5 ml of ethanol, heat slightly and gradually dilute to 100 ml DDW. Store in refrigerator and use 2.0 ml/l of the stock solution or a concentration level as required.

Growth substances based on the indole nucleus are referred to as auxins. Indole3-acetic acid (IAA) is a naturally occurring plant growth hormone produced by the growing apices of stems and roots. It migrates away from the top to the base, to the zone of elongation. It has been demonstrated that the biosynthesis of IAA proceeds from the aminoacid tryptophan. Of some interest is the finding that epiphytic bacteria found in association with plants can also synthesize IAA. The concentration of IAA depends upon, among others, the balance between synthesis and breakdown of the auxin by oxidative enzymes like peroxidases. A mechanism for the control of IAA levels within the tissue is the formation of conjugates with glucose or aspartic acid and others. Conjugates represent inactive forms of IAA, which under proper stimuli increase the activity of enzymes, which degrade the conjugate and release free IAA. It can cause different effects in different plants at different times. Auxins may initiate or promote cell division from tissues cultured in vitro, can stimulate shoot growth and inhibit root growth, control vascular system differentiation, regulate apical dominance, delay senescence, promote flowering, fruit setting and, ripening. This is an example of multiplicity of effects induced by a single hormone.

Auxins, as plant growth regulators are mostly used for plant cell, tissue and organ culture and are normally added to culture media in concentrations varying from 10^{-8} to 10^{-5} g/ml depending upon the purpose of the cultivation and the kind of material under use. They are usually added to culture media from concentrated stock solutions. For the culture of plant tumour tissue, for example crown gall, exogenous auxin is not required. The two synthetic auxins commonly in use are α-naphthaleneacetic-acid (NAA) and 2,4-dichlorophenoxyacetic acid (2, 4-D) at a concentration of 10^{-6} M with majority of tissues. High concentrations of 2, 4-D inhibit growth completely. Auxin effect is not destroyed on autoclaving, as they are thermostable, unlike natural auxins like IAA, that under the influence of a low pH, exposure to

light, oxygen and peroxides may be degraded, losing its activity. Filter-sterilized IAA is also destroyed if the medium is exposed to light. Hence IAA-containing cultures are grown in the dark or in diffuse light. It is seldom used as the sole auxin in a culture medium.

Synthetic auxins added alone in the medium may not evoke any response unless cytokinins (kinetin, zeatin or BAP 10^{-6}-10^{-7} M) are added as synergistic stimulants. 2, 4-D is widely used, alone or in combination with low levels of a cytokinin, to obtain a callus and for its maintenance as callus growth. Coconut milk in consort with 2, 4-D in the medium is stimulatory to many monocotyledonous tissues in the initiation of a callus, which is later transferred to simpler media without the addition of any undefined plant extracts for growth and maintenance. For evoking organ morphogenesis in a callus, 2, 4-D-deprived culture medium is used to avoid repression by the auxin.

Cytokinins

Cytokinins are now fully recognised as one of the major groups of endogenous plant hormones. It is a general term proposed to cover all compounds having similar activity. Several cytokinins are adenine derivatives and occur in plants as nucleosides and nucleotides. The following compounds are of special importance in plant tissue culture.

Kinetin (6-furfurylaminopurine): a specific highly active, cell division-inducing factor obtained from autoclaved herring sperm DNA (Miller et al. (1955).BAP (N^6-benzylaminopurine), a related analogue is synthesized.

IPA (N^6-isopentylaminopurine)-2iP or 6-(γ,γ-dimethylallyl aminopurine).

Zeatin 6-(4-hydroxy-3-methylbut-trans-2-Enylaminopurine)- a cytokinin isolated in a crystalline form from the immature kernel of maize. Further studies by Letharn (1973) resulted in the isolation of zeatin riboside, zeatin ribotide and others, substituted purines with an isoprenoid side chain.

PBA [6 - (Benzylamino)-9-(2-Tetrahydroy-pyranil)-9-H-purine].

Adenine, a cytokinin characterised by its ability to stimulate cell division especially in conjunction with an auxin to induce lateral buds, delay senescence of detached leaves and stimulate germination of certain seeds (overcoming dormancy). All natural cytokinins are substituted adenine derivatives. Cytokinins are dissolved in a small volume (2-ml) of 0.5 N.HCL by gentle heating and gradual dilution to 100 ml DDW and stored in a refrigerator. Sometimes these substances are dissolved in dimethylsulfoxide (DMSO). Zeatin being thermolabile should not be autoclaved. High concentrations (1-10 mg/l) can induce shoot proliferation and root inhibition.

Most reports in literature indicate that cytokinins inhibit rooting while auxins promote this process, although these two hormones may well interact. The inhibitory effect of cytokinin-free bases on rooting has been confirmed (Drewes and Van Staden, 1989). Cytokinins have been found to induce haustoria formation in segments of *Cuscula* vine (Ramasubramanian *et al.*, 1988).

The site of synthesis of cytokinins in the plant seems to be the roots. Although cytokinins are of widespread occurrence in plants, kinetin has not been isolated from any plant tissue. It is found as a degradation product of DNA or in fresh nucleic acid preparations that have been autoclaved. The degraded DNA showed marked stimulation of cell proliferation. The active substance was identified as 6-furfurylaminopurine and named kinetin. Subsequently, the first synthetic analogue, 6-benzylaminopurine was found to be effective as a cytokinin. The substance has shown growth regulatory effects, indicated by bud induction in both tobacco stem segments and Callus (Skoog and Tsui, 1948; Miller and Skoog, 1953). It continues to be used in

all plant regeneration studies in the range of 40 to 80 mg/1. Used as a sulphate, it is more soluble in water. Its addition to a cytokinin containing medium reinforces regenerative responses like somatic embryogenesis, adventitious shoot formation in callus (indirectly) or directly from explants, and axillary shoot proliferation, depending on the source of explants and the genotype. But its use inhibits rooting. Inhibitory effects of adenine are also reported, as for instance, shoot formation in tuber discs of potato (Jarret *et al.*, 1980) and inconsistent growth of shoot tip cultures of carnation (Davis *et al.*, 1977).

Cytokinin antagonists such as 2, 6-diaminopurine, 8-azaguanine and 8-azaadenine have been reported to inhibit growth of callus of soybean, grown in kinetin medium.

Gibberellins

Gibberellins are also used in tissue culture studies but are not obligatory. Of the 50 or so designated gibberellins, which are structurally different and vary in biological activity, GA_3 forms an important, naturally occurring growth hormone in higher plants. They are a large family of cyclic diterpene acids freely soluble in water. Vegetative parts of plants in general contain lower amounts of GA_3 than reproductive tissues. GA_3 shows a wide range of activity on plant growth and metabolism. When it is applied to genetic dwarf plants it caused hyperelongation of stems and stimulated flowering in long day plants, besides showing various other effects such as internode elongation, sub apical cell division, induction of parthenocarpy. Stimulation of α and β-amylase etc. It is a potential growth regulating substance in agriculture and sometimes is necessary for plantlet regeneration, though not for the initiation of callus.

Experiments conducted on the stability of the growth substance, as observed from its biological activity, report that autoclaving reduced the gibberellin activity of the GA_3 solution by 90% (Van Bragt and Pierik. 1971) at pH 4.8 to 5.3 or 6.0. Monocot tissues were inhibited by GA_3 without exception (Butenko, 1968). As GA_3 is found to be frequently inhibitory in growth and morphogenesis invitro, chemicals having antigibberellin properties have been added to the medium to block endogenous gibberellin synthesis. Where natural-gibberllin concentrations are supra optimal. Chemicals blocking biosynthesis of endogenous levels of the hormone are added to restore promotary effects. Compounds having anti-gibberellin properties such as chloromequat (CCC), Argo 1618, and ancymidol have been used.

Abscisic Acid

Abscisic Acid (ABA) occurs naturally in plant tissues and evidence suggests its synthesis within the plastids. It is an unusual sesquiterpene containing both 2-cis and 2-trans isomers, each as the dextrorotatory enantiomer and possesses a growth retarding function. It is an acidic substance which could hasten the process of abscission in a bioassay and originally, termed abscisin 11. A physiological function attributed to ABA in its natural form is the closure of stomata in vivo during water stress. ABA is regarded as the major growth inhibitor in plants, in a hormonal sense. Its physiological effects are manifested in accelerating the process of abscission of leaves, dormancy in buds, retardation of germination and bud development and hastening senescence (Wilmar and Doornbos, 1971). Both the isomers of ABA are stable to autoclaving. Its action as a growth factor has not been demonstrated, but probably, the mode of action is by inhibiting nucleic acid and protein synthesis. Low concentration of ABA may stimulate somatic embryogenesis as in Citrus senensis (Spiegel-Roy and Kochba, 1980) and in suspension cultures of Pennisetum on transfer to low levels of ABA (Basil and Vasil, 1981b). ABA might act by modifying cytokinin synthesis or activity.

Ethylene

Ethylene (ethene. C_2H_4), a gaseous hormone is one of the most unusual gaseous hormones synthesized in cultured cells, fungi and bacteria, and an event which explains at least some of the actions of high concentrations of auxin. Throughout ontogenesis, ethylene intervenes in the metabolism and development of higher plants. It exerts a strong regulating influence on most aspects of plant growth. Flower development and ripening of fruits are known to be due to the presence of small amounts of the gas, ethylene (Abeles, 1973). There appear to be no specialized sites of synthesis of the gas in plants but sites of action are identifiable in terms of whole organs, through movement as dissolved molecules. Depending on the plant material and on the stage of development, it may inhibit growth processes.

Stimulation of ethylene production by auxins has been reported (Yu and Yang 1979) indicating an interaction of ethylene and other phytohormones, but the physiological significance of ethylene in cell cultures is, by and large, obscure. Recent reports suggest that ethylene plays an important role in somatic embryogenesis, morphogenesis and plant regeneration from in vitro cultures (Miller and Robers, 1984). The hormone is also known to cause abnormal plant growth. Ethylene's releasing synthetic chemicals such as ethephon (2-CEPA or 2-chloroethyl-phosphonic acid) are used in tissue culture experiments. The rate of ethylene production is entranced in callus and suspension cultures, if the tissues are subjected to stress (high NaCI) levels (Garcia and Einset, 1982, 1983).

Significant accumulation of ethylene in varying levels is observed in invitro culture vessels or flasks entirely closed. In some instances as in tobacco culture, a close correlation between the rates of growth and ethylene production is reported to exist (Huxter *et al.*, 1979; Bridger and Lineberger, 1981), but its role in morphogenesis appears to be dubious. Ethylene may

promote organ initiation in some and inhibit in others, much depending on its concentration, other growth regulators in the medium and the genotype involved. It is also reported that organogenesis/embryogenesis is considerably influenced in conifer tissues (Kumar *et al.*, 1986).

3.2.5 Vitamins

The medium requires to be supplemented by the various vitamins of the B complex for healthy growth of tissues in culture: B-vitamins are water soluble and their individual roles in tissue culture remain to be elucidated. They are apparently synthesized in sub-optimal quantities by callus tissues. Of the B-vitamins, thiamine HCl is an essential ingredient but its biochemical role is not well understood. Vitamins play a catalytic role in cell metabolic apart from being a factor in accessory food supply, but their requirements vary from species to species. Generally, addition to culture medium after filter sterilization is preferred to autoclaving. They can be added either individually or as a mixture, to enhance growth. Of all the vitamins, only thiamine may be required for most tissue cultures. Vitamins are prepared x 100 concentrates and the solutions stored in the freezer at -20°C. High concentrations of vitamin C are used as an anti-oxidant. The plants synthesize vitamins in vitro and hence, addition of a mixture of several vitamins may not be necessary. Together with magnesium, vitamins act as a cofactor in decarboxylation in the respiratory chain, thereby stimulating respiration.

Biotin (vitamin H), folic acid (vitamin B_c), riboflavin, p-aminobenzoic acid (vitamin B) choline chloride, ascorbic acid, cyanocobalamin (B_{12}), adenine (vitamin B4) are also added in special cases but their nutritive contribution is not well established. Addition of a mixture of vitamins is essential for protoplast culture (Kao, 1977; Gamborg *et al.*, 1981).
Myo-inositol (sometimes described as meso-inositol) which is a cyclic alcohol and a constituent of CCM is also routinely added to the culture

medium in small amounts and is understood to play a role in many biosynthetic pathways and a growth actor for several callus tissues (Risser and White, 1963). Addition of 100 mg/l of the substance improves cell growth.

3.2.6 Sucrose as energy source

A variety of carbon sources are used in the tissue culture media, the disaccharide, sucrose being the most standard. Glucose and fructose can also be used but somewhat less suitable. Other sugars such as pentoses, hexoses, trisacharides, sugar alcohols, glycols and glycosides are also in use singly or in consort for experimental purposes in order to ascertain the nature of carbohydrate nutrition of the tissue.

Sucrose is a necessary component in the culture media as most tissue cultures are not autotrophic.Glucose , maltose and raffinose are also used but fructose and galactose are less effective while mannose and lactose are almost without any effect. Fructose and maltose could be good substitutes for sucrose in special cases (Straus and La Rue, 1954; Sievert and Hildebrandt, 1965). A partial degradation of sucrose on autoclaving leads to the production of sucrose acids which reacts with other constituents in the medium (Pierik, 1971).

Sucrose as a precursor in reduced concentrations, serves to minimise the quantum of alcohol production. Not only is it an energy source but acts as the main osmoticum. Mannitol can replace sugar as an osmoticum. Optimal shoot formation is favoured in the presence of low concentrations of sucrose (Thorpe, 1980).

Cultured tissues of a few plants are able to metabolise sugars supplied in the form of starch, possibly by action of extracellular amylases released into the medium. While sucrose is incorporated in the medium usually in the optimal range of 2-4% (w/v) for the growth and morphogenesis of most tissues, organogenesis in individual cases may require altered levels of

sucrose. It has been shown that action of cytokinins on cell division and the effective utilization of nitrate and ammonium ions are largely dependent upon the availability of assimilable sugar, sucrose (Gamborg *et al.*, 1974). Sometimes addition of less easily metabolised sugars, in place of sucrose have been known to stimulate embryogenesis (Kochba *et al.*, 1971).

3.2.7 pH

The pH of the medium is taken, in order to determine the alkalinity or acidity of the final solution, as it greatly influences the uptake of ingredients, solubility of salts and gelling efficiency of agar. Although the pH of the medium is altered during culture, an initial pH is selected before autoclaving. The pH is adjusted using NaOH or HCI using a pH meter. A pH of 5.6-5.8 has been found suitable for maintaining all the salts in a near buffered form. Despite the initial adjustments, the pH of the medium usually changes after autoclaving and has an effect on medium composition too. Although plant tissue culture media are poorly buffered, nevertheless pH is stabilised to some extent as tissues are cultured in media containing both nitrate and ammonium ions.

Additions of phosphate buffers may alter the medium composition, not conducive to normal growth of explanted tissue. At a low pH, the agar may not gel and the medium may have to be prepared a fresh. Buffering may also prove toxic. Bonga and Durzan (1982) advocate the use of MES 12-(N-morpholino) ethane sulphonic acid and TRIS (Tris (hydroxymethyl) methylamine) buffers for plant tissue culture.

3.2.8 Gelling agents

It is a natural plant product and is used as a gelling agent, being inert. Difco-bacto agar (0.6 to 0.8%) is generally used by plant tissue culturists. For routine work, inferior grade agar may be used where small amounts of

impurities may not be toxic. Gelatine or silica gel and more recently certain types of acrylamide gels have been used, but not widely Sometimes, Difco Noble agar or agarose (for culture of protoplasts) is also used. Agar manufactured by different companies contains organic and inorganic contaminants but cause no deleterious effects on tissue growth. Alginate, gelrite (Merck, USA) etc. may also be used. Because of the possible occurrence of impurities in agar, physiological and biochemical evaluation concerned with nutrition and metabolism of tissues is carried out using stationary suspension cultures which do not entail any nutrient grades, as in the case of callus growth on agrified medium.

3.2.9 Aseptic Tissue Transfer

The transfer chamber may be an ordinary bacteriological glove box made of fibreglass or plexiglass fitted with accessories, or, the more sophisticated. Laminar flow cabinets (horizontal or vertical) available in various sizes (Klenzaids Engineers Pvt. Ltd., Bombay; Killoskar Electrodyne Pvt. Ltd., Pune; Thermodyne Pvt. Ltd., Faridabad, Haryana, India). A cubicle of size 1.2 m x 1.2 m x 2.1 m fitted with glass on the upper half, work bench with sunmica top end to end, a small sink and a door, would suffice. All types of transfer cabinets are fitted with a germicidal UV lamp for inside sterlization and cool white fluorescent tubes for uniform illumination. They are installed in one corner of the room away from direct drought. The laminar flow chambers give an enclosed area in which the air is circulated through dust and microbe screening filters ("Vertical flow unit" or "horizontal flow unit") over the working surface, at a uniform rate. These are built to keep the transfer area under positive pressure.

The advantage of using such chambers with a continuous air flow system is that they could be used for long periods, which is not so with the simpler glove boxes or fume hoods or cubicles, where aseptic environment cannot be maintained for a prolonged time. The entire internal surface of the transfer

chamber is hand sprayed with either 70% ethanol or 20% aqueous solution or phenol prior to use, for elimination of airborne contaminants, the door tightly closed and the UV switched on for ½ to 1 h for inside sterilization. At the start of operations, the illuminating tubes are switched on. Since ozone build up inside the chamber may be nauseating, some time is allowed to elapse before the actual commencement of operations. The filtered air flows from the whole of the top, or the back portion of the, cabinet, whichever forms the filter surface, at a uniform velocity 80-100 ft/mm/min positive pressure. Filtration is achieved in two or sometimes three stages (depending on the make), the third stage being submicron.

Units meant for bench mounting are also custom made. A series of such transfer cabinets can be installed in special transfer rooms for aseptic manipulations, where, individual workers can use them without being hustled into quitting before completion, of work or causing the least disturbance to others. Before entry into the transfer room, it would be advisable for the researcher to wear a clean lab coat, don a sterile cap, use a facemask, scrub his hands with ethanol and change into new canvas shoes as a precautionary measure just as for surgery.

Flasks, inoculated containers and vials are arranged on shelves, which are specially designed to store cultures, and the fluorescent tubes fitted with reflectors are fixed at the level of the media. So that the explanted tissue may get the maximum illumination. Illumination may also be arranged from above, with the tubes fixed to the base of each shelf above. The lights are controlled by automatic time clocks although each is provided with a manual on-off switch The cultures are subjected to a 12-16 hr photoperiod per day or exposed to diffuse light or grown in dark at a relative humidity of 50-60%.

3.2.9.1 Incubation of culture

The constant ambient temperature of 25+/-2^{0}C (for the tropics) is maintained in the incubation room by an air-conditioner of 1-2 tone capacity, but certain cultures may grow better in above or below these temperatures. A temperature recorder may be installed externally to monitor temperature fluctuation. To ensure good air circulation a ceiling fan at low speed may be fitted. It may be practical to maintain strict photoperiodic diurnal cycles. Provision may be made to blow cool air downwards over the fluorescent tubes. A walk in refrigerator cold room (-10^{0}C to 10^{0}C) enables one to store plant material in bulk to be drawn as needed throughout the year. A low level of lighting of ca 100-1000 lux would suffice for maintenance of tissue cultures of different plants. But regenerated plantlets are grown at ca 3000 lux prior to soil transfer under green house conditions. In temperate regions, illumination is provided with a mixture of cool white fluorescent tubes and incandescent bulbs but then optimal environmental conditions vary with genotype and the objective of the experiment. In tropical climate, incandescent bulbs may be dispensed with.

DNA Mediated Gene Transfer (DMTG)

There are two approaches of DNA delivery, firstly 'indirect' methods which are based on manipulating natural system *e.g. Agrobacterium tumefaciens* and plant DNA viruses to deliver DNA into the cell. These systems have a number of restrictions including limited host range however, these systems have an advantage of transferring DNA in more controlled and targeted manner. The second approach is to use 'direct' methods of transfer including microprojectile particle bombardment, electroporation, PEG mediated transformation and arrange of novel approaches. Direct approaches tend to overcome host range limitations but DNA transfer is less controlled.

3.3 Indirect gene transfer technique.

Agrobacterium tumefaciens, nature's most effective plant genetic engineer, has been extensively modified by researchers to allow faster and more specific addition and manipulation of desirable plant genetic traits. In the natural environment *Agrobacterium* introduces its T-DNA into compatible host plant cells and via highly evolved molecular mechanisms stably integrates the new DNA into the plant genome.

3.3.1 *Agrobacterium* mediated transformation

Agrobacterium tumefaciens is a plant pathogenic bacterium that is able to cause tumerous growth on infected plants called as crown gall disease. The disease was first described long ago, and the involvement of bacteria was described as early as 1907 (Smith and Townsend 1907). It was subsequently shown that the crown gall tissue represents true oncogenic transformation; callus tissue can be cultivated in vitro in the absence of bacterium and yet retain its tumerous properties. These properties include the ability to form an overgrowth when grafted on to a healthy plant, the capacity for unlimited growth as a callus in the tissue culture in media devoid of plant hormones necessary for invitro growth of normal plant cells, and synthesis of opines, which are unusual amino acid derivatives not found in the normal tissue. These growth responses result from a natural genetic engineering event, in which the specific DNA from a Ti (tumour inducing) or Ri (root-inducing) plasmid is transferred from *Agrobacterium* to plant cell. This transferred DNA (T-DNA) is integrated and expressed in the nuclear genome of plant cells. It encodes enzymes responsible for biosynthesis of phytohormones and /or proteins affecting the sensitivity of plant cells to phytohormones. The expression of these genes results in the development of tumours or hairy roots. The T-DNA also encodes genes specifying enzymes involved in the production of opines (derivatives of amino acids) which the *Agrobacterium* utilizes as a food source. *Agrobacterium* itself does not appear to express genes on the T DNA.

The importance of *Agrobacterium* for plant genetic engineering is its natural ability to transfer a segment of DNA into plant cells. Over the last ten years plant scientists have capitalized on molecular biology technology to manipulate the T-DNA of *Agrobacterium* for the development of gene vectors to produce transgenic plants. To use *Agrobacterium* for plant transformation a number of experimental steps must be optimized in both the bacteria and the plant material. These include:

(1) The identification of an *Agrobacterium* strain which 'infects' the appropriate plant genotype.

(2) The design and construction of modified T-DNA to allow gene expression in plant cells.

(3) The transfer and maintenance of the modified T-DNA in a specified *Agrobacterium* strain.

(4) The frequency of T-DNA transfer events to plant cells being high enough to be detected.

(5) The selection and regeneration of transformed plant cells.

3.3.2 Host Range of *Agrobacterium*

A wide range of plants are susceptible to tumour and hairy root formation induced by *Agrobacterium*. Though the host range includes mostly dicotyledonous plants, but few gymnosperms and several monocotyledonous plants are also included. The strain specificity for plant genotypes and tissue types has been well documented. Examples include peas, soybeans (Byrne *et al.*, 1987), pine and *Brassica* (Charest *et al.*, 1989).

The nature of the interaction between bacteria and plant cells is still unclear. It is important to screen a series of *Agrobacterium* strains on a range of crop genotypes for virulent strain genotype combinations. There are many hypotheses to explain the inability of *Agrobacterium* to induce tumours or hairy roots in most monocotyledonous species (and other non-susceptible plants). These include: lack of binding of *Agrobacterium* to plant cell walls, reduced activity of T-DNA promoters, inhibition of *vir* gene induction and

'abnormal' auxin-cytokinin balance in monocotyledonous cells. The lack of a pronounced wound response is thought to be an important factor, since only plants and tissues, which proliferate large populations of wound adjacent cells, are considered competent for efficient transformation.

3.3.3 Target cells for transformation

The main aim of any gene transfer procedure is to stably insert DNA into the nuclear genome of the cells capable of giving rise to a whole transformed plant. Transformation without regeneration, and regeneration without transformation, are of limited value. In most species it is often difficult Ti identify such target cell types; several possible transformation routes exist which are outlined in the table no1 given below:

Table 1: Theoritical routes for production of transgenic plants

Target cell type	Method of obtaining transformed plants
Cultured cells	Organogenesis or embryogenesis via callus phase
Immature embryos or organ meristem cells	Invitro plant regeneration from transformed cell lineage's following continued development of the embryo or organ.
Cells in immature embryos, shoots and flower meristems	Continued development of embryo, shoot or flower to give rise to a chimeric plant. Pollen derived from transformed cell lineage's used to produce transformed seeds via normal fertilization.
Pollen	Direct production of transformed plants via fertilization with DNA-treated developing, mature or germinating pollen.
Zygote	Direct development of transformed plant.

Plant cells are totipotent and can be stimulated to regenerate into whole plant in vitro via organogenesis or embryogenesis. However, in vitro plant regeneration imposes a degree of 'genome stress' especially if plants are regenerated via a callus phase. This may lead to chromosomal or somaclonal variation.

3.4 Vectors used for transformation

Vectors used for genetic transformation of eukaryotic cells have several features in common and the plant transformation vectors are no exception. Most vectors carry 'marker' genes, which allow the recognition of transformed cells, by either selection or screening. Selectable markers are dominant, usually of microbial origin, and placed under the control of strong, constitutive, eukaryotic promoters, often of viral origin.

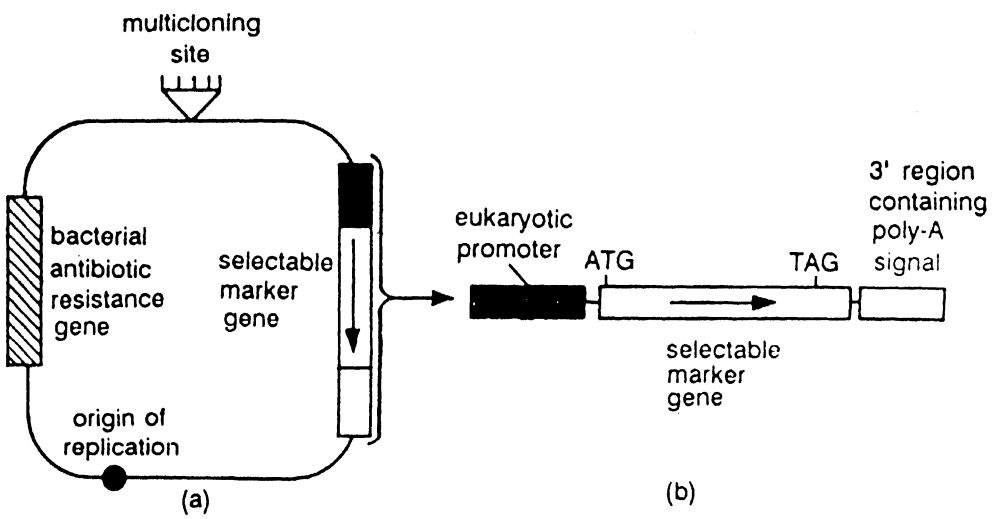


Fig 1.0 (a) Generalised vector for the transformation of eukaryotic cells; (b) details of the selectable marker genes. (Redrawn from D. Grierson, *'Plant Genetic Engineering'*, 1991).

3.4.1 Selectable marker genes

The usefulness of a particular resistance marker depends on the characteristics of the selection agent, the resistance gene and the plant material. The selection agent should fully inhibit the growth of untransformed plant cells; however the influence exerted by the dying, untransformed cells on the transformed cells should be minimal. Therefore, the lowest concentration of the selection agent that suppresses growth of untransformed cells is generally used. The sensitivity of plant cells to the selection agent depends upon the following factors:

(1) The genotype

(2) The explant type

(3) The developmental stage

(4) The tissue culture conditions

These include constructs affording resistance to antibiotics such as kanamycin or methotrexate, and genes, which allow growth in the presence of herbicide such as glyphosate or bialaphos. For successful selection, the target plant cells must be susceptible to relatively low concentrations of the antibiotics or herbicide in a non-leaky manner. The most popular selectable markers genes used in plant transformation vectors are listed in the table no 2 given below.

Neomycin phosphotransferase II (NPTII)

Aminoglycoside 3'- phosphotransferase II (APH (3')-II), or NPTII, is the most widely used selectable marker for plant transformations. The enzyme is coded by the NPTII (or neo) gene, derived from the transposon Tn5, and inactivates a number of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (G418), and paromycin by phosphorylation. Kanamycin is mostly used as a selective agent, normally in concentrations ranging from 50 to 500 mg/l. G418 is generally more toxic than kanamycin. The gene for

NPTII enzyme is often used with nos promoter, which drives its synthesis. For the enzyme assay of NPTII (Resis *et al.*, 1984) the enzyme is first fractionated using non-denaturing polyacrylamide gel electrophoresis (PAGE). Since the enzyme detoxifies kanamycin by phosphorylation, radioactively labelled ATP (p32) is used with kanamycin in an agar layer, which is used to cover the gel containing the enzyme. The whole set is incubated at 35^0C and the phosphorylation leading to incorporation of 32p in kanamycin can be detected by autoradiography.

Hygromycin phosphotransferase

Hygromycin B is an aminocyclitol antibiotic that interferes with protein synthesis. A hygromycin phosphotransferase gene (hpt),originally derived from a *Escherichia coli*, was modified for expression in plant cells and has since found wide application as a resistance gene. Hygromycin B is usually more toxic than kanamycin and kills sensitive cells more quickly. Hygromycin resistance can be checked by a nondestructive callus induction test.

Table 2: Important selective markers used for plant transformation

Gene	Enzyme coded	Selective agent
Antibiotic Ble	Enzymatic activity not known!	Bleomycin
Dhfr	Dihydrofolate reductase	Methotrexate Trimethoprim
Hpt	Hygromycin phosphotransferase	Hygromycin
Npt II	Neomycin Phosphotransterase	G418 Kanamycin Neomycin
Herbicides Als	Mutant forms of Acetolactate synthase	Chlorsulfuron Imidazolines
Aro A	5Enolpyruvyl sikimate 3-phosphate synthase	Glyphosphate

Bar	Phosphinothricin acetyl Transferase	Phosphinothricin

3.4.2 Screenable marker ('reporter') genes

The principle of using reporter genes in studying molecular processes in the living cell means that in the natural gene, a synthetic modification is introduced (or the protein coding sequence is deleted and replaced by another gene) in order either to simplify the detection of the gene product or to distinguish it from similar or identical genes in the genome. The use of the reporter genes requires the method of gene transfer either transient or stable. Screenable marker genes are obviously only useful if enzymes with comparable activities are not present in non-transformed plant tissues. The utility of any particular construct as a transformation marker varies depending on the plant species and the explant involved. As for now Kanamycin resistance is probably the most widely used selectable marker phenotype, and β-glucuronidase is a versatile reporter gene. Screenable markers ('reporter genes') tend to be developed from bacterial genes coding for easily assayed enzymes, such as chloramphenicol acetyl transferase, β-glucuronidase, luciferase, nopaline synthase and octopine synthase.

Qualities of an Ideal reporter protein for plants

* Strong signal
* Low back ground activity
* No detrimental effects on metabolism
* Short to medium half life, to enable induction /down regulation experiments
* Scoring the reporter signal should be sensitive, nondestructive and inexpensive

Use of the reporter genes

Confirmation of DNA delivery and stable transformation

* Developing a DNA delivery technique
* Optimization of the DNA delivery technique

* Identification of the chimeric transformants
* Quantification of gene expression
* Comparison of promoters and the other gene regulatory elements
* Fusion to other engineered proteins that are difficult to assess in plant hosts

Table 3

Comparative advantages and disadvantages of major genes for plant use

Reporter Protein	Advantages	Disadvantages
β-Glucuronidase	-No specific equipment required -Relatively inexpensive -Good quantification assay	-Assays are toxic or destructive -Leaky staining procedure -Background activity -Microbial false positives -Methylation of the coding sequence
Firefly Luciferase	-Non toxic visualization -Sensitive quantification -Good quantification assay -Short halflife	-Expensive detection and quantification apparatus -Differential subatrate uptake -Cost of the substrate
Modified GFP's	-No substrate required -No assay required -Differential accumulation of plant cells -No leakage in cells -Fluorescence can be visualized in culture containers	-Reports of toxicity in whole plant -Quantification not resolved -Cost of the fluorescence microscope

Other components common to most modern eukaryotic transformation vectors include features required for various recombinant DNA manipulations. Multiple unique restriction sites (polylinker), bacterial origin s of replication (e.g., Col E1) and prokaryotic selectable markers for plasmid selection and maintenance in *Escherichia .coli* (e,g. antibiotic resistance) are present in all vectors. Plasmids containing these common components, plus a specific selectable marker gene engineered for expression in plants, may be used directly as transformation vectors in physical DNA delivery strategies. Such vectors do not have any features which facilitate

their transfer to plant cells or integration into the plant nuclear genome. Some of the vector systems developed for use with the natural gene transfer method based on *Agrobacterium* virulence genes and T-DNA border sequences have wide host range replication and transfer functions to allow conjugation from *E. coli* to *Agrobacterium* and plasmid maintenance in both bacterial hosts.

Table below presents a list of common reporter genes and some of their properties.

Table 4: Reporter genes and their properties

Gene	Enzyme coded	Substrate(s) and assays
CAT	Chloramphenicol Acetyl transferase	(^{14}C) Chloramphenicol and Acetyl-CoA. TLC seperation of acetylated (^{14}C) Chloramphenicol detection by autoradiography.
Lac Z	β-Galactosidase(X-gal)	Colour of the colony.
GUS	β- Glucuronidase	Glucuronides (PNPG, X-GLUC, NAG, REG) Fluorometric, colourimetric and Histochemical techniques available.
Lux	Luciferase bacterial insect	Decanal and $FMNH_2$, ATP + O_2 + luciferin. Bioluminescent assays: Quantitative tests on extracts or *in-situ* tissue assays with activity detected by exposure of X-ray film
NptII	Neomycin phosphoryl transferase	Kanamycin and (^{32}P) ATP. *In-situ* assay on enzyme fractionated by non-denaturing PAGE; enzyme detected by autoradiography.
OCS	Octapine synthase	Arginine + Pyruvate + NADH Assayed by electrophoresis
NOS	Nopaline synthase	Arginine + Ketoglutaric acid+ NADH Detected by electrophoresis.

β-glucuronidase (GUS) gene

During the past couple of years the bacterial gene *uid A*, encoding β-glucuronidase (GUS), has become the most frequently used reporter gene for the analysis of plant gene expression. *uid A*, encodes a soluble enzyme of molecular mass of approximately 68 kd and a optimum P^H of 7-8, being in its active form a homotetramer. Its wide acceptance has mainly resulted from the

availability of highly sensitive non-radioactive assay using the fluorogenic 4-MUGluc and of a histochemical assay using X-Gluc, that allows a qualitative analysis of cell and tissue specific expression, in addition to low endogenous activity in most plant species.

***Agrobacterium* hosts for transformation**

3.4.3 Disarmed Strains

Wild type *Agrobacterium* Ti and Ri plasmids are capable of transferring to a plant cell genes that encode hormone biosynthetic activities. Obviously cells synthesizing high levels of auxin and cytokinin will be difficult, if not impossible, to regenerate into whole plants. In general there have been two approaches to separating these hormone genes from a foreign gene of interest. The simplest has been to disarm the Ti plasmid. Using homologous recombination, the hormone genes can easily be removed from the Ti plasmid, leaving behind a "disarmed" *Agrobacterium*. This recombination can be accomplished to remove the entire T-DNA, as is the case for LBA4404. A Ti plasmid with its entire T-DNA removed can then be used in conjunction with virtually any binary transformation vector. Alternatively, a portion of the T-DNA (not including the hormone genes) can be left behind as homologous DNA for cointegrating vectors. This is the approach used in the SEV (split end vectors) system of Fraley *et al.* In either case, the essential *vir* functions remain intact in the *Agrobacterium*, and the T-DNA transfer can occur.

3.4.4 Nondisarmed Strains

An alternate approach to seperating the hormone biosynthetic genes from the gene of interest can be used with binary vectors. When two independent T-DNAs are present within a single *Agrobacterium*, as in the case of the cell containing a wild type Ti plasmid and a binary vector, there is a high

propobility that the two DNAs will become integrated into a single plant cell. However some transformed cells will contain only one of the T-DNAs. Since some selective agent such as kanamycin is always used during the transformation/regenaration protocol, only the cells transformed with the binary T-DNA will survive. Some of the cells will contain only the binary T-DNA, while others will contain both T-DNAs. The problem of this approach is that the cells transformed with the wild type T-DNA will synthesize phytohormones that can interfere the regeneration protocol. The main advantage is related to host range effects observed with different *Agrobacterium* strains.

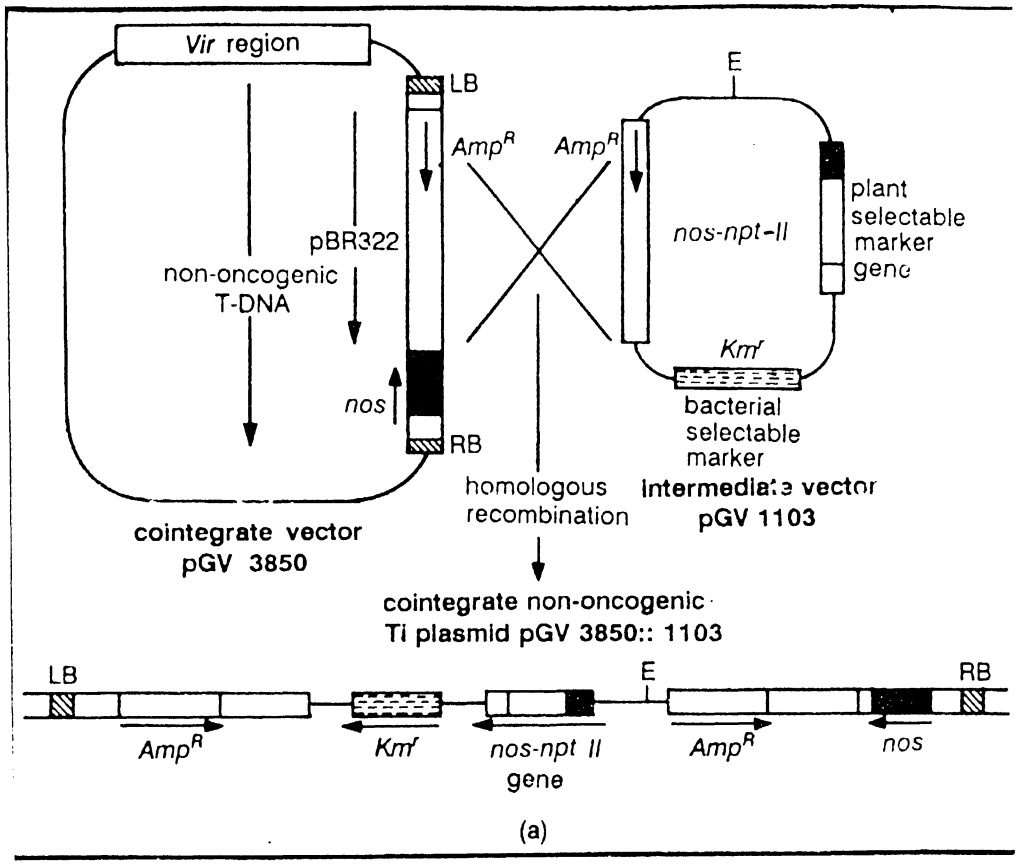
Plant transformation vectors based on *Agrobacterium* can generally be divided into two categories: those that cointegrate into a resident Ti plasmid and those that replicate autonomously (the binary vectors).

3.4.5 Cointegrating Vectors

Cointegrating transformation vectors must include a region of homology between the vector plasmid and the Ti plasmid. This requirement for homology means that the vector is capable of integrating into a limited number of Ti plasmids. The vector is usually designed to cointegrate into one or a few specific Ti plasmids. Two cointegrating systems are in use today. The first utilizes the disarmed *Agrobacterium* Ti plasmid pGV3850. In this plasmid the phytohormone genes of the C58 plasmid have been excised and replaced by pBR322 sequence. Any plasmid containing the pBR322 sequence homology can be cointegrated into the disarmed Ti plasmid. The border sequences as well as nopaline synthase are part of Ti plasmid, and the cointegration places the sequence between the T-DNA borders.

A different approach to a integrating vector was used by Fraley *et al.* In this system, the right border and all the phytohormone genes are removed from the Ti plasmid. A left border and a small part of the original T-DNA, referred to as Limited Internal Homology (LIH), remain intact. The vector to

be introduced into *Agrobacterium* contains the LIH region for homologous recombinant as well as a right border. The cointegrating DNA reconstructs a functional T-DNA with a right border and a left border.



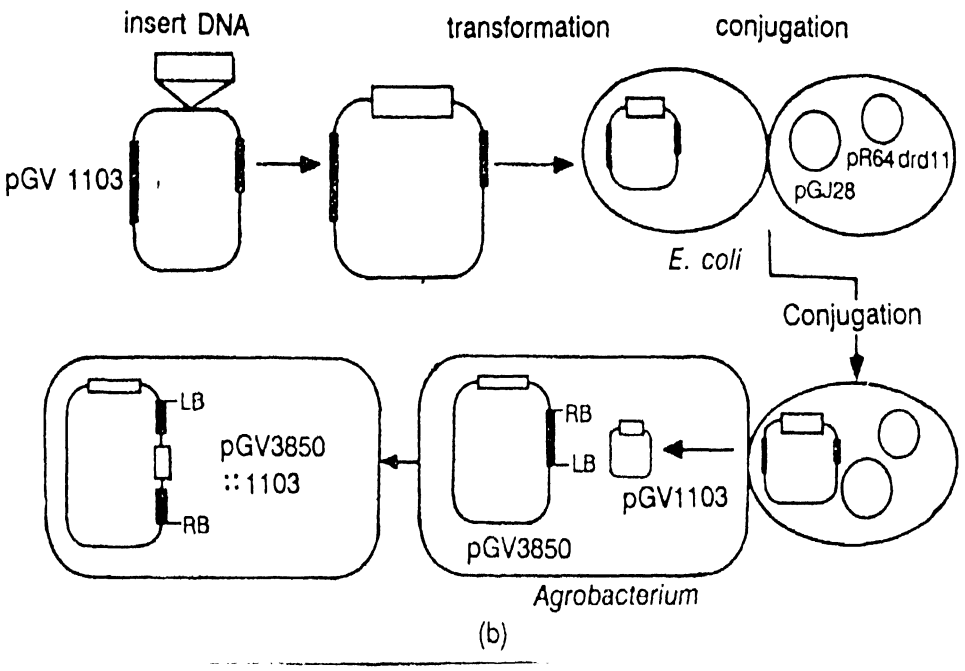


Fig1.1 (a) Use of the cointegrate vector pGV3850 with the intermediate pGV1103. (b) Preparation of a cointegrative vector for the transfer of a desired DNA segment. (Redrawn from D Grierson *Plant Genetic Engineering*, 1991).

This system has been used extensively for introduction of many genes into plants. The cointegrate systems, while more difficult to use, do offer advantages. Once the cointegrate has been formed, the plasmid is stable in *Agrobacterium* and is virtually impossible to be lost. Binary vectors, on the other hand, are not completely stable in *Agrobacterium* in the absence of drug selection.

Binary Vectors

Binary vectors are based on the principle that *vir* genes may be located on a '*helper*' Ti plasmid having the whole of T-DNA deleted (e.g. pAL4404), because *vir* genes can function even in trans configuration.

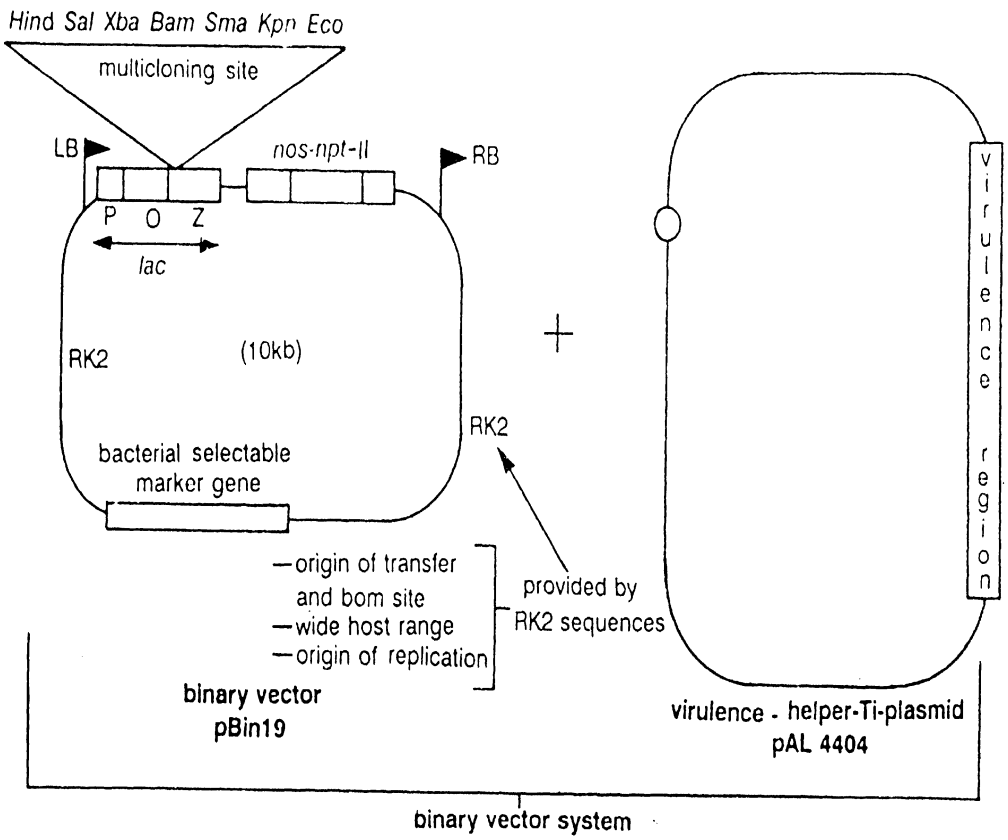


Fig 1.2 Typical binary vector strategy. (Redrawn from D. Grierson. '*Plant Genetic Engineering*, 1991).

In this case, T-DNA is found on a separate vector (binary vector) designed to replicate in both *E.coli* and *Agrobacterium and* capable of conjugal transfer between these two bacterial species. Many *binary* vectors have been developed, which differ in size and the source of 25bp repeat sequences, plant selection marker, bacterial selection marker and cloning sites. PBin19, which was designed in 1984, is still popular and is based on wide host range replicon pRK252. This vector contains the following elements: kanamycin resistance gene (APH-1) for selection in bacteria, T-DNA borders derived from pTi37, a plant-selectable transformation marker (npt-II) isolated from transposon, Tn5 (this marker is associated with promoter and polyadenylation signal derived from nopaline synthesis gene), a multiple cloning site derived from pUC19 and housed with lac Z (β-galactosidase gene) region. Bacterial colonies containing pBin19 are recognised by loss of blue colour on IPTG/GAL plates.

3.5 Transfer of modified T-DNA

3.5.1Basis for crown gall and hairy root formation

Bacteria of the genus *Agrobacterium* are gram-negative rods that belong to the bacterial family of Rhizobiaceae. They are classified according to their phytopathogenic characteristics as follows: *Agrobacterium tumefaciens*, which induces crown gall disease, *A. rhizogenes*, which induces hairy root disease and *A. radiobacter*, which is avirulent. The characteristics of *Agrobacterium* induced neoplastic growths are determined by the Ti *or* Ri plasmid content of *an Agrobacterium* strain. Saprophytic strains without Ti or Ri plasmids are also common and are avirulent with respect to crown gall and hairy root induction. A tumorigenic strain is not necessarily designated *A.tumefaciens,* or a hairy root strain A.*rhizogenes,* since transconjugants can be selected in which any strain can harbour a Ti or Ri plasmid. For this reason it is preferable to designate *Agrobacterium* strains with their plasmid complement.

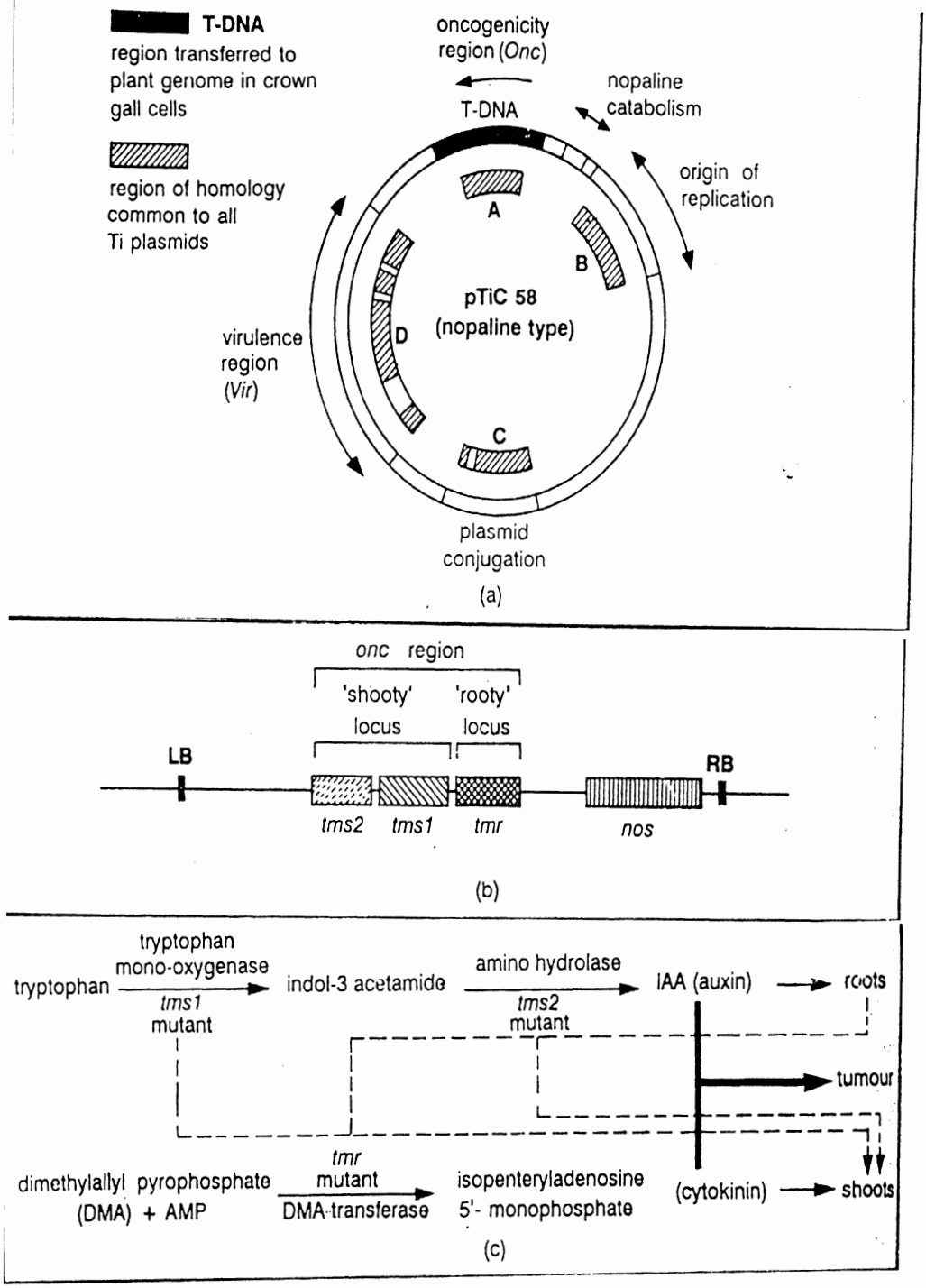


Fig 1.3 (a) General features of Ti plasmids; (b) general structure of the T-DNA; (c) phytohormone biosynthesis in crown gall tissues. (Redrawn from D. Grierson *'Plant Genetic Engineering'* 1991).

The Ti and Ri plasmids are named according to the *Agrobacterium* strain from which they were originally isolated. They are large plasmids and range in size from 140 to 235 kilobase pairs. The T-DNA region which is transferred to plant cells ranges in size from 14 to 42 kb, and is bordered by conserved 25 base pair sequences. Any sequences between these borders may be integrated into plant nuclear DNA. There are two other important regions on Ti and Ri plasmids.

One is the virulence region which encodes the genes responsible for the excision, transfer and integration of T-DNA from *Agrobacterium* into the genome of plant cells. The other region encodes genes responsible for the catabolism of opines, thus allowing *Agrobacterium* to utilise the opines secreted in tumours and hairy roots. The opines are secreted from transformed plant cells into the intercellular regions of a tumour or rhizosphere of hairy roots where *Agrobacterium* lives. These compounds cannot be metabolised by the plant cells but serve as a carbon and nitrogen source for the bacteria. The opine synthase genes carried by each Ti and Ri plasmid determine the opine type produced by the tumour or hairy root cells. A strain carrying specific opine synthase genes on its T-DNA also carries genes for the catabolism of these specific opine synthase genes on its T-DNA and also carries genes for the catabolism of these specific opines elsewhere on its Ti or Ri plasmid. For this reason, *Agrobacterium* strains and their Ti and Ri plasmids are often classified on the basis of opine types

Table 5: Opine types classified on a biological basis

Opine type	Representative opines present in neoplastic growths	Agrobacterium strain,examples
Ti plasmids Octopine	Octopine, Octopinic acid, Lysopine, Histopine, Agropine, Agropinic acid, Mannopine, Mannopinic acid.	B6, ACH5
Nopaline	Nopaline, Nopalinic acid, Agrocinopine A	C58, T37
Agropine	Agropine, Agropinic acid, Mannopine, Mannopinic acid, AgrocinopineA.	AT1, AT4
Succinamopine	Succinamopine, Succinamopine lactam,	Eu6, 181
Grapevine	Octopine, Cucumopine	K305, K308
Ri plasmids Agropine	Agropine, Agropinic acid, Mannopine, Mannopinic acid, Agrocinopine A	A4, TR105
Cucumopine	Cucumopine	2655, 2657
Mannopine	Mannopine, Mannopinic acid, Agrocinopine C, Agropinic acid.	TR7, 8196

3.5.2 T-DNA

During tumour formation a defined sequence of Ti plasmid, the T-DNA, is transferred to the plant cell and integrated into the plant nuclear genome. The T-DNA is stable within the plant genome and hybridization of a Ti plasmid-specific probe to tumour DNA has shown that the T-DNA found in the plant cell is colinear with the T-DNA found in the Ti plasmid of the *Agrobacterium,* indicating that no major rearrangements of the sequence take place during establishment of the tumour. One or more copies of the T-DNA can be present in the plant DNA and, although multiple T-DNA copies can occur in tandem repeats, they can also be separated and linked to different

regions of plant DNA. The site of integration of T-DNA into plant DNA is apparently random.

3.5.3 Tumorogenic and rhizogenic genes

Invitro cultures of tumours and hairy roots continue to grow and proliferate after excision from the site of inoculation. This growth occurs without exogenously supplied hormones and is due to the expression of stably integrated T-DNA genes. These genes are either responsible for phytohormone production (the *onc* (oncogenity) genes of Ti plasmids) or increased sensitivity to auxins (the *rol* (root locus) genes of Ri plasmids). Tumour tissues continue to proliferate as a disorganised callus culture, whereas hairy roots proliferate into a highly branched plageotropic root system.

Homologous DNA sequences have been found in nopaline T-DNA and octopine TL DNA. These regions of sequence similarity contain the tumour producing phytohormone genes encoding enzymes in the pathways for the production of isopentenyl-AMP (a cytokinin) and a indole-acetic acid (an auxin). A recently characterized onc gene (6b) has also been found to have growth inducing properties. This gene induces tumours on *Nicotiana glauca, N.rustica Kalanchoe tubiflora* and grapevine, and may act by reducing the inhibitory effects of high auxin concentrations, there by maintaining cells in an undifferentiated state.

The T-DNA of Ri plasmids has a series of four *rol* genes known as *rol* A, B, C and D. The transfer to, and expression of, these genes in plant cells leads to the hairy root phenotype. The exact cellular effects of the *rol* genes are still not clearly understood; however, they are known to enhance the sensitivity of plant cells to endogenous auxins. In agropine Ri plasmids, the TR-DNA contains auxin biosynthetic genes, which may play a major role in the development of hairy roots (Gelvin *et al.*, 1988).

3.5.4 T-DNA border sequences

The T-DNA regions of *Agrobacterium* Ti and plasmids are flanked by border sequences that define the segment of DNA to be transferred to recipient plant cells. These border sequences are 25-bp imperfect direct repeats and are the only elements required in *cis* orientation for mobilization of the DNA into the plant cell (Zambryski *et al.*, 1989). Any DNA sequences placed between these borders can be transferred into plant cells.

Deletion of the first 6 bp or the last 10 bp of the 25-bp sequence blocks T-DNA transfer (Wang *et al.*, 1987). The right border is a key element as T-DNA transfer is initiated at this site. Consequently the correct orientation is important; inversion results in a substantial reduction in efficiency of T-DNA transfer. Neighbouring the right border just outside the TDNA) is a 24-bp enhancer element, which contributes to the efficiency of T-DNA transfer. This element, known as **overdrive (ode)** is located 13 -14 bp from the right border, although it is active up to 7 kb away, and can function in either orientation.

3.5.5 The virulence region of the Ti and Ri plasmid

The virulence region of Ti and Ri plasmids occurs outside the T-DNA and is a segment of approximately 40kb consisting of six distinct operons *(virA, virB, virG, virC, virD, virE,* reading clockwise towards the T-DNA) encoding trans-acting factors essential for T-DNA transfer. There are varying numbers of open reading frames (ORFs) within each of these operons, which are strongly and coordinately induced by phenolic compounds leached from wound sites on plants (Stachel *et al.*, 1986). The ability of plants to produce these signal molecules may be important factor contributing to the host range of *Agrobacterium.*

The *virA and virG* loci are required for induction of the remainder of the *vir* operons by external signals. The *virA* protein is a periplasmic membrane

protein that senses specific phenolic compounds (such as acetosyringone and alpha hydroxy acetosyringone) synthesized by the wounded cells. Some opines enhance the induction of vir operons by phenolic compounds.

The *virA and virG* proteins form part of a two component positive-regulatory system (*i.e.* sensor and activator). The *virA* protein appears to be a transmembrane protein, and therefore, is in the correct position to act as the primary signal receptor. Once the *virA protein* binds the phenolic inducer, the resulting complex is thought to act as a kinase phosphorylating the *virG* protein. This converts the *virG* protein to a form capable of binding to specific DNA consensus sequences of various *vir* gene promoters. In this manner the activated *virG* protein increases transcription of the *virB, virC, virD and virE* operons (Zambryski *et al.*, 1989).

Two proteins encoded by the *virD operon, virD1 and virD2* have an endonuclease activity capable of generating single stranded site specific nicks between the third and fourth base pairs on the bottom strand of the T-DNA borders repeats. The *virD2* protein attaches to the 5' terminus of the nicked right border T-DNA and a replicative process synthesizes a single stranded DNA molecule known as the T-strand (Zambryski *et al.*, 1989). The overdrive enhancer element (ode) stimulates T-strand formation by interacting with the *virD2* protein and one or both proteins encoded by the *virC* operon. A non-specific single-stranded DNA binding protein, the *virE2* protein is thought to bind to and protect the T-strands. This T strand protein complex is believed to be an intermediary for T-DNA transfer to plant cells, which must be exported by the *Agrobacterium.*

The *virB* operon encodes at least 11 proteins thought to form membrane associated structures that may form a channel(s) through which the T-strand protein complex is exported. Other accessory virulence genes may be present in specific Ti or Ri plasmids and may help to determine host range for specific plant species. For example, in octopine strains there is a *virF* locus encoding a 22.4-kDa protein necessary for tumour formation on *Nicotiana*

glauca. Nopaline strains lack the *virF* locus, but instead contain a TZS gene encoding biosynthesis of the cytokinin trans-zeatin in *Agrobacterium*

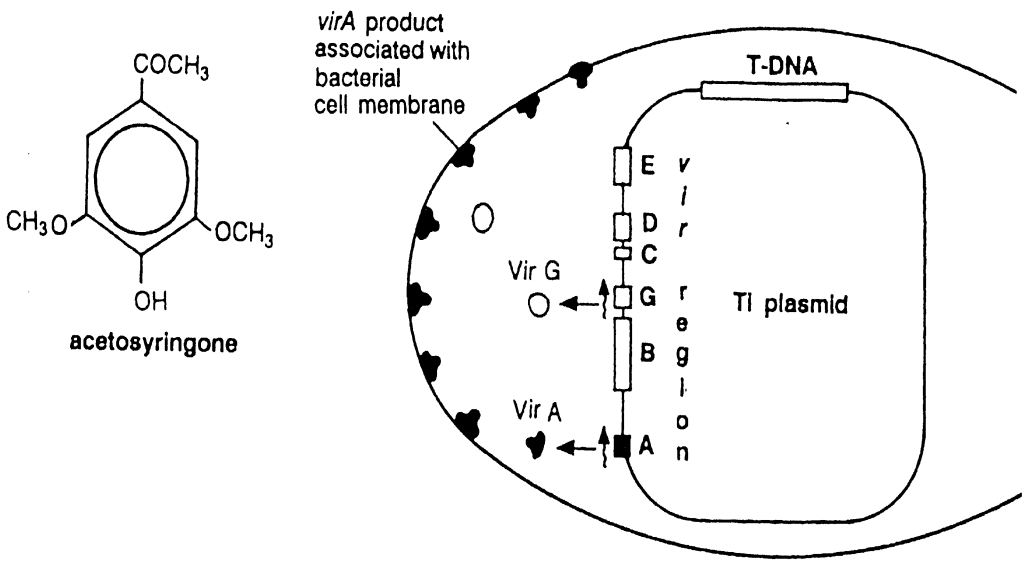


Fig 1.4 Ti plasmid gene expression in vegetative *Agrobacterium* prior to *vir* gene induction by the plant phenolic compound acetosyringone.

(Redrawn from D. Grierson '*Plant Genetic Engineering*', 1991)

5.6 Molecular events involved in T-DNA transfer

An early event in the T-DNA transfer process is the nicking of Ti plasmid at two specific sites, each between the third and fourth base of the bottom strand of each 25bp repeat. This initiates DNA synthesis from the nick in the right hand 25bp repeat sequence in 5'-3' direction, thus displacing a single T-DNA strand. This T-DNA single strand forms a complex with protein *virE* and gets transported to the plant nucleus. The *virD* an endonuclease produces the nicks in the border sequences. Several gene products of the *virB* operon have been identified in the bacterial envelope, a

location, which suggests that they may play a role in directing T-DNA transfer extracellularly. The functions of several other *vir* gene products are largely unknown. Apart from the role of Ti plasmid, the genes located on *Agrobacterium* chromosome also help in virulence. These genes are involved in the synthesis and secretion of glucons, cellulose fibrils and cell surface proteins. These loci are constitutively expressed and are also found in other soil bacteria associated with higher plants. Thus, these loci play a more general role in the virulence of *Agrobacterium*, and thus also in the *Agrobacterium* mediated gene transfer. The first step toward gene transfer by *Agrobacterium* is the attachment of the bacteria to host plant cells at wound sites.

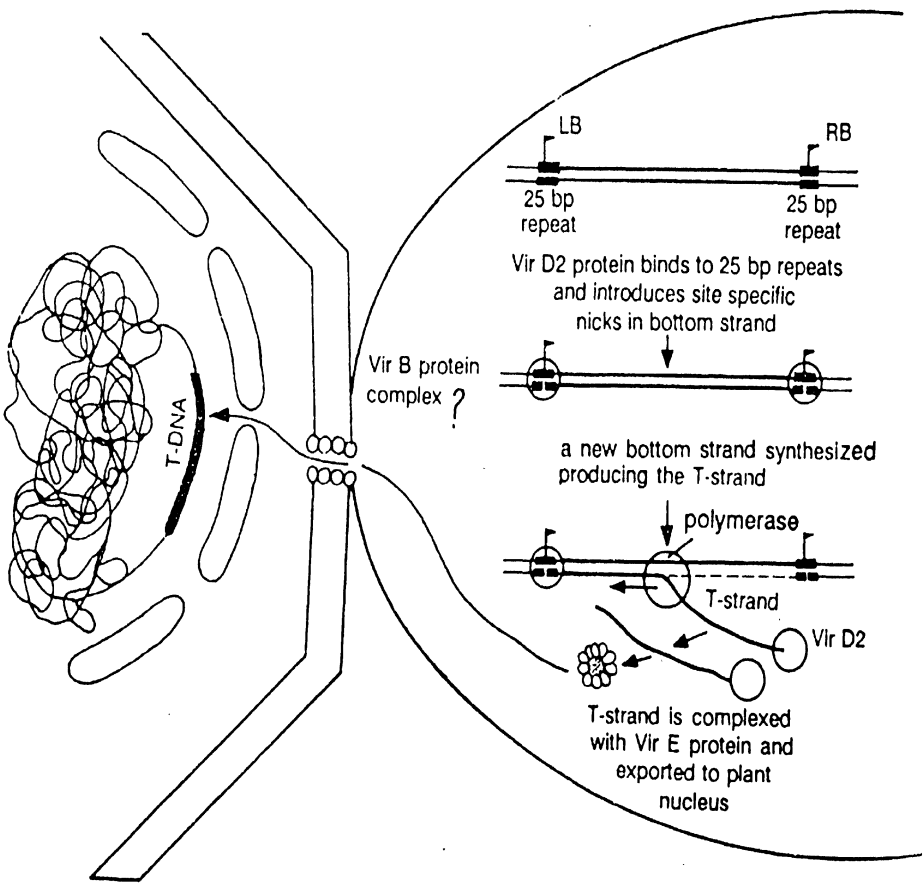


Fig 1.5 Molecular events in the transfer of the T-DNA to plant cells. (Redrawn from D. Grierson. *'Plant Genetic Engineering'* 1991).

The nature of the plant cell receptor to which *Agrobacterium* binds is unknown, but the availability of a receptor for attachment is considered to be one factor important in determining in host range of *Agrobacterium* The plant cell is believed to be a passive partner in the attachment process. *Agrobacterium* plays a more active role, with several constitutively expressed chromosomal virulence genes being important. These genes *(chva, chvb, psca, att)* affect the biosynthesis and secretion of polysaccharides such as P-1,2 glucan and succinoglycan. Mutations in any of these chromosomal loci result in defective plant cell attachment and consequent lack of virulence.

3.6 Selection and regeneration of transformed plant

3.6.1 Prerequisites for production of transgenic plants

The important requirements for transformation by *Agrobacterium* are following 1) The plant explants must produce acetosyringone, or other active compounds, in order to induce the *vir* genes; where these compounds are absent *Agrobacterium* may be preinduced with synthetic acetosyringone, 2) The induced *Agrobacteria* must have access to competent plant cells that are capable of regenerating adventitious shoots or somatic embryos at a reasonable frequency. Unfortunately, the specific factors affecting the competence of plant cells for transformation are largely unknown, but there is reasonable evidence to suggest that for gene transfer to occur cells must be replicating DNA or undergoing mitosis (Firoozabady and Galbraith, 1984; Meyer *et al.*, 1985; Okada *et al.*, 1986). The majority of transformation experiments utilize either freshly explanted tissue sections, protoplasts in the process of reforming a cell wall and entering cell division, or callus/suspension cultured cell clumps wounded by chopping/pipetting and stimulated into rapid cell division by the use of nurse cultures (Draper *et al.*, 1988).

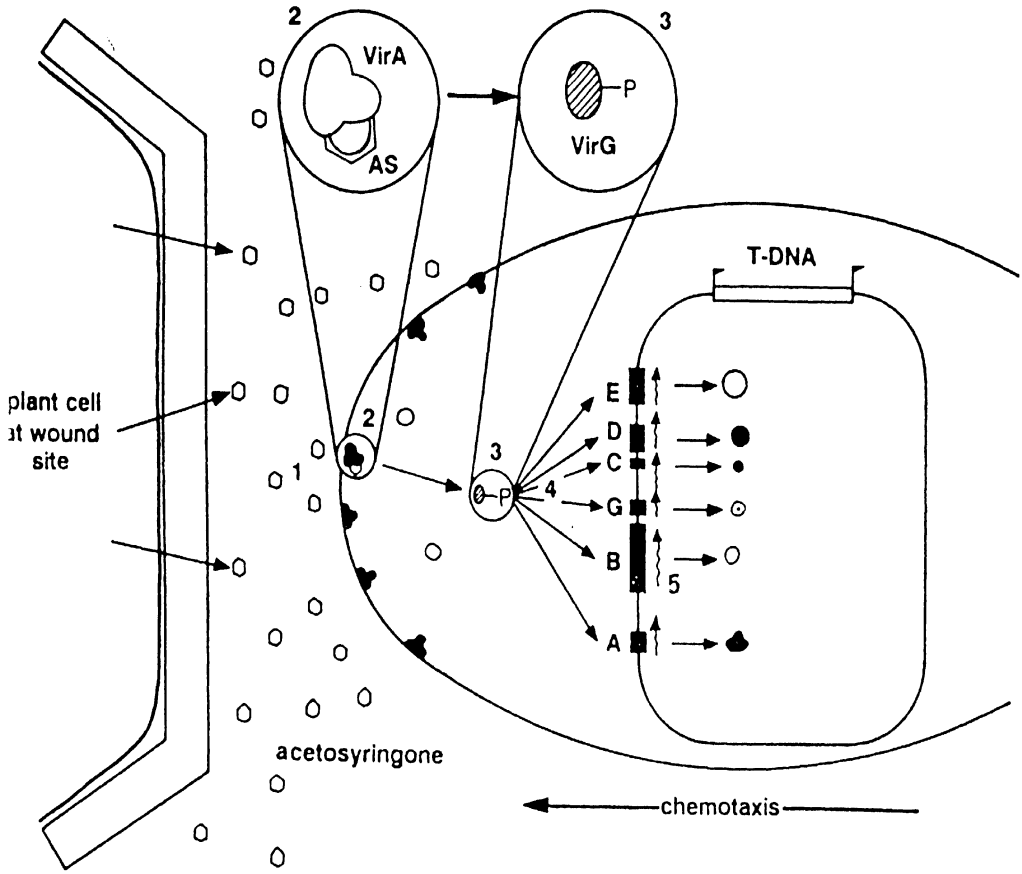


Fig 1.6 Key events in the induction of the Ti plasmid *vir* genes by acetoyringone and other wound-related compounds: 1. acetosytringone released by plant cells at the wound site; 2, acetosyringone activated VirA protein located in the bacterial membrane; 3. a vated VirA modifies VirG protein, possibly by phosphorylation; 4. modified VirG

3.6.2 Explants used for transformation by *Agrobacterium*

Explants for transformation by *Agrobacterium* vary enormously in size, and range from individual cells (usually protoplasts), suspension-cultured cells and callus cell clumps (undifferentiated and proembryogenic), thin cell layers

(e.g. epidermal strips), tissue slices (e.g., carrot phloem and potato tuber storage parenchyma) to whole organ sections (leaves, roots stems and floral tissues). Many of the explants used for the transformation experiments would be easily obtained from aseptically germinated seedlings or micropropogated shoots.

Explants of high quality are essential for optimal transformation efficiency. The quality of the explant is influenced by the environmental conditions under which the source material is grown. Nutrient composition and concentration, lighting intensity and quality, the temperature all can effect subsequent transformation efficiency. The developmental ages of the explant also have a major effect on transformation. Transformation responsiveness is influenced by several factors associated with the explanting and resulting wound sites. These factors are: plant defence mechanisms that cause phenolic production, cell division frequency, and the regeneration competence of the cells at the wounded sites.

3.6.3 Co-cultivation with *Agrobacterium*

The most widely used method for *A. tumefaciens* is the co-cultivation of disarmed strains carrying a modified T-DNA with explants of plant tissue. For *A. rhizogenes* mediated transformation the usual approach is to co-cultivate with strains harbouring binary vectors and select transformed hairy roots from which complete plants are regenerated in culture. Other approaches not involving the use of tissues culture include the co-cultivation of *Agrobacterium* with imbibed seeds, e.g. *Arabidopsis thaliana* and the injection of *Agrobacterium* into germinating seeds, e.g. soybean (Chee *et al.*, 1989). When transformation occurs using the latter methods the resulting plants are expected to be chimeric for sectors of untransformed tissues and sector of transformed tissue that may have arisen from one or more independent transformation events. Progeny of the next generation can be

screened for totally transgenic plants resulting from individual transformation events.

The initial studies on transfer of foreign genes to plants involved the cocultivation of plant protoplasts with *Agrobacterium.* A major technical advance was the demonstration that transgenic plants could be regenerated from the leaf discs following cocultivation with *Agrobacterium.* Subsequently, transgenic plants have been produced from many families using this approach, or modifications of it. Virtually every plant source has been co-cultivated with *Agrobacterium* and transgenic plants obtained. Such explants include cotyledons, leaves, thin cell layers, cotyledonary petioles, peduncles, hypocotyls, stems, microspores and proembryos.

The explant cocultivation method involves dipping explants into a culture of modified *Agrobacterium,* blotting on sterile filter paper, and culturing on callusing or regeneration media. After 24-72 hrs of incubation, explants are transferred to similar medium containing an antibiotic (e.g., cefotaxime or carbeniciffin) to suppress *Agrobacterium* growth. Selection for transgenic cells, by inclusion of the selective agent in the culture medium is usually initiated at this time. In some instances it is preferable to delay selection until 6-8 days after cocultivation, e.g., in potato (Conner *et al.,* 1991). The concentrations of the selective agent used vary widely depending on the sensitivity of the plant species and/or explant source. In *Brassica napus* 15 mg/1 of Kanamycin was used, where as 300mg/1 was necessary in petunias, tobacco and tomato. Cell colonies and/or shoots growing on the selection medium are transferred to fresh medium as required for the development of complete plants. The selective agent is usually maintained in all culture media throughout plant development. In some instances it may inhibit shoot regeneration or root initiation and it may become necessary to omit or reduce the concentration of the selective agent, e.g. in potato (Conner *et al.,* 1991). Transformation of dicotyledonous plants with *Agrobacterium tumefaciens* is well established, and produced stable transgenic plants expressing a number of foreign genes. This has not been the case for

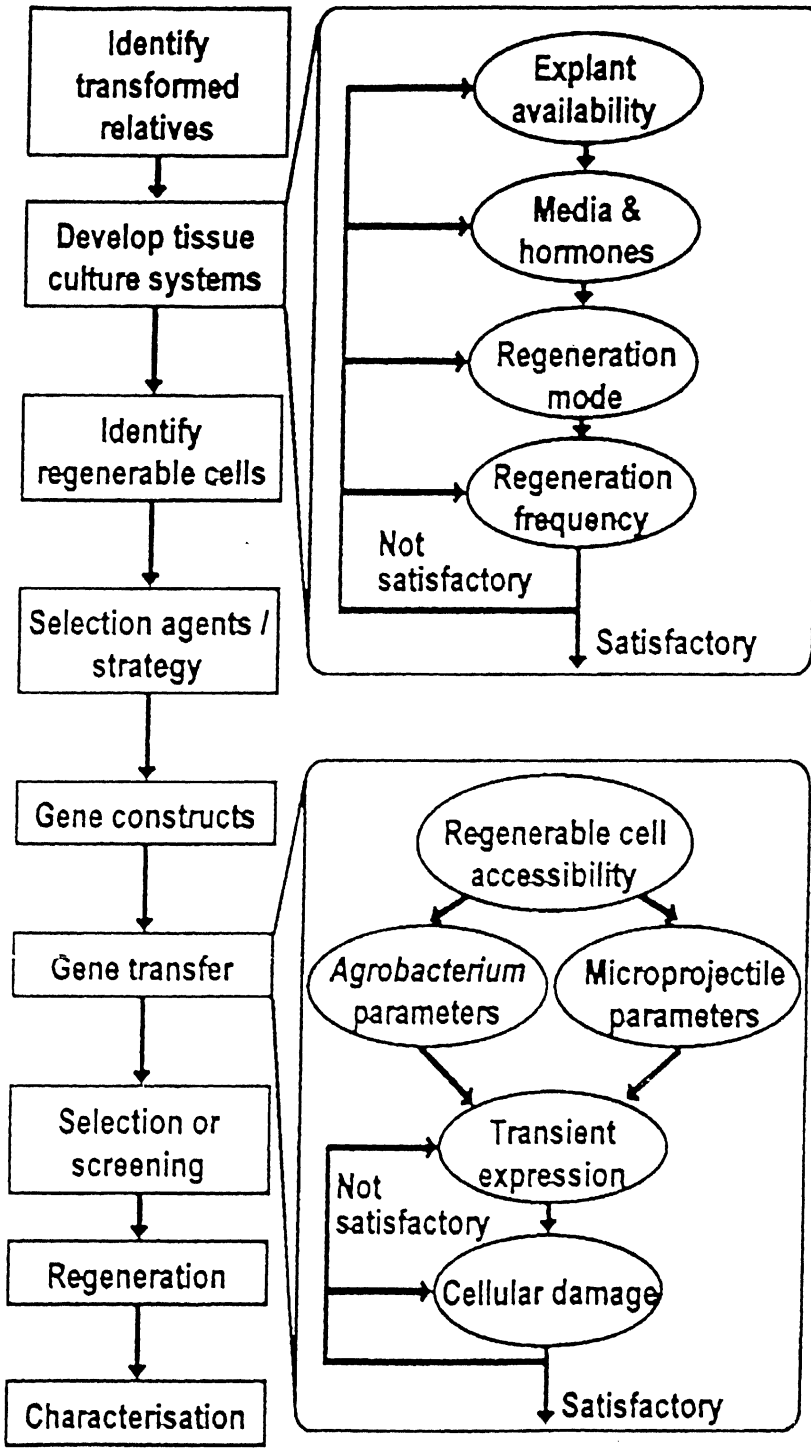


Fig 1.7 A generalized approach to development of transformation systems for recalcitrant plan species.

monocots in general. To date only a few representatives of the families Liliaceae, Amaryllidaceae, Iridaceae, Dioscorea and Gramineae have been reported to be transformable by *Agrobacterium.*

3.7 Direct DNA delivery methods

Agrobacterium mediated gene transfer has already had a tremendous impact on the plant sciences, but this is somewhat tempered by the fact that the cereals (and therefore the most important food crops) are not easily amenable to transformation. Additionally, the tissue culture techniques required for the regeneration of transformed shoots from other groups of crop plants, such as the majority of seed legumes, are still very rudimentary; this has led to an intensive search for alternative gene transfer techniques. Physical methods for the delivery of transforming DNA to eukaryotic cells have been available since the late 1970s. Most of these techniques were derived firom studies examining the expression of virus genomes in tissue cultured animal cells.

Besides the prerequisite of efficient DNA uptake, any direct gene transfer system must protect the vector firom nuclease or mechanical degradation. Such systems should also take into account any plant cells competence, be non-cytopathogenic and retain reasonable cell viability after DNA uptake. In general, physical methods for DNA transfer can be grouped according to the type of the target cell. Some, such as the chemically stimulated endocytosis of plasmids or DNA loaded liposomes and electroporation, are limited for use with protoplasts. Theoretically techniques such as microinjection and microprojectiles can be used with a much wider range of explants. Although it is possible to transform protoplasts from any host species, the efficient regeneration of transformed plants from protoplasts derived tissue, especially that of cereals remains problematic. Though recent progress with rice and maize is encouraging (Rhodes *et al.,* 1988), it remains to be seen if such techniques are applicable to a wide range of varieties and are free from fertility problems.

DNA delivery methods which are not limited only to protoplasts (microinjection, microprojectiles and macroinjection) aim to manipulate immature embryos, organ meristems, gametes and zygotes without adversely affecting their normal development. These techniques are currently under rigorous evaluation and although some are promising, there is as yet insufficient evidence to suggest that this emerging technology is fully reproducible and efficient enough for general use.

Direct gene transfer techniques generally use small *E.coli* cloning plasmids as vectors. In theory, prokaryotic vectors are suitable for direct gene transfer with only addition of an antibiotic resistance gene engineered for plant expression. The majority of these selection marker constructs have already been tested *via Agrobacterium* transformation and are known to be expressed efficiently in transformed cells; indeed many small intermediate vectors have been used successfully for direct gene transfer. The delivery of vector DNA into synchronized protoplasts at S phase and mitosis improved transformation efficiencies by several fold (Meyer *et al.*, 1985; Okada *et al.*, 1986); transformation of immobilised protoplasts by microinjection was also much improved if they were allowed to reform a cell wall for a day or so and thus reactivate the cell cycle prior to injection (Crossway *et al.*, 1986; Reich *et al.*, 1986). These data again strongly suggest that competence for transformation may be related to genome replication and repair in stressed cell reentering cell division. Competent plant cells, like animal cells, will integrate any type of DNA introduced into the nucleus, and therefore specific DNA sequences designed to direct preferential integration of particular vector fragments rarely do so. Plants transformed by direct techniques often contain multiple copies of the vector DNA, or even whole linearized vector sequences, integrated into one or a few sites in the genome, either singly or as concatamers (e.g. Potrykus *et al.*, 1985; Riggs and Bates, 1986). The selection of transformants is only possible where transformed cells integrate at least one copy of the marker gene, which has not been scrambled during DNA uptake and integration. There is some suggestion that more efficient

transformation can be achieved using linear DNA vector DNA was found to be integrated in long concatamers (Riggs and Bates, 1986). Under these circumstances the structure of the inserts was more predictable, and thus by linearising vectors at positions distant firom the selection marker gene and passenger DNA it may be possible to predetermined, to a certain extent, the configuration of transferred DNA.

3.7.1 Gene Transfer By Particle Bombardment

Gene transfer into intact cells and tissues by particle bombardment has become an important tool in plant molecular biology. In addition to its application for the production of genetically transformed plants, particle bombardment has been used in transient expression experiments for the functional analysis of promoter elements that confer regulation to environmental and tissue specific factors. Gene transfer by particle bombardment provides a means of circumventing the use of protoplasts in transient assay systems. The expression of reporter genes therefore can be monitored directly in tissues, which should exert proper regulation upon the introduced gene.

Birch and Bower (1994) suggest that ideal components of a system for production of transgenic plants by particle bombardment are

1. To ensure the proportion of cells in the target area, are both regenerable and competent for integration of introduced DNA.
2. To minimize cell damage but still maintains a high frequency transfer of an appropriate DNA load.
3. Efficient selection for transformants.
4. Minimal frequency of undesired genetic change.

The commercially available device (Biolistic PDS-1000, *Du Pont)* uses gun powder or helium (Williams *et al.*, 1991) discharge, whereby a nylon membrane macroprojectile (coated with microprojectiles) is shot down a

barrel towards a stop plate. Although using the same basic principle, the Particle inflow gun (PIG) differs in that it is easier to prepare experimentally, as cost effectiveness compared to the commercial device. An inexpensive and easy to assemble particle gun was first described by Takeuchi *et al.*, (1992). The 'flowing helium gun' accelerated particles directly in a stream of low pressure helium macrocarriers did not support or carry the particles and the force necessary to accelerate the particles was therefore reduced. The absence of macrocarriers reduced consumables, cleanup time and cycle time. In addition, this device gave transient transformation of a variety of different plant tissues. The flowing helium gun was used as the basis for development of the particle inflow gun (PIG). Several new features were added to the basic design of the flowing helium gun to make it more efficient and compatible with biological targets: l. A vacuum chamber was used to reduce the drag on the particles and also lessen tissue damage. 2. A timer relay driven solenoid replaced the manual syringe stopcock (Morikawa *et al.*, 1989). The solenoid provided more consistent accelerations by permitting better control of the amount of helium released through the use of a timer relay. The amount of helium released could be further controlled using a pre-chamber, upstream of the solenoid. By reducing the amount of helium used to accelerate the particles, tissue damage or displacement was also reduced. Unlike a membrane rupture system (Williams *et al.* 1991) no preparation was required with the solenoid and it functioned at low helium pressures, which was less damaging to the target tissue.

The success of microprojectile bombardment depends on the penetration of plant tissues and transfer of DNA. To optimize delivery and minimize tissue damage, several gun parameters can be modified including DNA attachment to microprojectiles, microprojectile size, velocity of gas flow (including the effect of pressure, aperture and pulse time in the modified PIG system), and distance to the target tissue.The adsorption of DNA directly to the surface of the microprojectile (which is most commonly tungsten or gold) is essential for efficient DNA delivery. Tungsten can produce toxicity

to cells, and therefore decrease recovery of stable transformants (Russell *et al.*, 1992) reported a four fold increase when gold was used instead of tungsten in tobacco transformation. Franks and Birch (1991) include the following features required for microprojectile particles:

1. defined particle diameter (usually 1 μm)
2. high density for cell wall penetration
3. sufficiently inert to eliminate the chance of explosive oxidation
4. nonreactivity with components of the precipitating mixes and DNA
5. low toxicity to plant cells.

Both calcium chloride and spermidine are necessary for good DNA precipitation onto tungsten particles. The concentrations of these are critical and it has been found that the optimum ranges are 0.24 to 1.9 M for $CaCl_2$, and 100 mM for spermidine (Klein *et al.*, 1988). In contrast, DNA has generally been precipitated onto gold particles using ethanol and dried onto carrier surface before acceleration (McCabe *et al., 1988).* Small differences in diameter influence the ability of the microprojectile to penetrate cell walls and membranes. Klein *et al,* (1988) used tungsten particles of diameters of 0.6, 1.2 and 2.4 μM and reported that highest transient expression was obtained when using microprojectile particles with an average diameter of 1.2 μM.

The velocity with which the microprojectile are propelled toward the target cells and the distance that they travel before striking the target, both affect the extent of cell injury. Velocity can be controlled by altering the accelerating force (such as gas pulse pressure), and the vacuum in the target chamber or the distance travelled by the microprojectile particles (Franks and Birch, 1991).

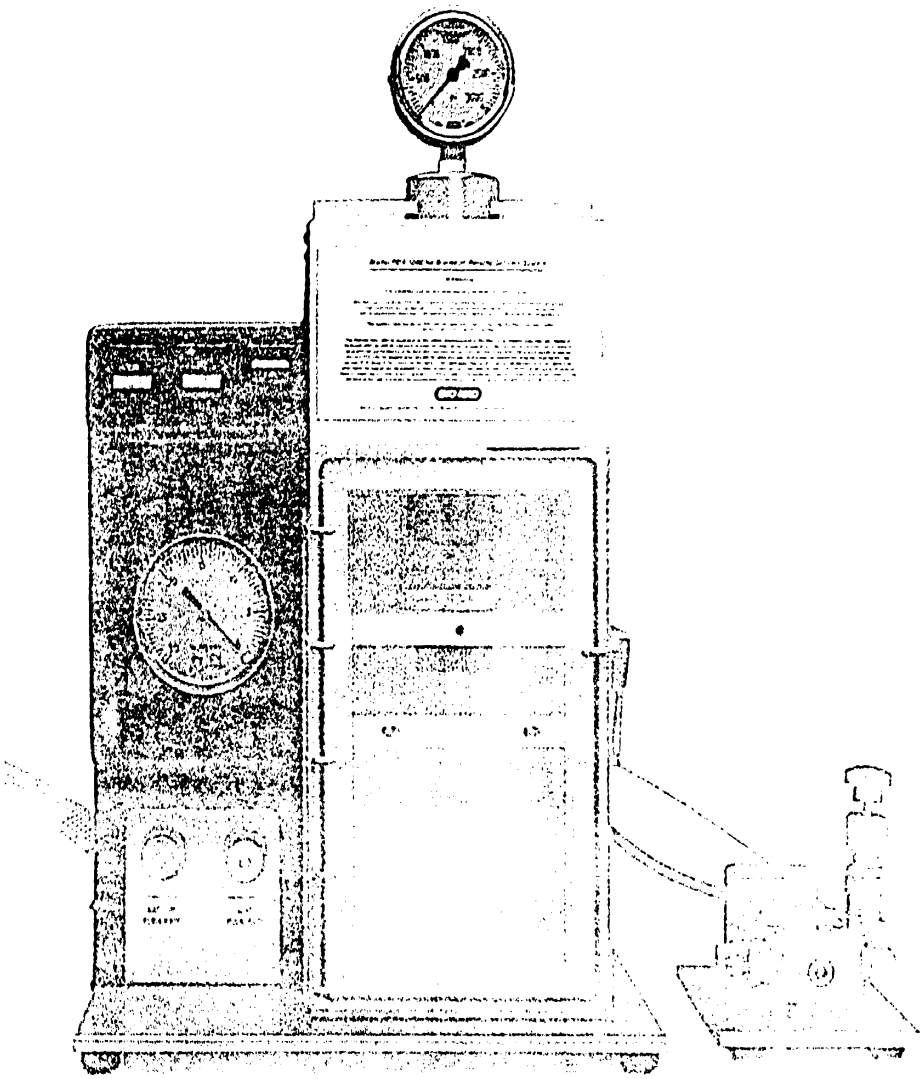
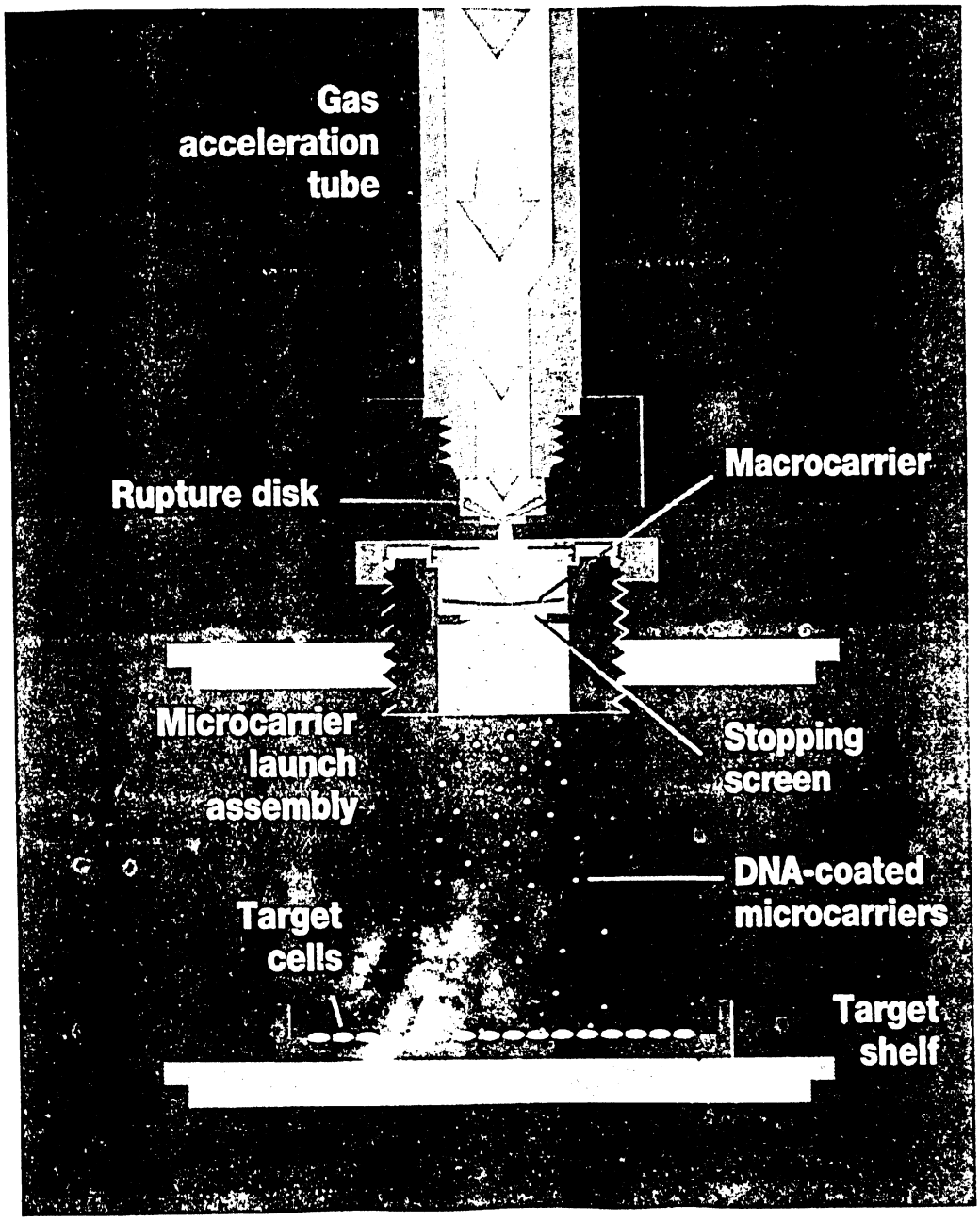


Fig 1.7

The Biolistic PDS-1000/He unit. *Main chamber containing the microcarrier launch assembly and the bombardment helium pressure gauge (on top). The central gauge (in the left side of the instrument) monitors the vacuum within the chamber, and the two lower knobs adjust the vacuum flow and vent rates. The helium metering valve is next to the main chamber (lower right).*

Fig 1.8 Schematic representation of the PDS-1000/He system during activation (The arrows indicate the direction of helium flow)



The helium pressure and vaccum circuits in the PDS-1000/He system effectively accelerate the microcarriers into the target cells. After all the materials are in place, the chamber door is closed and a vaccum is applied. The vaccum reduces the frictional drag on the DNA-coated microcarriers and provides asafety interlock; the instrument cannot be activated unless a vaccum is drawn.

Activating the fire switch allows helium to flow into the gas acceleration tube at a rate regulated by the helium metering valve and monitered by helium pressure gauge. The gas is held until the burst pressure of the rupture disk is reached. This generates the helium shock wave into the bombardment chamber.

The shock wave hits the microcarrier launch assembly and propels a plastic macrocarrier holding DNA- coated microcarriers towards the target cells. A stopping screen retains the plastic disk, while allowing the coated microprojectiles to pass through and transform the target cells.

Applicability of Biolistics

1. It is easy to handle
2. One shot can lead to multiple hits (transfer of genes into many cells)
3. Cells survive the intrusion of one (?) particle.
4. The genes coated on the particle have biological activity.
5. Target cells can be as different as pollen, cell culture cells, cells in differentiated tissues and meristems
6. They can be located at the surface or in deeper layers of organs.
7. This method depends on physical parameters and so on.

3.7.2 Electroporation

A rapid and simple method for introducing cloned genes into a wide variety of microbial, plant and animal cells is electroporation. This technique depends upon the original observation by Zimmerman *et al.*, (1983) that high voltage electric pulses can induce cell plasma membranes to fuse. Subsequently, it was found that when subjected to electric shock (typically a brief exposure to a voltage gradient of 4000-8000V/cm), the cells take up exogenous DNA from the suspending solution, apparently through pores momentarily created in the plasma membrane. These pores appear to be at least 30 nm in diameter and persist for several minutes after pulse and a proportion of these cells becomes stably transformed (Newman *et al.*, 1982, Potter *et al.*, 1984), and can be selected if a suitable marker gene is carried on the transforming DNA. This technique, therefore, is not dependent upon special characteristics of the cell and can be used with virtually any cell type.

Stably transformed cell lines have been produced in a number of monocotyledonous species by electroporation, including maize (Fromm *et al.*, 1986), Wheat (Lorz *et al.*, 1985) and rice (Uchimiya *et al.*, 1986). Recently it has proved possible to regenerate plants, which are in some cases fertile, from transformed cell lines of several cereals (Rhodesetal., 1988; Zhangetal.,1988; Shimamoto *et al.*,1989).

The efficiency of transformation by electroporation is influenced by number of factors

1. The strength of the applied electric field
2. The length of the electric pulse
3. Temperature
4. Conformation and concentration of DNA
5. Ionic composition of the medium

Electroporation of *E.coli* is the most effective method to obtain transformants. With many *E.coli* strains, a transformation frequency of at least 10^8 transformants per μg DNA can be obtained

3.7.3 Detection of transformed plants

There are several ways of demonstrating the presence of foreign DNA in plant tissue. In order to facilitate this, transformation experiments include a reporter gene, a selectable marker gene or both. Selectable marker genes generally code for herbicide or antibiotic resistance. The bar gene has already been described. The other commonly used selectable marker genes include the NPTII gene, conferring resistance to the antibiotics, kanamycin, G418, paromomycin, and hygromycin. Efficient selection requires incorporation of the transgene into the plant cell genome (stable transformation), and sufficiently high level of expression of the gene to allow selection. Tissue surviving selection is not necessarily transgenic, escapes being possible. This means other proofs are required.

Reporter genes express a protein whose presence can be conveniently assayed. Ideally, assays for reporter genes should be sensitive, unambiguous and cheap. It should be possible to visualize the expression in the tissue and to qualify the level of expression. Reporter genes are used in transient expression experiments. These may be aimed at optimizing gene transfer or for testing a promoter activity. The most widely used reporter gene in the plants is the *uid* A gene encoding β-glucuronidase. It is commonly known as GUS gene.

There are two ways to test the presence of a transgene at the DNA level. These are PCR amplification of the part of the insert, and DNA:DNA hybridization. PCR amplification is never sufficient proof of transformation because of the risk of false positives. Nevertheless it can be useful for quick check. The definitive proof of transformation is DNA:DNA hybridisation. Cells and tissue expressing B-glucuronidase will turn blue when incubated at 37^0C overnight.

False positives caused by endogenous GUS like activity is some times a problem, although this is generally less intense and less discrete than real GUS activity. The blue colour from the GUS assay can also be masked in green tissues. This can be solved by clarifying the chlorophyll with ethanol after staining.

3.7.4 Stability of Inserted Genes

The genetic stability of any particular gene inserted into a plant genome is expected to be a function of:

1. The particular gene
2. The physicogenectic location of the gene in the genome
3. The physical structure of the introduced, inserted DNA segment that carries the gene in the plant genome
4. The level of gene expression vis-à-vis the phenotype.

Basic genetic research over the year has established that gene stability depends both on its own nature and on its environment in the genome. Engineered genes integrated into a plant genome are, of course, not immune to the basic principles governing stability of "natural" genes. Thus a gene comprised of an inherently unstable DNA sequence will, in all likelihood, be similarly unstable upon reintroduction into a plant genome.

The most commonly used transformation procedure involving *Agrobacterium* infection, introduces genes to many sites in the genome, meaning that the same gene can be introduced to a variety of genetic environments and be subject, in theory, to the effects of DNA sequences surrounding the insertion sites. In addition, the segment of DNA that is integrated into the genome may be physically organized as either a single copy, a tandem direct repeat of two or more copies, or a tandem inverted repeat of two or more copies. Interestingly, the unstable insertion is the most complex integration event, namely, an array of five tandem copies organised in three inverted repeats and one tandem repeat. This insertion appears to be unstable both somatically and germinally and to produce deletion derivatives that are themselves also highly unstable. It appears that tandem repeats occur frequently is a characteristic that apparently varies among strains of *Agrobacterium*, and so the choice of strain can have an indirect effect on the stability of introduced genes. This technology will permit us to more clearly determine the effect of DNA structure and genetic location on the stability of introduced genes.

3.8 Uses of gene transfer technology

The majority of genetically transformed plants studied to date have been generated via organogenesis in tissue culture. In many instances, such plants have been used to examine a particular aspect of gene expression or molecular biology, and there have been few comprehensive studies on the genetic stability of such plants. However, this is set to change now that the commercial sector has begun field trials to evaluate the performance of

genetically engineered plants. In most instances the aim of genetic engineering experiment is to transfer a single gene to an existing plant variety without altering its major characteristics. Apart from uncontrollable genetic changes associated with tissue culture, individual transformed plants vary with regard to the transferred gene(s) copy number, organisation and expression.

The number of transferred DNA inserts is variable with both *Agrobacterium* mediated and direct gene transfer techniques (electroporation, microprojectile bombardment *etc*), although the former is generally more predictable, usually varying between one and three copies. Multiple gene copies are often present as large concatemeric inserts and in terms of genetic linkage, these will generally acts as a single locus, even though several copies of the transferred DNA are present. Generally, direct gene transfer methods afford less control over the scrambling of genes during transfer, and this may lead to problems in identifying the active loci, except by genetic analysis. It is even possible that problems with stability could occur as a result of recombination between homologous sections of the transferred DNA. However, to date most transferred genes have exhibited normal Mendelian inheritance.

Although transformed plants have been obtained in many species, reasonably efficient transformation procedures exist for only a few (e.g. alfalfa, *arabidopsis,* carrot, potato, tobacco and tomato). The host range for transformation is still largely related to the availability of tissue culture techniques for any particular species and there is still room for improvement in many other crops besides the obvious bottlenecks of the seed legumes and cereals.

The number of 'useful' traits that might be engineered into crop plants is growing slowly, but in the near future an enormous amount of effort will be required to develop novel strategies for identifying genes controlling many important crop characteristics, for example disease resistance, flowering characteristics, fertility, incompatibility and plant morphology.

Currently, a major application for transgenic plants relates to their utility for the detailed analysis of the regulatory elements that control gene expression; specialised promoterless 'reporter gene' vectors have been developed to assist these types of experiments (e.g., An, 1986; Jefferson *et al.*, 1987). These experiments commonly involve the construction of a transcriptional gene fusion between a DNA sequence with putative regulatory properties (e.g. tissue-specific or environmentally regulated) and a reporter gene coding for β-glucuronidase or chloramphenicol acetyl transferase enzymes for which a simple and sensitive assay procedures exists. Such studies have identified regions upstream (5') of several genes which confer developmentally and environmentally regulated gene expression (e.g. Herrera-Estrella *et al.*, 1984; Sengupta-Gopalan *et al.*, 1985).

Besides studying the regulation of expression of individual genes in a heterologous genomic background, transgenic plants also enable the investigator to study the effects of manipulating particular enzymes, or other proteins, on basic plant biochemistry and metabolism. Transgenic plants have applications in the study of protein transport in plants. In particular, hybrid genes with altered N-terminal transit signal and peptides have been constructed to examine protein targeting into organelles (Kuntz *et al.*, 1986; Boutry e*t al*; 1987) and routing of seed storage proteins through the endomembrane system into protein bodies (Iturriaga *et al.*, 1989).

A more specialised use of *Agrobacterium* transformation is the delivery of virus genomes into intact plants by a process known as 'agroinfection' (Grimsley *et al.*, 1987). In these experiments the T-DNA contains a dimer of the viral genome, which, when delivered to the plant cell, produces an active viral genome, either by homologous recombination and excision from the T-DNA or by synthesis of molecules which act as replicative intermediates. Although agroinfection depends on a functional *vir* gene system. T-DNA integration into the plant nuclear genome is not required. Indeed agroinfection studies using a dimer of maize streak virus provided the first evidence that T-DNA delivery into the cytoplasm of cereals

was possible (Grimsley *et al.*, 1987). From a commercial point of view, agroinfection techniques can be used to introduce rapidly viral genomes to a large number of host plants to test for their susceptibility to infection by particular strains. This technique is particularly useful with viruses which are normally transmitted by an insect vector, or which are normally impossible to inoculate by any techniques other than grafting.

Transgenic plant biotechnology offers at least two basic techniques for the isolation of genes via insertion mutagenesis. The random insertion of modified T-DNA sequences might be used to inactivate particular genes. This approach requires either the transformation of large numbers of single cells and selection of a particular mutant trait in clonally derived transformed colonies, or the regeneration of large numbers of independently transformed plants and their screening/selection for particular mutant phenotypes.

From the above discussion, it can be seen that transgenic plants play an important role in the future, not only in gene expression work, but also in helping to provide methods for finding genotype to phenotype in order to identify new genes for both fundamental and commercially oriented studies.

Materials And Methods

4. MATERIALS AND METHODS

4.1 Organogenesis and somatic embryogenesis

JL24 was the principle genotype used for the present study. Kernel of groundnut was removed and the seeds were surface sterilised using 0.1% aqueous mercuric chloride and kept for about 6-7 minutes on an orbital shaker at 200 rpm. Seeds were then thoroughly washed 3-4 times with sterile distilled water in aseptic conditions. The seeds were stripped off from the seed coat after 4-5 hours of soaking and were incubated on the surface of the MS agar medium in a culture room under 24,48, 72, 96 hour (d_1, d2, d3, d4) photoperiod conditions, under 3000 lux illumination at 25 to 29^0C. The cultures were examined daily for contamination and infected cultures were discarded. The seeds thus obtained were placed on a sterile petridish with forceps and were cut open using a surgeon's scalpel fitted with a number11 surgical blade. The leaflets and the embryos were excised and placed on the surface of MS media having different concentration of phytohormones and sucrose (as shown in the table below). The pH of the media was adjusted to 5.8 before autoclaving. The leaflets and somatic embryos were checked for caulogenesis and somatic embryogenesis respectively after two weeks.

Media used for Organogenesis

Experiment I (varying NAA concentration)

I MS+BAP(3mg)+NAA(1mg)

II MS+BAP(3mg)+NAA(0.5mg)

III MS+BAP(3mg)+NAA(0.2mg)

Experiment II (varying BAP concentration)

IV MS+BAP(1mg)+NAA(1mg)

V MS+BAP(2mg)+NAA(1mg)

VI MS+BAP(3mg)+NAA(1mg)

VII MS+BAP(4mg)+NAA(1mg)

Media for somatic embryogenesis

1 MS+BAP(1mg)+2,4D(90μM)+6% Sucrose

2 MS+2,4D(90μM)+6% Sucrose

3 MS+2,4D(90μM)+5% Sucrose

4 MS+2,4D(90μM)+4% Sucrose

5 MS+ 2,4D(90μm)+3% Sucrose

6 MS salts+B5 organics + Kinetin(1ml) + 2,4D(90μM) (D90 media)

Note: 20mg/l 2,4D = 90μM

***Agrobacterium* mediated transformation**

4.2 Culture conditions

The recombinant *Agrobacterium* colonies of pCAMBIA:1301:GRAVcp plasmid containing hygromycin phosphotransferase (hpt) and β-glucuronidase (GUS) were cultured by adding 200μl of culture in 200ml YEB medium supplemented with 20μl (50mg/l) of selective agent. The inoculated culture was incubated overnight at 37^0C. 25ml of the fresh overnight culture was then subjected to plasmid isolation.

4.3 Plasmid isolation

Plasmid DNA was extracted by the alkaline lysis method (Brinboim and Dolly; 1979, Ish Horowitz and Burke; 1981). 25ml of the culture was centrifuged at 5000rpm. The supernatant was removed and the pellet was resuspended in 200µl of GTE buffer. The suspension was then transferred to a microfuge tube and kept on ice for 5min. 400µl of freshly prepared lysis buffer was added in the tube and inverted several times and stored on ice for 5min. 300µl of ice cold 5M potassium acetate was added and vortexed vigorously and stored in ice for 5min. The tube was centrifuged for 5min at 4^0C and the supernatant was transferred to a fresh tube. 600µl of isopropanol was added to precipitate the DNA. It was allowed to stand for 2min at room temperature and centrifuged at 12,000rpm for 15min. The supernatant was discarded and the pellet was washed in 1ml of ice-cold 70% ethanol. The pellet was air dried and dissolved in 60µl of TE 8 containing free Rnase (5µg/ml) and incubated at 37^0C for 60minutes. This treatment eliminates RNA that can mask small fragments of DNA in agarose gels. The quality of the DNA was checked on 0.8% agarose gel. Quantity and purity were determined spectrophotometrically by measuring the absorbance at 260nm and 280nm with a SHIMDZU UV 160A spectrophotometer.

4.4 Restriction digestion of plasmid DNA

5µl of isolated pCAMBIA:1301:GRAVcp and PRT99 G plasmid DNA was subjected to digestion using 1-2 units of restriction enzyme Pstl using in appropriate buffer. The mixture was kept at 37^0C for 2-3 hours and the reaction was stopped using a loading buffer. The plasmid was loaded into the well made of 0.8% agrarose gel using TBE. DNA molecular marker HindIII-

digested λ DNA was loaded into another lane and electrophoresis was carried out at 65 volts for 3 hours

The gel was then stained in 0.5μg /ml solution of ethedium bromide for 15-30 minutes and visualized on UV illumination. The size of the insert DNA was compared with Hind III marker.

4.5 *Agrobacterium* mediated transformation of tobacco

The mature leaf of tobacco was surface sterilized using 15% chlorax and was placed over a sterile petridish. Leaf discs were made using a sterilized disc borer and dipped into a cocultivation media (5ml of the overnight grown *Agrobacterium* culture media centrifuged at 5000rpm and the bacterial pellet was dissolved in ½ MS media containing ½ the amount of the MS salts) for few seconds. The disc was transferred to surface of MS_4 agar medium containing cephotaxime (250mg/l). After 3 days these discs were transferred on to a selection medium containing MS_4 along with cephotaxime (250mg/l) and hygromycin (5mg/l). The leaf discs survived showed shooting after a week. The shooting discs were cut ands transferred into sterile tubes containing MS_4, cephatoxime (250mg/l) and hygromycin of two different concentrations i.e. 5mg/l and 10mg/l to check the sensitivity of plantlets. Small pieces of the plantlets from each tube were taken and dipped in 200μl of GUS buffer and incubated at 37^0C. After 24 hours GUS expression was observed.

4.6 *Agrobacterium* mediated transformation of groundnut

Sterilized seeds of groundnut (JL24) were taken and the leaflets were excised. The basal part of the leaflet was wounded and dipped into the cocultivation media containing the suspended *Agrobacterium*. The *Agrobacterium* infected leaflets were then transferred on to the surface of MS media containing cephatoxime (250mg/l). After 3 days these leaflets were

transferred on to a selection medium containing definite amount of phytohormones (BAP 4mg + NAA 1mg), media which showed best regeneration through tissue culture experiments) in MS media along with cephatoxime (250mg) and kanamycin 50mg/l. Small pieces of the survived explants from each plate were randomly selected and dipped in 200µl of Gus buffer and incubated at 37^0C. After 24 hours checked for blue coloration.

4.7 Particle bombardment using Biolistic gun

4.7.1 Preparation of Microcarriers

About 60mg of microprojectiles (gold particles) were weighed in 1.5ml microfuge tube. 1ml of freshly prepared 70% ethanol was added, vortexed on a platform vortexer for 3-5min and incubate for 15min. The tube was spinned for 5sec and the supernatant was discarded. The pellet so obtained was washed thrice by adding 1ml of sterile water, vortexing it for 1min & allowing it to settle for 1 min and then spinned for 2 seconds to pellet the microparticles. 1ml sterile 50% glycerol was added to bring the microparticle concentration to 60 mg/l. The particles so obtained can be stored at room temperature for upto 2weeks for later use.

4.7.2 Coating DNA onto microcarriers

Microcarriers prepared in 50% glycerol (60mg/ml) were vortexed for 5min on a platform vortexer to resuspend the agglomerated particles. 50µl (3mg) of microcarriers were transferred to the microfuge tube. While vortexing 5µl (1µg/µl) of PRT99G plasmid containing NptII (Neomycin phosphotransferase) and GUS (β-glucuronidase), 50µl $Cacl_2$ (2.5M), 20µl spermidine (0.1M) was added. Vortexing was continued for 2-3 minutes. Microcarriers were allowed to settle for 1minute. The centrifuge tube was spinned for 2 seconds. Supernatant was discarded and 140µl of 70% ethanol was added without disturbing the pellet. 140µl of 100% ethanol was added

after discarding the supernatant making sure the pellet was not disturbed. After discarding the supernatant 48µl of 100% ethanol was added to gently resuspend the pellet. Tapping the side of tube several times, and vortexing at low speed was carried out for 2-3 seconds 6µl aliquots of microcarrier were taken and transferred on to macrocarrier to carry out bombardment.

4.7.3 Explants used for bombardment

Various plates of different age leaflets (d0, d1, and d2), shooting explants and embryoids were used for bombardment. The explants to be bombarded were placed at the center in a 2cm-diameter area on the surface of LMS5 agar media.

4.7.4 Particle Bombardment

Inner side of the biolistic gun was sprayed with 70% ethanol to ensure the aseptic conditions. The microcentrifuge tube containing the gold/plasmid DNA mix was vortexed using the microcentrifuge rack in order to resuspend the mix. Immediately following the action 2µl of the mixture was placed over the mylar film used as the macroprojectile or macrocarrier. The remaining mixture in the cetrifuge tube was placed back on ice. Macroprojectile was placed in the dissembled syringe filter unit along with the metal screen. The filter holder was reassembled and screwed into place (finger tight is adequate). Leaf tissue in a petridish is placed on adjustable shelves at distances between 14-23cm from the screen in the syringe filter unit. Vaccum of 28-30 Hg was applied and the high pressure chamber is pressurized at 1500psi with helium gas. The membrane (rupture disk) which restrains the helium is then ruptured. The resultant shock wave of helium accelerates the macroprojectile, which is positioned 9 mm below the rupture disk. The macroprojectile was stopped by the steel screen placed 10 mm below the launch point. The macroprojectile continue onward to penetrate the cells

which are placed in the third slot below the microprojectile screen. Selection for kanamycin resistant explant was performed two days post bombardment on modified MS media (MS + BAP4mg + NAA1mg) containing 50mg/l kanamycin. Somatic embryos were placed on D90 media containing 50mg/l kanamycin. Resistance explants were observed for Organogenesis and somatic embryogenesis after 6-7 weeks. Small pieces of the randomly picked explants were checked for GUS expression using X-GLUC.

Results And Discussion

5.0 RESULTS AND DISCUSSION

Mature embryo derived leaflets of different ages (do-d4) were used to initiate organogenesis using Murashige and Skoog's (MS) medium with various levels of NAA (0.2-1mg/l) and BAP (1-4mg/l) of JL24 cultivar of peanut.

The results obtained are as below.

5.1 Organogenesis :

Effect of different concentration of NAA and BAP on induction of shoot regeneration from different aged leaflet explants

Experiment I (varying NAA concentration)

Media I

Table 6: MS+BAP (3mg/l) +NAA (1mg/l)

Age of the leaf	Number of explants responded (x)	% of response (x/24x100)	Total number of shoots	Average number of shoots/explant
D_0	24	100%	552	552/24=23
D_1	24	100%	480	480/24=20
D_2	21	87.5%	480	480/21=22.8
D_3	20	83.3%	425	425/20=21.2
D_4	20	83.3%	421	421/20=21

Media II

Table 7: MS+BAP (3mg/l) + NAA (0.5mg/l)

Age of the leaf	Number of explants responded (x)	% of response (x/24x100)	Total number of shoots	Average number of shoots/explant
D0	23	95.8 %	138	138/23=6
D1	21	87.5%	110	110/21=5.2
D2	21	87.5%	99	99/21=4.7
D3	20	83.3%	62	62/20=3.7
D4	20	83.3%	54	54/20=2.7

Media III
Table 8: MS+ BAP (3mg/l)+NAA (0.2mg/l)

Age of the leaf	Number of explants responded (x)	% of response (x/24x100)	Total number of shoots	Average number of shoots/explant
D0	0	0	0	0
D1	0	0	0	0
D2	0	0	0	0
D3	0	0	0	0
D4	0	0	0	0

Experiment II (varying BAP concentration)

Media IV
Table 9: MS+BAP (1mg/l)+NAA (1mg/l)

Age of the leaf	Number of explants responded	% response	Total number of shoots	Average number of shoots/explant
D0	23	95.8%	462	462/24=20
D1	21	87.5%	437	437/21=20.8
D2	21	87.5%	397	397/21=18.9
D3	21	87.5%	360	360/21=17.1
D4	21	87.5%	362	362/21=17.2

Media V
Table 10: MS+BAP (2mg/l) + NAA (1mg/l)

Age of the leaf	Number of explants responded	% response	Total number of shoots	Average number of shoots/explant
D0	24	100	480	480/24=20
D1	23	95.8	414	414/23=18
D2	23	95.8	416	416/23=18
D3	22	91.6	374	374/22=17
D4	23	95.8	352	352/23=15.3

Media VI
Table 11: MS+BAP (3mg/l) + NAA(1mg/l)

Age of the leaf	Number of explants responded	% response	Total number of shoots	Average number of shoots/explant
D0	23	95.8%	542	542/23=23.5
D1	24	100%	491	491/24=20.4
D2	24	100%	490	490/24=20.4
D3	23	95.8%	492	492/23=21.3
D4	23	95.8%	475	475/23=20.6

Media VII
Table 12 : MS+BAP (4mg/l)+NAA (1mg/l)

Age of the leaf	Number of explants responded	% response	Total number of shoots	Average number of shoots/explant
D0	24	100%	560	560/24=23.3
D1	24	100%	561	561/24=23.3
D2	22	83.3%	522	522/22=23.7
D3	21	87.5%	489	489/21=23.2
D4	20	83.3%	440	440/20=22

The cultivated groundnut is known to be relatively recalcitrant in tissue culture (Cheng *et al* 1992). Nevertheless, there are several recent reports of successful in vitro regeneration of groundnut via organogenesis from cotyledons, immature leaflets, and other seedling explants (Mroginski *et al* 1981 Seitz *et al* 1987, McKently *et al*;1990,1991; Baker and Wetzstein 1992,Chengetal *et al* 1992, Durham and Parrott 1992, Gill and Saxena 1992, Ozias-Akins *et al.*1992). Most widely used explant of cultivated groundnut for plant regeneration is the immature leaflet isolated from very young seedling (Cheng *et al.*1992).

Manipulation of nutrient medium and culture conditions selectively influence genes resulting in biochemical, biophysical, physiological, and structural changes in the cultured tissue. Phytohormones are important

factors, which can selectively influence the genes to trigger differentiation of cells in cultures. In this study we report caulogenesis from the base of the mature embryo derived leaflets of groundnut by manipulating the hormonal composition of the media. When cultured on MS basal medium supplemented with various levels of BAP (1-4mg/l) (table 9-12) most peanut explants showed shoot regeneration. After 2 weeks on basal media with both

NAA and BAP shoot primordia were induced without a callus phase.(Fig 1.9) A combination of NAA 1mg/l and BAP 4mg/l (table 12) was optimum for both frequency of induction and number of buds per explant. This experiment describes the synergistic activity of NAA and BAP on the cells of peanut leaf base.

In the present study BAP/NAA induced shoot primordia appeared within 15 days of culture with explant (Fig 1.9 C). The basal region of the explant is the most regenerative part of the leaflet and possibly contains pre-existing meristems (Fig 1.9 A). With high levels of BAP and very low levels of NAA white masses appear along with callus. However shoots arise directly from explants without intermediary callus growth. Shoots were induced earlier or around the same time as callus.

Leaflets with different ages i.e. D0 to D4 were cultured in MS with combination of BAP and NAA. It is clear that many shoots were induced with 4mg /l BAP and 1mg/l NAA for D0and D1 leaflet explants (table 12). This shows that juvenile explant has a competitive cells and BAP + NAA induced regeneration systems appear to be repetitive since several cycles of shoots were produced from the initial explants. Such a cyclic-regeneration systems is ideally suited for gene transfer research because of ease of imposing antibiotic selection on transformed tissue and denovo induction of shoot buds from such tissue without an intervening callus phase.

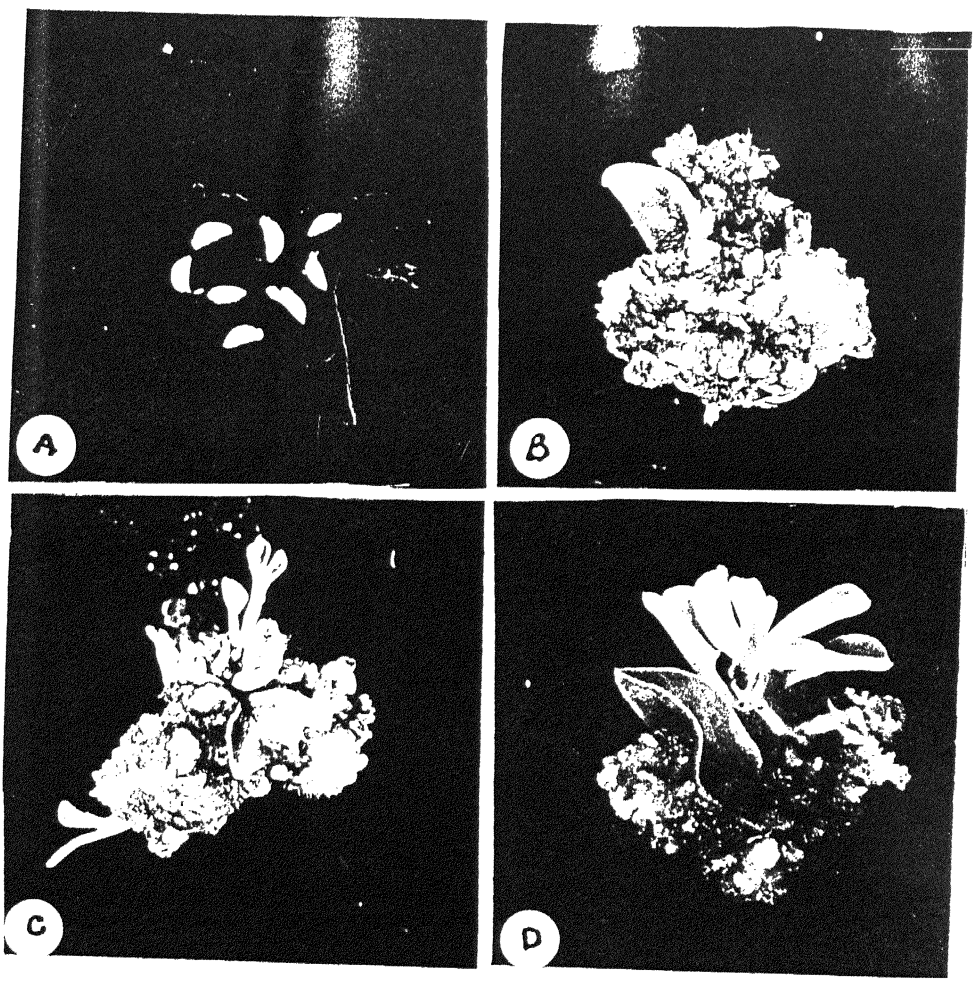


Fig 1.9: Regeneration of shoots from embryo derived leaflets of groundnut
A) Excised leaflet explants of groundnut B) Leaflet explants showing adventitious shoot buds after a week C) Development of shoots after two weeks D)Elongated shoots after 30 days

5.2 Somatic embryogenesis

Table 13: Effect of sucrose conc. on the induction of somatic embryogenesis from embryo axis explants of groundnut var. JL24

Media	Number of explants responded (x)	% response (x/10x100)	Total number of embryoids	Average number of embryoids/explants
MS + BAP (1mg/l) + 2,4D(20mg/l)+ 6% Sucrose	5	50%	35	35/5=7
MS + 2,4D (20mg/l) + 6% Sucrose	4	40%	28	14/3=4.6
MS + 2,4D (20mg/l) + 3% Sucrose	3	50%	14	28/4=7
MS + 2,4D (20mg/l) + 4% Sucrose	5	50%	24	27/5=4.8
MS + 2,4D (20mg/l) + 5% Sucrose	5	60%	25	25/5=5
MS salts + B5 organics+ Kinetin + 2,4 D (20mg/l)	6	60%	37	37/6=6.1

In embryogenesis somatic embryos like naturally occuring zygotic embryos develop as bipolar structures bearing both root and shoot apex. Thus both meristems neccesary for complete plant development are initiated simultaneously thus somatic embryogenesis has now been observed in many plant taxa. Somatic embryos can develop either from callus or directly from organs without involving any intermediate callus stage. In the present work attempts were made for direct embryogenesis.

In vitro regeneration of plants via somatic embryogenesis has much potential for the use in plant propogation and gene transfer (Ammirato 1987, Parrott *et al.*1991, Senaratna 1992). However, the efficient conversion of somatic embryos into plants remain a problem (Ammirato 1987). To be of practical value, somatic embryogenesis should culminate in the formation of plantlets. Recently, there has been a great deal of interest in in vitro regeneration of peanut (Hazra *et al.* 1989, Ozias-Akins 1989, Durham and Parrott 1992, Baker and Wetzstein 1992, Reddy and Reddy 1993) via somatic embryogenesis. Direct somatic embryogenesis from immature zygotic embryos and 50% conversion was reported (Hazra *et al* 1989). A similar

report (Ozias-Akins 1989) also indicated problems in convertion of somatic embryos to plants. From then several experiments were carried out using various explants like leaflets (Chengalrayan, Sulekha Hazra *et al* 1994), hypocotyl (Venkatachalam, *et al;* 1996) using different concentrations of auxins. Successful induction and maturation of somatic embryos was achieved on the medium supplemented with 2,4 dichlorophenoxyacetic acid (S.Hazra, *et al*)

In the present work mature zygotic embryos of groundnut var.JL24 were used as explants. 2,4D at the concentration of 20mg/l along with various concentration of sucrose, BAP and kinetin were checked for embryogenesis (table 13). Sucrose level showed no drastic effect on embryo morphology but the mean number of embryos/explants differed and the media containing 3% Sucrose induced more number of embryoids (Fig 2.0 B). Similar results were obtained by Reddy and Reddy, 1993 where 2% sucrose induced more number of somatic embryos as compared to 6%. Higher concentration of sucrose might have increased osmotic stress on explants and possibly decreased embryogenesis. However only 50-60% of the embryoids showed positive response and the rest of them showed browning of the tissue. This might be due to the high auxin concentration. Incorporation of BAP and Kinetin in the medium was not much effective in improving the frequency of somatic embryo formation similar to the results of Chengalrajan et al 1994. However among the cytokinins used BAP showed better response than kinetin.

The regenerable cultures thus obtained which could be aseptically maintained provide a continuous supply of tissue for transformation experiments and offer a considerable advantage in terms of treatable units and selection strategies but the disadvantages are the genotypic differences in in vitro response and the amount of time required for plant regeneration from embryonic cultures which is nearly 4-5 months.

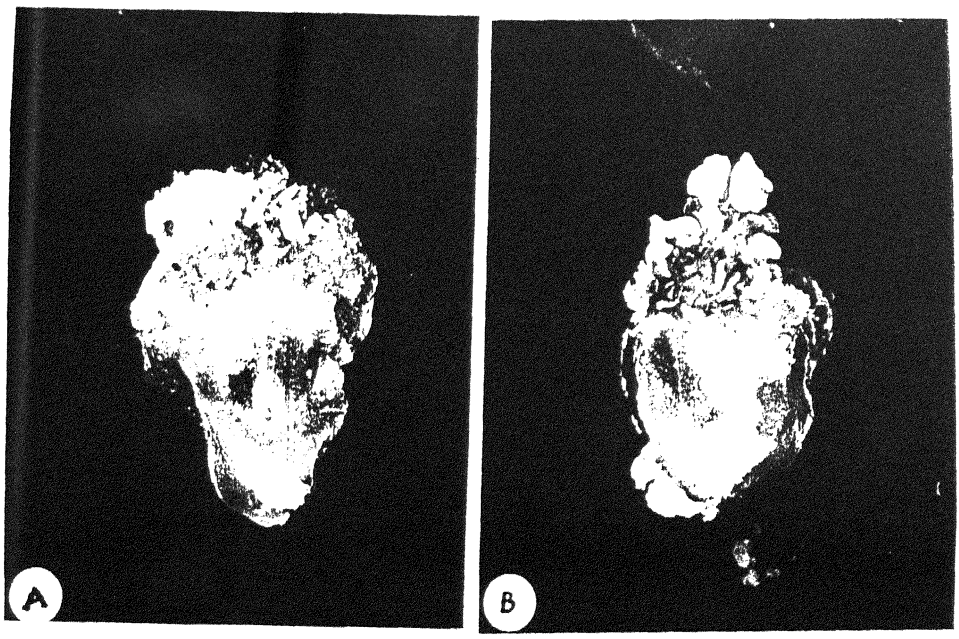


Fig 2.0 Stages of groundnut embryo maturation on MS medium containing 2,4 D and Sucrose A) Embryo turning green in the process of getting defined into embryoids B) Induction of multiple embryoids in the excised embryo after 2 weeks on the medium

5.3 Plasmid isolation

Ethedium bromide stained 0.8% agarose on which the isolated plasmid DNA samples were analyzed showed clear bands characteristic of plasmid DNA without any RNA contamination.(Fig 2.1 and 2.2) The OD ratio of 260/280 which is around 1.8 conforms the purity of the DNA sample isolated.

5.4 Restriction digestion of plasmid DNA

Plasmids contain sequences that can be cut specifically by restriction enzymes. Fragments of known length are generated by digestion of the plasmid with restriction enzymes, and running the fragments on agarose gel, the size of the fragment can be compared to expected fragment size according to the restriction map (Fig 2.1 and 2.2). Molecules of linear, duplex DNA travel through gel matrix at a rate that is inversely proportional to the $\log_{10}$ of their and molecular weight. Thus the complete digestion of the plasmid was evident by the appearance of the electrophoresed DNA on 0.8% agarose gel matrix after ethedium bromide staining and in UV illumination and the presence of the insert DNA was conformed when compared with the Hind III marker.

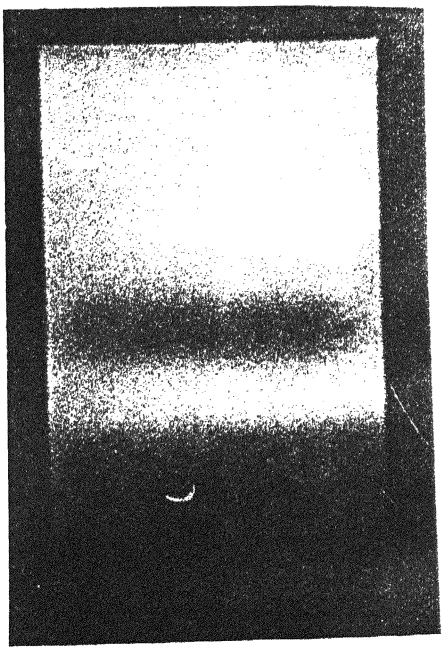


Fig 2.1: Restriction digest analysis of plasmid pCAMBIA:1301:GRAVcp : Lane1: Uncut plasmid, Lane 2 1301:GRAV + Hind III, Lane 3 1301:GRAV + pstI, Lane 4 1301 GRAV + XhoI, Lane 5 1301 GRAV + XhoI + Sal I, Lane 6 1301 GRAV + EcoRI + Sal I, Lane 7 1301 GRAV + EcoRI,Lane Hind III – digested λ DNA.

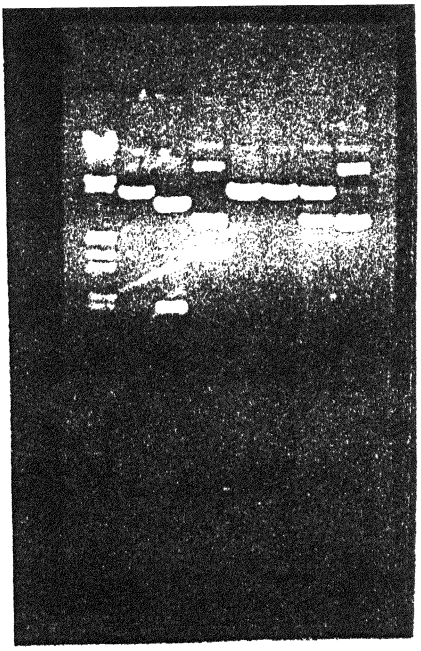


Fig 2.2: Restriction digest analysis of plasmid pRT 99 G plasmid DNA: Lane1. λ Hind III-EcoRI size marker, Lane2. pRT 99 + Xho I, Lane3. pRT 99 + pst I, Lane4. pRT 99 + Hind III, Lane5. pRT 99 + EcoR I, Lane6. pRT 99 + Sma I, Lane7. pRT99 + Xba I, Lane8. pRT 99 uncut

5.5 *Agrobacterium* mediated transformation

With the advances in plant transformation and regeneration (Zambriski *et al* 1983; Fraley *et al* 1985), the techniques of advanced molecular biology offer new opportunities to alleviate various biotic constraints through the production of genetically engineered plants. Several reports on *Agrobacterium*-mediated transformation were obtained in different plants like in Glycine max (Hinchee et al; 1988), Vigna unguiculata (Garcia et al; 1986) and Bean (Leon *et al.*1990; Bustos *et al.*1991; Genga *et al.* 1991) and in many other plant species (Chyi et al., 1987; Chabaud et al.,1988; Dong and Mc Hughen,1991; Sangwan et al., 1991,1992; Pawlicki et al.,1992;De Bondt et al.,1994). Later some Brazilian cultivars of groundnut were shown to be susceptible to *Agrobacterium* spp. (Lacorte *et al*;1991). This study confirmed that groundnut is permissive host for the acceptence of genes from specific gene vectors. Experiments of genetic transformation were later carried out in *Arachis hypogea* like introduction of viral coat protein gene (Hull and Davies 1992), Bacillus thuringiensis endotoxin gene (Feitelson et al, 1992) and protease inhibitor gene (Ryan 1990). Recently transient and stable expression of β-glucuronidase (GUS) activity have been observed in the callus(Clemente et al., 1992; Cheng et al., 1994,1996; Eapen and George,1994) and successful production of transgenic peanut plants were reported (Brar et al., 1994; Mc Kently et al., 1995; Cheng et al., 1996)

In the present work the response of mature embryo derived leaflet and 4-7 days old aseptically raised seedling leaflets of groundnut cultivar JL24 (Fig 2.3:A) to infection by *Agrobacterium* strain C58 containing the plasmid pCAMBIA 1301:GRAVcp harbouring hygromycin phosphotransferase (*hpt II)* and β-glucuronidase (*gus*) as marker genes were investigated. *Nicotiana tobaccum* was used as a model system. The leaflet explants from presoaked seeds were cocultured with the bacterial strain on MS medium containing 4mg/l BAP and 1mg/l NAA (table12) for 3 days, and subsequently subcultured on the selection medium containing the above medium +

250mg/l cefotaxime + hygromycin (5mg/l, 10mg/l). Two weeks after inoculation, shoots were observed on both tobacco and groundnut at the points of infection (wounded region) (Fig 2.3 and 2.4B). Cefotaxime inhibited the growth of *Agrobacterium.* The shoots produced from the leaf discs of tobacco continued to grow when excised and cultured on hormone free MS medium containing cefotaxime and hygromycin(Fig 2.3 B). When checked for sensitivity hygromycin concentration of 5mg/l supported the plant growth where as bleaching of leaflets was seen at concentration of 10mg/l. The shoot regeneration process in leaflets of JL24 being slow was not possible to be checked in two months of dissertation time allotted but the transient GUS expression was clearly seen in transformed *Arachis hypogea* leaves (Fig 2.4D). The leaves from in vitro growing shoots were histochemically analysed for the presence of the GUS gene (Jefferson 1987). Leaf pieces were incubated for 12-14 hours at 37^0C in a reaction mixture containing X-gluc, which is substrate for enzyme β-glucuronidase, and cleared of chlorophyll by passing the leaf segments through 70-90% ethanol for atleast 8 hours, mounted in glycerol, and observed under a light microscope.

Transformed leaves of both tobacco and JL24 cultivar of groundnut were checked for transient GUS expression wherein blue coloration of explant was clearly seen in X-GLUC solution. Out of the 25 randomly picked transformed explants of tobacco 19 turned blue in the X-GLUC buffer. Out of the 30 transformed explants 10 were randomly selected for checking the GUS expression where 4 of them responded positively. Thus 40% efficiency of *Agrobacterium* mediated transformation was seen in groundnut variety tested.

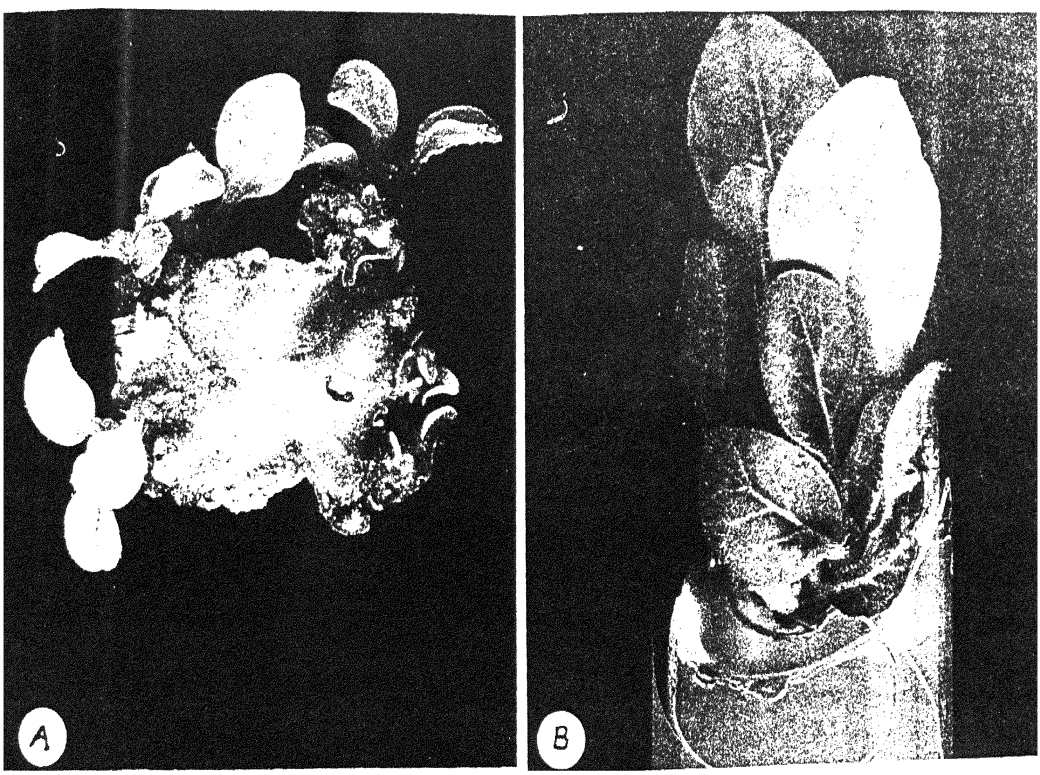


Fig 2.3: Stages in leafdisc transformation of tobaccoA) Shoot formation of the transformed leafdises from the injured parts on the selective medium B) Regenerated tobacco shoots growing on the medium containing Kanamycin

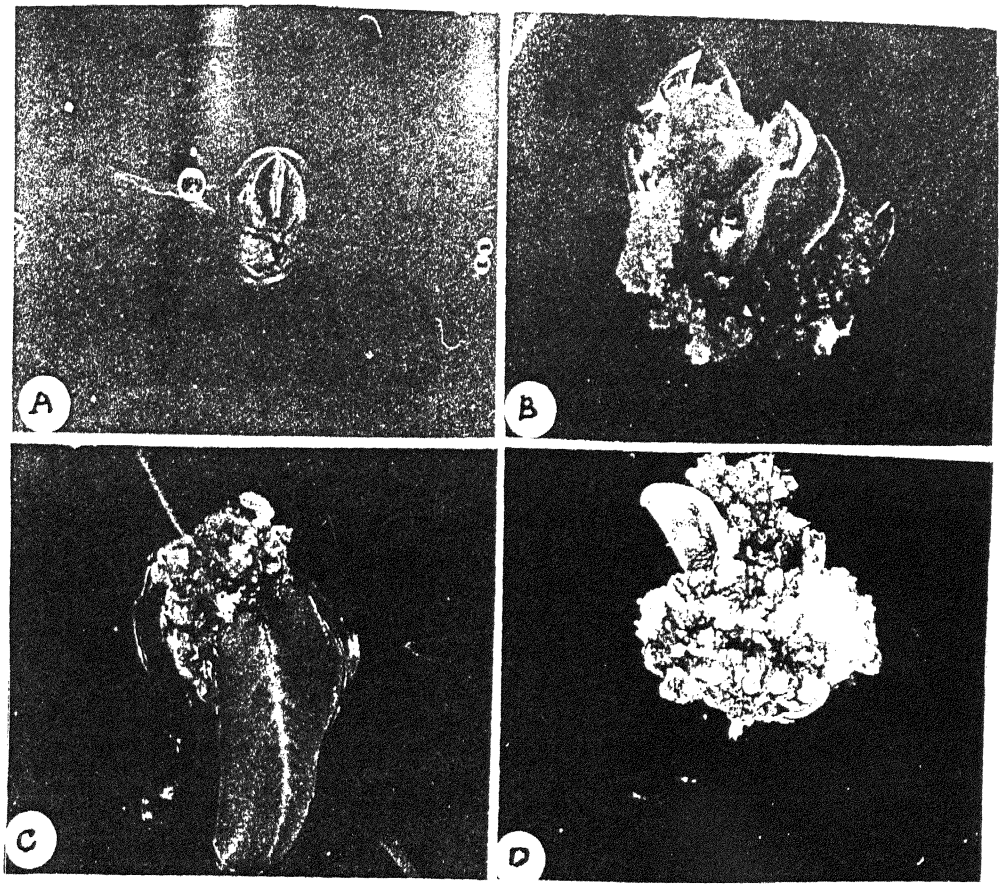


Fig 2.4: Stages in *Agrobacterium* mediated transformation of groundnut A) The leafbase injured Agrobacterium infected leaflet of groundnut B)Transformed leaflets in the developing stage C) Transformed leaflets showing shhot regeneration after a week D) Transformed leaflets showing GUS expression

5.6 Transformation using Gene gun

As *Agrobacterium*-mediated transformation has ben shown to be highly strain and cultivar-specific microprojectile bombardment became a powerful method of transformation of recalcitrant plant species (Birch and Franks, 1991). Bombardment of the tissue that is capable of regeneration and inducing plant formation under selection was done using suitable explant. An ideal target for particle bombardment should contain a high proportion of penetrable cells capable of integrating introduced DNA and regenerating to intact plants. In model plants like tobacco, direct DNA transfer using microprojectile bombardment was carried using leaf discs (Tomes *et al* 1990) Clemente *et al*; 1992 reported stable transformation of callus formed from bombarded leaflets of groundnut seedlings. However, efficiency was low and no transformed regenerable tissue or transgenic plants were obtained. Regeneration of target cells following microprojectile-mediated gene transfer was shown by optimizing the various parameters (Gordon-Kamm *et al.* 1990; Christou *et al* 1991; Bower and Birch 1992). Recently, the first transgenic groundnut plants were produced following particle bombardment of 1-2 year old embryonic callus (Ozias Akins *et al*; 1993).

Mature embryos, de-embryonated cotyledons and embryonic leaflets from mature embryos were shown to be closest to ideal to be used as explants (D.Malcolm Livingstone, Robert G.Birch 1995). Thus for the present study immature leaflets and mature embryos of groundnut variety JL 24 were used as targets for microprojectile-mediated transformation as they could readily undergo oganogenesis and somatic embryogenesis.

pRT99G Plasmid containing *NPTII* (Neomycin phosphotransferase) as selectable marker and *uidA (*gus;βglucuronidase) reporter gene is used as it could best determine the transient expression frequencies in bombarded tissue. Embryonic leaflets and zygotic embryos bombarded with pRT 99G Plasmid were allowed to proliferate on MS media containing 4mg/l BAP and 1mg/l NAA (table 12) and 1mg/l BAP, 20mg/l 2,4D, 6% sucrose (table 13) as these media show maximum production of shoot primordia and embryos repectively. Kanamycin (125mg/l) was used as a selective agent.

In the present study bombarded embryonic material is currently proliferating in the presence of kanamycin(Fig 2.5). Out of 5 plates containing nearly 200 explants (immature leaflets and mature embryos) 60% survived the kanamycin selection. And out of the 20 randomly selected explants only 14 of them showed the GUS expression *i.e.* only 70% of them were transformed. Further regeneration studies to test the stable integration of the genes are yet to be carried.

Conclusions

6.0 CONCLUSIONS

Improved transformation techniques have resulted in increasing the number of crop plants transformed to date. Even the plant species which were earlier thought to be recalcitrant and difficult to transformation have responded to the new transformation techniques invented. The Biolistic or Particle gun transformation method which delivers the foreign genetic material to the target plant tissue irrespective of genotypic boundaries have revolutionized the crop improvement methods. Though *Agrobacterium*-mediated transformation have number of restrictions like limited host range, this system have an advantage of transferring DNA in more controlled ans targetted manner.The major conclusions of the present stidy are as follows:

1) Synchronous multiple shoot induction has been obtained from the base of the leaflet explants after culturing for two weeks on MS medium containing definite concentration of BAP and NAA. Each of these shoots can be regenerated into complete plants.

2) Somatic embryogenesis was successfully carried out using tested concentration of 2,4 D and Sucrose.

3) Both organogenesis and somatic embryogenesis were successfully used for *Agrobacterium*-mediated and Biolistic-based transformation studies where in transient GUS expression was clearly seen in the transformed groundnut (var JL 24) explants

4) When compared to 70% efficiency in microprojectile-mediated transformation the *Agrobacterium*-mediated transformation showed 40% transformation efficiency *i.e.* the former method is nearly twice more efficient in producing the transient gene expression.

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Appendices

8.0 Appendix I

MS media

NH4NO3.....10ml
KNO3.....20ml
MgSO4 7H2O....10ml
KH2PO4.....10ml
CaCl2.....10ml
MS minor.....10ml
Fe-EDTA.....10ml
MS Organics.....10ml
Myo-inositol.....10ml
Sucrose.....30gm
pH.....5.8
Agar.....8gm

D90 medium

NH_4NO_3.....10ml
KNO_3.....20ml
$MgSO_4$.....10ml
KH_2PO_4.....10ml
$CaCl_2$.....10ml
MS minor.....10ml
Fe EDTA.....10ml
Myo - inositol.....10ml
B5 Oganics.....2.5ml
Sucrose.....30gm
Kinetin.....1ml (1mM)
2,4-D.....20mg
pH.....5.8
Agar.....8gm

MS organics	**1 litre**
Nicotinic acid	50 mg
Pyridoxine HCl	50 mg
Thiamine HCl	10 mg
Glycine	200 mg

MS minor	**1litre**
KI	83 mg
H_3BO_3	620 mg
$MnSO_4$	2230 mg
$ZnSO_4$ $7H_2O$	25 mg
Na_2MoO_4 $2H_2O$	25 mg
$CuSO_4$ $5H_2O$	2.5 mg
$COCl_2$ $6H_2O$	2.5 mg

B5 Organics	**250ml**
Nicotinic acid	50mg
Pyridoxine monohydrochloride	50mg
Thiamine hydrochloride	500mg

Fe-EDTA	**1 litre**
EDTA 2H2O	3.73 gm
FeSO4 7H2O	2.74 gm

Dissolve in hot water

Appendix II

LB (Luria Bertani)medium
Peptone.....10g/l
Nacl.....10g/l
Yeast Extract....5g/l and adjust the pH to 7.0

LB agar
Peptone.....10g/l
Nacl.....10g/l
Yeast extract.....5g/l
pH.....7.0
Agar.....15g/l

YEB medium
Peptone.....5g/l
Yeast extract.....1g/l
Beef extract.....5g/l
Sucrose.....5g/l
$MgSO_4.7H_2O$....0.5g
PH.....7.0

TE buffer
0.1M Tris.Cl, 5mM EDTA (pH8.0) di-sodium salt

TAE buffer(50X)
242 g Tris.Base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA di-sodium salt and make the volume to one litre

GTE buffer
50 mM Glucose
25mM Tris. Cl (pH 8)
10mM EDTA(pH 8) disodium salt and autoclaved at 15lb/sq in an liquid cycle for 15 min and stored at 4^0C

Lysozyme
10mg/l in 10mM Tris.Cl (pH 8)

Potassium acetate solution
5M Potassium acetate solution...60ml
Glacial acetic acid.....11.5 ml
Water.....28.5 ml

GUS buffer
X-gluc 1mM
Sodium phosphate...100mM
EDTA.....10mM
Triton X-100.....0.1%

Running buffer – Tris-acetate
40mM Tris acetate (pH 7.6)
1mM Na_2EDTA

Loading buffer(SBX- 6X buffer)
40% (w/v) sucrose
0.25%(w/v) bromophenol blue
0.25 %(w/v) Xylene cyanol

Appendix III

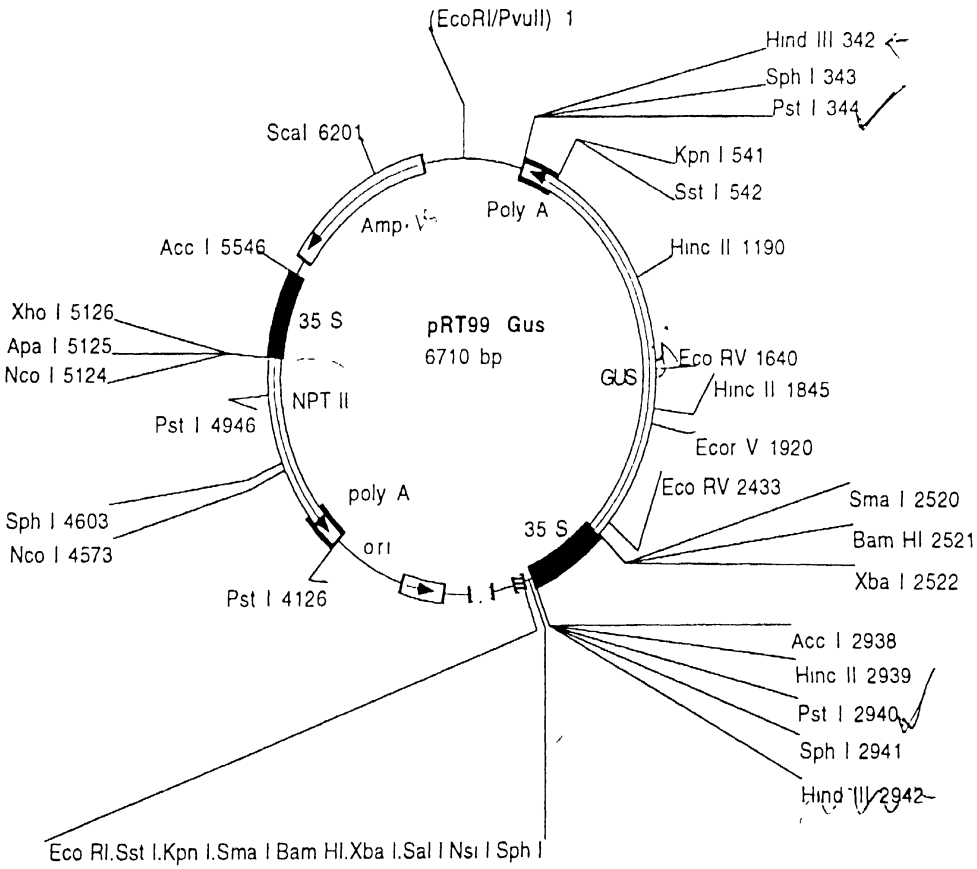
Table I : List of plasmids (pCAMBIA) used for transformation

pCAMBIA VECTORS	NUMBER	REPORTER GENE	ANTIBIOTIC SELECTION IN BACTERIA	IN PLANTS
1381T-DNA	C4	GUS	Kan(50µg/ml)	Hyg
1391 T-DNA	C8	GUS	Kan	Hyg
1381ZT-DNA	D6	GUS	Kan	Hyg
1391ZT-DNA	D7	GUS	Kan	Hyg
1301 T-DNA	B4	GUS	Kan	Hyg
2301 T-DNA	B6	GUS	Kan	Kan
3301T-DNA	B8	GUS	Kan	PPT
1302 T-DNA	C1	GFP	Kan	Hyg
1303 T-DNA	C2	GUS+GFP	Kan	Hyg
1304 T-DNA	C3	GUS+GFP	Kan	Hyg

GUS- β-glucuronidase

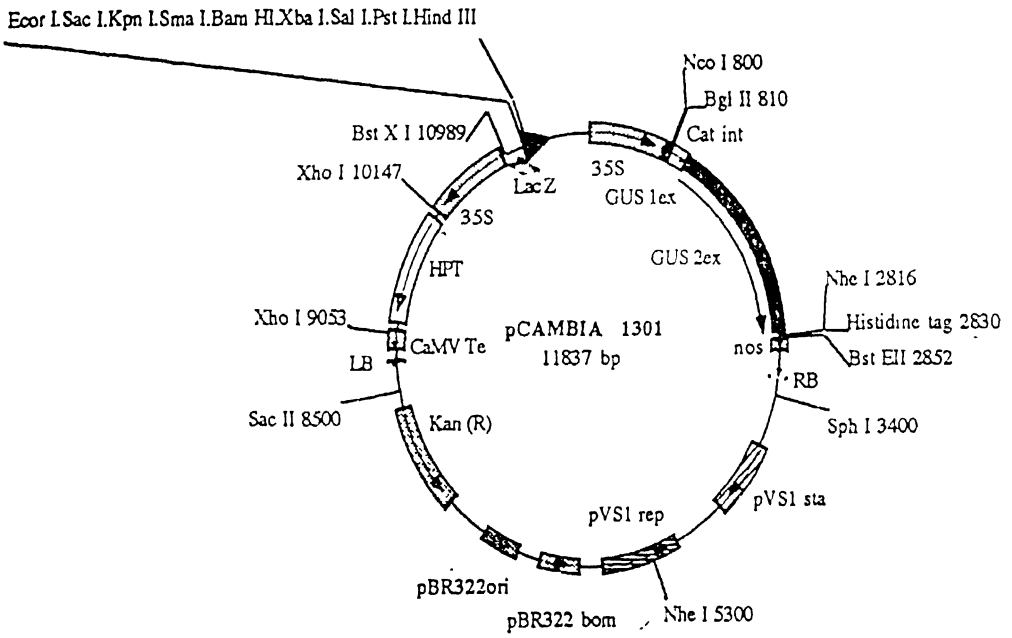
GFP- Green flourescent protein
Kan- Kanamycin
Hyg -Hygromycin
PPT -Phosphinothricin

Appendix IV



Plasmid name: pRT99 Gus
Plasmid size: 6710 bp
Constructed by: Messing & coworkers
Construction date: 1985
Comments/References: Yanisch-Perron, C., Vieira, J., & Messing, J (1985) Gene, 33:103-119. MacPlasmap default startup plasmid.

Appendix V



Plasmid name: pCAMBIA 1301

Plasmid size: 11837 bp

Constructed by: Richard Jefferson, CAMBIA

Construction date: 1996

Comments/References: General purpose Binary vector with GUS-Int and HPT genes Has a multicloning site in the Lac Z alfa for cloning novel genes. Kanamycin resistance is used for bacterial selection. Histidine tag enables the "HexaHis" column purification of GUS protein.