DEVELOPMENT OF TRANSGENIC PLANTS OF GROUNDNUT (*ARACHIS HYPOGAEA* L.) FOR INDUCED RESISTANCE TO GROUNDNUT ROSETTE DISEASE

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Certificate

This is to certify that the thesis / dissertation entitled **Development of Transgenic Plants of Groundnut (*Arachis hypogaea* L.) for Induced Resistance to Groundnut Rosette Disease** that is being submitted by **Mrs. Sunita Daniel** in partial fulfillment for the award of **Ph.D.** degree in **Biotechnology** to the Jawaharlal Nehru Technological University (JNTU), Hyderabad, is a record of bonafide work carried out by her under our supervision and guidance

The results embodied in this thesis have not been submitted to any other University or Institute for the award of any degree or diploma

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Declaration

I hereby declare that the thesis has been composed by me and all the work presented in this thesis is a record of my own work unless specifically stated otherwise, and it has not been presented previously in partial or complete fulfillment for any other degree or professional qualification. The research was conducted at ICRISAT, Patancheru under the supervision of Dr. Kiran K. Sharma as a partial fulfillment for Ph.D. degree of Jawaharlal Nehru Technological University, School of Biotechnology, Hyderabad, under the guidance of Dr. M. Lakshmi Narasu.

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Abstract

Improvement of disease resistance in groundnut (*Arachis hypogaea* L.) by biotechnological approaches has been limited due to the lack of efficient protocols to regenerate whole plants through in vitro regeneration of adventitious shoot buds from transformed tissue. The primary objective of this work was to develop efficient protocols for producing transgenic groundnut plants for induced resistance to groundnut rosette disease (GRD), which is caused by a complex of three viruses that include groundnut rosette virus (GRV), groundnut rosette assistor virus (GRAV) and a satellite RNA (sat RNA). Amongst various seedling explants, shoot morphogenesis was induced from the petiolar cut end of leaflet explants derived from 1 d-old in vitro grown seedlings of groundnut genotype ICGS-44. A combination of 13.3 μM N^6-benzyladenine (BA) and 5.3 μM α-naphthalene acetic acid (NAA) in the Murashige and Skoog's medium (MS) was optimum for inducing multiple shoots. The shoot bud differentiation occurred in a polar fashion from tissues of the petiolar cut end within 2 weeks of culture in over 92% of the cultured explants.

The development of shoot buds into elongated shoots and their rooting was achieved on a hormone-free MS medium. The rooted shoots could be readily acclimatized in pots containing in vitro regenerated plantlets covered with polythene bags, and maintained in a glasshouse with over 90% success rate. In the glasshouse plants exhibited normal growth and morphology, and upon maturity produced viable seeds. The method for regeneration of whole plants developed in this study is widely applicable to different genotypes of *Arachis hypogaea* L.

To study the ontogeny of shoot formation the histological events associated with shoot primordia formation in cultured leaflet explants were examined. Cytological changes were observed within 1 d after culture of the leaflet explants on shoot induction medium. By 2 d mitotic activity which was initially random, became organized and restricted to the vascular parenchyma cells, and sub-epidermal cell layers in tissues that were in contact with the culture medium. A nodular mass of meristematic and cytoplasmically dense cells developed by 5 d at the proximal cut end continued to divide and accumulate starch grains in cells of the cortex. The cells in the peripheral region of the nodular cell mass differentiated further into meristematic zone by 7 d after culture and shoot bud initials with vasculature were formed by 10 d. Within this meristematic zone, shoot bud primordia, and eventually multiple shoot buds with well-developed apical meristems were formed by 14 d.

A reproducible and efficient transformation protocol was developed for groundnut from immature leaflet explants by using *Agrobacterium tumefaciens*. Binary vectors based in *A. tumefaciens* strain C58 carrying *npt*II and coat protein gene (cp) of GRAV (pROKII:GRAVcp) or *hpt*, *uid*A and *GRAVcp* genes (pCAMBIA1301:GRAVcp) were used for co-cultivation of 1 d-old leaflet explants. The putatively transformed shoots developed with the plasmid pROKII:GRAVcp were selected on 100 mg/l kanamycin. The selection system for recovering transgenic shoots was based on the gene *npt*II selection marker encoding neomycin phosphotransferase, which provides resistance to the antibiotic kanamycin or *hpt* gene encoding hygromocin phosphotransferase that provides resistance to the antibiotic hygromycin.

A large number of putative independent transformants (over 60) were sucessfully transferred to the glasshouse. Integration of the transgene and stable genetic transformation in the progeny was confirmed by PCR amplification of the 700 bp fragment of *npt*II and 384 bp fragment of *GRAVcp* genes, and Southern blot hybridization for the introduced genes in the T_0 generation of transgenic plants. Analysis of 40 T_1 generation transgenic plants showed the segregation of single copy transgenes in a ratio of 3:1 thus suggesting Mendelian inheritance of the introduced genes. The transgenic plants generated during this study could not be tested in the glasshouse for their efficiency in providing resistance to GRAV or GRD. This is due to the fact that the causal agents for GRD are present in Africa and could not be imported in India. This study will be taken up by ICRISAT in collaboration with NARS partners in sub-Saharan Africa. In conclusion, the method for shoot regeneration and the production of transgenic plants reported here is highly efficient for introducing novel genes into groundnut for the agronomic improvement of this important crop of the Semi-Arid Tropics.

Contents

Abbreviations

µg / l	Micro gram per litre
µl	Micro litre
BA	N^6-Benzyladenine
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
°C	degree Celsius
Cef	Cefotaxime
cm	Centimeter
cp	Coat protein gene
d NTP	Deoxy nucleotide triphosphate
d	Day
DEAE-cellulose	Diethyl amino ethyl cellulose
dia	Diameter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent assay
GRAV	Groundnut rosette assistor virus
GRD	Groundnut rosette disease
GRV	Groundnut rosette virus
GUS	ß-glucuronidase
GUSINT	GUS gene containing an intron
h	Hour
hpt	hygromycin phosphotransferase gene
IAA	Indole-3-acetic acid
KN	Kinetin
LB	Luria Broth
mg / l	Milli gram per litre
min	Minutes
ml	Milli litre

mm	Milli meter
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog's medium
NAA	∝-Napthalene acetic acid
NARS	National Agricultural Research System
*npt*II	Neomycin phosphotransferase gene
PCR	Polymerase chain reaction
PEG	Polyethlene glycol
PGR	Plant growth regulator
PPM	Plant Protection Mixture
psi	Pounds per square inch
PTGS	Post-Transcriptional gene silencing
rpm	Revolutions per minute
RNA	Ribonucleic acid
SAT	Semi-Arid Tropics
SEM	Shoot elongation medium
SIM	Shoot induction medium
Sat RNA	Satellite RNA
SSC	Sodium chloride and Sodium citrate
TDZ	Thidiazuron
Tris	Tris (hydroxymethyl) methylamine
TGS	Transcriptional gene silencing
*uid*A	β-glucuronidase gene
Vir	Virulence gene
w / v	Weight per volume
X-gluc	5-bromo-4-chloro-3-indoly β-D-glucuronide
YEB	Yeast extract broth

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INTRODUCTION

1.1 Introduction

Legumes are important sources of dietary protein and fats in developing countries of the semi-arid tropics (SAT) where groundnut (*Arachis hypogaea* L.) is one of the important food legume crop. It is a rich source of protein (23 %) and edible oil (43 %) (Norden, 1980) and is considered as one of the principle economic crop of the world (Cobbs and Johnson, 1973). The genus *Arachis* belongs to the sub-family *pappillonacea* of the family *leguminoseae*. It is native to South America and comprises of diploid (2n = 20), tetraploid (2n = 40) and octoploid (2n = 80) species.

Groundnut is a seed propagating, self-pollinating crop originating from Brazil. It is perennial or annual legume with tetra-foliate, stipulate leaves, pappillonate flower, tubular hypanthus, underground fruit, prostrate, and leaves abruptly bipinnate, adenate to the petiole at the base, axillary spike, sessile at the leaf axil, bracteolate, calyx tube filiform, petals and stamens inserted at the apex of the tube (Smart, 1994). The geographical classification of groundnut is delineated into six regions: the America, Africa, Asia, New East Asia, Europe and Oceania (Gregory et al., 1980). The total area under groundnut cultivation is over 19.8 million hectares and the world production is over 18.9 million metric tons per year, with an average yield of 985 kgs per hectare (Cummins and Jackson, 1982). On the global scale, India is a major producer of groundnut with a total production of 8.9 million tons per year. Groundnuts are utilized in several ways; the edible oil is an importance source for human consumption and the meal is used for livestock feed. It is also used directly for food in industrial countries including USA, Canada, and the European Union.

Since the mid-1970's edible groundnuts have increased in both domestic consumption and export trade. In contrast, the production in Africa has declined by 17 percent from last two decades. The major reasons for such low productions are various abiotic and biotic stresses (Cummins and Jackson, 1982). Groundnut crop is prone to several virus diseases that result in heavy economic losses annually (Reddy, 1991). Among the virus diseases, peanut clump virus (PCV) and groundnut rosette disease (GRD) cause major losses to the crop yield and production. Groundnut commodity and quality is adversely affected by the disease caused by *Aspergillus flavus* that produces aflatoxins for which no adapted resistant groundnut genotype is available. Foliar diseases such as early and late leaf spots caused by *C. arachidicola* and *C. personatum* respectively, are most damaging diseases (Subramanyam et al., 1985). Amongst the insect pest, spodoptera, legume pod borer, aphids and thrips cause the greatest losses to groundnut crop (Wightman and Ranga Rao, 1993).

Virus diseases are amongst the most important biotic constraints to groundnut crop, especially in Asia and Africa (Reddy et al., 1994). Peanut clump disease and groundnut rosette disease (GRD) continues to cause significant losses to groundnut in Africa. Groundnut is the only known natural host of the three agents of the rosette disease complex. These include groundnut rosette virus (GRV), groundnut rosette assistor virus (GRAV) and the satellite RNA (sat RNA). Rosette epidemics are sporadic and unpredictable, but when they occur, the yield losses are severe and the impact on rural economy is profound. About 6800 groundnut germplasm accession originating from South America, Africa, and Asia were evaluated for resistance to rosette disease (Subramanyan et al., 1998). Of these, 116 germplasm accessions, including 15 short-

duration Spanish types, have shown high level of resistance to groundnut rosette disease, but all were susceptible to GRAV (Nigam and Bock, 1990). Genetic resistance to GRV is available in long duration Virginia type cultivars that is governed by a homozygous recessive gene. However in Africa, Spanish type short-duration cultivars are preferred because groundnut is a rainfed crop. Since the maturity and resistance to GRV is governed by two independent homozygous recessive genes, it is difficult to develop resistant genotypes by conventional breeding. Recently, (Subramanyam et al., 2001) showed that several accessions in different wild species of the genus *Arachis* possess resistance to all the components of groundnut rosette. This indicates that it should be possible to breed groundnut cultivars with combined resistance to all the three components of GRD.

Genetic transformation of plants has become a source of agriculture innovation in plant biotechnology. Genetic engineering of plants came into existence from the time natural genetic engineers like *Agrobacterium tumefaciens* was identified which can transfer segment of its DNA (T-DNA) to the host cell for integration. Plants with new traits such as resistance to herbicides, insect pests, viruses, fungi have been genetically engineered by using genes from diverse species (Chilton et al., 1977; Krens et al., 1982; Pappu et al., 1995; Birch, 1997; Sharma and Ortiz, 2000). This ability to transcend species barrier brought worldwide interest in genetic engineering of plants for alleviation of many biotic stresses, for which there is no background resistance in the available germplasm.

Integrated disease management involves combining factors (growing resistant cultivars, optimum spacing, crop rotation, intercroping, recommended use of appropriate

biological or chemical insecticides at an appropriate time, etc.) that could result in substantial reduction in losses due to pathogens or pests. Integrated management of rosette disease was demonstrated by A'Brook (1964) that involves careful adjustment of sowing dates and phytosanitary methods. Such interventions can reduce the spread of the rosette disease besides effective control of aphids has been demonstrated by (Davis, 1975). Although, chemical control of the vector and cultural practices are known to reduce the risk of rosette incidence, the improvement in the yield is not substantial enough to meet the demand of production. The application of biotechnological methods holds great potential for the agronomic improvement of groundnut crop (Sharma and Ortiz, 2000). Cultivation of disease resistant genotypes would probably be the cheapest and effective method of controlling disease. Advances in molecular biology of plant and plant viruses coupled with the development of techniques for the genetic transformation of plants has made it possible to produce transgenic plants for induced resistance to specific plant viruses by expressing genes that interfere with some stage in viral life cycle (Draper et al., 1988). The most successful approach that has aided in the development of virus resistant plants is by using the coat protein gene of the virus itself which is based on the cross-protection phenomenon. This occurs when prior inoculation with a mild strain of a virus protects a plant against the effects of subsequent infection by a second, related virus (Sherwood, 1987). While the host plant resistance is the most effective strategy, the introgression of genetic characteristic such as pest resistance might not be possible between unrelated species through conventional breeding (Hadley and Openshaw, 1980). The introduction of plant biotechnology into integrated pest management would be beneficial in improving the yield of the crop through genetic transformation with novel

pest resistant genes. However, for the effective application of this technology, it is imperative to have the ability to transfer genes into crop plants in an efficient and reliable manner. Therefore, the present study was undertaken to develop efficient tissue culture regeneration and transformation methods to introduce novel genes into groundnut for inducing resistance to the Groundnut Rosette disease (GRD). The major objectives of this study were as follows:

1.2 Objectives

1. To develop an efficient shoot regeneration protocol by using leaflet explants of groundnut.
2. To study the ontogeny of shoot bud differentiation from leaflet explants.
3. To optimise an efficient protocol for genetic transformation of groundnut by using the coat protein gene of groundnut rosette assistor virus (GRAV).
4. Molecular and genetic characterisation of groundnut transgenics for integration and inheritance of the coat protein gene of GRAV.

REVIEW OF LITERATURE

2.1 Plant Tissue Culture

Plant tissue culture or the aseptic culture of cells, tissue and organs is an important tool in basic and applied studies. The foundation for in vitro plant regeneration is that somatic plant cells, or protoplast from these cells, are capable of proliferation and organisation into tissues, and eventually developing into a complete plant or organism. This capability is termed 'cellular totipotency' (Vasil and Hildebrandt, 1965). Totipotency of the cell is manifested through the process of differentiation of plant cells into well defined organs viz., roots, shoots, or somatic embryos, where the plant growth regulators (PGR) play a important role in altering the cellular functions or 'inductive stimulus'. It is generally recognised that not all cells subjected to in vitro culture are capable of expressing totipotency even if the culture as a whole is regenerable (Vasil, 1988; Potrykus, 1990). The capability of a plant cell or group of cells to respond to an inductive stimulus for a developmental process is referred to as competence (Mein and Binns, 1979). Tissue explants are mixture of cells varying physiologically, biochemically and developmentaly (Lindsey and Yeomm, 1985). Certain cells in an explant are competent to respond to the in vitro culture condition as a result of cellular heterogenity (Potrykus, 1990). Various processes and factors that affect the cellular totipotency are as follows:

Morphogenesis and cellular differentiation: Competence is the first step in the dedication of one or more undifferenciated cells towards morphogenesis. The second stage of dedication is the induction of determination in competent cells. Individual cell or groups of cells are said to be determined when they have become committed to follow a particular genetically programmed developmental pathway. Morphogenesis from cells which are already committed to follow a developmental pathway are called permissive,

while that from cells induced to become morphogenesis by endogenous or exogenous growth regulators, are called inductive (Smith and Krikorian 1988) The three major factors affecting in vitro plant regeneration are genotype, explant source and culture conditions (including culture media and physical environment)

Explant source and ontogenetic stage The successful culture of plant material in vitro is greatly influenced by the age of tissue or organ that is used as initial explant Explants taken from juvenile plant tissues particularly from seedlings are highly resposive The immature organs or meristematic and undifferentiated tissues are most responsive and reliable explant sources (Vasil and Vasil, 1986) The reason for the stage-specific response may be due to genetic epigenetic or physiological changes that occur in mature cells (Vasil, 1988) Other factors to consider include size, orientation in culture, pre-treatment and inoculation density (Brown and Thorpe, 1986)

Culture conditions Composition of culture medium is an important factor in the successful establishment of tissue cultures Each tissue type requires different formulation, depending on whether the objective is to obtain optimum growth rate or induce organogenesis Several media have been developed by various workers to suit particular requirements of a cultured tissue (White 1942, Murashige and Skoog 1962, Linsmaier and Skoog, 1965, Gamborg and Veligh, 1968) A standard or basal medium consists of balanced mixture of macronutrients and micronutrients (Salts of chlorides, nitrates, sulphates, phosphates, iodides of calcium, magnesium, potassium, sodium, iron, mangnese, zinc and boron), vitamins, carbon source, organic growth factors (aminoacids, urea and peptons), source of reduce nitrogen supply and plant hormones The inorganic salts are supplied in two groups, as macro salts and micro salts, the salts needed in high

amounts are called macro salts. Nitrogen is mostly provided in the form as nitrates and as ammonium compounds. In most media, iron is chelated as (Fe-EDTA). Vitamins used in the culture media are myoinositol, nicotinic acid, pyridoxin, thiamine etc; and carbohydrates is supplied usually as sucrose (Thorpe, 1980). The most commonly used amino acid is glycine. In addition, phytohormones (auxins and cytokinis) or their synthetic counter parts are required either singly or in combination to initiate and maintain cell division. The concentration and ratio of hormones may vary from plant to plant and should be standardised for particular plant tissue. The auxins that are commonly used in culture media are IAA (indole-3-aceticacid), 2,4-D (2,4-dichlorophenoxyaceticacid), NAA (∝-napthalene acetic acid) and IBA (indole-3-butyricacid). The cytokinins are kinetin (6-furfuryl aminopurine), BA (N^6-Benzyl adenine), zeatin, 2-iP (2-isopentenyladenine). The hormones are physiologically active in very small quantities. The pH of the medium is adjusted in order to determine the alkalinity and acidity of the final solution, as it greatly influences the uptake of ingredients, solubility of salts and gelling efficiency of agar. A pH of 5.6 to 5.8 has been found suitable for maintaining all the salts in a near-buffered form. Physical conditions also have a major role in in vitro culture. These include light (intensity, quality and photo period), temperature and culture container (including container size, permeability of gas exchange). Cultures can be maintained on semi-solid media or as suspensions in liquid with shaking (Evans et al., 1981). Plant regeneration in in vitro cultures occurs via two developmental pathway, organogenesis and somatic embryogeneis.

Organogenesis: Organogenesis is a developmental pathway in which shoots or roots are induced to differentiate from a cell or group of cells. In vitro plant regeneration

involves induction and development of the shoot from the explant tissue (with or without) an intervening callus stage, followed by transfer to a medium to induce root formation and development. If the root or shoot is induced and develops directly from the explant without undergoing an initial callus phase, it is termed as direct or adeventitious organogenesis (Thorpe, 1970; Christianson and Warnicke, 1988). The first major advancement in the control of organogenesis can be attributed to Skoog and Miller (1957), who reported that alteration of the auxin and cytokinin ratios where sufficient to control morphogenesis in tobacco. High cytokinin: auxin produces shoots (caulogenesis), low cytokinin: auxin ratio produced roots (rhizogenesis), and equal concentrations of these phytohormones were found to result in callus proliferation. Organogenesis is most widely used route for in vitro plant regeneration that has a wide applicability in genetic transformation studies.

Embryogenesis. Somatic embryogenisis is a developmental pathway in which embryos are induced from a somatic cell or a group of somatic cells. Somatic embryos can occur directly from the cells of the explant tissue without an intervening callus phase (Conger et al., 1983) or indirectly from a proliferated callus, is generally more common (Williams and Maheswaran, 1986). During the initiation of embryogenic cultures the exogenously supplied auxin will induce both cellular proliferation and the embryogenic pathway. The degree of morphogenesis depends primarily on auxin concentration, following removal or reduction of the auxin supply. The embryogenic development in the cultures can proceed to the maturation and germination steps (Ammirato, 1984). In vitro regeneration of plants via callus phase has the drawback of increased risk of introduction of variations such as polyploidy and aneuploidy (Vasil, 1986).

2.2 Plant Transformation

Plant transformation is the introduction and stable integration of alien genes into the genome of recipient plant. With transformation, the gene pool available for improvement of a crop is unlimited, where genes from virtually any organism can be used. Techniques for gene transfer to plants have been reviewed (Birch, 1997; Sharma and Ortiz, 2000).

Vectors for gene transfer: Vectors used for genetic trasnformation of plants carry "marker gene" besides the gene(s) of interest which allow the recognition of the transformed cells by selection or screening. These genes are dominant, usually of microbial origin and placed under the control of strong constitutive promoter, often of viral origin. The most popular marker genes used include those affording resistance to antibiotics such as kanamycin and hygromycin. For successful selection, the target plant cell must be susceptible to relatively low concentration of the antibiotics or herbicides in a non-leaky manner. Table 1 and 2 list the important selectable marker genes and reporter genes used in plant transformation vectors. The utility of any gene construct as a transformation marker depends on the plant species and explant involved. To date kanamycin resistant gene *npt*II (Reiss et al., 1984) and *uid*A gene (Jefferson, 1987), are the most commonly used selectable and screenable markers. The plant transformation vectors have features for recombinant DNA manipulations that include multiple unique restrictions sites, bacterial origin of replication, selectable marker gene for plasmid selection, and maintenance in *Escherechia coli,* in addition to selectable marker gene/reporter gene for expression in plants. The large size, and consequent absence of restriction enzyme sites, and the tumorogenic properties of Ti and Ri plasmids preclude their direct use as transformation vectors.

Table I

List of important selectable marker genes used in plant transformation vectors.

Gene	Enzyme encoded	Selective agents	References
dhfr	Dihydrofolate reductase	Methtrexate	Herrera-Estrella et al., 1983
hpt	Hygromycin phosphotransferase	Hygromycin B	Van den et al., 1985
*npt*II	Neomycin phosphotransferase	Kanamycin	Herrera-Estrella et al., 1983
als	Acetolactate synthase	Chlorsulfuron	Haughn et al., 1988
*Aro*A	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate	Shah et al., 1986
Bar	Phosphophinothricin acetyltransferase	Phosphothricin	De Block et al., 1987

Table 2

Lists of reporter genes used in plant transformation vectors.

Gene	Enzyme encoded	Reference
CAT	Chloramphenicol acetyl transferase	Herrera-Estrella et al., 1983
LacZ	ß -Glcuronidase	Helmer et al., 1984
GUS	ß-Gucuronidase	Jefferson, 1987
Lux	Luciferase	Koncz et al., 1987

Development of "disarmed" or "non-oncogenic" vectors have paved the way for plant genetic transformation (Lichtenstein and Fuller, 1987). Non-oncogenic vectors that are currently used can be divided into two types, cis or trans depending on whether the T-DNA flanked by 25 base pair repeat sequence are carried on the same replicon as the *vir gene or* on separate plasmid. The former (cis-acting *vir* genes) are referred to as co-integrative vectors, while the later with trans-acting *vir* genes, as binary vectors. Binary vectors have considerable advantage over co-integrative system, since they can be used in conjuction with any *vir* helper strain; as many plants species are susceptible to *Agrobacterium* strain (Byrne et al., 1987).

Although several diverse approaches have been tried successfully for integrative transformation (Potrykus, 1990), only three are widely used to introduce genes into a wide range of plant. a) direct DNA transfer into isolated protoplast. b) micro projectile bombardment with plasmid DNA or Biolistic gene transfer, and c) *Agrobacterium*-

mediated gene transfer. These methods and key components of genetic transformation of plants are discussed as follows:

Direct DNA transfer into isolated protoplasts: Protoplasts are separated single cells, which have been stripped off their cell walls thus facilitating the transfer of foreign genes through the plasma membrane. The advantage of using protoplasts for genetic transformation is that the transgenic plants regenerated from them will have a uniform genetic make-up. Various chemical treatments have been used to stimulate DNA uptake by protoplast but PEG is the most common treatment used to stimulate DNA uptake into protoplasts of both dicots and monocots (Negrutiu et al., 1987). Gene transfer into protoplasts by electroporation is based on the use of short electrical pulses of high field strength to facilitate DNA uptake by increasing the permeability of protoplast membrane (Shillito et al., 1985). Virtually every protoplast system has proven transformable, although with different efficiencies. Transformation of protoplast by electroporation produced stably transformed cell lines in monocots such as maize (Fromm et al., 1990), and rice (Uchimiya et al., 1986). Unfortunately, there are problems with the recovery of transgenic plants from the transformed protoplast in most cases (Potrykus and Shillito, 1989). Hence, the use of this approach is limited to crops where regeneration from protoplast is readily available.

Biolistics gene transfer: The term 'biolistic' was coined to describe the name of the delivery of foreign DNA into living cells or tissue through "bombardment" with a biolistic device (Klein et al., 1987; Sanford,1990). Acceleration of heavy microprojectiles (1 to 3 μm diameter tungsten or gold particles) coated with DNA has been developed into a technique that carries genes into virtually every type of cell and tissue. This method

allows the transport of genes into many cells at nearly any desired position in a plant without much manual effort. This technology basically involves loading tiny gold or tungstun particle (1 to 1.5 μm; micro carrier) with vector DNA and then spreading the particle on the surface of the mobile plate (macro carrier). Then, under a partial vacuum, the microprojectile is fired against a retaining plate or mesh, by a shock wave caused by helium under pressure. The microprojectile decelerates instantly, whilst the momentum and small size of the dense microprojectiles causes them to be thrown from the surface of the microprojectile and to penetrate the target plant tissue. This technique, although not as efficient as *Agrobacterium*-mediated gene transfer, has a distinct advantage in that virtually any type of meristematic totipotent cells/tissues/organs and monocots that are not amenable to agroinfection can be used with reasonable success rate. The real advantage of the biolistic technique lies in its application in transcint gene expression studies in differentiated tissues. To date, impressive success has been achieved in many crops including rice, wheat, sorghum (Cao et al., 1990).

Agrobacterium-mediated gene transfer: *Agrobacterium* occurs as a soil-borne plant pathogen that causes crown gall disease in more than 200 dicotyledonous plant species by genetically transforming plant cells. The tumor inducing capability is due to the presence of large Ti (tumor-inducing) plasmid in virulent strains of *Agrobacterium*. Likewise, Ri (root-inducing) megaplasmids are found in virulent strains of *Agrobacterium rhizogenes*, the causative agent of 'hairy root' root disease. Ti and Ri plasmids and molecular biology of crown gall and hairy root induction have been studied in great detail (Lichtenstein and Fuller, 1987; Klee et al., 1987; Zambryski et al., 1989). In response to wounded tissue of the host plant, *Agrobacterium* responds by transfering a

segment of plasmid called the T-DNA to the plant cell (Chilton et al., 1977). The T-DNA is the discrete segment of DNA flanked imperfect direct repeat sequence (right and left borders) of 25 bp length (Thomashow et al., 1980), borne on 200 kb Ti plasmid. Integration of T-DNA into the plant genome and expression of the genes carried by wild type T-DNA causes proliferation of the plant tissue to form a gall. T-DNA contains genes which encode for the over production of auxins (Akiyoshi et al., 1984) and cytokinins (Barry et al.,1984), and thereby induce gall formation. Futher genes codes for the production of enzymes involved in the synthesis of opines (Bomhoff et al., 1976). *Agrobacterium* strains are classified depending on which opine synthases are encoded by their T-DNA, such as octopine (oct), nopaline (nop) (Hooykaas and Beijersbergen, 1994). The initial infection process involves chemotaxis followed by cell-cell adhesion and *Agrobacterium* host cell recognition (Matthysse, 1986). Bacterial chromosomal virulence genes and appropriate plant-cell wall components were shown to mediate this process (Lippincott et al., 1984; Neff et al., 1987; Binns, 1990). The next stage is the transduction of signals from the wounded plant tissue to *Agrobacterium* and the induction of *vir* genes. Following this, a transferable copy of the T-DNA is produced and the protein-T-DNA strand complex is formed prior to the transport into the plant nucleus and the T-DNA integrated into the plant nuclear genome. The transfer of T-DNA to the plant cell is dependent upon the virulence genes present on the Ti plasmid *(vir* genes) and the bacterial chromosome (*chv* A, *chv* B genes) (Zambryski, 1992). The 35 kb virulence region on the Ti plasmid contains seven major loci *(vir*A, *vir* B, *vir* D, *vir* E, *vir* F, *vir* G, *vir* H), which encode proteins that are involved in the response to signals to the plant tissue and mediate transfer of T-DNA to the plant cell. The signals released from wound

tissue specifically induce expression of *vir* genes; the wounded plant tissue excretes sap with characteristic acidic pH (5-5.8) and various phenolic compounds (Stachel et al., 1985). The signals released by wounded plant cells are recognized by *virA / vir* G, a two component regulatory system initiating a signal transduction pathway leading to the induction of various *vir* genes. The *vir* D1 and *vir* D2 proteins are involved in processing the T-DNA and creating an active single stranded transfer intermediate called T-strand, the synthesis of T-DNA is thought to occur in 5'-3' direction, initiating at the right border of the T-DNA, the T-strand complexes with *vir* E2 while the *vir* D2 protein becomes bound at the 5' end of the T-complex, passage of the T-complex through the bacterial membrane is thought to be facilitated by *vir* B proteins (Zupan et al., 1998). The T-strand integrates into the plant genome at random sites (Mayerhofer et al., 1991). One of the major limitations of this method of gene transfer is the host range of the *Agrobacterium*

Several factors have been thought to be involved in determining the host range, such as, The *virA / vir* G components (Yanofsky et al., 1985; Leroux et al., 1987; Winans et al., 1994) and *vir* F loci (Jarchow,1991), T-DNA border sequences (Paulus et al., 1991). The chromosomal virulence genes (Citovsky et al., 1992; Zambryski, 1992), plant physiology (age, tissue type etc), predominantly *via* the wound response (Godwin et al., 1992) and its effect on *vir* gene induction. Even though *Agrobacterium* infects most dicot plant species, different strains display different degrees of virulence to different hosts *e g.* soyabean (Owens and Smigock, 1988), pea (Puonti-Kaerlas et al., 1989), chrysanthemum (Van Wordragen et al., 1991). Evidence suggests that efficient *Agrobacterium*-host interaction is determined by the genetic, physiological and physical components of the bacterium-plant interaction (Godwin et al., 1992). Several reports have shown that the host range is

dependent on the protocol used to infect the plant tissue with *Agrobacterium* and have suggested that by manipulation of in vitro conditions, it is possible to modulate the plant-bacterium interaction to obtain high efficiencies of transformation. Some of the factors which proved to have pronounced effects on in vitro transformation efficiencies in several plant species are:

Induction of *Agrobacterium* is one of the main factors for efficient T-DNA transfer depends on the level of *vir* gene induction and expression. Induction of *vir* genes can also be achieved in vitro by culturing *Agrobacterium* in medium containing signal molecules such as acetosyringone (Stachel et al., 1985) or others phenolics like vanillin, caffeic acid or sinapic acid (Bolton et al., 1986; Melchers et al.,1989). An acidic pH (<5.7) was shown to be required for *vir* gene induction (Stachel et al., 1985; Alt-Moerbe, 1998). Other chemical addition to bacterial culture medium such as inclusion of osmoprotectants like proline and betain (Vernade et al., 1988) in the culture medium were also shown to enhance the transformation efficiency. A temperature below 30 °C (Stachel et al., 1985) is required during the *Agrobacterium* culture and co-cultivation. The number of days under co-cultivation influences the rate of transformation (Cai et al., 1998). Pre-culture of explants can also enhance transformation (Jansen and Gardner, 1989).

Factors affecting the efficiency of transformation: The transformation efficiency of pea explants was found to be influenced by the explant type and maturity. Puonti-Kaerlas et al. (1992) and De Kathen and Jacobsen (1995) have reported that explant maturity, pre-culture and presence of growth hormones during co-cultivation conditions affect transformation efficiency. Successful protocols for *Agrobacterium*-mediated transformation of recalcitrant crops were developed by identifying the host and bacterial

genotype combination giving the highest levels of infectivity (Hinchee et al 1988, Van Wordragen et al , 1992) The importance of *Agrobacterium* strain and the plant genotype was demonstrated in different legumes e g alfa alfa (Desgagnes et al , 1995), soyabean (Owens and Smigock, 1988), and pea (Lulsdorf et al , 1991) The susceptibility of groundnut to *Agrobacterium* infection was demonstrated by (Mansur et al 1993)

The majority of transformation experiments utilize either freshly expanded tissue sections, protoplast in the process of forming a cell wall and entering cell division or callus/suspension-cultured cell clumps wounded by chopping and stimulated into rapid cell division by the use of nurse cultures (Draper et al , 1988) Adventitious shoot production in vitro is most commonly employed in most systems However the major problem in the development of transformation system is providing induced *Agrobacterium* with access to cells capable of dedifferentiation followed by regeneration

The most widely used method for the genetic transformation of plants is based on the interaction between the plant pathogenic soil bacteria *Agrobacterium tumefaciens* and wounded plant cells *Agrobacterium* mediated gene transfer is the method of choice since it allows stable integration of a well-defined DNA segment in one or a few copies The value of *Agrobacterium* mediated plant transformation is measured primarily by the number of independently transformed plants carrying the gene of interest per explant, which can be a function of genotype of the species, *Agrobacterium* strain virulence, the selectable marker, regeneration capacity of the target cell, and the accessibility of the bacterium to the regenerable cells (Sharma and Anjaiah, 2000) However, many monocotyledenous plants, including the cereals are recalcitrant to *A tumefaciens* gene transfer Alternative transformation methods have been developed, that include biolistic

bombardment, direct DNA transformation of plant protoplast using PEG or electroporation (Negrutiu et al. 1987; Cao et al., 1991).

Charecterization and screening of transgenic plants: Once the target cells have been transformed by one of the above methods the transgenic cells or cells produced are selected on a selection medium. Selection medium gives a selective advantage to those cells that have stably incorporated the transgene construct, and therefore resistant to the selective antibiotic. The putative transformants after selection are propagated in vitro by rooting and transfer to the containment glasshouse for further molecular evaluation and production of seeds from subsequent sexual generation. Successful integration of the transgene into plant genome is a pre-requisite for obtaining transgenic plants. However, the insertion of a gene does not mean that the introduced gene will be expressed at the desired level in the transformed plant or its progeny. Only plants displaying desired levels of expression of the new gene are useful in breeding programs. In most of the studies in which independent transgenic plants were analyzed, a high variation in transgene expression was observed (Peach and Velten, 1991; Nap et al., 1993; Meyer, 1995). This variation in expression, or inactivation (silencing) of the transgene may result from several factors such as (1) site of integration of the transgene (Van-der-Hoeven et al., 1994), (2) number of copies and orientation of the introduced gene (Hobbs et al., 1993), and (3) mutation or rearrangement of the introduced DNA sequence in the transgenic plants either during in vitro culture or at later stages (Maessen, 1997). The variation in transgene expression is reviewed by Phillip et al. (1994) and Meyer, (1995) that results from various factors. The first of these factors is position effect which depends on the influence of host DNA sequence at or near the site of integration (Peach and Velten,

1991; Van der Hoeven at al., 1994). If they become inserted into euchromatin, expression may be maintained (Koncz et al., 1989; Herman et al., 1990; Kertbundit et al., 1991). Insertion of the T-DNA in or near transcriptionally inactive heterochromatin may result in silencing or reduction of transgene expression levels (Meyer et al., 1993; Meyer, 1995). Expression of introduced DNA can be silenced soon after their introduction, at particular stage of development, in a random manner (Finnegan and McElroy, 1994; Matzke and Matzke, 1998). Factors associated with silencing are multiple copy insertion, repeat sequence (Finnegan and McElroy, 1994; Matzke et al., 1994), and the homology of transgene sequence to endogenous sequence (Matzke and Matzke, 1991).

A second factor associated with gene inactivation is the number of copies of the transgene inserted at the same or different sites in the genome. The integration of single T-DNA copy is common, but integration of higher copies has also been observed. Data from several different transgenic dicotyledons species showed an average of 3 T-DNA inserts, with occasionally up to 20-30 copies in some plants (Hobbs et al., 1993; Jorgensen et al., 1990; Vaucheret, 1993). Silencing of transgene may occur either before transcription (transcription gene silencing; TGS) or after initiation of transcription (post-transcriptional gene silencing; PTGS). The TGS results from promoter inactivation and PTGS occurs when the promoter is active but the mRNA fails to accumulate, involving sequence-specific RNA degradation. Unlike the meiotically heritable silencing frequently observed with TGS (Park et al., 1996), PTGS is fully reversed during meiosis (Stam et al., 1997; Depicker and Van Montagu, 1997; Vaucheret et al., 1998). DNA methylation has been shown to mediate both the kinds of gene silencing (Kooter et al., 1999). It has known to have evolved as a defense mechanism against invasive nucleic acid molecules

including viruses, transposable elements and viroids (Matzke and Matzke, 1998). PTGS and a subset of TGS have been shown to be triggered by aberrant RNA molecules followed by degradation (Fire, 1999; Hamilton and Baulcombe, 1999). Apart from the above-mentioned factors, pleiotrophic effects from transgene, somaclonal variations in the regenerated transgenic plants, or environmental effects on promoter driving the transgene expression are responsible for non-expression or low expression of the transgene in a transgenic plant (Matzke and Matzke, 1993; Stam et al., 1997).

Inheritance of the transgenes: Successful genetic transformation of any plant involves not only the production of primary transformants showing stable expression of inserted gene but also the inheritance of introduced trait. Inheritance studies have been carried out using resistance marker such as in petunia (Deroles and Gardner, 1988 a, b; Ulian et al., 1994); *Arabidopsis (*Kilby et al., 1995); tobacco (Matzke and Matzke, 1993); maize (Walters et al., 1992). Most of the studies have shown that the marker genes segregate as dominant loci in a Mendelian fashion (3:1). Recent work suggests variability in these inheritance pattern based on the detection of the transgene expression (Ulian et al., 1994). Skewed segregation of the introduced genes, during meiosis leading to non-mendelian inheritance may be caused by various factors such as linkage to a recessive lethal gene, mutational effect of T-DNA insertion and chromosomal rearrangement (Meassen, 1997). The transgenic status of the plant is confirmed by assaying for expression of the transgenes inserted. Stable integration and number of copies of inserted DNA are confirmed by Southern hybridization while gene expression is confirmed by Northern hybridization or RT-PCR and protein synthesis (translation) by Western

hybridization. Further evaluation of the transgenic plants is done under agronomic conditions by carrying out field assessment (Levin and Strauss, 1991).

2.3 Groundnut Rosette disease

Groundnut rosette disease is the most damaging disease of goundnut and occurs sporadically in severe epidemics, particularly in West Africa. It is endemic to Africa and is limited to the African continent and its offshore islands, and is transmitted by the Aphid, *Aphis craccivoer* (Storey and Bottomley, 1982) in a persistent manner. Rosette disease of groundnut was first described from Tanganyika (Zimmermman, 1907) and was subsequently found to be widely distributed throughout west, east and southern Africa and off shore islands, including Madagasar. The 1975 epidemic in northern Nigeria destroyed an estimated 0.7 million hectares of groundnut, causing losses of US$ 0.9 million (Anonymous, 1996). The 1995 epidemics in the eastern provision of Zambia affected over 43,000 hectares causing loss of US$ 4.89 million (Anonymous, 1996).

Epidemiology: The epidemiology of groundnut rosette disease is reviewed by (Naidu et al., 1998). A complex of three agents causes ground rosette disease, *viz* ground rosette virus (GRV), satellite RNA (sat RNA) and groundnut rosette assistor virus (GRAV). GRAV is a member of the genus *Luteovirus* (Casper et al., 1983; Murant, 1990), while GRV belongs to the genus *Umbravirus* (Murrant et al., 1995; Taliansky et al., 1996). GRV is mechanically transmissible and replicates independently in plants, where as the satellite RNA depends entirely on GRV for its replication (Murant et al., 1988; Blok et al., 1994). Either GRAV or GRV alone causes no obvious symptoms or only a mild transient mottle in groundnut. The satellite RNA is largely responsible for

the different types of rosette symptom (Fig. 1). Variants of satellite RNA were shown to cause the chlorotic and green forms of the disease (Murrant et al., 1988; Murrant and Kumar, 1990). The inter-dependence and interactions among the three agents of rosette disease complex where GRAV replicates autonomously in plants and is transmitted only by aphids, mainly by *Aphis craccivora*. By contrast, satellite RNA depends on GRV for replication and GRV depends on satellite RNA for aphid transmission. Both, in turn, depend on GRAV for their packaging in GRAV coat protein and subsequent transmission by an aphid vector (Murrant, 1990). In nature, all three agents must occur together for rosette disease to be transmitted successfully by aphid vector. Recent advances in understanding the causal agents of groundnut rosette disease at the Scottish Crop Research Institute, (SCRI), Dundee, UK suggest several possible routes to the production of novel resistance to rosette disease (Robinson, 1988).

Rosette disease occurs as two predominant forms based on symptoms; chlorotic rosette and green rosette (Gibbons, 1977; Murrant and Kumar, 1990). Chlorotic rosette is widely distributed where as green rosette has so far been reported only in West Africa and Uganda (Subramanyam et al., 1997). Plants affected by either green or chlorotic rosette are severely stunted and of bushy appearance due to shortened internodes and reduced leaf size (Fig. 1 A). Leaves of green rosette affected plants appear dark green in color when compared to unaffected plants (Fig. 1 B). Leaves of chlorotic rosette affected plants are curled and puckered and show a bright chlorosis. Early infection with green or chlorotic rosette results in severe or total yield loss, where as late infection causes less drastic decrease in the number and size of pods.

Resistance to GRD in the cultivated groundnut was discovered in local land races in Burkina Faso (de Berchoux, 1960). These land races belong to the Virginia variety group and are late maturing with poor yield. It was also shown that this resistance was controlled by two independent recessive genes. It was observed that resistant lines were not immune and that individual plants could become infected with GRV when subjected to inoculation by massive number of aphids. This resistance apparently operates equally against both chlorotic (de Berchoux, 1960) and green (Harkness, 1977) rosette. A low recovery of resistant plants from Virginia x Spanish crosses suggested that the double-recessive plants may succumb to heavy inoculation pressure in the early stages of growth. It was also suggested that double-recessive genotypes might not confirm resistance in all nuclear backgrounds. Infrequent recovery of short-duration resistant plants in crosses between Virginia and Spanish parent were reported. In Africa, Spanish type short-duration cultivars are preferred because groundnut is a rainfed crop. Since the maturity and resistance to GRV is governed by double homozygous recessive gene, it is difficult to develop resistance genotype by conventional breeding. The low frequency of recovery of short-duration genotypes from crosses between rosette-resistant Virginia parent and susceptible Spanish lines could be due to the mode of inheritance of both characteristics and their possible linkage. Recent studies (Murant et al., 1988) have shown that this resistance is directed against GRV and brings with it resistance to the satellite RNA. Since the plants were fully susceptible to GRAV which alone induces no obvious symptoms. Recently, (Subramanyam et al., 2001) showed that several accessions in different wild species of the genus *Arachis* possess resistance to all the components of

groundnut rosette. This indicates that it should be possible to breed groundnut cultivars with combined resistance to all the three components of rosette disease.

Several opportunities exist for utilizing biotechnological approaches (Wilson, 1993) to incorporate pathogen-derived resistance to one or more of the rosette disease agents. Possible constructs could be based on the coat protein gene of GRAV (Scott et al., 1996), the RNA-dependent RNA polymerase gene of GRV (Taliansky et al., 1996), or sequence of a variant satellite RNA that down regulates GRV replication (Taliansky et al., 1998).

2.4 Strategies for engineering virus resistance in transgenic plants

Most crop plant species are susceptible to a number of different pathogens, some of which may cause severe systemic infection resulting in significant crop losses. For most crop plants, evolutionary forces in the pathogen population act in time to subvert the effectiveness and durability of resistance. Consequently, the identification of new source of resistance is a major challenge. Most new resistance genes are identified amongst the wild relatives of crop species but many cannot be incorporated into conventional breeding programs because of barriers to sexual compatibility. Fortunately, over the past decade, advances in molecular genetics and in particular, the development of plant transformation technologies, have made it possible to engineer high level resistance to viral pathogens in plants by using transgenes from wide variety of sources (Draper et al., 1988). Crucially, because the transgenic approach for generating virus-resistant cultivars involves a single step introduction of individual highly characterized transgenes, the recovery of plants that retain the original cultivar traits is relatively straight forward

(Jongedijk et al , 1992) and the genetic engineering for virus resistance is one of the major success stories in plant biotechnology (Beachy, 1997; Table 3)

Table 3

Summary of plant species and viruses for which genetically engineered resistance has been attempted.

Resistance mediated by	Plant	Virus(es) resistance	Reference
Coat protein	Alfalfa	AIMV	Hill et al., 1991
	Barley	BYDV	Lister et al., 1994
	Cucumber	CMV	Gonsalves et al., 1992
	Maize	MDMV	Murry et al , 1993
	Oats	BYDV	Lister et al., 1994
	Papaya	PRSV	Fitch et al., 1992
	Potato	PLRV	Kaniewski et al., 1994
		PVX	Hoekema et al , 1989
		PVS	MacKenzie et al., 1991
	Tobacco	TSWV, TMV	Hayakawa et al., 1992
		PRSV	Powell-Abel et al., 1986
		CMV	Kim et al , 1994
		AIMV	Ling et al., 1991
	Tomato	TMV	Cuozzo et al , 1988
		TMV	Loesch-fries et al , 1987
		AIMV	Nelson et al., 1988
		ToMV	Tumer et al , 1987
		TSWV	Sanders et al., 1992

	Groundnut	IPCV	Sharma and Anjaiah, 2000
Antisense /untranslatable RNA	Potato	PLRV	Kawchuk et al , 1991
	Tobacco	TSWV	De Haan et al , 1992
	Tobacco	TGMV	Day et al , 1991
Satellite RNA	Tobacco	CMV	Harrison et al , 1987
	Tomato	CMV	Yie et al , 1993
Coat protein and satellite RNA	Tobacco	CMV	Yie et al., 1992
Replicase	Tobacco	TMV	Golenboski et al., 1990
		CMV	Anderson et al , 1992
		PEBV	MacFarlane and Davies, 1992
		PVX	Braun and Hemenway, 1992
	Potato	PVY	Chiang et al , 1994

Source: Modified from H R. Pappu, C.L. Niblett and R F. Lee, vol II· 1995. AIMV = alfalfa mosaic virus; BNYV = beet necrotic yellow virus; CMV= cucumber mosaic virus; PRSV= papaya ring spot virus; PEBV= pea early browning virus; PVS= potato virus S; PVX= potato virus X; PVY= potato virus Y; IPCV= Indian peanut clump virus; PLRV= potato leaf roll virus; TBRV= tomato black ring virus; TMV= tobacco mosaic virus; TSWV= tomato spotted wilt virus; ToMV= tomato mosaic virus; TSV=tomato streak virus.

Potential for transgenic Interference: Potential for transgenic interference exists at each stage of the infection cycle of a virus in an infected cell. The first stage in a viral infection is the uncoating of virus particles. One of the mechanisms proposed to explain cross-protection was that protein from the protecting virus prevented uncoating of the particles of the challenge virus (Sherwood and Fulton, 1982). Transforming plant cells with a viral coat protein gene has proven to be an effective way of protecting plants from virus infection (Powell-Abel et al., 1989). Once the virus particle in uncoated, it must be translated by ribosome's to produce proteins necessary for replication. Translation of mRNA in plant cells can be inhibited by the presence of complementary RNA sequences or antisense RNA (Ecker and Davis, 1986) Anti sense sequences are a part of genes that have been reversed in the transformation vector so that the RNA transcripts made in the transformed cells have sequences complementary to that of the target mRNA. Anti sense and target RNA hybridize together thus inhibiting the replication of the virus.

Ribozymes are also RNA molecules that have stretches of sequence complementary to the target RNA, and bind to the target RNA by hybridization (Haseloff and Gerlach, 1988). The nucleotide sequence in the center of the ribozyme between the regions that bind to target RNA has catalytic activity that results in hybridization to a ribozyme and subsequent cleavage of the target RNA. The next step in the development of a viral infection is the transcription and replication of virus RNA by replicase enzyme(s). Plants transformed with antibody genes have also been shown to produce serologically active antibodies (Hiatt et al., 1989). Hence, the expression of antibodies to the virus replicase enzymes might be an effective way to inherit virus replication

Replication is probably the stage at which satellite RNA is active in suppressing virus RNA synthesis. Satellite RNA multiplication diminishes that of the helper virus RNA. DI (defective interfering) RNA molecules are similar to satellite RNA, except they are derived from the viral genome RNA. DI RNA and satellite RNA molecules are encapsidated by the helper virus coat protein and may compete with genomic RNA molecules for coat protein during the particle assembly stage of the infection cycle. The final stage of virus multiplication is its spread to other cells. Many viruses are disseminated by being specifically carried by a vector with some persistent viruses, binding to internal organs of the vector (Harrison and Murrant, 1984). Thus, if plants are transformed to express a molecule that mimics the binding of either the virus particle protein or the helper component, this could compete with infective virus and thus dilute the inoculum carried by the vector.

Cross-protection phenomenon is a promising source of potential resistance. Cross protection is a phenomenon in which a plant that has been infected with mild strain of virus that produces few or no symptoms is protected from super infection by a severe strain of a related virus. Cross protection differs from induce systemic resistance in that the host is not resistant to either the mild (protecting) or severe (challenging) virus alone and the protection is specific to related viruses. Cross-protection has been used in agriculture to protect crops for which no other source of resistance or control measures was available (Fulton, 1986). A successful example is the control of tristeza virus disease of citrus trees, (Costa and Muller, 1980). However, there are potential drawbacks to the widespread use of cross-protection since the protecting strain might mutate to more severe form thus leading to great losses. There is a possibility of conditioning on entire

crop for a severe synergistic reaction by unrelated viruses or the protecting virus might spread to other crops on which its effects may be more severe. Powell-Abel et al. (1986) suggested that these objections might be overcome if cross-protection could be obtained as the result of expression of a single virus gene in a transgenic plant. Several mechanisms that have been proposed to explain cross-protection (Palukites and Zaitlin 1984); Sherwood (1987) have suggested ways in which virus-resistant transgenic plants might be produced. The presence of an excess of coat protein of the protecting-virus in a cell could prevent uncoating the challenging virus and replications of its nucleic acid. It is hypothesized that the challenging (+ve sRNA) virus could initiate replications by synthesis of a negative-strand RNA. The negative strand could hybridize to previously synthesized positive strand of the related protecting virus (negative-strand capture), thereby preventing it from acting as a template for synthesis of positive strands of the challenging virus.

Sherwood and Fulton (1982) showed that the nature of the capsid protein of the challenge strain was important in the protection mechanism. In their studies, they encapsidated challenger TMV-RNA with the capsid protein from bromo mosaic virus and observed protection that resulted due to accumulation of the coat protein. A gene encoding the viral coat or capsid protein of tobacco mosaic virus (TMV) was the first pathogen-derived transgene to be tested for its cross protection potential in transgenic plants (Powell-Abel et al., 1986). The resulting plants were found to be resistant to TMV infection, where those expressing the highest level of coat protein showing the highest level of resistance. The general applicability of this strategy for engineering virus resistance has since been widely documented for members of at least 20 different RNA

viruses (reviewed in Hull and Davies, 1992). Typically, coat protein mediated resistance manifested as a reduction in the number of lesions on inoculated leaves, a reduced rate of systemic disease development and very low levels of virus accumulation, in transgenic compared with control plant following deliberate inoculation with the parent virus. In many instances, the strength of coat protein-mediated resistance can approach near immunity to infection even with high concentration inocula.

Several lines of evidence suggest that the underlying mechanism responsible for the resistant phenotype differs depending on the viral group or viral transgene been studied. In the case of TMV (Powell-Abel et al., 1986), AIMV (Loesch-Fries et al., 1987), and PVX (Hemenway, 1988) the strength of resistance corelated positively with the levels of coat protein in transgenic plants. Indeed plants that accumulated only coat protein transcript of TMV or AIMV and not the coat protein itself were not resistant. However in the case of potato virus Y (PVY) (Farinelli and Malnoe, 1993) and potato leafroll virus (PLRV) (Baker et al., 1993) resistance corelated with the levels of coat protein transcript and not with levels of coat protein. The specificity of coat protein-mediated resistance has been extensively studied in several systems. Transgenic tobacco plants expressing the coat protein gene of soyabean mosaic virus (SMV), for which tobacco is non-host species, were found to be resistant to two serologically unrelated potyvirus, PVY and tobacco etch virus (TEV) (Stark and Beachy, 1989). The coat protein of SMV, TEV and PVY share approximately 60 % amino acid sequense homology. Similarly, transgenic plants expressing coat protein of TMV also showed significant levels of resistance to infection with viruses whose coat protein shared 60 % or greater amino acid homology with the TMV coat protein (Nejidar and Beachy, 1990). Significant levels of homologous

resistance have also been reported in other virus groups (Nakajima et al., 1993; Dinant et al., 1993). Field testing of virus-resistant transgenic plants is vital in order to test the durability of resistance under natural conditions and to determine whether important agronomic characteristics of the original cultivar have been retained following the transformation procedure. Because the coat protein-mediated resistance strategy has been widely applied since the mid 1980's many large scale field trails of transgenic virus resistance potato (Kaniewski et al., 1990; Jongedijk et al., 1992), (Gonsalves et al., 1992) lines have now been completed after several years of evaluation. These studies confirm the durability of the resistance under field conditions and further show that commercially available, highly resistant, true-to-type lines can be obtained.

Non-coat protein transgene: The possibility of engineering resistance using viral genes other than the coat protein gene include genes coding for functions such as I. The RNA-dependent RNA polymerase or replicase protein involved in virus replication and viral protease involved in processing polyprotein gene product.

Replicase-mediated resistance: The initial discovery that expression of a viral replicase transgene could confer resistance to infection came from experiments designed to test whether the hypothetical 54 KD subunit of the replicase of TMV was involved in viral replication (Golemboski et al., 1990). Transgenic tobacco plants containing a cDNA copy of this portion of the replicase gene were found to be highly resistant TMV infection even though the 54 KD protein products could not be detected. There are several reports of similar "replicase mediated" resistance to different viruses: PVX (Braun and Hemenway, 1992; Longstaff et al., 1993), PVY (Audy et al., 1994), pea early browning virus (Macfarlane and Davis, 1992), CyRSV (Rubino et al., 1993). Replicase-

mediated resistance can confer virtual immunity to infection. The mechanism of replicase-mediated resistance is unclear, depending upon the particular system under investigation, there can either be an inverse relationship between the degree of resistance and the quantity of the expressed viral gene product or no relationship at all. In many cases the translational product of the transgene cannot be detected in plants that show high levels of resistance. This suggests that resistance may involve RNA transcripts of the transgene rather than the replicase protein itself.

Polyprotein protease-mediated resistance: In viruses of the PVY group the viral genome codes for a single large protein, the polyprotein, rather than several discrete protein products. Following its translation, the polyprotein is proteolytically processed to produce the mature functional gene products. Studies in transgenic plants of factors controlling the proteolytic processing of the polyprotein, led to the unexpected finding that plants engineered to express the virus protease domain of either PVY (Maiti et al., 1993) or tobacco vein mottling virus, exhibited a high degree of resistance to the respective viruses. The resistance was very strain-specific and presumed to be due to interference with normal processing of the primary polyprotein product, thus resulting in disruption of the viral life cycle.

RNA based strategies for engineering symptom attenuation or resistance: Satellite RNAs are small viral RNAs (300 nucleotides) that cannot infect or replicate by themselves and are essentially molecular parasites of replication-competent viruses. In this capacity they can act either to increase or decrease the severity of the symptoms produced by the helper virus (Palukaitis and Zaitlin,1992). The first indication that viral RNA sequence could be used to engineer resistance came from studies on transgenic

plants that expressed cDNA copies of the symptom attenuating satellite RNAs of CMV (Harrison et al., 1987; Gerlach et al., 1987). In both cases, the inoculation of transgenic plants with the parent virus led to the amplification of the satellite RNA transcripts to very high levels. This in turn conferred protection against the otherwise severe effects of infection with the parent virus alone. Protection was thought to be based on RNA: RNA interactions, since satellite RNA do not contain open reading frame, but the precise mechanics is not known.

Anti sense and sense RNA: An alternative RNA based strategy that has been successfully employed to down-regulate the expression of numerous plant genes, is based on anti sense RNA. Anti sense-mediated resistance has been reported for geminivirus tomato golden mosaic virus and for TMV. Transgenic tobacco plants expressing anti sense transcript of the *AL1* gene TGMV showed significantly reduced symptoms development when challenged with the virus, the reduction showing a broad corelation with the level of anti sense transcript (Day et al., 1991; Nelson et al., 1993).

2.5 Tissue culture and genetic transformation of groundnut

Reliable system for whole plant regeneration in tissue culture is a pre-requisite for genetic transformation studies. Transformation of plants includes the stable introduction of foreign DNA sequence into the nuclear genome of cells capable of giving rise to transformed plants. The very basis of regeneration in tissue culture is that somatic cells are totipotent and can be stimulated to regenerate into whole plant in vitro, *via* organogenesis or somatic embryogenesis under optimal hormonal concentration and nutritional conditions (Skoog and Miller, 1957). The incorporation of foreign genes into

plants using *Agrobacterium*-mediated transformation depends upon the ability to successfully regenerate whole plants from single or group of transformed cells. An efficient in vitro shoot regeneration protocol compatible with genetic transformation methods would thus be very useful in the rapid development of transgenic plants of groundnut.

There are numerous reports in the literature on tissue culture and regeneration of groundnut from various explants (Bajaj, 1984). Sexually functional plants have been regenerated from immature embryos (Ozias-Akins, 1989), leaves of young seedlings (Mroginski et al., 1981; Pittman et al., 1983; Seitz et al., 1987; Mckently et al., 1991; Sharma et al., 1993; Livingstone et al., 1995; Chengalrayan et al; 1995), cotyledons (Bhatia et al., 1985; Kanyand et al., 1997; Ponsamuel, 1998), (Sharma and Anjaiah, 2000), embroyo axis (Atreya et al., 1984; Baker et al., 1998; Hazra et al., 1989), leaf explant (Eapen and George, 1993; Cheng et al., 1994, 1997; Rohini and Rao, 2000). Earlier reports on *A hypogaea* L. in vitro regeneration have indicated that differentiation of shoots from callus can be induced from a variety of explants (Narasimhulu and Reddy, 1983) while callus tissue has been recovered following protoplast release, but no shoots regenerated in any of these sutdies (Oelck et al., 1982).

In vitro regeneration of groundnut occur through embryogenesis or organogenesis. Regeneration by primary organogenesis occur by the development of shoots directly on the cultured explants or by intervening callus phase i.e. the development of shoots directly from calluis tissue, young leaflets, and leaf tissue were capable of producing shoots at higher frequency during the primary culture stage, while no shoots regeneration was obtained from fully expanded leaflets (Mroginski et al., 1981; Chengalrayan et al.,

1995). Shoots grow readily from excised shoot tips cultured under suitable conditions (Kartha et al , 1981). Prolific organogenesis can be obtained from seed parts mostly the cotyledons or tissue surrounding the cotyledonary node (Kanyand et al , 1997). High frequency regeneration from mature cotyledon was achieved in groundnut (Sharma and Anjaiah, 2000). Regeneration through somatic embryogenesis occurs when bipolar shoots and roots are initiated and follow a development pathway similar to zygotic embryos. Development of somatic embryos has been reported by using immature leaflets, cotyledons, callus (Ozias-Akins, 1989; Baker and Wetzstein, 1992; Chengalrayan et al, 1997).

The success with transplantation is over 90 % with cotyledons (Sharma and Anjaiah, 2000). In groundnut cultures the in vitro response appears to be strongly influenced by genotype, hormone content of culture media, age of source explant (Mroginski et al., 1981). A combination of 2, 4-D, NAA or IAA with BA or kinetin in a defined culture medium in sufficient to induce shoot formation Rooting of shoots generally occurs on basal medium or medium supplemented with auxin. A variety of auxins have been observed to elicit somatic embryogonesis when applied to the explants. Various explants such as petiole, epicotyl, hypocotyl, mesocotyl, cotyledon, mature and immature embryos and whole seed, immature leaflets have been employed successfully to regenerate shoots. (Mckently et al., 1990, 1991; Mckently, 1995; Baker and Wetzstein 1992, 1998; Cheng et al., 1992, 1996; Gill and Sexena, 1992; Ozias-Akins et al , 1992; Chengalrayan et al., 1994, 1997, 1998; Eapen and George, 1993; Lacorte et al., 1991; Li et al , 1993, 1994, 1997; Kanyand et al , 1994, 1997, Ponsamuel et al , 1998, Zhuang et al , 1999; Rohini and Rao, 2000, Sharma and Anjaiah, 2000) Besides the cotyledon

system well established in groundnut, leaflet explant was used in the present study to develop efficient regeneration and transformation protocol. Table 4 represents in vitro plant regeneration in *Arachis* species.

Table 4

Reports on morphogenic responses in direct shoot regeneration methods previously reported in groundnut (*Arachis hypogaea* L.).

Explant	Response	References
An, IL	Org	Mroginski et al., 1981
IL	Org	Pittman et al., 1983
Epi	Org	Cheng et al., 1992
IE	Org / Emb	Eapen and George, 1993
EC	Emb	Ozais-Akins et al., 1993
IL	Org	Chengalrayan et al., 1995
S	Org	Li et al., 1994
ME	Emb	McKently et al., 1995
IL	Emb / Org	Livingstone and Birch, 1995
C	Org	Lacoite et al., 1991
IL	Org	Utomo et al., 1996
IE, C	Emb / Org	Cheng et al., 1996
IE	Org	Kanyand et al., 1997
Emb cal	Emb	Livingstone and Birch, 1999
E	Emb	Zhuang et al., 1999
Plu	Org	Ponsamuel et al., 1998

Org = Organogenesis; Emb = Embryogenesis

Anthers = An; Immature leaves = IL; Cotyledons = C; Callus = Cal;

Embryoaxes = E; Embryogenic callus = Emb cal; Seed = S; Plemule = Plu

Groundnut tissue is susceptible to infection by wild type strains of *Agrobacterium* (Lacorte et al., 1991). Although several reports on efficient regeneration from diverse explants of groundnut have been published, not much success with genetic transformation of *Arachis* species has been achieved. This is due to the lack of efficient protocols to regenerate whole plants through in vitro regeneration of adventitious shoot buds from the transformed tissue. Transformation of protoplast may not be useful for groundnut (Shillito et al., 1985), because plant regeneration from protoplast has not been reported. Although direct DNA delivery is an alternative to *Agrobacterium* transformation where bombarded leaflets gave rise to slow growing brown callus, which did not respond on further sub-culture. Microprojectile bombardment of immature groundnut leaflet produced stably transformed callus lines but no transformed plants were obtained (Clemente et al., 1992). Bombardment of leaflet gave rise to slow growing brown callus and green clusters, although plants have been regenerated from this experiments through organogenesis, none were stably transformed (Schnall and Weissinger, 1995).

Transgenic plants produced by bombardment of embryogenic cultures, produced transgenic shoots at a frequency of 0.1 % (Ozias-Akins at al., 1993), while the shoot meristem of mature embryogenic axis produced transgenic plants at low frequency of 0.9 to 1 % (Brar and Cohen, 1994). Similar results were obtained by (Livingstone and Birch, 1999) where 1 % frequency of the transgenic plants were produced through biolistics from leaflet explants. Preliminary evidence suggests that groundnut embryogenic axes co- cultivated with *Agrobacterium* have been stably transformed (McKently et al., 1995). *Agrobacterium*-mediated transformation protocol using leaf explants was developed by Eapen and George, (1994) where transformation frequency of 2 % was observed. Cheng

et al. (1997) produced transgenic plants using leaf segments with 0.3 % frequency. More recently, a very efficient system based on cotyledon explants has been developed by Sharma and Anjaiah (2000) that results in transformation frequency > 55 %. Table 5 represents current status of groundnut transformation via *Agrobacterium* and particle bombardment methods.

For successful genetic transformation or modification by the production of transgenic plants, effective regeneration and transformation system is imperative. The key feature of this protocol is the regenerability of the cells wounded during excision of the cotyledons and these cells are mainly surface cells, readily accessable to the bacterium. The above report shows transformation frequency over 55 %. With the availability of efficient transformation and regeneration protocols that have been developed in the recent years, it should be possible to genetically engineer important agronomic traits for the improvement of groundnut.

Table 5

Reports on genetic transformation of groundnut (*Arachis hypogaea* L.) through *Agrobacterium* and particle bombardment methods.

Explant	Method	Transformation frequency	Reference
Lef	PB	Nil	Clemente et al., 1992
Emb cal	PB	1 %	Ozias-Akins et al., 1993
Lef	At	1-3 %	Eapen and George, 1994
ME	ACCELL	0.5 –1 %	Brar and Cohen, 1994
SE	PB	0.5 %	Yang et al., 1998
Emb cal	PB	1 %	Schnall and Weissinger, 1993
Emb cal	PB	1-2 %	Singsit et al., 1997
Lef	At	0.3 %	Cheng et al., 1996
Lef	At	0.3 - 0.9 %	Cheng et al., 1997
C	At	2 – 3 %	Li et al., 1997
Emb cal	PB	0.9 %	Livingstone and Birch, 1999
E	At	2 %	Rohini and Rao, 2000
C	At	55 %	Sharma and Anjaiah, 2000

PB = Particle bombardment

At = *Agrobacterium tumefasciens*

C = Cotyledons; E = Embryo; Emb cal = Embryogenic callus; ME = Mature embryo; Lef = Leaf.

MATERIALS and METHODS

3.1 Shoot morphogenesis and plant regeneration

3.1.1 Plant material

The seeds of genotype ICGS-44, IGGC-11 and cultivar JL-24 were used in the experiments since these genotypes are widely cultivated due to their desired agronomic traits. Mature seeds of these genotypes (Fig. 2) were used as source of explants for initiating tissue culture. Unless mentioned otherwise, genotype ICGS-44 was used in all experiments.

3.1.2 Preparation of Explants

Aseptically germinated seedlings served as the source of leaflet explants. The seeds were surface sterilized in 0.1 % mercuric chloride (w/v) with 1 to 2 drops of tween-20 for 7 min on a rotary shaker. Seeds were rinsed 3 times with sterile distilled water and soaked in sterile water for 3 to 4 h before use. After removal of the seed coat 5 to 8 seeds were implanted in each petri-plate (90 mm x 16 mm dia) containing MS basal medium (Murashige and Skoog, 1962; Table 6), 3 % sucrose and 0.8 % agar for 1 d. For the preparation of leaflet explants the seed was split open and the immature leaflets were excised at the petiolar region of the embryo axis and cultured on shoot induction medium (SIM). At this stage the leaflets measured 2 to 3 mm (Fig. 4A). The petiolar cut end of the explant was placed on culture medium with its abaxial surface in contact with the medium. The explants were cultured at a density of 8 per petri-plate and sealed with parafilm.

Table 6
Composition of Murashige and Skoogs (MS) tissue culture medium.

Salt	Concentration	Stock	Use (per / l)
MAJOR SALTS (50X)			
NH_4NO_3	1650	33.0 g / 200 ml	10 ml
KNO_3	1900	38.0 g / 400 ml	20 ml
KH_2PO_4	170	3.40 g / 200 ml	10 ml
$CaCl_2$	440	8.80 g / 200 ml	10 ml
$MgSO_4$	370	7.40 g / 200 ml	10 ml
MINOR SALTS (100X)			
KI	0.83	83.0 mg / l	
$MnSO_4\ 4H_2O$	22.3	2230 mg / l	
$ZnSO_4.\ 7H_2O$	8.6	860 mg / l	
$Na_2MoO_4.\ 2H_2O$	0.25	25 mg / l	5.0 ml
$CuSO_4.\ 5H_2O$	0.025	2.5 mg / l	
$CoCl_2$	0.025	2.5 mg / l	
Iron (100X)			
$Na_2EDTA\ 2H_2O$	37.3	3.7 g / l	10 ml
$FeSO_4.2H_2O$	27.8	2.78 g / l	
ORGANICS (100X)			
Glycin	2.0	200 mg / l	
Nicotinic acid	0.5	50 mg / l	
Thiamine HCl	0.1	100 mg / l	10 ml
Pyridoxin HCl	0.5	50 mg / l	
Myo-inositol	100	5.0 mg / 500 ml	

3.1.3 Culture medium and conditions

All the experiments were conducted by using MS basal medium supplemented with different phytohormones that included kinetin (KN), N^6-benzyladenine (BA), α–naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA). The culture vessels used were (90 mm x 16 mm dia) sterile disposable petriplates for regeneration. Test tubes (150 mm x 25 mm) plugged with non-absorbent cotton were used for elongation of shoots and rooting. The medium and tubes were autoclaved at 15 psi pressure at 121°C for 15 min. The culture medium was gelled with 0.8 % (w/v) Difco-Bacto agar and pH of the medium was adjusted to 5.8 prior to autoclaving. All the cultures were maintained at 26 ± 2 °C under continuous light having 100 $\mu Em^{-2}S^{-1}$ irradiance provided by cool day light fluorescent lamps.

3.1.4 Regeneration of plants

The leaflet explants were cultured on SIM with the petiole cut end embedded into the medium. The SIM comprised of MS basal medium containing 3 % sucrose, 13.3 μM BA and 5.3 μM NAA, the medium was gelled with 0.8 % Difco-Bacto agar and the pH was adjusted to 5.8 prior to autoclaving. The cultures were observed over a period of 2 to 3 weeks for shoot morphogenesis from leaflet explants. The adventitious shoots regenerated from leaflet system were transferred to shoot elongation medium (SEM) that comprised of MS + 3 % sucrose + 2.3 μM KN + 2.2 μM BA in test tubes for 10 to 15 days, followed by three passages on MS basal medium. For rooting the elongated shoots were transferred to MS basal medium for 2 weeks after which the plants with well developed roots (Fig. 4F) were transplanted to autoclaved sand : soil (1:1) mixture in plastic pots. The plants were covered with polythene bags and maintained at 26 ± 2 °C

with about 80 % relative humidity for 2 weeks prior to their transfer to 19 cm (dia) pots containing autoclaved field soil. The plants were maintained in the glasshouse and periodically irrigated with tap water.

3.2 Ontogeny of shoot bud differentiation from leaflet explants

To study the histogenesis of shoot bud differentiation from leaflets, the explants were cultured on SIM for different periods (1, 2, 5, 7, 10, 14 d) and the regenerative tissue was processed for plastic sectioning according to the procedure described by (O'Brien and McCully, 1981) as follows:

Fixation: The leaflets were fixed in ice-cold fixative comprising of 2 % gluteraldehyde + 2 % formaldehyde in 0.05 phosphate buffer at pH 6.8. During fixation and dehydration the vials were kept at 4 °C and cold solutions were used at each change. Fixation was carried out overnight at 4 °C followed by 3 changes of phosphate buffer before proceeding further.

Dehydration and infiltration: The fixed material was dehydrated in ethanol series comprising of 50, 60, 70, 80, 90, 100 % ethanol for overnight at 4 °C for each change. The dehydrated specimens were then infiltrated with Historesin by giving several changes in the activated historesin at 4 °C until the material was ready for embedding and sectioning.

Embedding: The specimens were embedded in the activated historesin containing the fardner solution. The plastic blocks were filled partially with the historesin and then specimen was placed in it in the desired orientation. The blocks were tightly capped with

metal holders to avoid air bubbles and stored over night at room temperature for polymerization.

Sectioning and staining: Plastic blocks containing the specimen were trimed with a fine scalpel blade prior to sectioning. Serial section of 3 to 5 µm thickness were cut using glass knives on a Reichert–Jung autocut microtome. The sections were placed serially on pre-cleaned glass slides and stained for 1 min with Toluidine Blue O (TBO) by placing the slide in the dye for 1 min, rinsed in distilled water until the plastic was nearly free from stain (Yeung and Law, 1987), observed under a microscope, and photographed.

3.3 Genetic transformation of groundnut by using *Agrobacterium tumefaciens*

3.3.1 Plant material and explant preparation

Mature seeds of groundnut genotype ICGS-44 were used as a source of explant for transformation experiments. Surface sterilization, explant preparation, culture medium and conditions were similar to regeneration procedure as described in section 3.1.

3.3.2 Bacterial strains and cultures

Agrobacterium strains carrying the binary plasmids C58 pROKII:GRAVcp and C58 with pCAMBIA1301:GRAVcp (Fig. 3 A and B) were used for all the studies. *Agrobacterium* strain C58 harbouring the binary plasmid pROKII:GRAVcp (kindly provided by Dr. A.F.Murrant, Scottish Crop Research Institute, Dundee, UK) contains coat protein gene of GRAV (600 bp) driven by a CaMV 35S promoter and *nos* terminator and a neomycin phosphotransferase coding *npt*II gene under the control of *nos* promoter and terminator within the T-DNA borders. The plasmid contains kanamycin resistance gene for bacterial selection. The plasmid pCAMBIA1301:GRAVcp (constructed by Dr. Kiran K Sharma)

was constructed by cloning 1640 bp *Hind* III fragment containing 2X 35S promoter-GRAVcp-35S terminator into the *Hind* III site of the binary vector pCAMBIA:1301, that also has *hpt* and *uid*A-INT genes as selectable and screenble marker and are driven by 35S promoter and *nos* terminator within the T-DNA borders. The plasmid contains kanamycin resistance gene for bacterial selection. *A tumefaciens* strain C58 also has resistance to rifampicin. Thus, the strains were periodically maintained on YEB (Sambrook et al., 1989) agar plates containing 50 µg/ml kanamycin and 25 µg/ml rifampicin. For long term storage 1 ml of overnight grown culture containing 50 % sterile glycerol were transferred to an appendroff tube, frozen in liquid nitrogen (-196 °C) and stored at -80 °C.

3.3.3 Bacterial culture and harvest

Single colony of *Agrobacterium culture* was streaked on YEB medium containing appropriate antibiotic. The cultures were grown at 28 °C for 2 d and the freshly grown culture was used for transformation experiments. The strains were sub-cultured at 3 week intervals. A single colony from freshly grown culture of *Agrobacterium* of each strain was inoculated into a conical flask containing YEB (20 ml) medium with 50 µg/ml kanamycin. The cultures were grown overnight on a shaker at 250 rpm until they reach an OD of 0.9 to 1.0.

3.3.4 Co-cultivation with *Agrobacterium tumefaciens*

The bacterial culture (10 ml) was centrifuged in polypropylene tubes (30 ml) at 5000 rpm for 6 min and the pellet was collected under aseptic conditions. The pellet was resuspended in 30 ml of ½ strength MS liquid medium (1:6) containing 3 % sucrose. The suspension medium was stored at 4 °C for 1 to 2 h prior to its use for co-cultivation

Excised leaflet explants from groundnut genotypes ICGS-44 were taken and their petiole cut ends were briefly dipped into the bacterial suspension and immediately cultured on SIM with a filter paper. The explants were co-cultivated with the *Agrobacterium* for 72 h in dark and transferred to SIM supplemented with filter sterilized 500 mg/l cefotaxime. Care was taken to embed petiole cut ends into the medium Plating density was maintained at eight explants per petri-plate. The cultures contaminated with *Agrobacterium* were rescued in the initial experiments using 500 mg/l cefotaxime but a new antifungal and antibacterial mixture called PPM (Plant Protection Mixture; Plant Cell Technology) was used at a concentration of 10 mg/l in later part of the work. This compound proved to be very effective in controlling bacterial and fungal contamination.

3.3.5 Plant regeneration and selection of putative transformants

The leaflet explants after treatment with *Agrobacterium* were maintained on SIM containing filter-sterilized cefotaxime 500 mg/l for 3 weeks when multiple shoot buds appeared on at least 70 % of the explants (Fig 4C) while shoot buds continue to form. Before choosing the stringent selection, different concentrations of antibiotics kanamycin and hygromycin were tested on non-transformed groundnut shoots to determine their natural resistance to these antibiotics, lethal dose LD_{50} of each selection agent and the efficiency of selection. Kanamycin was used at 50 mg/l, 75 mg/l and 100 mg/l while hygromycin was tested at 4 mg/l, 6 mg/l, and 8 mg/l. The explants cultured on SIM free of selection agents were used as controls. After 3 weeks of culture the efficiency of selection was scored. After 2 weeks the green surviving shoots were further transferred to test tubes containing elongation medium with selection pressure for 2 to 3 passage of selection. The elongated shoots were cultured on hormone-free MS medium for rooting.

90 % of the shoots produced multiple adventitious roots within two weeks. The base of the stem was excised and the shoot cultures were inserted inside the medium to ensure good contact with the medium for adequate uptake of nutrients and selection pressure. Occasionally, callusing or browning of tissue at the base of the shoot clusters was removed before placing them on fresh selection medium.

3.3.6 Cultivation of putative transgenic plants

The rooted plants were transferred to the pots containing autoclaved sand : soil (1:1) mixture and maintained under high humidity 85 % at 26 ± 2 °C in a growth cabinet. After two weeks they were transfered to glasshouse, allowed them to flower and set seed. The mature seeds were collected and analysed for the presence and expression of the introduced genes. The primary transformants upon transfer to the containment glasshouse were termed as T_0 generation, while those from subsequent seed generation were termed as # T_1, # T_2 and so on.

3.3.7 Histochemical localization of *uid*A gene in putative transformants

Gus extraction buffer: Sodium phosphate buffer (0.1 M) was prepared by mixing equal concentrations (0.05 M) of NaH_2PO_4 and Na_2HPO_4 stock solutions. pH of the buffer was adjusted to 7.0 and solution was stored at 4 °C. The X-gluc solution was prepared by dissolving 10 mg of the X-gluc in 100 µl of dimethylformamide (DMF) and the volume was made up to 5 ml with 0.1 M sodium phosphate buffers, pH 7.0. The contents were further mixed with 25 µl of 200 mM potassium ferrocyanide + 25 µl of 200 mM potassium ferricyanide + 50 µl of 1 M Na_2EDTA + 100µl of 0.1 % triton-X and 200 µl of 50 mg/l sodium azide and the tube containing X-gluc mixture was wrapped in the aluminium foil to protect from light and stored at 4 °C. Freshly prepared solution was

used for all the assays. ß-glucuronidase (GUS) enzyme activity was detected histochemically in unfixed leaves and petiole of the in vitro growing plants. 200 µl of the X-gluc assay mixture was added to the tissue sample and infiltrated for 3 to 5 min, the sample was incubated at 37 °C for 4 to 24 h in dark. The reaction was stopped by removing the mixture and dehydrating the sample by sequentially changing in 70 to100 % ethanol until tissue was devoid of chlorophyll. The tissue was then observed under the microscope.

3.4 Molecular analysis of the putative transformants

3.4.1 Extraction of genomic DNA from groundnut leaves.

Total genomic DNA was extracted from fresh leaves of the putative transformants (T_0, T_1, and T_2). The DNA extraction was done following the method of (Sharma et al., 2000). Young leaf tissue from putatively transformed plants growing in the glasshouse were collected and ground to fine powder under liquid nitrogen and was transferred to 25 ml polypropylene tubes to which 15 ml of extraction buffer (100 mM EDTA, 50 mM Tris and 500 mM NaCl) and 1 ml 20 % SDS was added, mixed gently and incubated in water bath at 65 °C for 15 min. The samples were brought to room temperature and 5 ml potassium acetate at pH 4.8 was added, mixed gently and incubated on ice for 30 min. The mixture was centrifuged at 13,000 rpm for 20 min and the supernatant separated and precipitated with 0.6 volumes of isopropanol and the mixture was kept at –20 °C for 30 min before centrifugation at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed in 70 % ethanol and air-dried. To the dry pellet 700 µl TE (pH 8.0) and 10 µl of RNase (10 mg/ml) was added and incubated at 37 °C for 1 to 2 h. The DNA solution was stored at -20 °C, until further use

3.4.2 Purification of the genomic DNA

Two to three volumes of distilled water was added to the DNA solution and 1ml of DEAE-cellulose suspension (Sharma et al , 2000) and mixed gently for 3 min to keep DEAE-cellulose suspended, there by maximizing interaction between nucleic acids and DEAE-cellulose. The mixture was centrifuged for 30 sec at 3,000 rpm to sediment DEAE-cellulose to which nucleic acids have bound The supernatent was carefully removed and the pellet was resuspended in 1 2 ml wash medium to eliminate proteins, polysaccharides and secondary metabolites not bound to DEAE-cellulose. The mixture was centrifuged for 30 sec at 3000 rpm and the supernatent removed. This step was repeated at least once. 0.5 ml of elution medium was added to DEAE-cellulose pellet and gently mixed to elute nucleic acids, prior to centrifugation the supernatant was collected and 0.3 ml of elution medium was added to DEAE-cellulose, mixed, and centrifuged. All the supernatants were pooled and 0.6 ml of isopropanol was added to supernatant, mixed and centrifuged at 10,000 rpm for 10 min at room temperature. The pellet was air dried and dissolved in 100 µl of TE buffer pH 8.0. The DNA solution stored at –20 °C until futher use for analysis.

3.4.3 Quantification of genomic DNA

The concentration of DNA samples was determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. One 260 unit is equivalent to 50 µg/ml for DNA. The 260/280 nm ratio was used as an indication of purity of the nucleic acids samples and should be no less than 1.8.

3.4.4 PCR analysis of the introduced genes

Putative transformants were screened by polymerase chain reaction (PCR) for the presence of *npt*II and *GRAVcp* genes. The 700 bp region of *npt*II gene was amplified by using 21-mer oligonucleotide primers (primer I 5'-GAG GAT ATT CGG CAT TGA CTG -3' and primer II 5'-ATG GGG AGC GGC GAT ACC GTA-3') as previously reported (Hamill et al., 1991). A 1200 bp region of *uid*A gene was amplified using 21-mer oligonucleotide primers (primer I 5'- GGT GGG AAA GCG GGT TAC AAG - 3' and primer II 5'- GTT TAC GCA TTA CTT CCG CCA – 3'). A 384 bp coding region of *GRAVcp* gene was amplified by using 21-mer oligonucleotide primers (primer I 5'-CCT CAA CCA AAC AGC-3' and primer II 5'-ATG TAG CGA CCC ATC CAG -3'). The PCR reaction was performed in a total volume of 50 µl containing 1X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mix, 10 µM primer I and 10 µM primer 2, 1.25 units of Taq DNA polymerase (GIBCO-BRL) and 3 µl of template DNA (200 ng of genomic DNA). The PCR amplification reaction was carried by using a Techne PHC3 thermocyler under the following conditions for *npt*II gene (700bp); 93 °C for 3 mins (1 cycle), 92 °C for 1min (denaturation), 54 °C for 1min (annealing), 72 °C for 1.5 min (extension) for 32 cycles and final extension for 72 °C for 4 min (1 cycle). The PCR amplification reaction was carried under the following conditions for *uid*A gene (1200bp); 93 °C for 3 mins (1 cycle), 92 °C for 1 min (denaturation), 58 °C for 1 min (annealing), 72 °C for 1.5 min (extension) for 32 cycles and final extension for 72 °C for 4 min (1 cycle). The PCR amplification reaction was carried under the following conditions for *GRAVcp* gene (384 bp); 93 °C for 3 mins (1 cycle), 92 °C for 1 min (denaturation), 54.2 °C for 1 min

(annealing), 72 °C for 1.5 min (extension) for 32 cycles and final extension for 72 °C for 4 min (1 cycle).

The amplified product were analysed on 1.2 % agarose gel, followed by visualization under UV transilluminator to identify the fragments of expected lengths. To verify the fidelity of the amplicons, the fragments resolved on the agarose gel was transferred to Hybond N^+ nylon membrane (Amersham) by Southern blotting and probed with *GRAVcp* fragment from the respective plasmid (Fig. 3). The blot was hybridized with *GRAVcp* labelled with non-radioactive AlkPhos direct system (Amersham). In all PCR reactions, genomic DNA from untransformed groundnut plants was used as a negative control for the transgene and the binary plasmid DNA carrying the corresponding transgene as the positive control.

3.4.5 Southern blot analysis

Southern blot analysis was done following the procedure of Sambrook et al. (1989). For Southern analysis of putative transformants 15 µg of the genomic DNA of each sample was digested with 5 to 10 unit of *Eco* RI restriction enzyme that has single internal site pROKII:GRAVcp (Fig. 3) to ascertain the integration pattern and number of copies of insert DNA based on size. The reaction was carried out in a final volume of 20 µl in 1.5 ml ependorff tube containing 1X reaction buffer at 37 °C overnight. Genomic DNA of untransformed plants served as a negative control and the DNA of binary plasmid (0.05 ng) carrying the corresponding transgene to be detected was used as positive control. Both the controls were digested with same enzyme with which the putative transformants were digested. The digested samples were separated on a 0.8 % agarose gel prepared in 1X TAE buffer. The DNA samples were mixed with 5 µl of loading dye and

electrophoresis was performed at 50 volts for 3 to 4 h. The DNA fragments were separated on the gel and visualized by staining the gel with 0.5 mg/l ethidium bromide in distilled water. The gel was washed with several changes of water before processing the gel for southern blotting. The gel was depurinated with 0.25 M HCl for 20 mins with gentle shaking. HCl creates nicks in the double standard DNA, breaking large DNA fragments in to smaller ones, which results improved transfer on to the membrane. The DNA was denatured by soaking the gel in denaturation solution (0.5M NaOH, 1.5 M NaCl) for 30 min with gentle shaking. After a single wash with sterile distilled water the gel was incubated in neutralization buffer (1 M Tris-Cl, 1.5 M NaCl, pH 7.4) for 30 min followed by soaking the gel in 20X SSC (3 M NaCl, 0.3 M sodium citrate) for 10 mins before blotting. The blotting apparatus was set up as follows. A glass plate was placed across a 20X SSC so that the ends hung down on the support and the exposed blotting paper surrounding the gel was masked with parafilm. Positively charged Hybond N^+ nylon membrane cut to the size of the gel was pre-soaked in 2X SSC before placing it on the gel. Air bubbles between the gel and the membrane was removed by rolling a glass pasture pipette over the surface. Another three layers of Whatman No. 3 paper cut to the size of the gel soaked in 20X SSC and placed on the membrane followed by a 10 to 15 cm stack of absorbed paper towels. A glass plate with about 1 kg weight was placed on top of the paper towels and the blotting was performed overnight. Subsequently, the blot was dried at room temperature for 10 min and the DNA was cross-linked by placing the DNA side down on a UV transilluminator for 2 min.

Non-radio labelling and detection of transgene stable integration: The DNA fragments required for generation of probes were obtained by PCR amplification of the respective

transgene from the binary vector as the DNA template. The amplified products were purified from gel by using Quaigen kit. Non-radio labelling of the probe, pre-hybridization, hybridization, post-hybridization washes and detection system were performed as per the instructions of Alkphos direct system (Amersham). Blots were wraped in polythene wrap and exposed to X-ray film.

3.4.6 Inheritance of transgenes in the progeny

A total of 50 seeds from the primary independent (7 lines) transformants (T_1 generation) and 24 seeds from T_1 progeny (T_2 generation) growing in the glasshouse were analysed to study the segregation pattern in the progeny of the transgenes. Genomic DNA was extracted from T_1, T_2 samples and analysed for transgene integration by using PCR for *GRAVcp*. The amplification reactions were carried out by using a Techne PHC3 thermocycler following conditions as described in 3.4.4.

RESULTS

4.1 Shoot regeneration

4.1.1 Shoot bud differentiation

Initially the leaflet explants from 1 d-old seedlings were cultured on media supplemented with different concentration of BA (5.5, 13.3, 18.3 μM) and NAA (7.9, 6.3, 5.3,1.0 μM) individually and in different combinations. In the absence of any growth regulator only roots were formed without any apparent sign of callus formation. Amongst the different media tested, MS containing 13.3 μM BA + 5.3 μM NAA (SIM) produced the highest frequency (92.3 %) of multiple adventitious shoot buds. The SIM (shoot induction medium) was originally optimized for variety ICGS-44 where the explants turned green, underwent considerable enlargement within two days of culture initiation. Adventitious shoots differentiated at the petiole cut end within 15 to 20 d in over 90 % of the explants (Fig. 4B). Leaflets cultured on medium containing 13.3 μM BA + 5.3 μM NAA was optimum for shoot bud formation where 4 to 6 shoots could be recovered from each explant.

4.1.2 Orientation of the explant

A general observation in leaf cultures during shoot formation was that margin of the expanded leaf curled towards the abaxial side of the explant lifting cut ends of the base away from the medium. If the petiole cut end of the leaflet lost contact with the medium, it did not form shoots. Thus the explant with the abaxial surface in contact with the medium and base embedded on the medium is essential for shoot bud differentiation.

4.1.3 Age of leaflet donor seedlings

In order to study how the age of the leaflet explant effects regeneration potential, leaflets were excised from 0 to 6 d-old seedlings and cultured on MS medium supplemented with 13.3 µM BA and 5.3 µM NAA (SIM). The percentage of cultures with shoot bud differentiation decreased with the age of the seedling explant where 1 d-old leaflets showed the maximum response (Fig. 4A) while those from 7 d-old seedling showed negligible regeneration. The older leaflets expanded, appeared green, produced callus which on subsequent sub-culturing failed to regenerate shoots. Therefore, leaflet explants from 1 d-old seedlings were used in further experiments (Table 7).

Table 7

Effect of age of explant donor seedlings on shoot bud differentiation from leaflet explants of *Arachis hypogaea* genotype ICGS-44 cultured on SIM containing MS + 13.3 µM BA and 5.3 µM NAA.

Age of donor seedlings (days)	Regeneration (percentage)
0	79.1
1	92.2
2	83.5
3	79.1
4	70.1
5	70.1
6	65.0

4.1.4 Efficacy of various cytokinins and auxins for shoot regeneration

To study the role of cytokinins and auxins on the regeneration of shoot buds, the immature leaflet explants from mature imbibed seeds of groundnut variety ICGS-44 were cultured on MS medium supplemented with 13.3 μM BA along with different concentrations of NAA (1.0, 5.3, 6.3, 7.9 μM) or MS containing 13.3 μM BA and IAA (1.14, 2.28, 5.7 μM). The effect of BA on shoot bud differentiation was compared with two other cytokinins, viz., KN and TDZ. All the cytokinins induced shoot bud differentiation but with different frequencies (Fig. 7). BA was the most effective cytokinin in terms of number of cultures forming shoots and number of shoots per explant. Leaflets cultured on MS with BA and IAA turned pale yellow with in 8 d; very few explants showed shoot bud differentiation. Instead, white fluffy callus was observed which on further subculture did not show any sign of morphogenesis. Leaflets cultured on MS with varying concentrations of BA (5.5, 13.3, 18.3 μM) showed more number of shoot buds with higher BA concentration along with NAA but the shoots were of inferior quality. After two to three weeks, shoots arising from the petiolar cut end of the leaflets were counted, and percentage regeneration was averaged for each treatment (number of explant forming shoots / total explants cultured x 100). Amongst the different treatments tested MS containing 13.3 μM BA in combination with 5.3 μM NAA produced the highest frequency (92.3 %) of multiple adventitious shoot buds that later developed into healthy shoots (Fig. 4B and 4D).

4.1.5 Elongation of shoots

For elongation of adventitious shoots, the whole leaflet explant with organogenic shoots were transferred to test tubes containing MS with 2.3 μM KN and 2.2 μM BA (SEM) for

week followed by 3 passages of 4 weeks each on MS medium for the development and elongation of adventitious shoot buds (Fig. 4E). The elongated shoots were rescued at the end of each passage. The shoots were micropropagated on SEM through nodal explants for clonal multiplication.

4.1.6 Rooting and transplantation to glasshouse

Regenerated shoots after elongation were transferred from SEM to hormone-free MS medium. Adventitious roots appeared on the elongated shoots within 2 weeks (Fig. 4 F) and developed further in 4 weeks. 80 % of the rooted shoots were transferred to autoclaved sand : soil (1:10) mixture in pots and maintained in growth chamber under continuous light at 26 ± 2 °C with 80 % humidity for 2 weeks prior to transfer to glasshouse. Upon transfer of plants to the glasshouse the plants showed normal growth, matured and produced 35 to 40 pods per plant within 4 months (Fig. 4G)

4.1.7 Genotype effect

To determine whether the in vitro shoot regeneration from leaflet explants of groundnut is genotype specific, groundnut cultivars ICGS-44, ICGS-11, JL-24 which belong to Virginia and Spanish types were tested for their organogenic response. The SIM was originally optimized for variety ICGS-44. On SIM 3 groundnut genotypes produced shoot buds with high frequencies (80 % to 92 %) and followed a similar pattern of growth and development. The regeneration system described is widely applicable to a range of groundnut genotypes

4.2 Ontogeny of shoot bud differentiation from leaflet explants

At the time of excision the leaflets measured 2 to 3 mm. Those placed on the shoot induction medium began to swell by 2 d and increased in length gradually up to 10 d. After 10 d culture, the petiolar cut end of the leaflets in contact with the medium acquired pronounced nodular appearance, after 14 d culture shoots appeared at the base of the explant.

Section of 0 d explants shows the epidermis, cortex and vascular elements. The cortical cells were isodiametric with vacuolated parenchyma containing one or two starch grains (Fig. 5A). Section of 1 d cultured explants on SIM for 24 h showed the petiolar cut end epidemal cells entering the dividing phase. A few starch grains in the cortical cells were observed (Fig. 5B). After 2 d culture on SIM the meristematic activity in the proximity of the vascular supply had considerably increased. In the transverse section periclinal and anticlinal divisions in the vascular parenchyma as well as in sub-epidermal regions were observed. Mitotic cell divisions with dividing nucleus could be seen (Fig 5C). Mitotic activity was localised mainly in the part of the leaflet that was near to or in direct contact with the culture medium. After 5 d culture on SIM, the explants showed the formation of lateral outgrowth and maturation of vascular elements (Fig. 5D). Mitotic activity in the vascular supply and parenchyma cells at the proximal end resulted in the formation of cytoplasmic nodular structures, which later become vascularised. One notable observation was the presence of starch grains in the sub-epidermal layers (Fig. 5B). By 7 d culture final differentiation followed by the development of the nodular mass occurred within the cells of the meristematic zone. This mass gave rise to definite meristematic zones (Fig. 6A). Meristematic zones further differentiated and developed

into shoot bud initials with well organized vasculature. The meristematic cells underwent more organised development towards the periphery by 10 d culture (Fig. 6B). After 14 d culture on SIM the broad meristematic zone gave rise to multiple growth centers producing shoot prinordia with organized apical meristem resulting in small shoots with organised vasculature (Fig. 6C). Each explant was capable of developing several meristematic nodules that ultimately resulted in the proliferation of multiple shoot buds from a single explant (Fig. 6D).

4.3 Genetic transformation of groundnut

Effects of various variables on transformation efficiency from the leaf explants were studied. The tissue culture and regeneration system optimized from the leaf explants was further used to develop a genetic transformation protocol for groundnut.

4.3.1 Effect of transformation on regeneration efficiency

Regeneration of shoots from the petiole cut end of immature leaflets following excision might increase the efficiency of transformation. The regeneration observed during the first 2 weeks of culture on shoot induction medium prior to applying any selection provides a good measure of explant vigour that is independent of amount of transformation taking place (Fig. 4C). Among the leaflet explants of ICGS-44, an average of 70 % of explants regenerated shoots following transformation (Table 8).

Table 8

Effect of *Agrobacterium* treatment on regeneration from leaflet explants of groundnut genotype ICGS-44 cultured on SIM containing 13.3 µM BA + 5.3 µM NAA + 500 mg/l cefotaxime.

Experiment No.	No. of explants inoculated	No. of explants producing shoot	% explants with shoots
Control (-*Agrobacterium*)	36	32	92.0
1	48	32	66.6
2	64	46	71.9
3	64	48	75.0
4	48	26	54.2
5	48	34	70.8
6	32	23	71.8
7	24	17	70.8
8	84	62	73.9
9	48	32	66.6
Average of all experiments	460	320	70.0

4 3 2 Effect of different selection agents on shoot regeneration

After 2 weeks of culture on SIM the shoots were subjected to selection on media containing the desired antibiotics to study their effect on shoot regeneration potential Based on visual observations the non-transformed control shoots were cultured on SIM containing media kanamycin and hygromycin Non-transformed control shoots originating from leaflet explant after 20 d culture on SIM were used for testing the selection agents Different concentrations of the antibiotics were tested These included 50 75 and 100 mg/l of kanamycin and 2, 4 and 6 mg/l of hygromycin After 3 weeks of culture the efficiency of selection was scored The non-transformed groundnut shoot cultures tested showed appreciable growth on selection medium upto 50 mg/l kanamycin The extent of bleaching was directly proportional to the inhibition of shoot growth Bleaching of shoots was evident at 75 mg/l and with further increase in kanamycin levels there was proportional increase in the explants exhibiting greater degree of bleaching Maximum inhibition of shoot growth with complete bleaching of the explant was obtained on 100 mg/l kanamycin In contrast, even low concentrations of hygromycin were deleterious for shoot growth Slight browning and necrosis was observed at the base of the shoot clusters in surviving explants at 6 mg/l When cultured on 10 mg/l hygromycin the shoots became necrotic For all experiments kanamycin at 100 mg/l and hygromycin at 8 mg/l was selected as stringent selection pressure represented in (Table 9)

Table 9

Effect of antibiotics kanamycin and hygromycin on the regeneration of shoots from leaflet explants of *Arachis hypogaea* genotype ICGS-44 was cultured on SIM containing 13.3 µM BA + 5.3 µM NAA.

Conc. of antibiotics (mg/l)	No. of explants cultured	Explants with shoots	Response				
			Green	+	Chlorotic ++	+	Necrotic ++
Control (-antibiotic)	36	32	32	-	-	-	-
Kanamycin							
50	64	48	10	30	8	-	-
75	48	40	-	22	18	-	-
100	48	40	-	8	22	-	10
Hygromycin							
4	48	32	20	-	-	12	-
6	48	32	34	-	-	18	10
8	48	32	-	-	-	22	10

4 3 3 Selection of transgenic groundnut plants by using the selectable marker

Only a small proportion of the target cells receive the DNA when treated with *Agrobacterium* It is therefore essential to select transformed cells among a large excess of non-transformed cells and to establish regeneration condition, allowing the recovery of intact plant derived from single or group of transformed cells Leaflet explants inoculated *Agrobacterium* strain were co-cultivated on shoot induction medium for 3 d in dark and then transferred to SIM containing cefotaxime 500 mg/l for 2 weeks Buds that developed at the petiolar cut end were transferred to stringent selection medium For selecting the transformed shoots, the explants with proliferating shoots on SIM containing cefotaxime 500 mg/l medium were moved to SIM medium with kanamycin concentration of 100 mg/l (Fig 4D) Subsequently, the small shoots were cultivated on the same medium after 2 weeks for their further development during the next two passages Occasionally callusing or browning of tissue at the base of the shoot clusters was removed before placing them on to fresh medium

Sub-cultures were carried out every 2 weeks depending on the cultured tissue At the end of each passage, the developing shoots were cultured on SIM with low selection pressure This was followed by elongation on MS basal medium with 75 mg/l kanamycin for 2 weeks At each stage before transferring to the selection medium the shoots were sub-divided and exposed to selection (Fig 4E) The base of the stem was excised and the shoots were inserted in to the medium to ensure good contact with the medium

Similar selection scheme was adopted for recovering putative transformants regenerated from leaflet explant co cultivated with *A tumefaciens* carrying the plasmid pCAMBIA1301 GRAVcp The shoots were placed on selection medium containing SIM

containing cefotaxime and 6 mg/l hygromycin for 2 weeks. The surviving shoots were subjected to 2 rounds of selection on SIM containing cefotaxime and 8 mg/l hygromycin for 3 weeks, followed by elongation on MS medium containing 4 mg/l hygromycin for 2 weeks (Table 10).

Table 10

Effect of selection for recovering putative transgenic groundnut shoots cultured on SIM containing 13.3 µM BA + 5.3 µM NAA along with the selected antibiotics kanamycin and hygromycin.

No. of explants initially cultured	No. of explants forming shoots	Shoots on stringent selection	Rooting	No. of Surviving shoots (%)
kanamycin	**90 mg/l**	**100 mg/l**	**50 mg/l**	-
64	48	28	24	19 (50 %)
48	32	18	14	13 (40 %)
22	16	14	14	8 (60 %)
64	41	22	20	18 (40 %)
48	30	22	10	6 (40 %)
Hygromycin	**6 mg/l**	**8 mg/l**	**4 mg/l**	-
24	21	16	7	6 (15 %)
32	24	18	10	10 (20 %)
56	48	32	23	19 (35 %)
54	40	22	20	16 (30 %)
48	32	20	14	11 (15 %)

4.3.4 Rooting of the transformants and transplantation

The putative transformants were rooted in hormone-free MS medium for 2 weeks, followed by sub-culture on MS medium for 3 weeks. Adventitious roots appeared on the elongated shoots within 2 weeks and developed further in 4 weeks (Fig. 4F). All the putative groundnut transgenic plants were established in the glasshouse with over 90 % success. The rooted shoots were carefully removed from the agar medium and rinsed in de-ionised distilled water to remove agar adhering to the roots. The plants were transferred to 10 cm (dia) pots containing sand : soil (1:1) mixture. The pots were covered with polythene bags to maintain high humidity to prevent desication of the plant. Hardening the plant was facilitated by gradual exposure to atmospheric conditions. After 10 d over end of the bag were cut open to facilitate aeration and to avoid excessive condensation on the walls of the bag while retaining sufficient level of humidity. The bags were removed once the shoots were actively growing. Plants of 10 to 15 cm length were transferred to 18 cm round pots containing autoclaved sand : soil : farm manure (2:2:1) mixture. Following this method, over 80 % of transplanted plants survived in the glasshouse and reached maturity. Over 60 independently transformed putative transgenic plants carrying *GRAVcp* gene were maintained in the glass house for further analysis. The transgenic plants appeared phenotypically normal and yield of the mature plants was 40 to 50 pods per plant (Fig. 4G). No morphological abnormality was observed in any of the in vitro produced plants.

4.4.1 Characterization and molecular analysis

Each putative independent transformant arising from a treated explant was numbered at the time of isolation and separately maintained for subsequent DNA analysis and progression of generations. Putative groundnut transformants obtained by using plasmid pCAMBIA1301 GRAVcp were analysed for histochemical *uid*A gene expression in the leaflets and petiole. β-Glucuronidase enzyme activity was detected histochemically in leaf sections and petiole regions using X gluc (5 bromo-4-chloro-3-indolyl-β-glucuronide) as the substrate (Fig. 8). The independent T_0 putative transformants grown in the glasshouse were tested for the presence of reporter gene *uid*A, amplification of 1.2 kb *uid*A gene fragment confirmed the presence of marker gene (Fig. 9A).

Over 60 independently putative transformed plants transformed with the plasmid pROKII GRAVcp were successfully transplanted to the glasshouse and then T_1 generation seeds (50) from 6 independent lines were collected and sown in glasshouse for molecular analysis. Over 24 of these T_1 plants from 4 independent lines have been advanced to T_2 generation. From the T_0 and T_1 generation plants growing in glasshouse the putative transformants were randomly selected for PCR amplification of *npt*II and *GRAVcp* gene fragments from the genomic DNA. Oligonucleotide primers specific to the coding regions of *npt*II and *GRAVcp* genes amplified the expected size of the respective gene fragment from at least 60 % of the analysed putative transformants (Fig. 9B and Fig. 10A, B). To ascertain the fidelity of amplification in PCR reaction the PCR products were transferred to nylon membrane for Southern hybridization and probed with non-radio labeled fragments of the respective genes from the plasmids shown in Fig. 3.

Transgenic nature of all the selected plants was confirmed by the expected hybridization patterns of the respective gene amplicons (Fig. 10A and 10B).

To ascertain the integration and copy number of the *npt*II and *GRAVcp* genes, genomic DNAs of 8 putative transformants obtained after transformation with the plasmid pROKII:GRAVcp were restricted with *Eco* RI that restricts the T-DNA only once. The blots were hybridized with *npt*II and *GRAVcp* gene probes separately. From the 8 selected plants four plants (lanes 1, 2, 3,5) showed positive hybridization for *npt*II (Fig. 11A). Two plants (lane 2, 3) showed integration of two copies. From the 8 plants tested for integration of *GRAVcp* only two (lane 1, 2) showed positive hybridization for *GRAVcp* with single insert (Fig. 11B). To ascertain the inheritance pattern of the introduced genes PCR analysis of *GRAVcp* gene in the genomic DNA from randomly selected 40 plants of T_1 progeny and 42 plants of T_2 progeny suggested the segregation in 3:1 Mendelian ratio that is characteristic of a single locus trait (Fig. 12 and Table 11).

Table 11

Status of the putative transgenics of groundnut containing *GR 11 cp* during T_1 and T_2 generations.

Plant No.	No. of progeny tested	PCR +ve	PCR -ve	χ^2 3:1 at $p=.05$ cal 3.26
T_0 generation				
Str 7-3	5 (a,b,c,d,e)	2	3	3.0
Str 7-8	5 (a,b,c,d,e)	4	1	0.066
Str 7-17	5 (a,b,c,d,e)	3	1	0.3
Str 7-19	4 (a,b,c,d)	2	2	1.2
Str 7-20	4 (a,b,c,d)	1	3	1.0
Str 7-25	5 (a,b,c,d,e)	0	4	8.05
T_1 generation				
Str 7-1-e	4 (1,2,3,4)	4	0	0.2
Str 7-8-a	4 (1,2,3,4)	3	1	0.5
Str 7-19-a	4 (1,2,3,4)	1	3	1.5
Str 7-20-a	4 (1,2,3,4)	1	3	4.5

DISCUSSION

5 1 Shoot morphogenesis from immature leaflet explant of genotype IC GS-44

The classical findings of Skoog and Miller (1957) that organogenesis in tissue cultures is governed by the balance of auxin and cytokinin in the medium is demonstrated in the present study in groundnut

The hormone content of culture media is a critical factor for induction of shoots from leaflets The influence of cytokinin alone or in combination with low levels of auxin has been demonstrated during de novo meristem formation in other species (Franklin et al 1991) Similar observation was made in the present study with leaflet explants where appropriate combination of BA and NAA was important for obtaining successful shoot bud differentiation with high frequency (92 %) The regeneration protocol described in this report shows 3 to 4 fold increase in shoot induction from leaflet explants over that reported by Cheng et al (1991) when they used BA (25 mg/l) and NAA (3 mg/l) Kanyand et al (1994) with 30 mg/l thidiazuron, McKently et al (1991) with BA (40 mg/l), and Ponsamuel et al (1998) with brassin Mroginski et al (1981) found that developmental age of source explant had profound effect on leaflet explants derived from young seedlings Leaf developmental stage had been shown to affect the shoot regenerability in other species (Baker and Wetzstein, 1998)

In the present study morphogenic potential was maximum at the petiole cut end of 1 d-old leaflet explants there by showing that the stage of explant development is a critical factor for induction of adventitious shoot buds However, these results are not in agreement with those obtained in other plant species such as tomato (Kartha et al , 1976) and *Solanum laciniatum* (Davis and Dale 1979) where mature and fully developed leaves were used to regenerate plants The reason would be that the mature leaf from groundnut

have a different physiological status of development or have lost some promoting factors that were present during the early stage of development

Although shoot organogenesis has been reported from leaflet systems and leaf-derived callus tissue in groundnut (Mroginski et al , 1981, Pittmann et al , 1983, Seitz et al , 1987, Mckently et al , 1991, Lapen and George, 1993, 1994, Cheng et al , 1992, 1994, Kanyand et al , 1994, Chengalrayen et al , 1995, Livingstone and Birch, 1995, Baker and Wetzstein, 1998 the frequency of plant recovery has been low Chengalrayan et al (1995) reported callusing caulogenesis and flowering from the base of embryo leaflets depending on hormonal composition In most of the earlier reports the shoots were transferred to media containing an auxin for rooting In the present regeneration system concurrent occurrence of adventitious roots at the base of shoots on hormone-free medium was achieved, which was similar to *Brassica juncea* (Sharma and Bhojwani 1990)

Several alternative regeneration systems have been tested in groundnut Cotyledon explants have been shown to be excellent explants for transformation and regeneration of fertile plants in groundnut (Sharma and Anjaiah, 2000) and from several other crop species such as *Brassica juncea* (Sharma et al 1990) *Brassica napus* (Moloney et al , 1989) However, the leaflet explants showed higher frequency of regeneration in the present study The regeneration system reported is preferred in transformation studies because of relative ease of obtaining explant from a ready source of axenically germinated seeds and high frequency of adventitious shoots under simple culture conditions The manipulations are simple The system is highly reproducible and requires only 12 weeks to obtain shoot regeneration and rooting It is advantageous to

both regeneration and transformation due to the following reasons i) readily available initial tissue, ii) a short regeneration period (15-20 days), iii) consistent regeneration, and iv) applicable to different genotypes

5.2 Ontogeny of shoot bud differentiation from leaflet explant

From the histological study, it is clear that shoot buds originated from parenchyma cells associated with the phloem and the inner epidermal cells in the young leaflet. The process leading to shoot formation involved mitotic cell division at every early stage (before 2 d) that produced a meristematic tissue. Within this meristematic zone, shoot primordia, and multiple shoots were formed sequentially. Such a developmental sequence has been observed in all systems examined histologically (Thorpe 1980, Sharma and Bhojwani 1990, Moloney et al., 1993).

On the basis of histological examination, it appears that the key feature is the time at which selection of leaflets took place i.e. when leaflets were still in a meristematic state of growth. In such a developmental state, the epidermis and the mesophyll tissue had not yet began to differentiate and, under the influence of exogenous BA and NAA the tissue was channeled into meristematic tissue at the base of the leaflet in contact with the medium. In vitro conditions and exogenous supply of hormones induced the cells at the cut end to under go a different developmental pathway leading to shoot bud differentiation upon culture on shoot induction medium. Similar observation was made in *Pinus radiata* by Cameron and Thomson (1969) and in *Brassica juncea* by Sharma et al. (1990). The immediate channeling of the physiologically competitive explant into meristematic tissue formation and altered morphogenetic pathway is a key to the high

shoot forming capacity of the present system Mitotic cell division in the leaflets during the first two days of the culture may render the cells susceptible to the applied growth regulators, and thus altering the developmental pathway One interesting feature of the shoot forming process is the accumulation of the starch in the cells directly involved in shoot bud formation as well as those in the surrounding regions The accumulation of the starch in the shoot-forming tissue and its utilization during shoot bud induction has been observed in many in vitro systems, since the initial observations of Thorpe and Murashige (1968) in tobacco callus (Thorpe 1981 Brown and Thorpe 1986 Sharma et al 1990) Mitotic divisions observed in the first 2 d of culture may render the cells susceptible to applied phytohormones thus altering their developmental pathway One noticeable cytological event was the pattern of cell division Initial cell divisions in the shoot-forming tissue were random followed by periclinal and anticlinal divisions Later stages of shoot primordial formation in groundnut leaflet explants are similar to those reported (Bonnett and Torrey, 1966, Sharma et al , 1990)

From the point of view of studying development there are advantages in using direct regeneration system, due to the rapidity of morphogenesis and no requirement of subculture onto regeneration medium, *de novo* production of shoot primordia is extremely rapid and initially synchronous with no prolonged period of cellular differentiation Such a regeneration system would favour easy accessibility of *Agrobacterium* to the meristematic cells, which are mainly surface cells during the initial co-cultivation stage of a transformation protocol (Sharma and Anjaiah, 2000)

5.3 Genetic transformation of groundnut

Transformation protocol for groundnut based on the *Agrobacterium*-mediated gene transfer into leaflet explants was developed during this work As in previous reports the choice of selectable marker was found to be important for the recovery of transformed plants (Lichtenstein and Fuller, 1987) Selectable marker confers dominant phenotype on transformed cells because they invariably result in the addition of a new trait not normally associated with untransformed cells Ideally, the LD_{50} (i e , the level at which 50 % growth inhibition is observed) for untransformed cells should be at least an order of magnitude lower than that of transformed cells (Desgagnes et al , 1995) The transformation system developed for groundnut leaflet explants makes use of the *nptII* selectable marker gene, conferring resistance to antibiotic kanamycin Similar strategy was used for selecting putative transformants that were transformed with pCAMBIA1301 GRAVcp, conferring resistance to hygromycin Progressive step-wise increase in the selection pressure combined with rapid induction of organogenesis during initial stages of selection was found to be vital for the successful recovery of transgenic shoots

There are cellular differences within an explant in competence for *Agrobacterium* transformation (DeBlock, 1993) Several authors have reported that one of the major limitations in developing *Agrobacterium* based protocols for recalcitrant species is the fact that usually the transformation-competence cells have poor regeneration capabilities (deKathen and Jacobsen, 1995) However, in the groundnut leaflet explants used in the present work, the regions which are competent for transformation have high regeneration potential The susceptibility of the leaf petiole cut end could be explained by actively

dividing status of the meristematic cells that was enhanced under the influence of the cytokinin activity

The inclusion of filter paper on the SIM during the co-cultivation has been found to improve the transformation efficiency in plant species Zhang et al (1997) reported a marked increase in the transformation frequencies in *V faba* and *V vulgaris* seedling explant when co-cultivated with a filter paper barrier The reason for enhanced transformation by the introduction of a filter paper during co-cultivation is not clear One possibility is that the phosphate starvation of *Agrobacterium* cells due to limitation of nutrient supply might enhance gene transfer (Li et al , 1994) The filter paper may also form a barrier to the supply of other nutrients for the vegetative growth of the *Agrobacterium* It was shown that the conditions which promote vegetative growth of *Agrobacterium* are not suitable for induction of virulence in *Agrobacterium* (Sheng and Citosky, 1996)

Previous studies with *Arachis hypogaea* L on genetic transformation by using *Agrobacterium tumafaciens*-based gene delivery system have resulted in a limited success due to inefficient in vitro regeneration protocols The biolistic-based systems for gene delivery into embryogenic calluses and embryo axes is labor intensive and requires the bombardment of a large number of explants to obtain a few transformed cell lines which are often chimeric (Ozias-Akins et al , 1993) The value of *Agrobacterium*-mediated plant transformation is measured by the number of independent transformed plants per explant use, which can be a function of genotype of the species, *Agrobacterium* strain virulence the selection pressure regeneration capacity of explant and accessibility of the bacterium to the regenerable cells (Moloney et al , 1989 Sharma

and Anjaiah, 2000) A key feature of this transformation procedure is spontaneous regeneration system in groundnut leaflet explants and regenerability of the cells wounded during excision of leaflet explants are mainly surface cells readily accessible to the bacteria This is also supported by the studies on shoot histogenesis as discussed in 5 2 Several authors have reported that one of the major limitations in developing *Agrobacterium* based gene transfer protocol is the poor regeneration capability of transformation competence cells The susceptibility of excised leaflet explants could be explained by actively dividing status of the meristematic cells enhanced under the influence of cytokinin activity Progressive step-wise increase in selection pressure combined with rapid induction of organogenesis during initial stage of selection was found to be vital for successful recovery of the transgenic shoots The manipulations used were simple compared with previous reports (McKently et al 1993 Biar et al 1994 Cheng et al 1996 Livingstone and Birch 1999) using leaflet explant where the reported transformation frequencies in the range of 0 1 to 0 9 % The method reported here has been used to optimize transformation in leaflet system that produces large number of shoots under short period with the transformation frequency of over 20 % More recently, Sharma and Anjaiah (2000) reported an efficient method for production of transgenic groundnut with 55 % frequency which is very much preferred The present study uses an alternative explant (immature leaflet) for the generation of transgenic plants The advantages of this system are as follows

i) Ready availability of the target tissue for transformation

ii) Highly reproducible and efficient regeneration system compatible with the transformation protocol

iii) Technically simple protocol without complicated manipulations

iv) Efficient selection system based on *nptII* and *hpt* marker gene

v) Relatively short time span to raise the transgenic plants (4 months) from treatment with *Agrobacterium* to acclimitization in the glasshouse

vi) Applicable to range of genotypes

Molecular Characterisation Each putative independent transformant arising from a treated explant was numbered and maintained separately for DNA analysis Transformation of leaflet explants by using *Agrobacterium tumefaciens* strain C58 harbouring pROKII GRAVcp plasmid led to the production of transgenic plants A large number of independently transformed plants (over 60) were successfully transplanted to the glasshouse Integration of the transgene and inheritance in the progeny was assessed by PCR amplification of the respective genes as described in section 4 4 1 The transgene integration copy number was assessed by Southern blot hybridization that suggested a transformation frequency of over 20 %, where two independent transformants showed two copies of *nptII* gene (Fig 11A) From the eight putative transformants tested for *GRAVcp* gene integration only two independent plants showed the positive hybridization with single insert (Fig 11B)

Similar integration pattern have also been reported recently in groundnut transformed with coat protein gene of Indian peanut clump virus (Sharma and Anjaiah, 2000) PCR amplification of respective transgenes in T_1 and T_2 progeny suggested the segregation in 3 1 Mendelian ratio that is characteristic of a single locus trait Similarly inheritance of introduced trait have been studied using antibiotic resistant markers in petunia (Deroles and Gardner, 1988 a, b), tobacco (Matzke and Matzke 1993) maize

(Walters et al , 1992) Most of the studies have shown that marker gene segregate as dominant loci in a Mendelan fashion (3 1)

Further glasshouse testing could not be done because GRAV is an African virus and cannot be imported into India Since the disease (GRD) is indigenous to Africa, further glasshouse studies and field testing can be done only in Africa Rosette disease is the most destructive viral disease of groundnut (*Arachis hypogaea* L) in sub-Saharan Africa Resistance to rosette was first discovered in groundnut land races originating from Burkina Faso (Nigam and Bock, 1990) Resistance to these lines was effective against both chlorotic and green rosette, and is governed by two independent recessive genes (de Berchoux, 1960) However, most of the resistance lines released to date are late maturing and they are not suitable for many production systems in Africa, due to short rainy seasons Since GRAV is the main component involved in Aphid transmission, identification of GRAV resistance sources could help to restrict the spread of the disease

Results of the recent study showed that several accessions in different wild species of the genus *Arachis* are free from all three components of rosette disease That indicates that it would be possible to breed groundnut cultivars with combined resistance to all three components of rosette disease (Subramanyam et al , 2001) Several routes have been investigated to introgress genes from wild diploid species in to polyploid but with low success rates Most plant resistance to the disease could be the most viable and substantial solution Genetic transformation offers opportunities for the utilization of wild *Arachis* germplasm irrespective of crosssability barriers Genetic transformation of groundnut crop with novel disease resistance genes would be an effective strategy in developing host plant resistance The prospects for being able to confer resistance on

agronomically valuable cultivars by transformation with viral genes are very promising. The method of transforming groundnut reported in this thesis will help in realising this promise.

CONCLUSIONS

I ollowing conclusions can be drawn from the present study

- Efficient regeneration of adventitious shoot buds from immature leaflet explants on simple medium containing MS + 13 3 µM BA + 5 3 µM NAA where a very high frequency (92 %) regeneration of shoots were obtained within 12 weeks
- Rapid and efficient regeneration of shoot buds developed without involving long-term culture and complex medium manipulation
- Efficient rooting was achieved on MS medium and the in vitro plants were transferred to glasshouse with high success rates (>80 %)
- The system provides phenotypically normal plants that produce normal viable seeds
- The regeneration system is highly reproducible and is applicable to a range of groundnut genotypes
- Histological events associated with the ontogeny of shoot bud differentiation from leaflet explants of groundnut showed direct regeneration system from surface cells that would favour easy accessibility to *Agrobacterium* infection
- An efficient and reproducible transformation protocol for groundnut has been developed from leaflet explants by using pROKII GRAVcp carrying *npt*II and the coat protein genes of groundnut rosette assistor virus (*GRAVcp)*
- Integration of transgene and stable genetic transformation in the progeny were accessed by PCR amplification of 700 bp fragment of *npt*II gene and 384 bp of *GRAVcp* gene and Southern blot hybridization in the I_0 generation of transgenic plants
- PCR analysis of genomic DNA of I_1 and I_2 progeny showed segregation of *GRAVcp* gene in 3 1 Mendelian ratio

- The transformation protocol developed during this study can provide transformants with frequency of tranformants over 20 %.
- The protocol reported here can be efficiently used to transfer different novel genes for developing resistance to both biotic and abiotic stresses as well as for the nutritional enhancement of groundnut crop.

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FIGURES

Figure 1. **Groundnut infected with groundnut rosette disease showing; (A) chlorotic lesions, (B) green rosette symptoms.**

Figure 1

Figure 2. **Seeds of groundnut genotypes used in the present work; (A) JL-24, (B) ICGS-11, (C) ICGS-44**

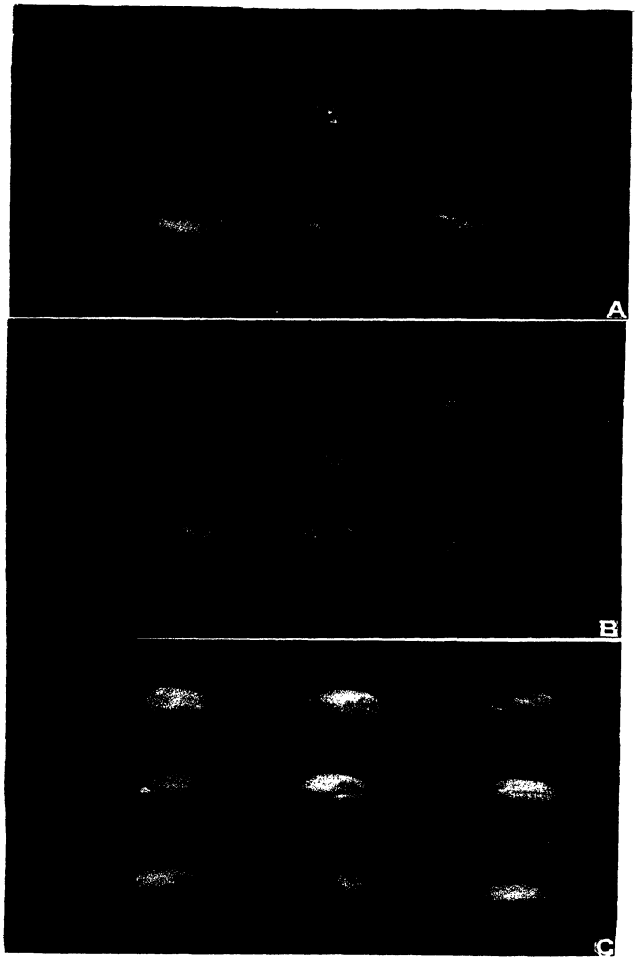

Figure 2

Figure 3. **Restriction map of T-DNA regions of binary vectors containing GRAV coat protein gene;**

(A) plasmid pROKII:GRAVcp, (B) plasmid pCAMBIA1301:GRAVcp.

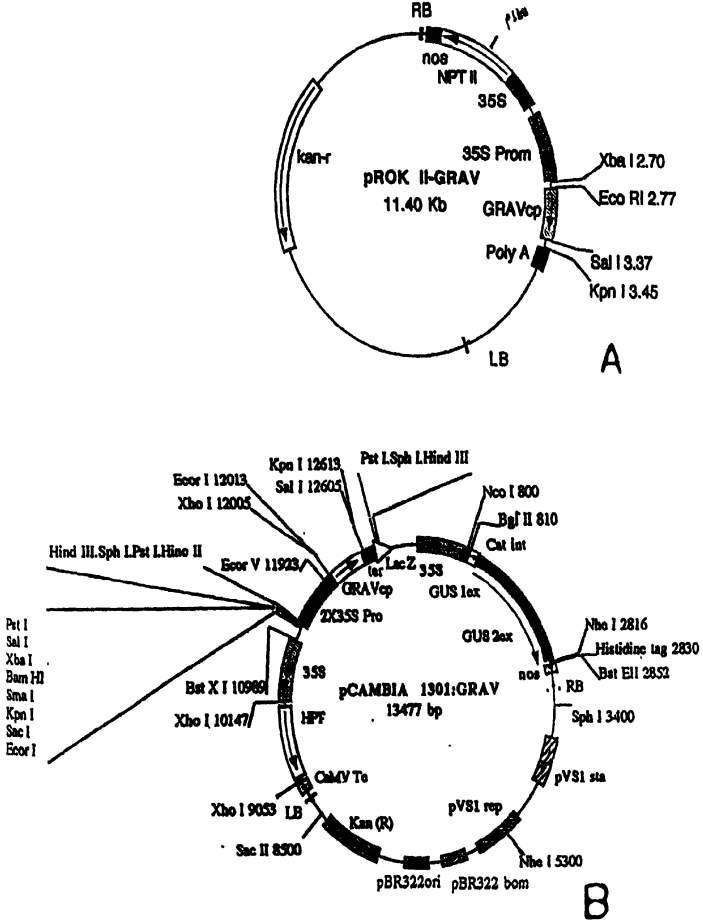
RB
nos
NPT II
35S
35S Prom
kan-r
pROK II-GRAV
11.40 Kb
GRAVcp
Xba I 2.70
Eco RI 2.77
Poly A
Sal I 3.37
Kpn I 3.45
LB
A
Kpn I 12613
Pst I.Sph I.Hind III
Ecor I 12013
Sal I 12605
Xho I 12005
Nco I 800
Bgl II 810
Cat Int
Hind III.Sph I.Pst I.Hind II
Ecor V 11923
ter
LacZ
35S
GRAVcp
GUS 1ex
2X35S Pro
GUS 2ex
Nhe I 2816
Histidine tag 2830
Bst EII 2852
Pst I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Ecor I
Bst X I 10989
35S
pCAMBIA 1301:GRAV
13477 bp
nos
RB
Xho I 10147
HPT
Sph I 3400
CaMV Te
pVS1 sta
Xho I 9053
LB
pVS1 rep
Kan (R)
Sac II 8500
Nhe I 5300
pBR322ori
pBR322 bom
B

Figure 3

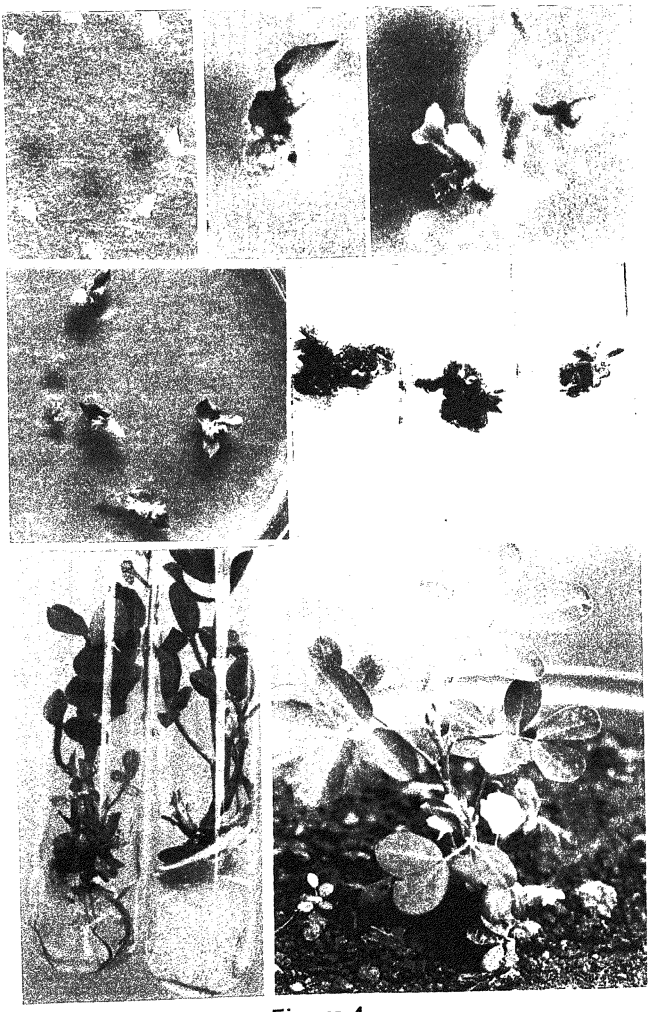

Figure 4

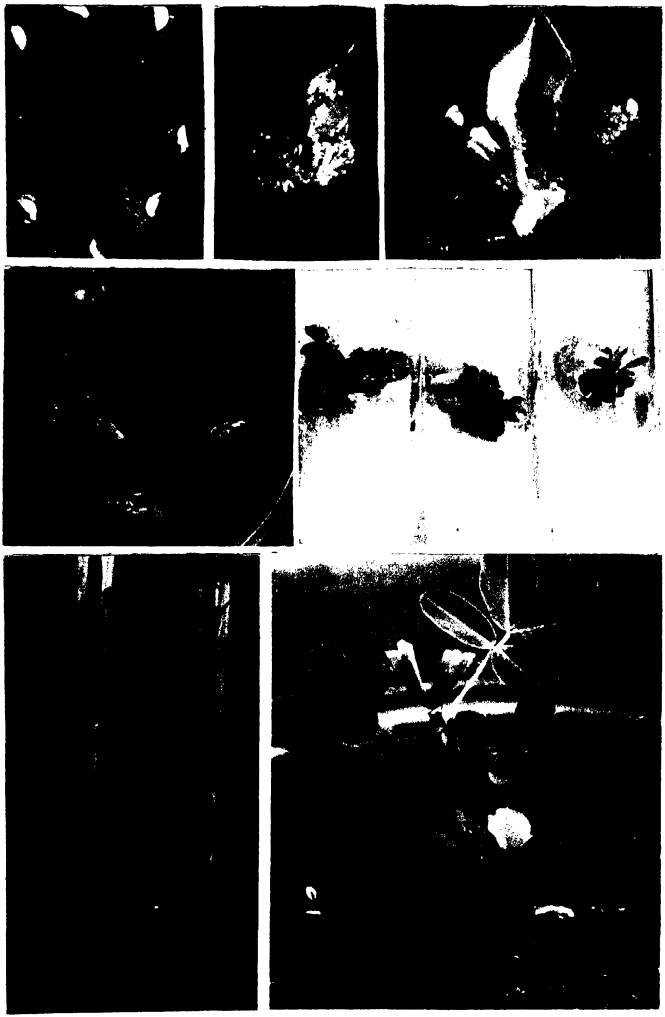

Figure 4

Figure 4. Regeneration of adventitious shoots from leaflet explants of A. hypogaea genotype ICGS-44;

(A) Leaflet explants at the time of culture initiation on shoot induction medium containing 13.3 µM BA and 5.3 µM NAA. Arrow indicates petiolar cut end.

(B) Induction of adventitious shoot buds from leaflet explants after 15 d of culture on SIM showing the differentiation of multiple shoot buds at the petiolar cut end (arrow).

(C) Induction of adventitious shoot buds from leaflet explants after co-cultivation with *Agrobacterium* on SIM and cefotaxime 500 mg/l.

(D) Selection of putative transgenic groundnut shoots after 20 d on MS + 13.3 µM BA and 5.3 µM NAA + 90 mg/l kanamycin.

(E) Elongation of groundnut shoots on selection medium after 4 weeks on MS + 2.3 µM BA + 2.2 µM kinetin (SEM).

(F) Rooting of putative transformants on selection medium containing MS + 50 mg/l kanamycin.

(G) Transformed plant regenerated from leaflet explant after transplantation to the glasshouse.

Figure 5 Ontogeny of shoot bud differentiation from leaflet explants of *A. hypogaea* genotype ICGS-44;

(A) Longitudinal section of day zero leaflet showing epidermis, cortex with nucleus and vascular bundles (arrow).

(B) Transverse section of explant cultured on SIM after 24 h showing dividing cells at the cut end and starch grains in the cortex (arrow).

(C) Section of leaflet explant after 2 d culture on SIM showing mitotic cell divisions, periclinal and anticlinal divisions at the cut end (arrow).

(D) Longitudinal section of 5 d culture explant showing maturation and rapid cell divisions in the proximity of vascular supply (arrow).

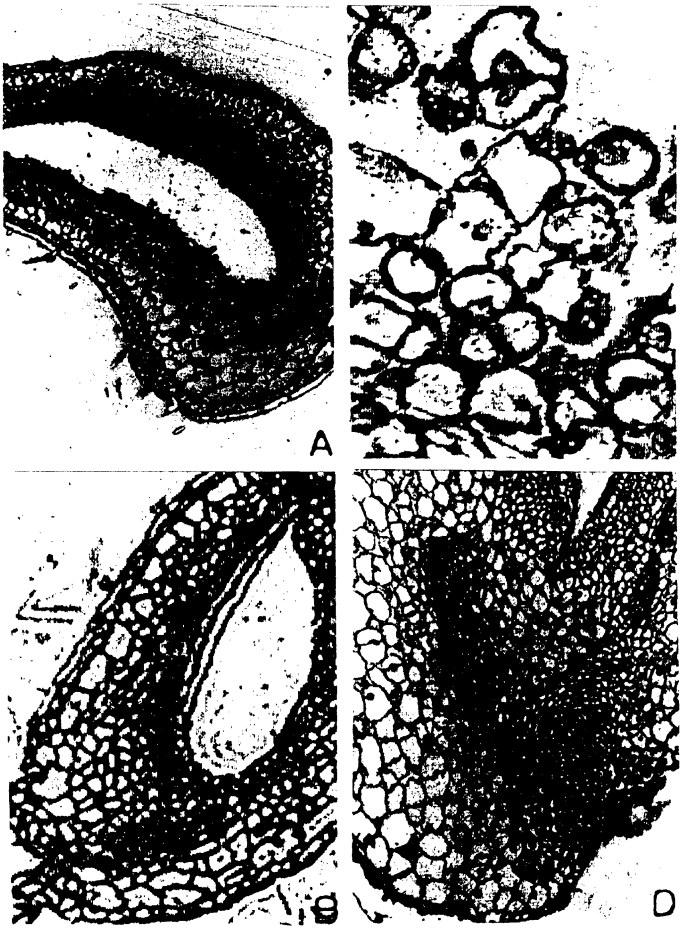

Figure 5

Figure 6 Ontogeny of shoot bud development from leaflet explants of *A. hypogaea* genotype ICGS-44;

(A) Longitudinal section of 7 d culture explant on SIM showing nodular cell masses with definite meristematic zones (arrow).

(B) Section of 10 d culture explant showing shoot bud initials with organized vasculature and vacuolated parenchymatous cells (arrow).

(C) Broad meristematic zones giving rise to multiple growth centers having shoot primordial and organized apical meristems (arrow).

(D) Transverse section showing multiple shoots with organized vasculature developed after 14 d culture on SIM.

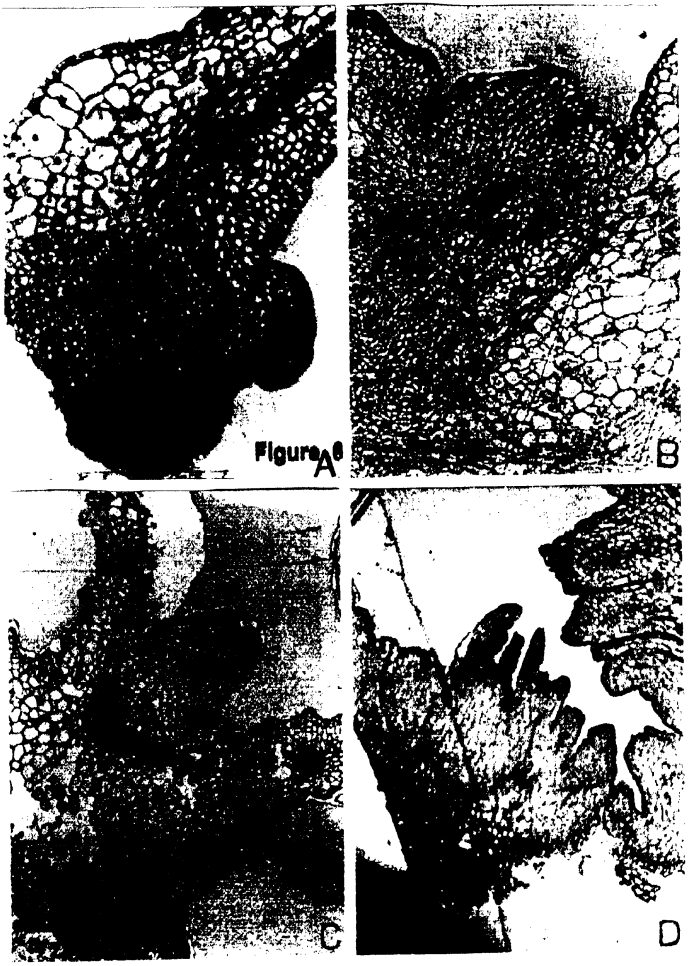

Figure 6

Figure 7 **Effect of NAA concentrations in combination with 13.3 μM BA on shoot regeneration from leaflet explants of genotypes ICGS-44, ICGS-11 and JL-24 derived from 1 d-old seedlings.**

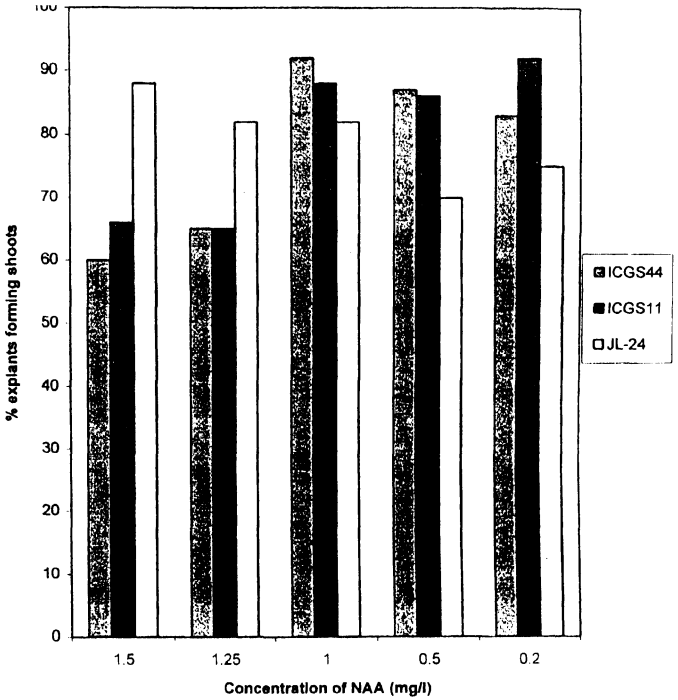
% explants forming shoots
100
90
80
70
60
50
40
30
20
10
0
1.5
1.25
1
0.5
0.2
Concentration of NAA (mg/l)
ICGS44
ICGS11
JL-24

Figure 7

Figure 8 Histochemical expression of *uid*A marker gene in leaves and petiole of independent putative T_0 transgenic groundnut plant transformed by using the binary plasmid pCAMBIA1301:GRAVcp.

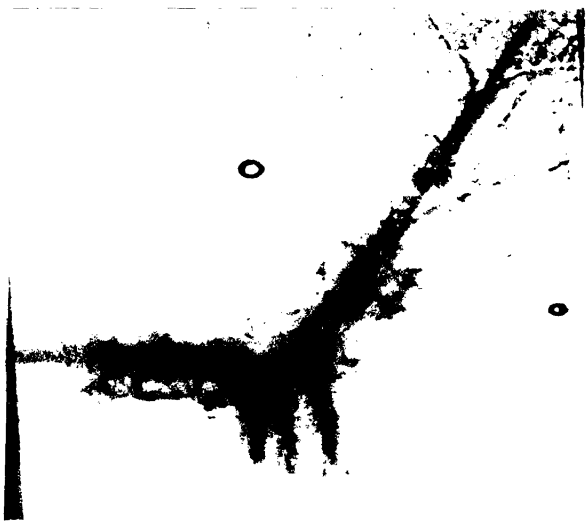

Figure 8

Figure 9 Molecular analysis of *npt*II and *uid*A genes in the genomic DNA of putative groundnut transformants, transformed by using plasmids pROKII:GRAVcp and pCAMBIA1301:GRAVcp in T_0 generation growing in glasshouse.

(A) PCR amplification of 1200 bp fragment of *uid*A gene in different transgenic lines.
1) 1301-1; 2) 1301-2; 3) 1301-3; 4) 1301-4; 5) 1301-5; 6) 1301-6; 7) 1301-7; 8) 1301-8; 9) 1301-15; 10) 1301-20; 11) 1301-22; 12) 1301-30; 13) 1301-38; 14) 1301-37; 15) 1301-39; 16) 1301-35; 17) negative control; 18) plasmid pCAMBIA1301:GRAVcp; 19) λ *Bst-I* marker.

(B) PCR amplification of 700 bp fragment of *npt*II coding region in different transgenic lines.
1) Str7-2; 2) Str7-5; 3) Str7-4; 4) Str7-26; 5) Str7-28; 6) Str7-10; 7) Str7-3; 8) Str7-7; 9) Str7-11; 10) Str7-1; 11) Str7-6; 12) Str7-8; 13) Str7-17; 14) Str7-19; 15) Str7-25; 16) Str7-20; 17) Str7-18; 18) plasmid pROKII:GRAV cp; 19) negative control; 20) λ *Bst-I* marker.

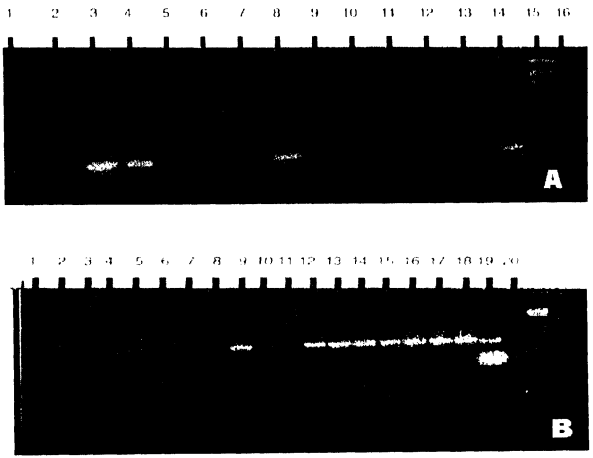
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B

Figure 9

Figure 10 Molecular analysis of GRAV coat protein gene in the genomic DNA of putative groundnut transformants transformed by using the plasmid pROKII:GRAV cp in T_1 generation plants

(A) PCR amplification of 384 bp fragment of GRAV coat protein gene in different transgenic lines.
1) Str7-1-a; 2) Str7-1-b; 3) Str7-1-c; 4) Str7-1-d; 5) Str7-1-e; 6) Str7-3-a; 7) Str7-3-b; 8) Str7-3-c; 9) Str7-3-d; 10) Str7-3-e; 11) Str7-8-a; 12) Str7-8-b; 13) Str7-8-c; 14) Str7-8-d; 15) Str7-8-e; 16) Str7-17-a; 17) Str7-17-b; 18) plasmid pROKII:GRAVcp; 19) negative control; 20) λ *Bst-I* marker; 21) Str7-17-c; 22) Str7-17-d; 23) Str7-19-a; 24) Str7-19-b; 25) Str7-19-c; 26) Str7-19-d; 27) Str7-20-a; 28) Str7-20-b; 29) Str7-20-c; 30) Str7-20-d; 31) Str7-25-a; 32) Str7-25-b; 33) Str7-25-c; 34) Str7-25-d; 35) Str7-25-e; 36) plasmid pROKII:GRAVcp; 37) negative control; 38) λ *Bst-I* marker.

(B) Southern blot hybridization of gel in figure 10A to verify the PCR product of *GRAVcp*.

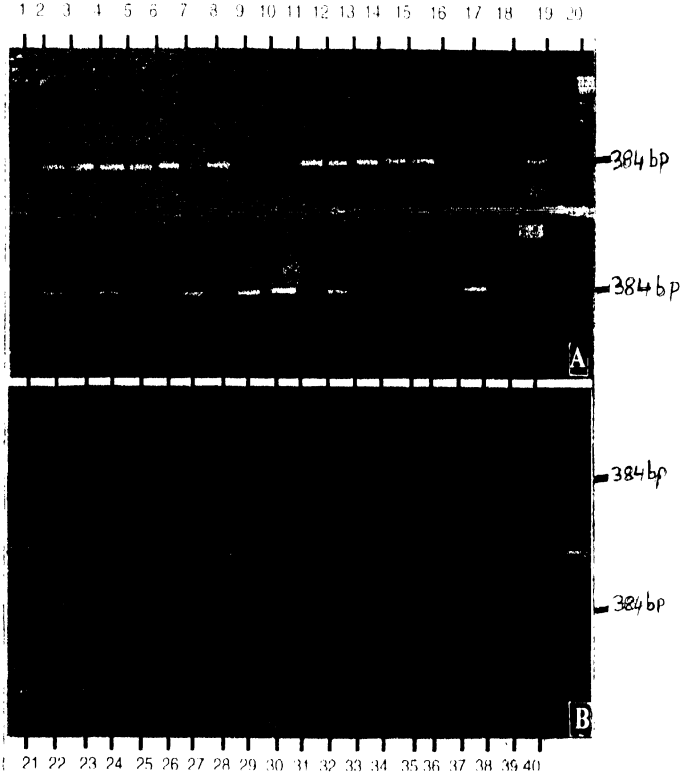
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
384 bp
384 bp
A
384 bp
384 bp
B
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

Figure 10

Figure 11 Southern blot analysis of *npt*II and *GRAVcp* gene in the genomic DNA of T_0 generation of groundnut transgenic produced by using the plasmid pROKII:GRAVcp.

(A) The DNA was digested with *Eco* R1 to provide single restriction within the T-DNA. The blot was probed with non-radio Alkphos-labled 700 bp PCR amplified *npt*II gene fragment.
1) Str7-1; 2) Str7-3; 3) Str7-8; 4) Str7-19; 5) Str7-18; 6) Str7-20; 7) Str7-25; 8) negative control; 9) plasmid pROKII:GRAVcp.

(B) The DNA was digested with *Eco* R1 to provide single restriction within the T-DNA. The blot was probed with non-radio Alkphos-labled amplified fragment of *GRAVcp* (384 bp).
1) Str7-1; 2) Str7-3; 3) Str7-17; 4) Str7-19; 5) Str7-18; 6) Str7-29; 7) negative control; 8) Str7-8; 9) plasmid pROKII:GRAVcp.

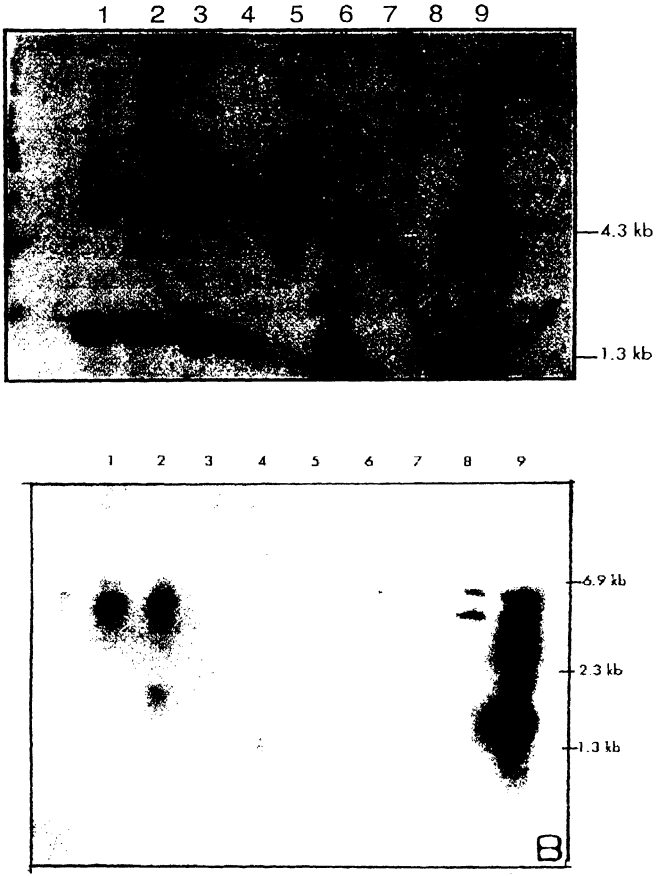

Figure 11