Agrobacterium-mediated transformation for the development of transgenic groundnut plants for resistance to fungal pathogens

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By

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CERTIFICATE

This is to certify that the project work entitled "***Agrobacterium*-mediated transformation for the development of transgenic groundnut plants for resistance to fungal pathogens"** done by Mr.***D.Vijaya Ramu*** for the award of degree of Master of Science in Biotechnology, submitted to the Bharathidasan University, Thiruchirapalli, is based on the results and studies carried out by him under our guidance and supervision.

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1. INTRODUCTION & OBJECTIVES

1. INTRODUCTION

Arachis hypogea an annual oil seed belonging to the family leguminosae and subfamily papillionacae is a legume native to South America but now grown in diverse environments in 6 continents between latitudes 40^0 N and 40^0 S. *Arachis hypogea* can grow in a wide range of climatic conditions (Gregory *et al.*, 1980).

Groundnut seed contains 50% high quality edible oil and 25% protein (Norden, 1980). The oil may be extracted and used for cooking and the residual cake is used most commonly in animal feeds and the shells may be ground and used as filler in animal feed (Nigam *et al.*, 1991). World groundnut production in 1980's averaged 19. 8 Mt from approximately 18.3 million hectares (Nigam *et al.*, 1991). Of about 100 countries involved in the production of groundnut India ranks first. About 80% of the worlds production of groundnut is from resource poor small farmers in developing countries who obtain low yields of 500-800 Kg / hectare. In many cases poor yields are because of diseases. Numerous diseases caused by fungi, bacteria and viruses drastically affect the yield of groundnut (Sharma and Mc Donald, 1990).

Of the various fungal pathogens of groundnut, *Cercospora arachidicola*, *Cercospora personata* and *Puccinia arachidis* are the most damaging which cause foliar diseases such as leaf spots and rust and it was reported to be a world wide problem (Subramanyam *et al.*, 1978). Other fungi causing serious disorders in groundnut include *Aspergillus niger* (Jackson and Bell, 1969), *Aspergillus Flavus* (Porter *et al.*, 1984), etc.

The fungal pathogens diminish plant growth and thereby reduce crop yield. There are several major strategies to eliminate the fungal pathogens. One major strategy is to develop plant resistance to fungal pathogens by the process of plant breeding. Breeding for pest resistance is an ongoing concern, because new strain of pests, always arise and attack the plants that were resistant to the old strains. Breeding multigene instead of single gene for resistance is a major objective of plant breeders.

The most direct strategy is to eliminate the pests and pathogens with chemicals. The chemical route as a mode of approach for pest control has been in use for a period of 50 years and is accompanied with several serious drawbacks. A more subtle approach is

biological control where by the population of pests can be brought back into the equilibrium with in the agricultural ecosystem. However, with the lack of genetic variability in the existing germplasm for some of the fungal pathogens, further break through in enhancing genetic resistance are remote (Sharma and Ortiz, 2000).

A novel approach is to make plants resistant to pests and pathogens, this approach necessitates gene transfer from other varieties *viz.* from more distant relatives or from unrelated organisms. Genetic engineering is greatly expanding the sources from which resistant genes can be obtained. Genetic engineering has paved the pathway for making plants resistant to fungal pathogens. Attempt to make plants resistant to fungus involves introducing the genes encoding fungus degrading enzymes into the plant which degrade mainly the fungal cell walls that consists of two polymers glucan and chitin that can be broken down by glucanases and chitinases (2 classes of enzymes) respectively. Chitinase catalyses the hydrolysis of chitin, a $\beta 1,4$ – linked polymer of N-acetyl glucosamine, that is a major component of cell walls of most filamentous fungi except the *Oomycetes*. Thus eliminating the fungal pathogens can be achieved by introducing such antifungal genes.

OBJECTIVES

Biotechnological approaches to evolve cell lines with special attributes and recovery of complete plants *invitro* have progressed to a stage where it has now become possible to obtain a number of disease resistant crops.

Attempts were made to develop transgenic groundnut plants conferring resistance to fungal pathogens with the following objectives

1. To transform cotyledon explants by *Agrobacterium tumefaciens* harboring the *chitinase* gene on a binary vector.
2. To select the putative transgenic groundnut plants in selection medium to obtain putative transformants.
3. To perform molecular analysis for the existing putative transgenic plants transformed with *nptII*.

2. REVIEW OF LITERATURE

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2.1.1 Plant biotechnology and its scope in crop improvement

Biotechnology offers a wide potential for the application of molecular biology techniques for human welfare. Plant biotechnology is an ever-emerging highly rewarding technology with large potential applications in crop improvement. Plant biotechnology has made rapid progress, which resulted from an increase in the understanding of how cells work at molecular, biochemical and physiological levels. It has steadily grown from the development of techniques, which allow the transfer of genes from one plant species to another or from other organisms such as bacteria. The development of improved crops includes those conferring resistance to fungal pathogens (Hirofumi uchimiya *et al.*, 1993) viruses (Van den Elzen *et al.*, 1989) and the nutritional improvement like the golden rice (Burkhardt *et al.*, 1997).

The objective of plant biotechnology is to identify the agricultural problems that need to be solved by complementing classical plant breeding and thus reducing the time scale required to produce a genetically enhanced germplasm. Plant biotechnology when integrated with traditional crop improvement programs enables a more efficient environmentally compatible and ultimately cost effective utilization of resources for improved agricultural production. The tools of biotechnology when provided to plant breeders present many opportunities for increased reliability in crop production while ensuring increased profitability and environmental compatibility (Sharma and Ortiz, 2000).

The development of transgenic plants depends on plant transformation, which relies on the introduction of plasmid construct or segments of plasmid constructs into the genome of the plant cell that confers resistance to diseases with the target gene of interest. Transgenic plants are regenerated from transformed cells, as most of the plant cells are totipotent they posses the ability to regenerate the whole plant from the single cell.

2.1.2 Transformation methods

Major contribution to biotechnology comes from the capability of genetic engineering. Lot of achievement has been made for the introduction of exogenous DNA into organisms and so also in their expression The transferred gene is called the transgene and the whole process is referred to as transgenesis. The physical barriers for DNA entry into cells vary for different groups of organisms. Plants have three physical barriers-cell wall, plasma membrane and nuclear envelope. The two basic approaches of DNA transfer in plants: 1. Artificial methods and, 2. Natural methods.

Artificial methods of DNA transfer include:

A. Physical methods

B. Chemical methods

A. Physical methods:

Various means of DNA transfer by physical methods include

1. Micro injection:

In this method DNA may be introduced into cells or protoplasts with use of very fine needles of 0.5 to 10 m diameter. Some of the DNA injected may be taken up by nucleus. Micro injected DNA may become stably integrated into chromosome of host cell through recombination and may be inherited in a mendelian manner. Transformation frequencies are considerably high and due to some specific advantages and application, method is used for artificial gene transfer in certain fields. Microinjection of DNA into plant nuclei (in protoplasts or tissues) is more difficult than for animal cells but now it is betterly developed. According to some reports 500-1000 cells can be microinjected per hour by skilled person having proper practice.

Advantages

1. Frequency of stable integration is far better than in other methods (20%)
2. Effective in transforming primary cells as well as cells in established cultures
3. Injected DNA appears to be subjected to less extensive modifications than transfected DNA.

Limitations:

1. More useful for animal cells

2. Costly
3. Skilled personnel required
4. This method is not used for walled cells and is used for protoplasts only
5. Rearrangements or deletions of host DNA adjacent to site of integration are common
6. Only 10^2 to 10^3 cells can be micro injected in the time in which 10^6 cells can be transfected. Thus method is slow.

2. Macro injection:

Macro injection is the method tried for artificial DNA transfer. In this method needles used for injecting DNA are with diameters greater than the cell diameter. DNA is injected with conventional syringe into region of plant, which will develop into floral tillers. Macro injection method was found to be successful with rye plants. It is also being attempted for other cereal plants.

Advantages:

1. Method does not require protoplast
2. Instrument will be simple and cheap
3. Method may prove useful for gene transfer into cereals which do not regenerate from cultured cells easily
4. Technically simple.

Limitations:

1. Less specific
2. Less efficient
3. Frequency of transformation is very low (2 in 3000).

3. Electroporation:

Electroporation can be used to introduce exogenous DNA to plant protoplasts (dicot and monocot). Fromm *et al.*, (1985) first reported gene transfer into maize protoplast be electroporation and Langridge *et al.*, (1985) first reported the stable transformation of carrot protoplast with DNA by electroporation. Electroporation can be used to increase efficiency of transformation or transfection of bacterial cell (by plasmid or phage respectively). Genes encoding selectable markers may be used to introduce genes using electroporation and range of transformed plants may be

selected. Equivalent procedure applied to suspension of cultured cells is referred to as "Electroinjection".

Advantages:

1. Method is applicable to variety of cell types
2. Method is quick
3. Less costly
4. Large number of cells can be treated simultaneously
5. High proportion of stable transformants are obtained
6. With in 10 minutes of pulsing stable transformants can be selected.

4. Sonication

In the process of sonication low frequency ultra sonic waves ranging between 1.0 MHZ-1.5 MHZ have the capacity to produce small pores in the cell which facilitate the entry of a plasmid containing the desired gene (Zhang *et al.*, 1991).

5. Biolistics

Among the physical methods for artificial transfer of exogenous DNA, biolistic transformation is relatively novel method. The term 'biolistic' (biological ballistics) was coined to describe the transfer of foreign DNA into living cells or tissues through bombardments with a particle gun. The method was developed to overcome the limitations of DNA delivery in other methods. It avoids the need of protoplasts and is better in efficiency. The method can be used for any plant cells, leaves, root sections, embryos, seeds, pollen. The method involves bombardment of particles carrying DNA of interest onto target cells using high velocity transfer mechanism. The method was first developed by Sanford *et al* in 1987 and in their model system onion epidermal cells were used. Klein *et al* in 1987 transferred genomic RNA of Tobacco Mosaic Virus (TMV) using this method and 30-40% of the epidermal cells of onion showed the signs of virus replication. The technique is finding universal application in the direct transformation of whole cells in culture, in tissue and in subcellular organelles such as mitochondria and chloroplasts.

In this method DNA of interest is coated on heavy microparticles of tungsten or gold DNA used for coating the particles is first precipitated with $CaCl_2$, spermidine and

polyethylene glycol. They are carried by nylon macroprojectile and are accelerated into living target cell at a very high initial velocity. Nylon macroprojectile is withheld in the barrel after a short while by the stopping plate while only DNA coated macroprojectile enters the aperture of stopping plate and continues to accelerate towards target cell and strikes it. This causes penetration of exogenous DNA through cell wall. For stable transformation to occur, the amount of DNA reaching the cells, thickness of the tissue being penetrated and potential of the target tissue or cell to regenerate into plant are the important factors. Biolistic transformation technique has been shown to be successful with papaya, sugarcane, soyabean, tobacco, etc. Genomes of subcellular organelles have been made accessible to genetic manipulations by this method. The particle gun has also been used with pollen, early-stage embryoids, somatic embryos and meristems. Some target tissues such as embryogenic suspension cultures (Fromm *et al.*, 1990) and meristematic tissue (Mc. Cabe *et al.*, 1988) have proven to be transformable and able to give rise to transgenic plants. When the biolistic process is applied to plant tissues plants regenerated from such tissue are usually chimeric in terms of introduced foreign gene due to random bombardment of a small number of cells in a multiple system.

Advantages:

1. Need of protoplast obtaining can be avoided
2. Walled intact cells can be penetrated
3. Genome of subcellular organelles can be manipulated.

Limitations:

1. Random integration.
2. The scope for the efficacy of this method is broader than that of *Agrobacterium* but less precise in its transgene integration pattern (Finer *et al.*, 1999).

B. Chemical methods of DNA transfer

Chemical methods of DNA transfer involves:

1. Use of Polyethylene Glycol (PEG)

Chemical agents such as PEG (Negrutiu *et al.*, 1987) increase the permeability of the cell membrane there by causing the transformation ability to the plant cells. This method has been successfully applied to Petunia, Nicotiana and other plant systems like maize, rice, etc.

2. Transfection using Calcium Phosphate

The process of transfection involves the admixture of isolated DNA (10-100 μg) with solution of calcium chloride and potassium phosphate under conditions, which allow fine precipitate of calcium phosphate to be formed. Cells are then incubated with precipitated DNA either in solution or in tissue culture dish. A fraction of cells will take up the calcium phosphate-DNA precipitate by endocytosis.

C. Natural means of DNA transfer

***Agrobacterium*-mediated DNA transfer**

Transformation of plants by *Agrobacterium*-mediated DNA transfer is currently the most commonly used means of accomplishing plant gene transfer. The natures genetic engineer contributes a lot to the rapid development of research through this mode of DNA transfer. *Agrobacterium*-mediated DNA transfer employs the transfer of a foreign gene (DNA) into the nucleus of the plant cell. Genetic engineers have developed gene vectors based on this natural process. The desired genes are inserted into this vector by standard molecular cloning techniques, re-introduced into the bacterium, which is then incubated with the plant tissue to be transformed. The specific piece of DNA containing the gene is then transferred to the plant cell nucleus and integrated into the chromosome. This system has worked out in a very broad range of species including a large number of crop plants

1.1.3 Molecular basis of *Agrobacterium* transformation

Agrobacterium is a gram-negative soil bacterium. It includes *Agrobacterium tumefaciens* which induces crown gall tumors (Smith and Townsend, 1907) and *Agrobacterium rhizogenes* which induces the formation of hairy root diseases in dicotyledonous plants (Delaine and Delay, 1976) and certain monocotyledonous plants (Hernal-steels *et al.*, 1984). Monocots are not easily transformed by *Agrobacterium tumefaciens* (Raineri *et al.*, 1986). Zaenen *et al.*, (1974) first noted that virulent strains of *Agrobacterium* harbor large plasmids. The ability to cause tumor lies with in the plasmids where by the T-DNA is transferred into the genome of infected plants (Vanlarebeke *et al.*, 1974). Series of classic experiments by Braun and coworkers demonstrated that once tumor formation has been initiated, the further presence of

Agrobacterium is not required for subsequent tumor proliferation (Braun and Stanier, 1958). The molecular studies on Agrobacterium and subsequent finding of Kerr (1971) were very useful to establish the central role of *Agrobacterium* plasmids in crown gall development.

Plant tumors resulting from *Agrobacterium* infection synthesize a variety of unusual amino acid derivatives called opines (Petit *et al.*, 1970) due to the expression of T-DNA genes encoding opine synthase enzymes (Watson *et al.*, 1975 and Bomhoff *et al.*, 1976) and nopaline synthase enzymes (Montoya *et al.*, 1977). Ti plasmids and the *Agrobacterium tumefaciens* strains harboring them can be classified according to the type of opines produced the three best studied are octopine, nopaline and agropine. The generation of tumors producing specific opines catabolyzable only by the inciting *Agrobacterium* strain is a central feature of the pathogenic relation ship between *Agrobacterium* and plant. Some of the plants regenerated from Nopaline containing tumour tissue continue to synthesize Nopaline (Schell and Van Montagu, 1977). The strains that utilize Octopine induce tumors that utilize only octopine and the strains that utilize Nopaline induce tumors that synthesize Nopaline (Bomhoff *et al.*, 1976 and Montoya *et al.*, 1977). Plasmids in the octopine group have shown to be closely related while those in the Nopaline group are in a diverse way (Sciaky *et al.*, 1978). Transformation using disarmed (non-tumorigenic) *Agrobacterium tumefaciens* plasmid vectors can result in transgenic plants of normal phenotype which express the introduced genes. The methods of transformation of intact cells or tissues with *Agrobacterium tumefaciens* have been developed using excised tissue of *Nicotiana* and *Petunia* spp. (Horsch *et al.*, 1985 and Rogers *et al.*, 1986). Nucleic acid hybridization studies have shown that octopine and nopaline plasmids are closely related having a wide stretch of homology in the T-DNA region (Willmitzer *et al.*, 1983)

The genetic elements encoded by *Agrobacterium* which are essential for T-DNA transfer are the T-DNA border sequences and the chromosomal virulence genes present on the Ti plasmid outside the T-DNA. The attachment of *Agrobacterium* to the plant cell is mediated by the chromosomal virulence genes (Douglas *et al.*, 1985, Thomashow *et al.*, 1987) which are constitutively expressed. The 25 base pair direct repeats flanking the T-DNA (Yadav *et al.*, 1982, Zambryski *et al.*, 1982) are the only part of T-DNA

important for transfer. The right border repeat is an essential cis acting element for transfer where as the left border repeat is thought to merely signal where the transfer of DNA normally ends. Subsequent steps in the T-DNA transfer require the proteins encoded by the *vir* region (*vir a, vir b, vir c, vir d, vir e, and virg*).

Agrobacterium tumefaciens infects only wounded actively dividing plant cells. The cells secrete wound specific compounds such as acetosyringone and alpha hydroxy aceto syringone. These phenolic compounds act as chemoattractants for *Agrobacterium* (Ashby *et al.*, 1987) and inducers of the *vir* genes (Stachel *et al., 1*985). Both processes are proposed to be mediated by the gene products of *vir a* and *vir* g (Stachel and Zambryski, 1986). The constitutively expressed *vir a* protein acts as a chemo receptor and transmits this information to *vir* g protein possibly by phosporylation mechanism (Jin *et al., 1*990). *vir g* transfectionally activates the *vir b, vir c, vir d , vir e and vir g* loci. A number of sugars act synergistically with phenolic compounds to enhance the *vir* gene expression. This induction pathway requires the gene products of chv c and *vir a* (Anken bauer and Nester 1990, Cangelosi *et al., 1*990). The genes on the Ti-plasmid and their functions were identified by transposon insertion and deletion mutagenesis (Holsters *et al.*, 1980, Garfinkel *et al.*, 1980, Ooms *et al., 1*981, Degreve *et al.*, 1982) methods.

The Ti-plasmid contains a well-defined T-DNA region encoding a series of genes responsible for the synthesis of auxinis and cytokinins in transformed plant cells (Akiyoshi *et al.*, and Inze *et al., 1*984), which causes over production of phytohormones and that cause tumor proliferation.

2.1.4 *Agrobacterium* derived vector systems

The disadvantage of DNA transfer using wild type *Agrobacterium* strains is the levels of phytohormones in the transformed tissue prevent the regeneration of cells into whole plants to over come this difficulty disarmed Ti-plasmids were constructed by deleting the oncogenic T-DNA genes. Two different types of vectors systems have been able to transfer foreign gene into plants by using *Agrobacterium.*

Co -integrate vectors: The first developed vector system was of the co-integrate type and made use of *Agrobacterium* strains with non oncogenic Ti-plasmids, most of its T-DNA genes have been removed and replaced by pBR 322 sequences. The genes to be

transferred to plant cells are cloned in pBR 322which can then be mobilized to *Agrobacterium* and co-integrated into the T-DNA region.

Binary vectors: The knowledge that the T- DNA and the *vir* region do not have to reside on the same plasmid paved the way for the development of binary vector systems (An *et al.*, 1985, klee *et al.*, 1985, Vanden elzen *et al.*, 1985, Simens *et al.*, 1986, Deblaere *et al.*, 1987). It is based on *Agrobacterium* strains containing two plasmids, one having the trans acting *vir* functions to transfer T-DNA while the second plasmid carries between T-DNA borders, the DNA sequences to be introduced into plant cells. This plasmid has the ability to replicate in two hosts, thus binary vectors systems are based on the separation of *vir* and T-DNA regions on two independent compatible plasmids.

Plant modification using recombinant DNA technology is the insertion of a known sequence of foreign DNA into the host plant genome. Thus it is quite distinct from mutation breeding because it is based on initial non-random DNA change and can cross species boundaries. The new genetic information is assembled as one or more gene 'cassettes' consisting of promoter region, coding region, and terminator regions. As it is impossible to screen for certain traits in individual transformants, selectable marker genes (genes conferring antibiotic resistance) are also co-introduced along with the primary target traits. In molecular breeding, premier varieties that have the most desirable innate traits are most amenable to tissue culture, that is the way by which the genetically modified crops have largely been produced using plant transformation systems utilizing tissue culture.

2.1.5 Selectable and screenable genes for plant transformation

Transformation in plants needs marker genes to distinguish between transformed and untransformed ones. In recombinant DNA research the marker genes are physically linked to the gene of interest. The *Agrobacterium* encodes genes nopaline synthase (Depicker *et al.*, 1982 and Bevan *et al.*, 1983) and octopine synthase (Degreve *et al.*, 1982) were the first ones to be used as markers for the isolation of transformed plants. Transformants can be sorted out and stabilized in their progenies especially when selectable marker genes are used (Finer and Mc Mullen, 1990). The marker genes can be classified as:

1. Selectable marker genes

A. Neomycin phosphotransferase II:(*npt II*): It is the most widely used selectable marker gene for plant transformation. It is derived from the transposon Tn5. *NPT II* codes for an enzyme that inactivates a number of aminoglycoside antibiotics such as kanamycin, geneticin (G418) and paromycin by phosphorylation. (Herrera-Estrella *et al.*, 1983).

B. Hygromycin phosphotransferase: Resistance to hygromycin is conferred by the selectable marker gene hygromycin phosphotransferase (Hpt) first isolated from *Streptomyces hygroscopicus* which is widely used as a selectable marker gene in genetic transformation experiments. Hygromycin prevents polypeptide elongation by interfering with aminoacyl t-RNA recognition and ribosomal A-site occupation (Cabanas *et al.*, 1978 and Hausner *et al.*, 1988). Hygromycin can lead to misreading during translation in vitro (Davies and Davies 1968, Gonzales *et al.*, 1978, Singh *et al.*, 1979) however; this effect was not duplicated *in vivo* (Baker, 1992).

2. Reporter genes (Screenable genes):

Reporter genes are coding sequences that upon expression in the transgenic plant provide a clear indication that genetic transformation has taken place. Herrera-Estrella *et al.*, (1988) and Schrott (1994) reviewed the literature on reporter genes upto 1986 and Schrott reviewed the literature till 1994. The most commonly used reporter genes are CAT (chloramphenicol acetyl transferase), GUS (β-glucuoronidase), Luciferase and GFP (Green Florescent Protein).

A. CAT gene: The CAT gene codes for an enzyme that acetylates the antibiotic compound chloramphenical. The assay is quite sensitive and semi quantitative (Scott *et al.*, 1988 Herrera-Estrella *et al.*, 1988). It was widely used in the past in plant transformation until more efficient reporter genes became available.

B. GUS reporter gene: Gus reporter gene was developed by Jefferson's group (Jefferson *et al.*, 1986, Jefferson 1987). The GUS gene encodes β-glucuoronidase, a soluble enzyme of molecular weight 68 KD and an optimum pH of 7-8 being in its active form. The Gus gene because of its highest efficiency as a reporter gene became the most frequently used reporter gene in genetic transformation of plants.

C. Luciferase reporter gene: The luciferase reporter gene system is based on luminisence reaction developed by De Wet and associates (De Wet et al., 1985) and was reviewed by Luchresen et al., 1992 and Schrott(1995). Ow *et al.*, (1986) demonstrated the utilization of the gene that encodes luciferase from the firefly *Photinus pyralis*. It mimics the *invivo* reaction that takes place in certain insects and bacteria by an enzyme luciferin 4-monoxygenase.

The genetic transformation generally involves 2 genes. One is the transgene that should be integrated in plant genome and expressed in the transgenic plant and the other is a selectable gene. Each of the two transgenes should thus have its own promoter and terminators. Futterer (1995) reviewed the promoters for genetic transformation of plants. The construction of chimeric genes allows the expression of any coding sequence under the control of 5' and 3' non-coding regions of genes expressed in plant (Herrera Estrella *et al., 1983*).

2.1.6 Molecular mechanisms influencing inter transformants variability

Molecular mechanisms that influence inter transformants variability are related to the process by which new genetic material is integrated into the host genome. Molecular events associated with genetic alterations are the position effects, insertional mutagenesis and pleiotrophy.

1. Position effects: Position effects are defined as variability in transgene expression due to structural and functional properties of chromatic regions flanking the DNA integration site. A property of the chromatin that may influence the expression level of integrated gene includes local and higher order structures (e. g., Looped domains) as well as neighboring regulatory sequence that might enhance or silence the activity of the introduced gene during the plant transformation. Foreign DNA is integrated at a random position in the genome, in most cases at a single locus either as a single copy or as a cluster of tandem copies. The randomness of integration could be perceived as exacerbating the problem of unexpected secondary effects however natural internal rearrangements of the genetic code through background transpositional events could also produce profound changes in molecular biology.

2. Insertional mutagenesis: Insertional mutagenesis is the modification or disruption of functional genes in the host plant at the site of foreign gene insertion. Inactivation of existing genes may occur when the incoming DNA inserts into the coding region.

3. Pleiotropy; Pleiotropy defined, as the ability of one gene to affect more than one trait is another source of unexpected or unintended effects seen in the progeny phenotype of breeding populations. The dramatic and unforeseeable effects of pleiotropy in traditional breeding were demonstrated by the male sterile lines of the Texas Cytoplasm maize hybrid (CMS-T). In the early 1970's the vast area in the USA dedicated to the CMS-T maize and was devastated by the southern corn leaf blight, caused by Bipolaris maydis race-T. The susceptibility to fungal disease was observed only with CMS-T maize.

2.2 Molecular analysis of plants transformed with *npt*II

2.2.1 Isolation of DNA

The isolation of good quality of DNA from groundnut is essential for the molecular characterization of putative transgenic plants. The problems encountered in the isolation of good quality DNA are the presence of phenolic compounds and polysaccharides. The acidic polysaccharides are inhibitory for *Hind*III enzyme restrictions of lambda DNA and it can inhibit classical two primer PCR (Demeke and Adams, 1992). Neutral plant polysaccharides are not inhibitory for *Hind*III enzymes restrictions of lambda DNA (Do and Adams, 1991) and they also do not inhibit PCR amplification of spinach DNA (Pandey *et al.*, 1996).

A protocol devised by Sharma *et al.* designated as the ICE proved superior to the other methods such as Dellaporta (Dellaporta *et al.*, 1983) GMD (Guillemaut and Marechal-Drouard,1992), RB (Rogers and Benedich,1985). The ICE method has yielded pure DNA of high molecular weight form different genotypes of peanut. The DNA thus obtained is consistently amplifiable by PCR and restrictable for Southern blot hybridisation (Sharma *et al.*, 2000).

2.2.2 PCR amplification

The Polymerase Chain Reaction (PCR) is an *invitro* method for replicating a defined (Target) DNA sequence so that its amount is increased exponentially. A single

gene could be amplified to a million copies with in a few hours by PCR. This amplification process is achieved with two synthetic oligonucleotide primers, a thermostable Taq DNA polymerase and the four deoxynucleoside triphosphates acting on the template DNA. (Erlich 1981, Gibbs 1990, and Bej *et al.*, 1991). The PCR procedure involves 3 steps each of repeated many times to produce cycles of amplification. The three steps involved in the reaction are denaturation, annealing and extension.

2.2.3 Restriction analysis

Restriction endonucleases recognise short DNA sequences and cleave double stranded DNA at specific sites with in or adjacent to the recognition sequences. Restriction endonuclease clevage of DNA into discrete fragments is one of the most basic procedures in molecular biology.

2.2.4 Southern analysis

Localization of particular sequences of DNA with in the fragments is accomplished by the transfer technique described by E.M.Southern (1975). DNA fragments that have been separated according to the size by electrophoresis through an agarose gel are denatured, transferred on to nylon membrane. The DNA attached to the nylon membrane is then hybridized to the probe and autoradiography is used to locate the position of any bands complementary to the probe. This technique can be used to identify the sequences inserted in the genomic DNA of plants (Botchan *et al.*, 1976, Jeffreys and Flavell, 1977).

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

The groundnut cultivars used in the study were of ICGS-44 variety, a high yielding Spanish variety provided by the Genetic Resources Division of ICRISAT.

Genetic transformation of groundnut was done by using disarmed Agrobacterium strains harboring pCAMBIA 1302:Rchit plasmid (Fig: 1). Molecular analysis was carried out with putative transgenic plants of groundnut developed in the Genetic Transformation Lab, ICRISAT by *Agrobacterium*- mediated transformation using plasmid pROKII containing *npt*II gene (Sharma and Anjaiah, 2000).

3.1.1 Explants used for genetic transformation studies

The cotyledons of groundnut have been used as explants for the development of transgenic plants. De-embryonated cotyledons have been widely used for *in vitro* regeneration because of their easy availability and ease of handling. A large number of shoots can be regenerated from a single cotyledon with in a short time thus making them good candidates for use in genetic engineering.

3.1.2 Preparation of *Agrobacterium* inoculum for transformation

A single colony of *Agrobacterium tumefaciens* harboring on binary vector was inoculated in 5 ml YEB on a rotary shaker at 28°C for over night. The over grown culture was centrifuged at 5000 rpm for 10 minutes. Under strict asceptic conditions the supernatant was discarded and the pellet was resuspended in 25 ml of half strength MS medium.

3.1.3 Sterilization of ground nut seeds

Groundnut seeds ICGS-44, were dipped in 0.1% HgCl2 with 0.1% tween solution (v/v). The seed coat from the cotyledon is removed and the embryo axis was removed surgically and each cotyledon was cut into vertical halves to obtain the cotyledon explants.

3.1.4 Agroinfection of *Arachis* explants

The cotyledon explants were infected by dipping for one minute in the *Agrobacterium* culture prepared as described above. The explants were placed on Shoot Induction Medium (SIM) such that the cut edges were embedded into the medium. The

SIM comprises the MS medium + B5 organics. The explants were plated at a density of six cotyledons explants per petriplate and sealed with parafilm. After 3 days of cocultivation, the explants were transferred into SIM (Shooot Induction Medium) containing cefotaxime. They were then transferred into SEM (Shoot Elongation Medium). The sensitivity of the explant to hygromycin is tested on shoot elongation medium containing different concentrations of hygromicin. The induced shoots that are not transformed become necrotic and bleached in the presence of hygromycin. Later they were placed on RIM (Root Induction Medium) for rooting. After the formation of roots the plants were transferred into pots and acclimatized to the glass house conditions.

3.1.5 Characteristics of binary plasmids used for transformation

Agrobacterium tumefaciens strain harboring a binary plasmid pCAMBIA 1302:Rchit used in the transformation studies was shown in the figure. The plasmid size was about 12.34Kb in size containing rice chitinase gene of 1.5Kb. The rice chitinase gene has been cloned and expressed in sorghum. The plasmid construct contains Hygromycin phosphotransferase as selectable marker and green flouresent protein as screenable marker. The *hpt* is driven by CaMV 35S Promoter and nos terminator.

The GFP is derived from Jellyfish and is driven by 35S promoter and nos terminator. The green florescent protein has the unique characteristic of florescing green when exposed to UV light. Transformed cells can be visibly selected on the basis of green florescence. Such an approach has the added benefit of increasing the efficiency of transformation.

3.1.6 Isolation of pCAMBIA 1302 from *Agrobacterium tumefaciens*

The plasmid was isolated by the slight modification of alkaline lysis method described by Sambrook et al., 1989. A Single bacterial colony was inoculated in 5 ml of YEB containing appropriate antibiotic (kanamycin) and it was grown on a rotary shaker for over night at 28 °C. The culture was centrifuged at 5000-rpm for10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 200 µl of GTE (Glucose, Tris, EDTA) and the cells were lysed with 400 µl of lysis buffer. 300µl of 5M potassium acetate (pH-5.2) was added. The eppendorf tubes were then centrifuged at 12, 000 rpm for 2 minutes. The supernatant was collected and the plasmid DNA was precipitated with

0.8 volume of isopropanol, mixed gently and centrifuged at 12,000 rpm for 10 minutes The supernatant was discarded and the pellet was washed with 70% Ethanol. The pellet was air dried and dissolved in 40µl of TE. The plasmid thus isolated was run on 0.8% Agarose gel and viewed on UV transilluminator.

3.1.7 Restriction analysis of the isolated plasmid

The isolated plasmid pCAMBIA1302 was restricted with restriction enzyme such as *Hind*III according to the instructions of the manufacturer. The restriction enzymes were chosen based on the restriction sites present on plasmid to confirm its size.

3.2.1 Isolation of genomic DNA from groundnut

Leaf tissue of groundnut (250mg) was frozen in liquid nitrogen and it was grinded to fine powder with the help of mortor and pistle. To the fine powder 10ml of the extraction buffer was added and vortexed gently. It was then transferred to a polypropylene tube and 1 ml of 20% SDS was added and mixed well by shaking it gently. It was incubated at 65^0c in a water bath for 10 minutes. 5ml of 5M-potassium acetate was added and kept on ice for 30 minutes. Later it was centrifuged at 14,000rpm for 20 minutes. The supernatant was collected and to it 0.6 volume of isopropanol was added and incubated at -20^0c for 30 minutes to precipitate the DNA. It was centrifuged at 10,000rpm for 10 minutes. The pellet was then washed with 70% ethanol and air-dried. It was dissolved in 500 µl of TE.

3.2.2Purification of DNA

To 500 µl of DNA taken, 1.4ml of DEAE cellulose suspension was added and mixed gently and centrifuged for 30 seconds at 5000 rpm there by increasing its interaction with the DNA and the supernatant was discarded. To the pellet 1.4 ml of wash buffer was added and was centrifuged at 5, 000rpm for 30-60 seconds. The supernatant was discarded and the DEAE cellulose was re suspend in 1.4 ml of wash buffer. This was done to eliminate the proteins, polysaccharides and metabolites that are not bound to DEAE cellulose. To this 600 µl of elution buffer was added and mixed well. This suspension was centrifuged at 5, 000 rpm for 30-60 seconds. 500µl of Supernatant was collected. To this another 350 µl of elution buffer was added and mixed

gently to elute the DNA. Supernatant was collected and pooled. A brief spin at 14000 rpm was given and 800 µl of the supernatant was collected. To this 1/10 volume of 3M Sodium acetate and 0. 8 vol of isopropanol was added and shaked gently. Later it was centrifuged at 10, 000 rpm for 10 minutes. Supernatant was discarded and the pellet was washed with 70% ice-cold ethanol. The pellet was air dried and dissolved in 70µl of TE.

3.2.3 Amplification with the help of PCR

PCR requires

1. A template molecule - DNA
2. Forward and reverse primers designed for the *npt* II [amplifies 700 bp of the coding region].
3. $MgCl_2$, Taq polymerase, dNTPs, PCR buffer.

The amplification reactions were carried out by using a thermo cycler under the following conditions – 94^0C for 4min [one cycle], 92^0C for 60S [denaturation], 58.5^0C for 45S [annealing], 72^0C for 90S [extension] and final extension at 72^0C for 5min.

3.2.4 Restriction analysis

Genomic DNA was restricted for over night using *ECoRI* enzyme which has a single restriction site in the plasmid transformed, and the restriction pattern was verified on 0.8% agarose gel.

3.2.5 Southern analysis

The restricted genomic DNA in the gel was subjected to depurination by immersing the gel in 250 mM HCl for 10 minutes. It was washed twice with demineralized water. It was then denatured using denaturation solution for 15minutes. This step was repeated again. The gel was washed twice with distilled water. The gel was then neutralised with neutralisation buffer for 15 minutes. This step was repeated again. An arrangement was made for the transfer of sequences from gel to nylon membrane by capillary action. A tray containing 20XSSC buffer was taken.A glass plate was placed over it. The nylon membrane was first dipped in 2XSSC. In the arrangement for Southern blotting 2 whatman filter papers were placed and above that the gel was placed in an inverted position and on the top of it the nylon membrane was placed. 2

whatman filter papers were placed on it. A stack of filter paper towels was placed over it. The entire set up was connected by paper wicks, which helps to draw buffer from the tray. As this process did not work well dot blot was carried out for detection of the positive transgenic plants. Southern hybridization was carried out using non-radio active direct AlkPhos labelling system (Amersham Pharmacia Biotech).

4. RESULTS AND DISCUSSIONS

4. RESULTS & DISCUSSION

4.1 *Agrobacterium*-mediated transformation for development of transgenic ground nut plants for resistance to fungal pathogens

4.1.1 Confirmation test for the presence of desired gene in the vector

The plasmid was isolated with slightly modified alkaline lysis method as discussed in materials and methods, and is viewed with the help of U.V transilluminator. The size of the uncut plasmid corresponded well with the exact size of the respective marker band. The plasmid restricted with *Hind* III released the *chitinase* gene where the molecular weight corresponded exactly with that of the marker (Fig: 2).

4.1.2 Tissue culture and transformation

I. Induction of adventitious shoots from the putative transgenic groundnut plants transformed with chitinase gene

The de-embryonated cotyledon explants when cultured on SIM (shoot induction medium){appendix A} produced multiple adventitious shoot buds. Out of 140 explants cultured on SIM, 79 of them responded for multiple adventitious shoot buds formation. On SIM the explants turned green and have under gone considerable enlargement with in 3 days of culture initiation. On these explants, multiple shoot buds differentiated at the proximal cut end, with in 15 days in all most all of the explants (Fig: 3).

II. Elongation of shoots in the putative transgenic plants

The explants bearing shoot buds were cut into two to four pieces and transferred into SEM (shoot elongation medium){Appendix A} for at least three passages of 4, a week each when elongated shoots were rescued at the end of each passage. A minimum of three to four shoots were recovered from each explant (Fig: 4). The shoots were vegetatively propagated on SEM through nodal explants for clonal multiplication and then transferred into root induction medium {Appendix A}.

III. Induction of adventitious roots from the putative transgenic plants

The adventitious roots appeared with in 2 weeks (Fig: 5) after the putative transgenic plants are transferred into the root induction medium and developed

further with in 4 weeks. They were then rooted in pots and transferred to the glass house and set for molecular analysis to be carried.

Invitro regeneration in crop plant is a prerequisite to achieve genetic transformation. In *Arachis spp.* cotyledons are a good source of shoots for *invitro* manipulation and their own nutrient storage is sufficient for initiation of shoots in large numbers. The cotyledon regeneration system proved to be an excellent vehicle for the production of large number of transgenic peanut plants in relatively short periods. These explants allowed *Agrobacterium*-mediated transformation to be targeted to regeneration competent tissue. Shoot formation was rapid and prolific, and large proportion of these shoots developed into phenotypically normal fertile plants (Sharma & Anjaiah, 2000).

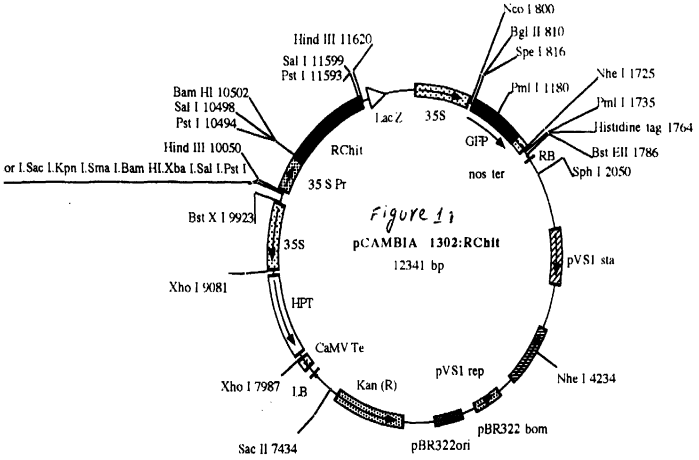
In legume tissue culture cotyledons have been established as explants for high frequency regeneration in many species and techniques have been developed to use de-embryonated cotyledons for high frequency of *Arachis spp* regeneration in ICRISAT (Sastri *et al.*1992). Preliminary results of regeneration were published in 1981 by Sastri *et al.* Cotyledons from mature seeds had greater propensity to form shoot buds and shoots at the nodal end directly. The use of de-embryonated cotyledons for high regeneration in *Arachis* is also practiced at the Scottish crops research institute for infection by avirulent & virulent strains of *Agrobacterium. Agrobacterium*- mediated transformation has proved to be successful in the generation of transgenic plants in peanut (Sharma *et al.*, 2000). The biological barriers which were earlier considered as hindrances are removed by means of *Agrobacterium tumefaciens* mediated transformation because genes from any biological source can be transformed by means of *Agrobacterium*-mediated transformation, Earlier studies have revealed the transformation of tobacco plants with bean chitinase gene conferred resistance to fungal pathogens by Agrobacterium-mediated transformation. Broglie *et al.* (1991) constructed a transformation cassette that contained the C-DNA of a bean endo chitinase gene under the control of 35S CaMV promoter. *Agrobacterium*- mediated transformation of tobacco led to transgenic plants that contained the bean chitinase gene. Some transgenic plants had a 2 - 44 fold increase of chitinase over control tobacco. When control and transgenic plants with increased chitinase, were infected with *Rhizoctonia solani*, the transgenic plants showed less

seedling mortality in infected soil than control plants. Infection with pythium affected equally control and transgenic plants because the latter fungus doesnot contain chitin in its cell wall. Transgenic *Brassica napus* expressing the pea chitinase showed tolerance to *R.solani*. Benhamou *et al.* (1993) analysed the cytology of transgenic canola plants expressing the chitinase transgene, after *Rhizoctonia* infection.

Howie *et al.* (1994) used a chitinase gene from the bacterium *serratia marcescens* and engineered the encoded protein to be either retained in the cells of the transgenic plants or extruded extracellularly. In both cases the gene was activated "constitutively" by the 35 S CaMV promoter. Both greenhouse and field experiment indicated that the system is functional. Thus it became evident that the transgenic plants that expressed the bacterial chitinase were tolerant to infection with *Rhizoctonia solani*. A more elaborate system was investigated by Zhu *et al.* (1994). They started with two tobacco transgenic lines. Into one they integrated and expressed and a chitinase gene (from rice) and in another line they integrated and expressed a gene for acid glucanase (from alfa alfa). They then crossed the two lines to obtain a progeny that is heterozygous for the two hydrolases and then selfed the plants to obtain homozygous plants for the two transgenes. It was found that the combination of the two transgenes provided a better protection against the fungal pathogen *Cercospora nicotianae* than when only one transgene for hydrolase was expressed in the transgenic plants. The same approach was followed by Lin *et al* (1995) but they performed their work with transgenic rice. These investigators used the cDNA of a rice chitinase and fused it downstream of the 35S CaMV promoter. They found that the degree of the resistance of the transgenic rice rice was correlated with the level of expression of the chitinase gene. Plants of one transgenic line did not show infection in their flag leaves.

A combined cDNA containing most of a tomato endo chitinase gene but a distal end from a similar tobacco chitinase gene was used by Grison *et al.* (1996) to produce transgenic *Brassica napus* plants. They tested the tolerance of the resulting transgenic plants to three fungal pathogens (*Cylindrosporium concetricum*, *phoma lingam* and *sclerotinia sclerotiorum*). Several transgenic lines exhibited an increased tolerance to those pathogens as compared to non-transgenic controls.

Figure 1. Plasmid construct map of PCAMBIA 1302:rice chitinase.



Plasmid name: pCAMBIA 1302:RChit

Plasmid size: 12341 bp

Constructed by: K.K. Sharma & coworkers

Construction date: 2000

Comments/References: Hind II fragment from pRT99 Gus:RChit inserted at the Hind III site of the MCS to produce pCAMBIA 1302:Rchit binary plasmid.

Figure 2. Restiction analysis of the plasmid DNA, pCAMBIA 1302:rice chitinase after restricted with *HindIII* 1500bp *chitinase* gene fragment was released.

Figure 2

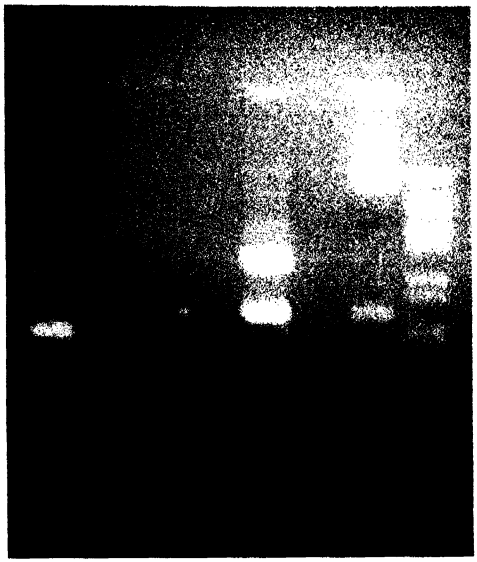
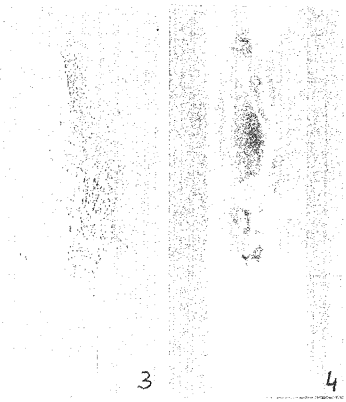


Figure 3: Production of adventitious shoots from the proximal cut part of the cotyledon explants of groundnut after 2 weeks of induction.

Figure 4: Elongated shoots derived from *invitro* formed shoot buds on the elongation medium containing hygromycin (15mg/l) after 3 weeks of culture.

Figure 5: Production of multiple roots on elongated shoots derived from cotyledons of groundnut after 3 weeks of culture on rooting medium.



3 4 5

Figure 6. DNA extracted from leaflet tissue of putatively transformed groundnut plant according to ICE method.

Figure 6

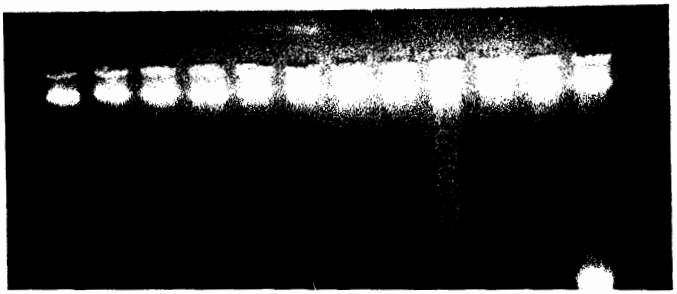


Figure 6(a): Isolated DNA according to ICE method

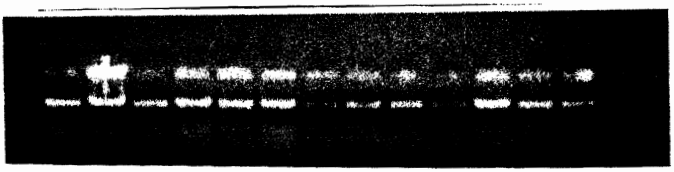


Figure 6(b): Purified DNA according to ICE method

Figure 7. PCR amplification of the 700bp fragment of *nptII* gene from the extracted genomic DNA of the putative transgenic groundnut plant.

Figure 7

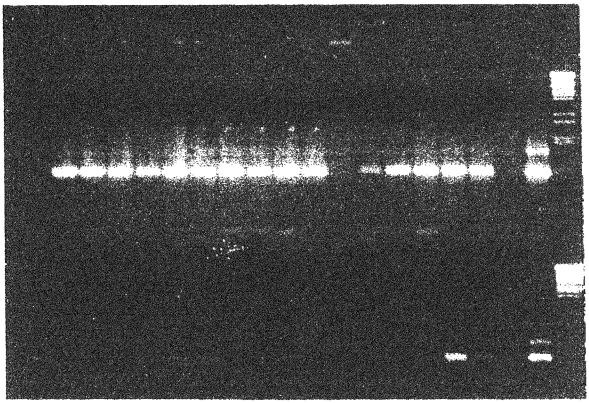


Figure 8. Restriction analysis of the genomic DNA of putative transgenic groundnut plants restricted with *Eco* RI

Figure 8

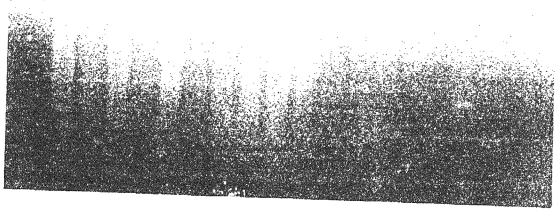
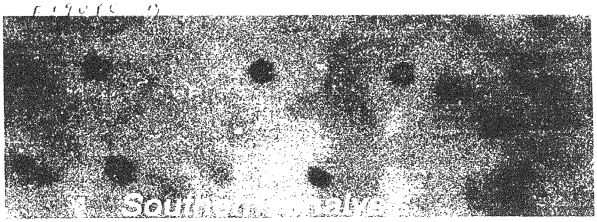


Figure 9. Southern analysis for the detection of transgenic groundnut plants transformed with *nptII*



In another study a chitinase transgene was introduced into tobacco plants by Jach *et al.* (1995). However, these investigators used three barley genes: a chitinase gene, a glucanase and a RIP gene. The first two genes were engineered for expression in the intracellular spaces of the transgenic tobacco plants where as RIP was expressed either in the cytosol or, after due engineering, in the inter cellular spaces. Transgenic plants expressing both chitinase and glucanase or both chitinase and RIP had an enhanced protection against *R.solani* – indicating a synergistic effect of the two transgenes. Thus the chitinase gene is used for eliminating the fungal pathogens.

4.2 Molecular analysis of putative transgenic plants transformed with *npt* II

4.2.1 Isolation of DNA

The DNA from putative transgenic plants transformed with *npt* II was isolated and purified according to the ICE method as described in materials and methods. The DNA samples were electrophoresed on 0.8% Agarose gel and no shearing was observed (Fig: 6). The DNA isolated was further subjected to PCR amplification and Southern analysis.

4.2.2 PCR amplification

Primers designed for the *npt* II gene amplified 700 bp of the putative transgenics confirming the presence of the *npt* II gene (Fig: 7).

4.2.3 Restriction analysis

The DNA from the PCR positive plants were selected and digested with *ECoRI* for overnight as described in materials and methods. The restricted samples were then electrophoresed on 0.8% Agarose gel. The restriction pattern of the genomic DNA further used in Southern analysis is shown (Fig: 8).

4.2.4 Southern analysis

Southern analysis was carried out as described in materials and methods using non-radio active direct Alk.Phos labelling system. As Southern analysis could not be carried out dot blot was performed and the putative transgenic groundnut plants transformed with *npt* II were detected (Fig: 9).

5. CONCLUSIONS

5. CONCLUSIONS

Many of the diseases in groundnut are caused by fungal pathogens that drastically decline the crop yield. Even though various approaches have been followed for the control of fungal pathogens, the novel approach is to utilize the tools of Biotechnology for crop improvement. The twin areas of plant cell culture and genetic engineering (Recombinant DNA technology) can bring the verge of a major revolution in the culture and propagation of plants and breeding for development of superior groundnut varieties conferring resistance to fungal pathogens. Tissue culture technology combined with classical breeding procedures would serve to attain the precise breeding goals envisaged at the commencement.

The transgenic groundnut plants have been produced by *Agrobacterium*- mediated transformation using cotyledon explants. The transformation efficiency was found to be high with *Agrobacterium*- mediated transformation and as large number of shoots have originated from the spliced cotyledon, the cotyledon explant system proved to be a better means for transformation of various genes in groundnut. The putatively transformed explants with multiple shoots were assayed for resistance to hygromycin (presence of HPT) and as many of the explants conferred resistance to hygromycin it tentatively suggested the integration of the chitinase gene.

Molecular analysis was carried out for transgenic groundnut plants transformed with *npt* II developed in the GTL (Genetic Transformation Lab), ICRISAT by *Agrobacterium* - mediated transformation. The process of molecular analysis involved several steps such as isolation of DNA, PCR amplification and Southern analysis. The putative transgenic groundnut plants were confirmed positive or negative by these steps.

Although various genes conferring resistance to fungal pathogens are characterized they are not yet cloned. The cloning of these genes would better serve the purpose of resistance against various fungal pathogens and this will help to increase the crop yield in groundnut.

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7. REFERENCES

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7. APPENDICES

APPENDIX - A

MS Major

MS NH_4NO_3	66.0g/400ml
MS KNO_3	38.0g/400 ml
MS $MgSO_4 7H_2O$	14.8g/400ml
MS Kh_2PO_4	6.80g/400 ml
MS $CaCl_2$	17.6g/400 ml

MS Minor

KI	83.0mg/1L
H_3BO_3	620.0mg/1L
$ZnSo_4 7H_2O$	2230mg/1L
2M NaCl + TE	860.0mg/1L
$Na_2MoO_4 2H_2O$	0.500mg/1L
$CuSO_4 5H_2O$	2.500mg/1L
$COCl_2 6H_2O$	2.500mg/1L

B_5 Organics

Nicotinic Acid	50 mg/250ml
Pyridoxine Monohydrochloride	50mg/250 ml
Thiamine Hydrochloride	500 mg/250 ml

Fe – EDTA

EDTA $2H_2O$	3.73g/1L
$FeSO_4 7H_2O$	2.78g/1 L

6.1 Shoot induction medium

The Shoot induction medium comprises of the above composition along with BAP 4.5mg/1L and 2,4 D 2.2mg/1L.

6.2 Shoot elongation medium

Shoot induction medium coinprises of above composition along with BAP 0.5mg/1L

6.3 Root induction medium

Root induction medium comprises of above composition along with NAA 4.5mg/1L

APPENDIX-B

Yeast Extract Broth (YEB)

Yeast extract	1.0g
Beef extract	5.0g
Peptone	5.0g
Sucrose	5.0g
$MgSO_4 7H_2O$	0.5g

Dissolve all the ingredients in 1 litre of distilled water. Adjust the medium pH to 7.0 by adding 1 M NaOH, dispense and autoclave for 25 min. at 121°C.

DNA Extraction buffer

50 mM EDTA

500 mM NaCl

100 mM Tris

ß Mercapto ethanol 18µl / 100ml

GTE buffer

50 mM glucose

25 mM trisHCl (pH 8.0)

10 mM EDTA di- sodium salt (pH-8), and autoclaved at 15 lb/sq in a liquid cycle for 15 min and stored at 4^0C.

Lysis buffer

0.2n NaOH (freshly diluted from a 10 N stock)

10% SDS

Distilled water.

Potassium acetate solution

5M potassium acetate solution 60ml

Glacial acetic acid 11.5ml

Water 28.5 ml.

RNAse free of DNAse

Pancreatic RNAse was dissolved in 10mM tris (pH 7.5), 15mM NaCl at conc. Of 10mg/ml, heated at 100°C for 15 minutes allowed to cool and placed at -20°C

DEAE cellulose

Wash buffer (400 mM NaCl + TE) (pH-7.5)

Elution buffer (2M NaCl+TE) (pH - 7.5)

TE (ph 8.0)

0.1 M tris Cl, 5mM EDTA (pH 8.0) di-sodium salt.

TAE buffer (50x)

242 g tris base

57.1 ml glacial acetic acid

100 ml. 0.5M EDTA di- sodium salt and make up the volume to one litre.