Studies on the development of groundnut (*Arachis hypogaea* L.) transgenics for resistance to *Aspergillus flavus*

Thesis submitted in partial fulfillment for the award of the degree of

Master of Technology
in
Biotechnology

By

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2007

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on the development of groundnut (*Arachis hypogaea* L.) transgenics for resistance to *Aspergillus flavus*"** submitted by **Sowmini Sunkara** for the award of degree of **Master of Technology** in **Biotechnology,** Center for Biotechnology, Institute of Science and Technology, JNTU is the original research work carried out, under our supervision at Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. No part of the thesis has been submitted for the award of any degree or diploma of any university or Institute.

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DECLARATION

I hereby declare that the dissertation entitled upon **"Studies on the development of groundnut (*Arachis hypogaea* L.) transgenics for resistance to *Aspergillus flavus*"**, is an original and independent record of research work undertaken by me at Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, during the period of my study under the supervision of Dr. M. Lakshmi Narasu, Professor & Head of the Department, Center for Biotechnology, Institute of Science and Technology, J.N.T.U, Hyderabad, except where specifically stated to the contrary, and it is not substantially the same as my thesis that has been submitted for any degree or diploma of any other University.

Date: 16-01-2007

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ACKNOWLEDGEMENTS

I express my deep sense of gratitude and sincere thanks to Dr. M. Lakshmi Narasu, Prof & Head, Centre for Biotechnology, IST, JNTU and my research supervisor for her constant guidance, supervision and valuable suggestions during the entire course of this study.

My heart full thanks and profound gratitude to Dr. Kiran .K, Sharma, Principal Scientist, Genetic Transformation Laboratory, ICRISAT and co- supervisor, Dr. Farid Waliyar, Principal Scientist, Mycotoxin Diagnostics and Virology Laboratory, ICRISAT, for their persistent efforts to perfect me as a researcher to be thorough, conscientious and logical with a scientific perception. I extol for the genuine co-operation offered to me for the guidance and ideas about the transformation of groundnut. Their endeavour to get the advanced techniques and scientific knowledge together and make the research applicable from the laboratory to field level made me to realize the purpose of investigation.

I specially thank Dr. Anjaiah Vanamala, Special Project Scientist, Genetic Transformation Laboratory, ICRISAT, for his help and suggestions received during the course of work. I express my sincere thanks to the research group at Genetic Transformation Laboratory ICRISAT, Dr. Mayank Rai, Dr. Arockiasamy, Dr. Arunachalam, Dr. Hameeda, and Dr. Sai Vishnu Priya who stood by me through out my research work. I also thank my colleagues Sreelatha, Pooja, Prashanthi, Kalyani, Madhurima, Padmini, Vani, Sujatha, Chander Rao, Swamy Krishna, Sreenivas Reddy, Sreedhar, Subramanian and Ramakrishna, for their co-operation and exchange of ideas during the course of study and made my work a pleasant experience. Without their help it would not have been possible to complete thesis in time.

It is my pleasure to acknowledge the services and support received from the Genetic Transformation Laboratory members, Yousuf, Pandary, Laxminarayana, Jagan Mohan Reddy, Laxmi, Manemma and Prabhakar Reddy, for their whole hearted co-operation extended through out my course of study at ICRISAT.

I sincerely thank the learning systems unit (LSU), ICRISAT for permitting me to avail the research and library facilities at ICRISAT. I also thank Mr. K.R.C. Bose, FESP, for his valuable suggestions during my stay at ICRISAT.

Last, but not the least, I wish to express my deepest appreciation to my husband and all my family members for their love, moral support and constant encouragement, without whose help it would not have been possible to complete the course of study.

Sowmini Sunkara

CONTENTS

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
BA	N^6-benzyladenine
BND	bud necrosis disease
bp	base pair
cDNA	complementary DNA
cef	cefotaxime
cp	coat protein gene
DAI	days after inoculation
DEAE-cellulose	diethyl amino ethyl cellulose
DNA	deoxyribosenucleic acid
dNTP	deoxyribosenucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GA_3	gibberellic acid
GRAV	*groundnut rosette assistor virus*
GRD	groundnut rosette disease
GRV	*groundnut rosette virus*
GUS	β-glucuronidase
GUSINT	GUS (*uid*A) gene containing an intron
HCl	hydrochloric acid
*hpt*II	hygromycin phosphotransferase gene
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
kb	kilo base
kinetin	6-furfuryl aminopurine
KN	kinetin
LB	luria broth
LD_{50}	lethal dose at 50% mortality
mol. wt.	molecular weight
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog (1962) basal medium

NAA	α-naphthaleneacetic acid
NaOH	sodium hydroxide
NARS	National Agricultural Research System
*npt*II	*neomycin phosphotransferase* II gene
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGR	plant growth regulator
PPM®	plant protection mixture
PTGS	post-transcriptional gene silencing
RIM	root induction medium
RNA	ribose nucleic acid
RNase	ribose nuclease
satRNA	satellite RNA
SAT	semi-arid tropics
SDM	shoot development medium
SDW	sterile distilled water
SEM	shoot elongation medium
SIM	shoot induction medium
SSC	sodium chloride and sodium citrate buffer
TAE	Tris acetate-EDTA buffer
T-DNA	transfer DNA
TDZ	thidiazuron
TE	tries-EDTA
TGS	transcriptional gene silencing
Ti plasmid	tumour-inducing plasmid
Tris	Tris (hydroxymethyl) methylamine
TSWV	*tomato spotted wilt virus*
*Uid*A	*β-glucuronidase* gene
uv	ultraviolet
vir	virulence gene
WAI	weeks after inoculation
X-gluc	5-bromo-4-chloro-3-indoly β-D-glucuronide
YEB	yeast extract broth

UNITS

°C	degree celsius
µg /l	microgram per liter
µl	micro liter
µM	micro Mole
cm	centimeter
d	day/s
dia	diameter
h	hour/s
ha	hectare/s
kg	kilogram
L	litre
M	molar
mg /l	milligram per liter
min	minute/s
ml	millilitre
mm	millimeter
pH	negative log of H^+ ion
psi	pounds per square inch
rpm	revolutions per minute
s	second(s)
t	tons
vol	volume
w/v	weight per volume
wk	week
wt	weight

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1. INTRODUCTION

Introduction

Leguminosae is a very important family of angiosperms comprising of many species in relation to human nutrition, pasture and fodder needs. Important protein rich seed bearing plants, mostly herbaceous, such as peas, lentils, beans collectively known as pulses are members of this family. They rank next to cereals in terms of human nutrition. In quantitative significance they are far behind the cereals, however, gaining some due importance as food additives in the recent years. Domination of the cereals in the food sector allowed only marginal increases in the overall yield of pulses. Recent concerns over the importance of these crops led to augmented efforts to improve the equality and quantity. Classical and modern breeding technologies resulted in limited success in interchange of the desirable characters in these crops. Biotechnological improvement has emerged as a potential supplement to these crops. Advances in plant tissue culture, genetic transformation methods and simultaneous improvements in molecular biology techniques and gene isolations gave an impetus to these efforts. Amalgamation of all these strategies laid foundation for many potential strategies for crop improvement.

Food and forage legumes

There are several species and subspecies classified as food legumes. But, only few (15 to 20) genera are very important. Hundreds of cultivars within these genera are included in the agricultural practices, each having some selected attributes. Most important of these species are *Glycine max, Arachis hypogaea, Cicer arietinum, Lens culinaris, Pisum sativum, Lathyrus sativus, Cajanus cajan, Vigna radiata, Vigna mungo, Vigna aconitifolia, Vigna umbellata, Vigna unguiculata, Phaseolus vulgaris, Macrotyloma nuflorum* etc. These species constitute over 80% of total food legume

output. Initially many of these species were thought to be recalcitrant in tissue culture and later advancements of biotechnological techniques gradually eased the technical difficulties. Micropropagation was relatively easier when compared to adventitious shoot regeneration, where the shoots originate from pre-existing meristems. Adventitious regeneration is a pre-requisite for a successful genetic transformation. Each species responded differently in tissue culture and some of the protocols were successfully used for genetic transformation.

Groundnut or peanut (*Arachis hypogaea* L.) is one of the principal economic crops of the world (Cobb and Johnson, 1973). The peanut well known worldwide as groundnut and to lesser extent as earthnut, monkeynut and goobersnut, is not a true nut but rather an annual legume crop. The genus *Arachis* belongs to the sub-family pappillonacea of the family *leguminoseae*. *Arachis hypogaea* has the widest distribution of any *Arachis* species. It is a major crop in tropical and sub tropical areas of the world. *Arachis hypogaea L.* with related species are currently being evaluated for farmer use (Moss, 1985). *Arachis villosulicarpa* has only been cultivated by Indians in the northwestern part of the Brazilian state of Mato Grosso (Gregory et al., 1973). *Arachis repens* and *A.rachis glabrata* have been grown in different parts of South America as ground covers in urban areas. Groundnut is native of southern Bolivia/north west region of South America and comprises of diploid (2n=40), tetraploid (2n=40) and octoploid species (2n=80).

The groundnut is one of the worlds most popular and universal legume crops, cultivated in more than 100 countries in all six continents. The geographical classification of groundnut is delineated in six regions: the America, Africa, Asia, New East Asia, Europe and Oceania (Gregory et al., 1980). It is currently grown on 25.2 million ha worldwide with a total production of 35.9 million metric ton (FAO,

2005). Developing countries account for about 97% of the world's groundnut area and about 94% of total production (Freeman et al., 1999). On the global scale, India is a major producer of groundnut with a total production of 8.9 million tons per year. The crop is largely grown by smallholder farmers under rain fed conditions in the semi-arid tropics (SAT). The crop play a significant role in the farmers livelihoods by providing the nutritional security and fetching cash revenue.

It is a seed propagating, self-pollinating crop. It is perennial or annual legume with tetra-foliate, stipulate leaves, papillonate 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.

Groundnuts are utilized in several ways; the seeds contain high quality edible oil (~50%), easily digestible protein (~25%) and carbohydrates (~20%) for human as well as animal consumption. Groundnut is used for different purposes: food (raw, roasted, boiled, cooking oil), animal feed (pressings, seed, green material, straw) and industrial raw material (Nwokolo, 1996) in industrial countries including USA, Canada and Europe. Crop improvement by conventional breeding in this important oilseed crop is not as rapid as envisaged to meet the demands of increasing population, especially in seed quality improvement and developing virus and insect-resistant varieties. There is an important need to improve several commercially grown varieties in India and else where. Tools of genetic engineering can be exploited as an additional method for introduction of agronomically useful traits into established cultivars. Major seed proteins of groundnut as well as of other leguminous crop species, are deficient in the essential sulphur containing amino acid methionine.

Groundnut-Constraints

Groundnut is an economically important oil and protein rich crop. It has a significant impact in tropical and sub-tropical regions of Asia, Africa, and North and South America. Worldwide, groundnut is cultivated on approximately 985 kg per hectares and is an economically important legume crop in the world. As compared to other oil seeds groundnut plants are relatively drought resistant, which makes them especially important in semi arid regions where precipitation exceeds evaporation for only 2-7 months per year (Bunting et al., 1985). Ground crop grows best in sandy, well- drained soils. The groundnuts are also cultivated in a wide range of field conditions form clays to sands and form acidic to alkaline soils.

Most groundnuts are used as a cash crop and even small farmers may sell their entire harvest. In addition to seeds being of high value, plant residues are extremely important as fodder for cattle in many regions of the world. Shells are also used for fuel, soil conditioners, fodder, chemicals, resin extenders, cork substitute and for hard board (Gibbons, 1980). The groundnut is becoming increasingly important as an income source in tree plantations, such as coconut, rubber, or banana. In Africa and Asia, many groundnuts are intercropped between maize, sorghum and pulses. Productivity of this crop increased significantly over years owing to the development and adoption of improved varieties and hybrids. However, there are several constraints to the productivity of the groundnut crop that result in great economic losses annually. Although some of the wild relatives of *Arachis hypogaea* have been identified as resistance source to several diseases and pests, the success in transferring the desirable traits to cultivated varieties has been limited due to reproductive barriers, and frequent failures in the interspecific crosses.

Groundnut is one of the most susceptible host crops to *A. flavus* invasion and subsequently aflatoxin production. After identification of the groundnut aflatoxin problem in 1963, (Bampton, 1963) many studies have been done in a number of groundnut-producing countries. It is evident that groundnut could be invaded by *A. flavus* and *A. parasiticus* and subsequently become contaminated with aflatoxin, before harvest and post harvest (Cole et al., 1982; Sander et al., 1985; Dorner et al., 1989). The extent of contamination varies with geographic location, agricultural and agronomic practices, storage and processing period. In some regions, contamination is predominantly preharvest while in others it is major post harvest (Swindale, 1987; Ahmed et al., 1989). Adopting some cultural practices, curing and drying and storage practices can minimize aflatoxin contamination. But these practices may not be suited to small-scale farming in developing countries, especially in tropical areas. Chemical control and removal of toxin have not yet been completely successful (Mehan et al., 1987). It was suggested that an effective solution to the problem would be the use of groundnut varieties that are resistant to infection by the aflatoxin-producing fungi, or resistant to aflatoxin production if colonized by the fungus (Mehan et al., 1987; Mixon, 1986; Petit et al., 1987).

A more recalcitrant plant/microbe interaction is that of seed-infecting fungi. These fungi are weak facultative pathogens and can exist as saprophytes on diverse non-living substrates and thus do not exhibit many of the attributes of obligate or hemi-obligate pathogens. A particularly troublesome seed colonizer is the genus *Aspergillus* that infects oil seeds, especially groundnut, maize and cottonseed, and contaminates them with aflatoxin or sterigmatocystin, two related carcinogenic mycotoxins. The presence of deteriorative fungi, *Aspergillus flavus* and *A. parasiticus*, with ability to produce mycotoxin of type "aflatoxins" in groundnuts

represents a great hazard for human and animal health, and it has been reported grown in all countries around the world (Williams et al., 2004). Aflatoxins are toxic, carcinogenic, teratogenic and immuno-suppressive substances. Aflatoxins, produced by *Aspergillus flavus* and *A. parasiticus*, are associated with both acute and chronic toxicity in animals and humans including acute liver damage, liver cirrhosis, induction of tumor and teratogenic effects. Studies during the past decade have shown the direct and indirect role of aflatoxins in immune suppression, interference with protein and micro nutrient metabolism, and synergistic action with Hepatitis B and C virus infection in causing liver cirrhosis and hepatocellular carcinoma (Turner et al., 2003; Williams et al., 2004).

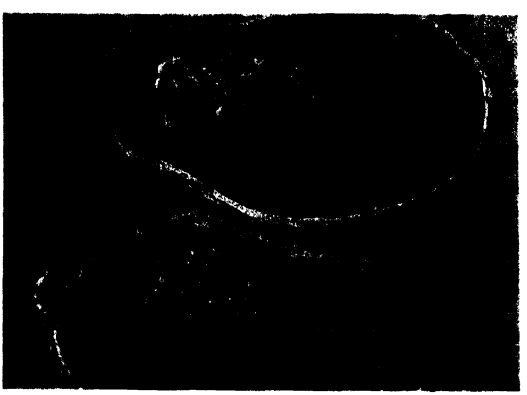

Figure 1: Groundnuts infected with *Aspergillus* fungi are the source of aflatoxin which is a primary problem for groundnuts worldwide. The toxin is carcinogenic, immune suppressing, anti-nutritional and is a major barrier to trade in groundnuts (Dely et al., 2005).

The frequency of pre-harvest infection of groundnut with *A. flavus /A. parasiticus* and aflatoxin contamination is very high in SAT, especially when end-of-season drought occurs (Waliyar et al., 2006). Although groundnut produced in all countries are prone to aflatoxin contamination, situation is particularly alarming in South Asia and Sub-Saharan Africa, as most of the groundnut is cultivated under semi-subsistence, rainfed conditions in poor soils that favor the fungal infection. About 100 countries have established regulations for controlling aflatoxins, and have set stringent limits on the levels of aflatoxins permissible in groundnut and groundnut-products (van Egmond and Jonker, 2002). For instance, the USDA has set 20μg kg^{-1} and European Union has set a limit of 2 to 4 μg kg^{-1}. Several other countries have set limits ranging form 10 to 30 μg kg^{-1}. Frequent aflatoxin contamination in groundnut produced in developing countries has drastically reduced groundnut exports for Asia and sub-Saharan Africa (Freeman et al., 1999; Ntare et al., 2005).

Althrough extensive efforts in 1980s on identification of resistance to *A. flavus/A. parasiticus* infection and aflatoxin contamination has resulted in the identification of resistance varieties. However, the levels of resistance are not sufficient to control aflatoxin to below permissible levels. At present, integrated crop management practice that can suppress *A. flavus/A. parasiticus* infection are being advocated as strategy to mitigate aflatoxin contamination (Waliyar et al., 2006). However, adoption of interventions of this kind depends on several socio-economic conditions and is not always possible for subsistence farmers to implement. Therefore there is an urgent need for easy-to-utilize strategies to limit pre-harvest aflatoxin contamination. Development of groundnut germplasm with good resistance to *A. flavus/A. parasiticus* invasion and aflatoxin production would be the most convenient and economical option for farmers. However, conventional resistance sources with

adequate levels of resistance in groundnut are not available. The introduction of antifungal genes via genetic engineering techniques to combat fungal diseases has been shown to be an effective strategy for rapid deployment of resistance to pathogens. Recently several antifungal genes have been identified that are involved in plant defense against fungal infection, and also the genes involved in the regulation of aflatoxin biosynthesis that can be exploited in the control of aflatoxin contamination in groundnut. In order to augment resistance to pre-harvest aflatoxin contamination similar approaches to deploy antifungal and anti-aflatoxin genes have been initiated and have shown the potential of such approaches in developing transgenic resistance to *Aspergillus* spp. and aflatoxin production (Niu et al., 2004; Sharma et al., Unpublished; Keller at al., Personal communication; Shah et al., DDPSC, Personal communication). Therefore, we undertook genetic engineering-based strategy to deploy novel antifungal and anti-aflatoxin production genes to incorporate durable and sustainable resistance to preharvest aflatoxin contamination in popular groundnut cultivars. Promising transgenic events will be selected and deployed for farmer cultivation and simultaneously, they will also be used as resistant donors to incorporate resistance into groundnut cultivars through conventional breeding programmes. ICRISAT has successfully developed the techniques for efficient transformation and regeneration of groundnut that has already resulted in the development of several transgenic groundnut events for various biotic and abiotic stresses and nutritional enhancement (Sharma, 2005). Work on the incorporation of antifungal genes such as '***rice chitinase***' and '***pea glucanase***' is ongoing at the Genetic Transformation Laboratory of ICRISAT. The use of novel anti-fungal/aflatoxin genes, such as '***defensin***', '13S and 9S LOX ***(lipoxygenase)*** genes' (Burow et al., 1997, 2000; Tsitsigiannis et al., 2005), '*rice chitinase*' (Anuratha et al.,

1991) and the use of the emerging RNAi technology against the fungal growth and aflatoxin production will provide a broad scope for developing good *A. flavus* and aflatoxin resistant groundnut events. The defense responses activated by groundnut-*Aspergillus flavus* interaction are illustrated in figure below.

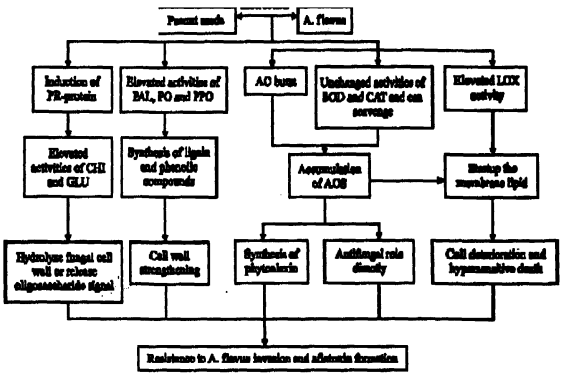

Figure 2: Schematic drawing of defense responses activated by the interaction of groundnut-*Aspergillus flavus*; PR: pathogenesis-related protein, PAL: phenylalanine ammonia lyase, PO: polyphenol oxidase, AO: active oxygen, SOD: superoxide dismutase, CAT: catalase, LOX: lipoxygenase, CHI: chitinase, PPO: polyphenoloxidase, AOS: active oxygen species, GLU: β-1, 3-glucanase (Liang, et al., 2005).

Anti-fungal/aflatoxin genes

1) Lipoxygenase

Aspergillus spp. grows and produces aflatoxin (AF) on lipid rich seed. The primary fatty acids found in seed are linoleic, oleic and palmitic acid. Unsaturated

fatty acids (i.e., linoleic acid) and their derivatives are known to affect sporulation, sclerotial production, cleistothecia production and mycotoxin production in *Aspergillus* species (Calvo et al., 1999). The primary effect is to induce asexual sporulation in Aspergillus spp., possibly by mimicking the effect of endogenous sporogenic factors called *psi* factors that are derived form linoleic acid. Depending on the configuration of the lipid moiety, it can either inhibit AF production or possibly extend AF production. For example, in plants, lipoxygenase (LOX) enzymes convert linoleic acid into either 9*S*-HPODE or 13*S*-HPODE hydroxylated derivatives.

An awareness that plant LOX enzymes are stress response enzymes induced by both abiotic and biotic factors has led to a series of studies investigating their role in plant defense (Bell and Mullet, 1991, 1993; Ohta et al., 1991; Farmer and Ryan, 1992; Melan et al., 1993: Ricker and Bostock, 1993; Geerts et al., 1994; Royo et al., 1996; Veronesi et al., 1996; Heitz et al., 1997; Rance et al., 1998). Each LOX produces different proportions of two stereo-specific linoleic (and linolenic) acid oxidation products: 9*S*-HPODE (9*S*-HPOTE form linolenic acid) and 13*S*-HPODE (13*S*-HPOTE form linolenic acid). Metabolites generated from the 13*S* pathway, particularly methyl jasmonate, were found to function as signals to induce expression of genes for defense response in plants (Farmer and Ryan, 1992) and a series of studies have shown that these metabolites are directly or indirectly involved in the response of plants to pathogen attack (Farmer and Ryan, 1992; Melan et al., 1993; Peng et al., 1994; Rance et al., 1998).

Recent reports have implicated the LOX pathway as playing a significant role in the *Aspergillus*/seed interaction. Studies have shown that C_6-$C1_2$ products of the LOX pathway inhibit *Aspergillus* spore germination (Doehlert et al., 1993; Zeringue et al., 1996) and that methyl jasmonate inhibits aflatoxin biosynthesis but not fungal

growth (Goodrich-Tanrikulu et al., 1995). We have found that 9*S*- and 13*S*-hydro peroxides differentially affect *Aspergillus* mycotoxin biosynthesis (Burow et al., 1997; Gardner et al., 1998) and that these same hydro peroxides act as *Aspergillus* sporulation factors (Calvo et al., 1999), suggesting that LOX isozymes play a role in regulating *Aspergillus* infection and aflatoxin contamination in oil seed crops. Of particular interest is the *in vitro* observation where exogenous 9*S*-HPODE extended the time of aflatoxin gene transcription whereas exogenous 13*S*-HPODE and 13*S*-HPOTE inhibited aflatoxin gene transcription (Burow et al., 1997).

The original interest in groundnut seed LOXs was due to their role in groundnut palatability and shelf-life (Sanders et al., 1975; Pattee and Singleton, 1977). The three groundnut LOX isozymes showed similar pH activity profiles to that of three major soybean LOX suggesting that one produced primarily 13*S*-hydroperoxy fatty acids, one primarily 9*S*-hydroperoxy fatty acids and one produced significant amounts of both products (Sanders et al., 1975; Pattee and Singleton, 1977).

2) Chitinases and β-1, 3-glucanase

The importance of chitinase and β -1, 3-glucanase as key enzymes responsible for fungal cell and sclerotial wall lysis and degradation has been reported (Cook et al., 1983). These enzymes have been shown to be produced by several fungi and bacteria and may be an important factor in biological control (Artigues et al., 1984; Elad et al., 1982; Ordentlich et al., 1988).

Plants express a wide variety of genes in response to pathogen/pest infection. Such genes are referred to as pathogenesis-related (PR) genes (Bowles 1990). The best-characterized genes belonging to this group are those that encode the hydrolytic enzymes known as chitinases (EC 3.2.1.14) and β-1, 3-glucanases (EC 3.2.1.39).

These hydrolytic enzymes inhibit the growth of many fungi *in vitro* by hydrolyzing the chitin and β-glucan of fungal cell walls. Furthermore, oligomeric products of digested chitin and β-glucan can act as signal molecules to stimulate further defense responses. These lytic enzymes have attracted much attention and have become very important resources in the genetic engineering of crop plants for disease resistance (Muthukrishnan et al., 2000).

In many plant–pathogen interactions, inducible pathogenesis related (PR) proteins have been well documented (Linthorst, 1991). However, in groundnut, very little has been done in the area of characterization of PR proteins and disease resistance. It has been reported that groundnut contains endogenous chitinases (Kellmann et al., 1996), although there are no reports of endogenous β-1, 3-glucanase in groundnut. In corn, PR proteins include hydrolases (chitinases and β-1,3-glucanases), which degrade structural polysaccharides of the fungal cell wall (Huynh e al., 1992); ribosome inactivating proteins (RIPs), which modify and inactivate foreign ribosomes (Guo et al., 1997; Walsh et al., 1991); and zeamatin which increases permeability of fungal cell membranes (Guo et al., 1997,Roberts et al., 1990). β-1, 3-glucanase is a well-known PR protein that is constitutively expressed at low levels in plants and can be dramatically induced when plants are infected by fungal, bacterial, or viral pathogens (Leubner-Metzger et al., 1999). Several experiments have demonstrated that β-1,3-glucanase was partially able to degrade the cell wall and inhibit mycelial growth or spore germination of certain pathogenic fungi (Leubner-Metzger et al., 1999). In corn seed, β-1,3- glucanase has been proposed as mechanisms for inhibiting the growth of *A. flavus* (Lozovaya et al., 1998; Neucere et al., 1995).

The physiological role of chitinases in the general metabolism of plant cells has not been documented. Indeed, a general role is difficult to envisage, because its substrate, chitin, does not occur in higher plants. It has been postulated that plants produce chitinase in order to protect themselves from chitin-containing parasites (Abeles et al., 1970; Bell, 1981; Boller, 1985) but, until recently, direct evidence supporting this hypothesis was lacking (Boller, 1985). Chitin, a linear polymer of *N*-acetyl glucosamine (NAG), is selectively degraded by the chitinolytic organisms and used as a carbon source for their growth and multiplication. However, in 1988, Roberts and Selitrennikoff reported that endochitinase purified form barley, was capable of inhibiting the growth of *Trichoderma reescei*, *Alternaria alternata*, and *Neurospora crassa*. In addition, Mauch et al. (1988) reported that in combination, chitinase and β-1, 3-glucanase act synergistically to inhibit fungal growth. These results, therefore, support the hypothesis that the in vivo role of these pathogenesis-related proteins is to protect the host form invasion by fungal pathogens and that, as such; they are an integral component of a general disease resistance mechanism.

OBJECTIVES

The objectives of the present study were to develop groundnut varieties with very low to non-existent levels of aflatoxin contamination conforming to the levels specified by various countries. The specific objectives were as follows:

1) Sub cloning of Lipoxygenase gene (*Pnlox 3*) from pTMK 12.6 along with 35*S* promoter and Poly A terminator into binary vector.
2) To develop transgenic groundnut with genes for resistance to *A. flavus* infection (*Rice chitinase)* and aflatoxin production (groundnut 13*S* and 9*S* LOX).

2. REVIEW OF LITERATURE

Review of literature

2.1. Plant biotechnology and its scope in crop improvement

Biotechnology offers a wide potential for 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 viruses (Van den Elzen et al., 1989) and the nutritional improvement like the golden rice (Burkhardt et al., 1997).

The objective of plant biotechnology was 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 Oritz, 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.

2.2. Current status of transgenic crops

Since the first introduction of Flavr Savr® tomato by Calgene Inc. in 1994, transgenic crops developed by introducing useful genes has become a general practice. During the last decade, worldwide cultivation of biotech crops has been increasing at a fast pace. During the nine-year period form 1996 to 2004, global area of biotech crops has increased more than 47-fold from 1.7 million ha to 81.0 million ha with an increasing proportion grown in developing countries. More than one-third (34%) of the global biotech crop area of 81 million ha in 2004, equivalent to 27.6 million ha, was grown in developing countries. The absolute growth in biotech crop area between 2003 and 2004 was, for the first time, higher for developing countries (7.2 million ha) than for industrial countries (6.1 million ha), with the percentage growth was almost three times as high (35%) in the developing countries of the South, compared with the industrial countries of the North (13%). The increased area and impact of the five developing countries (China, India, Argentina, Brazil and South Africa) growing biotech crops are an important trend with implications for the future adoption and acceptance of biotech crops worldwide (ISAAA, 2004).

2.2.1. Transgenic crop area by country

Countries that grow 50,000 ha, or more, of transgenic crops are classified as biotech mega countries. In 2004, there were 14 mega-countries, compared with 10 in 2003, with Paraguay, Spain, Mexico and the Philippines joining the mega- country group for the first time in 2004. This 40% increase in the number of mega-countries

reflects a more balanced and stabilized participation of a broader group of countries adopting biotech crops. The 14 mega-countries, in descending order of hectarage of biotech crops, were: USA with 47.6 million ha (59% of global total), followed by Argentina with 16.2 million ha (20%), Canada 5.4 million ha (6%), Brazil 5.0 million ha (6%), China 3.7 million ha (5%), Paraguay with 1.2 million ha (2%), India 0.5 million ha ((1%), South Africa 0.5 million ha (1%), Uruguay 0.3 million ha (<1%), Australia 0.2 million ha (<1%), Romania 0.1 million ha (<1%), Mexico 0.1 million ha (<1%), Spain 0.1 million ha (<1%), and the Philippines 0.1 million ha (<1%). Based on annual percentage growth in area, of the eight leading biotech crop countries, India had the highest percentage year-on-year growth in 2004 with an increase of 400% in Bt cotton area over 2003, followed by Uruguay (200%), Australia (100%), Brazil (66%), China (32%), South Africa (25%), Canada (23%) Argentina (17%) and the USA at 11 % (Clive James, 2004).

Tools of molecular biology and genetic engineering have provided with unprecedented power to manipulate and develop novel genotypes towards a safe and sustainable agriculture in the 21st century. However, it has now been realized that traditional plant breeding methods may not be sufficient to meet the increasing demand for food production (Mann, 1999). Many of the classical breeding methods are time consuming and labour intensive, and their success is constrained by limited variability in the available germplasm of different crops. Therefore, modern biotechnological tools in combination with traditional technologies hold great promise for augmenting agricultural productivity in quantity as well as quality. Gene transfer technologies developed during the last two decades have proved beyond doubt that agricultural production is going to be revolutionized in several ways. The efficacy of transgenic crop varieties in increasing production and lowering production costs has

already been demonstrated (Borlaug, 2000; Herrera-Estrella, 2000; Chrispeels, 2000; Prakash, 2001).

Development of transgenic crops during 1990s is an important landmark in the history of crop improvement. Since the first commercial release of transgenic crops in 1994 have registered steady increase in area (67.7 mha) and have slowly spread across 18 countries (James, 2003). Development and deployment of transgenic plants in an effective manner will be an important prerequisite for sustainable use of biotechnology for crop improvement. As a result of advances in genetic transformation and gene expression during the last decade (Hilder and Boulter 1999; Sharma, 2000; Sharma and Oritz, 2000) there has been a rapid progress in genetic engineering for crop improvement, of which protection of crops against the insects is a major goal. In addition to widening the pool of useful genes, genetic engineering also allows the use of several desirable genes in a single event and reduces the time to introgress novel genes into elite background. Research on transgenic crops, as is the case with conventional plant breeding and selection by the farmers, aims to selectively alter, add or remove a character of choice in a plant, bearing in mind the regional need and opportunities. It not only offers the possibility of bringing in a desirable character from closely related plants, but also of adding desirable characteristics from the unrelated species. After the transformation event, the transformed plant becomes a parent for use in conventional breeding programmes.

2.3. Tissue culture and regeneration

2.3.1. Tissue culture

The term tissue culture in general includes in vitro culture of various kinds of explants ranging from cells to tissues and tissues to organs. With the availability of

suitable conditions, each living cell of a multicellular organism is capable of ndependently developing into a complete plant or organism (White, 1963). This capability is termed as *'cellular totipotency'* (Vasil and Hildebrandt, 1965; Ritchie and Hodges, 1993). 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 an important role in altering the cellular functions or ' inductive stimulus'. The capability of a plant cell or group of cells to respond to an inductive stimulus for developmental process is referred to as competence (Mein and Binns, 1979). Tissue explants are mixture of cells varying physiologically, biochemically and developmentally (Lindsey and Yeomm, 1985). Development of an efficient regeneration system is a pre requisite for genetic transformation of plants and the transformation efficiency is generally been proportional to the efficiency of the tissue culture and gene transfer systems (Birch, 1997). The most important factors for efficient regeneration in the tissue culture system depends on the Cellular competence, and factors affecting in vitro cultures such as- type of genotype, source of explant, culture conditions and growth regulators.

Competence is the first step in the dedication of one or more undifferentiated 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).

Source of explant and ontogenic stage: The successful culture of explant 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 responsive. The immature organs or meristematic and undifferentiated tissues are most responsive and reliable explant source (Vasil and Vasil, 1986). The reason for the stage specific response may be due to genetic, epigenetic, or physiological changes that occur in mature cell (Vasil, 1988). Other factors to consider include size, orientation in culture, pre-treatment and inoculation density (Brown and Thorpe, 1986).

Culture conditions and growth regulators: Each type of tissue or explant requires different formulation, depending on whether the objective is to obtain optimum growth rate or induce organogenesis. Composition of culture medium is an important factor in the successful establishment of tissue cultures. 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 Eveligh, 1968). A standard or basal medium consists of balanced mixture of macro and micronutrients (salts of chlorides, nitrates, sulphates, phosphates, iodides of calcium, magnesium, potassium, sodium, iron, manganese, zinc and boron), vitamins, carbon source, organic growth factors (amino acids, urea and peptones), source of reduced 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 myo-inositol, nicotinic acid, pyridoxin, thiamine etc; and carbohydrates are supplied usually as sucrose (Thorpe, 1980). The

most commonly used amino acid is glycine. In addition, phytohormones (auxins and cytokines) 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 standardized for particular plant tissue. The auxins that are commonly used in culture media are IAA (indole-3-aceticacid), 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (α naphthaleneacetic acid) and IBA (indole-3-butyricacid). The cytokinins are kinetin (6-furfuryl aminopurine), BA (N6-benzyladenine), zeatin, 2-ip (2-isopentenyl adenine) etc. The hormones are physiologically active in very small quantities. The pH of the medium determines the alkalinity and acidity of the final solution, greatly influences the uptake of ingredients, solubility of salts and gelling efficiency of agar. Generally, a pH of 5.6 to 5.8 is found to be stable for maintaining all the salts in a near-buffered form. Physical conditions also play a major role in vitro culture. These include light (intensity, quality and photoperiod), temperature and culture container (including container size, permeability of gas exchange). Salas et al. (2001) reported the temperature influence greatly on the transfer mechanism and stable integration in the plant cells.

2.3.2. Pathways of regeneration

The rate of plant regeneration in tissue culture varies greatly from one species to another. Various cells, tissues and organs from numerous plant species can be successfully cultured aseptically to regenerate whole plants. Number of protocols for plant tissue culture has been developed during the past few decades and many plant species can now be regenerated in vitro. Several pathways have been put forward to depict the regeneration processes and the majority of them are either through organogenesis or embryogenesis.

Plant tissues regenerate *in vitro* through two pathways, namely 'organogenesis' wherein shoot buds are organized by concerted meristematic activity of a number of cells and 'embryogenesis', where usually single cell or a small cluster of cells undergo differentiation to produce somatic embryos similar to zygotic embryos. The regeneration of complete plants via tissue culture has made it possible to introduce foreign genes into plant cells and recover transgenic plants. Morphogenesis could occur directly from the explant or indirectly via the formation of a dedifferentiated callus. However the different pathways of regeneration, viz. organogenesis from callus (pathway I), embryogenesis from callus (pathway IV), organogenesis directly from explants (pathway II) and embryogenesis from explants in a direct mode (pathway III) vary in their amenability to different gene delivery techniques. The different pathways of regeneration and their amenability to the two major methods of gene delivery are shown in Figure 2.

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 medium to be induced and development directly from the explant, termed as organogenesis (Thorpe, 1970; Christianson and Warnick, 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. In general high cytokinin: auxin produces shoots (caulogenesis), low cytokinin: auxin ratio induces 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 wide applicability in genetic transformation studies.

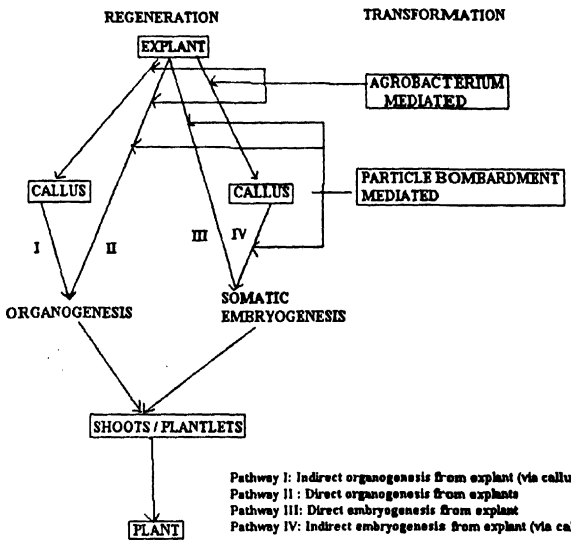

Figure 3: Flow chart showing different pathways of *in vitro* regeneration and their amenability to the two major methods of gene delivery (Atika et al., 2003).

Embryogenesis

Somatic embryogenesis is a developmental pathway in which embryos are induced from a somatic cell or a group somatic of 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 be proceeding 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).

2.4. Gene cloning and gene transformation methods

2.4.1. Gene cloning and vector constructs

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. Transferred gene is called the transgene and the whole process is referred to as transgenesis. One of the most important elements in recombinant DNA (rDNA) technology is cloning of gene into suitable vector. The gene cloning is the process of isolation and multiplication of an individual gene sequence by insertion of that sequence into a bacterium where, it can be replicated. A part of genomic DNA or cDNA segment or specific gene linked to a vector forms an rDNA molecule, which can be propagated in suitable host cells to a large number is a cloning vector. There are different types of cloning vectors for use with different types of host cells. The largest number exists for *Escherichia coli* and the best known of these is the plasmid vector. Most plasmid vectors in current use carry a replicon derived from the plasmid pMB1 (Ausubel et al., 1990). Plasmid vectors used for cloning have been specially developed by adding certain features like: a) Reduction in size of vector to a minimum; b) introduction of selectable

markers and synthetic polycloning sites; c) Incorporation of axillary sequences etc. The process of gene cloning has four essential components that include: 1) Cloning vehicles or vectors. 2) Enzymes for cutting and joining the DNA fragment into vector molecules. 3) DNA fragments, i.e., gene libraries. 4) Selection of a clone of transformed cells that has acquired the recombinant chimeric DNA molecule (Susman and Milman, 1984).

2.4.2. Gene transformation methods

Despite significant advances over the past decade, development of efficient transformation methods can take many years of painstaking research. Groundnut transformation like all other transformation system relies on the common key elements. The major components for the development of transgenic plants are: (1) the development of reliable tissue culture regeneration systems, (2) preparation of gene constructs and transformation with suitable vectors, (3) efficient techniques of transformation for the introduction of genes into the crop plants, (4) recovery and multiplication of transgenic plants, (5) molecular and genetic characterization of transgenic plants for stable and efficient gene expression, (6) transfer of genes to elite cultivars by conventional breeding methods if required, (7) evaluation of transgenic plants for their effectiveness in alleviating the biotic and abiotic stresses in the field condition. (8) biosafety assessment including health, food and environmental safety, and (9) deployment of genetically modified plants.

Transformation of plants involves the stable introduction of DNA sequences usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. Transformation without regeneration and regeneration without transformation are of limited value. The genetic transformation generally involves two genes. One is the gene of interest that should be integrated into plant genome and expressed in the

transgenic plant and the other is selectable gene. Each of the two transgenes should thus have its own promoter and Poly A signal. 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). The steps involved in gene cloning, regeneration and transformation are depicted in Figure 3.

Genetic transformation of plants is performed using a wide range of tools, the basic gene transfer techniques are grouped under two categories (Potrykus, 1985).

2.4.2.1. Direct DNA gene transfer

2.4.2.1.1. Physical gene transfer methods

a) Electroporation

b) Particle bombardment/ micro projectile/ biolistics

c) Microinjection

d) Macroinjection

e) Ultrasound mediated transformation/ Sonication

f) Liposome mediated transformation

g) Silicon carbide fiber mediated transformation

2.4.2.1.2. Chemical gene transfer methods

a) PEG mediated gene transfer

b) Transfection using Calcium Phosphate

c) The poly cation DMSO technique

d) DNA inhibition by cells, tissues, embryos and seeds

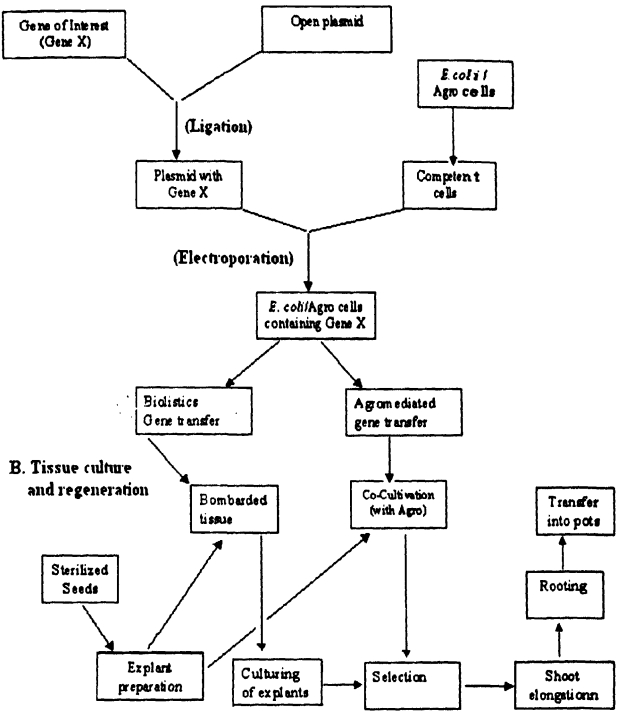

Figure 4: A schematic view of the various strategies for the genetic transformation of crop plant. A. Cloning and transformation; B. Tissue culture and transformation.

2.4.2.2 Vector mediated gene transfer

2.4.2.2.1. *Agrobacterium tumefaciens* mediated transformation

2.4.2.2.2. Virus mediated transformation

The status and problems of genetic transformation technology was reviewed in detail by Sharma et al. (2005). Amongst all these techniques, *Agrobacterium*-mediated transformation and particle bombardment/ micro projectile /biolistics are more popular and widely used for gene transformation due to their greater reliability.

2.4.2.1. Direct DNA gene transfer

2.4.2.1.1. Physical gene transfer methods

Various means of DNA transfer by physical methods include

a) 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 by 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 of 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 "Electro injection". Various advantages of this procedure are as follows:

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. Within 10 minutes of pulsing stable transformants can be selected.

b) 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 particle gun. The method was developed to overcome the limitation 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, and pollen. The method involves bombardment of particles carrying DNA of interest onto target cells using high velocity transfer mechanism. Sanford et al. first developed the method 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. Boynton et al. (1988) carried out particle bombardment on Chlamydomonas that could stably integrate transgenes in the chloroplast genome. Subsequently Sva'b et al. (1990) produced tobacco plants with stably transformed chloroplasts. The technique is finding universal application in the direct transformation of whole cells in culture, in tissue and in sub cellular organelles such as mitochondria and chloroplasts.

Biolistics have been proven to be the most effective means of transformation of plant organelles (Sva'b et al., 1990; Daniell, 1991). 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 (McCabe et al., 1988) have proven to be transformable and able to

give rise to transgenic plants (Finer et al., 1999). Transgenic plants through bombardment were produced from wide range of crops including both in dicots and monocots important of crop species. This include- from shoots apices of maize (Zhong et al., 1996); immature zygotic embryos of wheat (Becker et al., 1994); immature inflorescence of *Tritordeum* (Barcelo et al., 1994); leaves and pollen grains of tobacco (Tomes et al., 1990 and Stoeger et al., 1995) and embryonic cell suspension of soybean (Finer and McMullen, 1991). Stable transformation was reported in maize and soybean (Kamm et al., 1990), *Glycine max* (Hadi et al., 1996; Hu et al., 1999), *Gossypium hirsutum* (McCabe and Martinell, 1993), *Phaseolus vulgaris* (Russell et al., 1993; Zhang et al., 1997), *Arachis hypogea* (Schnall et al., 1993; Brar et al., 1994; Livingstone and Brich, 1995), *Oryza sativa* (Toriyama et al., 1988), *Zea mays* (Lowe et al., 1995), *Hordeum vulgare* (Ritala et al., 1994), sorghum (Devi and Sticklen, 2001), pearl millet (Devi and Sticklen, 2002). Although, the biolistic-mediated transformation technique has been shown to be highly successful with broad efficacy, due to its less precise in its transgene integration pattern, it may lead to multiple copy number and emergence of chimera plants limiting their further usage (Finer et al., 1999).

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. In this method DNA of interest is coated on heavy micro particles 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 macro projectile and are accelerated into living target cell at a very high initial velocity. Nylon macro projectile is withheld in the barrel after a short while by the stopping plate while only DNA coated macro projectile 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 for the tissue being penetrated and potential of the target tissue or cell to the regenerate into plant are the important factors. Biolistic transformation technique has been shown to be successful with papaya, sugarcane, soybean, tobacco, etc. Genomes of sub cellular organelles have been made accessible to genetic manipulations by this method.

Advantages

1. Need of protoplast obtaining can be avoided.
2. Walled intact cells can be penetrated.
3. Genome of sub cellular 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).

c) Microinjection

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 to tissues) is more difficult

than for animal cells but now it is bitterly 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 microinjected in the time in which 10^6 cells can be transfected. Thus method is slow.

d) Macroinjection

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).

e) 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 plasmid containing the desired gene (Zhang et al, 1991).

2.4.2.1.2. Chemical gene transfer methods

Chemical methods of DNA transfer involves:

a) PEG mediated gene transfer

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.

b) Transfection using Calcium Phosphate

The process of transfection involves the admixture of isolated DNA (10-100 ug) 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.

2.4.2.2. Vector mediated gene transfer

2.4.2.2.1. *Agrobacterium tumefaciens* mediated DNA transfer

Transformation of plants through *Agrobacterium*-mediated DNA transfer is currently the most commonly used means of accomplishing plant gene transfer (Gheysen et al., 1998). The nature's 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. rDNA method allowed us to develop gene vectors based on this natural process. The desired genes are cloned along with promoter into these vectors using standard molecular cloning techniques, re-introduced into the bacterium, which is then co-cultivated with the plant tissue to be transformed. The specific piece of T-DNA containing the gene of interest 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 larger number of crop plants.

Molecular basis of *Agrobacterium*- mediated 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 and certain monocotyledonous plants (Zaenen et al., 1974). The molecular studies on *Agrobacterium* and subsequent findings of Kerr (1971) were very useful to establish the central role of *Agrobacterium* plasmids in crown gall

development. Zaenen et al. (1974) first noted that virulent strains of *Agrobacterium* which harbor large plasmids. The ability to cause tumor lies within the plasmids where by the T-DNA is transferred into the genome of infected plants (Vanlarebeke et al., 1974). Series of the classic experiments by Braun et al. (1958) demonstrated that once tumor formation has been initiated, the further presence of *Agrobacterium* is not required for subsequent tumor proliferation.

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; Bomhoff et al., 1976) and nopaline synthase enzymes (Montoya et al., 1977). Tumor inducing Ti plasmids and the *Agrobacterium tumefaciens* strains harboring them can be classified according to the type of opines produced. The three best studied opines are octopine, nopaline and agropine (Vandequin–Dranrart et al., 1995). The generation of tumors producing specific opines catabolyzable only by the inciting *Agrobacterium* strain is a central future of the pathogenic relationship between *Agrobacterium* and plant. Some of the plants regenerated from nopaline containing tumor tissue continue to synthesize nopaline (Schell and Van Montagu, 1979). The strains that utilize octopine induce tumors that utilize only octopine and the strains that utilize nopaline induce tumors that synthesize only nopaline (Bomhoff et al., 1976; 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 by using disarmed (non-tumorigenic) plasmid vectors of *Agrobacterium tumefaciens* 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 *Nicotine* and *Petunia* spp. (Horsch et al.,

mediated by the gene products of *vir a* and *vir g* (Stachel and Zambryski, 1986). The constitutively expressed Vir a protein acts as a chemoreceptor and transmits this information to Vir g protein possibly by phosphorylation mechanism (Jin et al., 1990). The *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 e and *vir a* (Anken bauer and Nester, 1990; Cangelosi et al., 1990).

***Agrobacterium*-derived vector systems**

The disadvantage of DNA transfer using wild type *Agrobacterium* strains is the levels of phytohormones in the transformed tissue, which prevents the regeneration of cells into whole plants. To overcome this difficulty disarmed Ti-plasmids were constructed by deleting the oncogenic genes of T-DNA. Two different types of vectors systems have been used to transfer foreign gene into plants in *Agrobacterium*-mediated transform method. They are as follows:

1) 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 322 which can then be mobilized to *Agrobacterium* and co-integrated into the T-DNA region (Rogers et al., 1987; Mozo and Hooykaas, 1992).

2) Binary vectors:

The knowledge gained from transformation experiments revealed that the T-DNA and the *vir* region do not have to reside on the same plasmid which paved the

way for the development of binary vector systems (AN et al., 1987). Based on two plasmids, harboring in *Agrobacterium* strains one having the trans acting *vir* functions to transfer T-DNA while the second plasmid carries T-DNA carrying genes of interest to be introduced into plant cells. This plasmid has the ability to replicate in two hosts both in *E. coli* and *A. tumefaciens* thus binary vectors systems are based on the separations of *vir* and T-DNA regions on two independent compatible plasmids (Hamilton et al., 1996; Helens and Mullineaux, 2000).

Promoters and terminators

Promoters:

Futterer (1995) reviewed the subject of promoters for genetic transformation of plants. In the early years of genetic transformation of plants, investigators were merely interested in showing that integration and expression of transgenes is a reality in plants. So, initially promoters endogenous to the T-DNA were used. Soon it was observed that the promoters for opine synthesis were weak. Chua and his collaborators Odell et al., 1985 isolated the CaMV 35S promoter from turnip leaves infected with the Cauliflower mosaic virus (CaMV). This promoter was found to be many folds stronger and resulted in constitutive expression of the introduced genes. However, sub-domains of this promoter were found to be exerting tissue specific expression (Benfey and Chua, 1989). Since then this promoter became an attractive candidate for plant molecular biology research. Its fusion with part of mannopine synthase (MAS) promoter increased the potency of this promoter (Kay et al., 1987). Valuable information can be found in the reviews by Benfey and Chua (1989), Wang and Cutler (1995).

The above promoters were found to be more efficient in dicots and there was a distinguished interest for finding out the promoters for monocots. In the early studies with rice (Shimamoto et al., 1989) the CaMV 35S promoter was used to activate the selective and reporter genes. However, it was found that this promoter was more efficient in dicots. Combination of this promoter with other promoter segments and introns were even tried. This concept was followed by the usage of cereal alcohol dehydrogenase I *(Adh I)* gene (Callis et al., 1987; Kyozuka et al., 1990). A similar approach to integrate the first intron of the *Shrunken I* gene of maize was also followed in cereal transformation (Mass et al., 1991), but it became less popular in the subsequent years. Rice actin gene promoter *(Act I)* was found to be even more potent than the above two (Zhang et al., 1991). This promoter showed more or less similar potency as that of *Emu* promoter that is a recombinant promoter containing a truncated *Adh I* promoter with other elements (Last et al., 1991). The current most effective promoter is the *Ubiquitin I (Ubi I)* of maize (Christensen et al., 1992). This promoter was used successfully to transform wheat (Weeds et al., 1993), barley (Wan and Lemaux, 1994) and rice (Toki et al., 1992). Another promoter of the rice *Aldolase P (Ald P)* gene was found to be one of the better alternative (Kagaya et al., 1995).

Terminators:

Knowledge of the elements for gene expression is as important as the promoters. It is considered that fundamentally mRNA is stable unless destabilizing motifs are involved. Specific examples of the studies that handled the poly adenylation signals in plants are investigations of Mogen et al. (1990). Rothnie et al. (1994) studied the essence of the terminator regions and impact of 3'-end regions on the level of gene expression of octopine synthase gene and other gene constructs was studied by Ingelbrecht et al. (1989). Hence, the usage of terminator region at the 3'

end of the transgene was found to be essential. In practice, terminator of nopaline synthase gene or of the CaMV was fused into the respective chimeric gene.

2.5. Selectable and screenable genes for plant transformation

Plant modification using recombinant DNA technology is the insertion of a known sequence of foreign DNA into the host plant genome. 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 genes of interest. A suitable method for selection of transgenic plants is one of the most important aspects in any transformation system. 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.

Genetic transformation of plants requires a marker gene to distinguish between transformed and untransformed ones. In recombinant DNA research the marker genes are physically linked to the gene of interest. The *Agrobacterium* encoding genes of nopaline synthase (Depicker et al., 1982; Bevan et al., 1983) and octopine synthase (DeGreve et al., 1982) were the first ones to be used as markers for the selection of transformed plants. Transformants can be easily 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- selectable marker genes and reporter/screenable genes.

Selectable marker genes

The most popular selectable marker genes used in plant transformation vectors include *neomycin phosphotransferase* II (*npt*II) and *hygromycin phosphotransferase II* (*hpt*II) genes for antibiotic resistance to such as kanamycin and hygromycin, and *bar* gene for herbicides resistance such as phosphinotricin, glyphosate, bialaphos and several other chemicals (Wilmink et al., 1993).

Reporter/ Screenable genes

Reporter genes are the 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 (1995) reviewed the literature on reporter genes used upto year 1994. The most commonly used reporter genes are cat (chloramphenicol acetyl transferase), *uid* A (GUS-*β-glucuronidase*), Luciferase and GFP (Green Florescent Protein).

Removal of selectable marker genes following transformation and selection

Once the transgenic plant has established there is no further need for the marker gene in most applications. This would ease public concerns relating to transfer of resistance into non-target species such as weeds or microbes (Dale and Ow, 1991). So, a mechanism for removing the marker trait several techniques has been employed. The first of these involves the simultaneous but independent "co transformation" of plant cells with the marker gene as well as desired gene DNA molecule (De Block and Debrouwer, 1991). At some frequency, these genes will integrate into the plants chromosomal DNA with a possibility of genetically distinct loci. In such cases, it may be possible to eliminate the selectable markers genes during normal chromosomal segregation (De Block and Debrouwer, 1997). Another strategy has employed is the

sequence specific DNA excision using as a functions of plant transposable elements (Yoder and Goldsbrough, 1994). With the Ac/Ds transposon system, the Ac element encodes a functional transposase, the enzyme that recognizes the transposons inverted terminal repeat structures, cleaving the element from one location in the genome and inserting into a separate locus. Assuming the Ds embedded marker gene has transported to a separate chromosome or a locus sufficiently far away, it would be then be possible to remove the marker gene via genetic segregation such as multiple rounds genetic crossing and evaluation (De Block and Debrouwer, 1993). A third strategy involves the use of the bacteriophage P1 *Cre/lox* recombination system. In this system, *Cre* recombinase recognizes *lox* excision site DNA sequences and precisely clips them from the genome. When a selectable marker is introduced in a sandwiched fashion between two lox sites, the subsequent introduction of Cre recombinase, either by activation from inducible promoter (Wanggen et al., 2001) or by single genetic cross with a separate *Cre* expressing transgenic line (Dale and Ow, 1991), will cause excision of the marker gene (s) between the *lox* elements. The excised element is unstable and subsequently lost from all progeny cell. Though these strategies are well established for elimination of selectable markers from transformed plants, however, in practice they are very cumbersome.

2.6. Analysis of transgenic plants for expression of introduced transgene

2.6.1. Molecular characterization

The foreign genes / DNA sequence introduced into plant cells are then grown in vitro to regenerate into whole plants. The putative transgenic shoots initiation and elongation in- vitro followed by rooting and transfer to the containment glasshouse for

further evaluation and subsequent sexual generations. Selection and growth of plant cells on selective media provide initial phenotypic evidence for transformation. This includes resistance to antibiotics herbicides etc. Molecular evidences are essential to confirm the integration of introduced genes, for the presence and expression followed by genetical analysis for inheritance (Birch, 1997). Various techniques used in molecular characterization for analysis is depicted in figure 4. The number of copies of a transgene in transformants is variable and depends on the method of transformation. Majority of the reports suggests T-DNA insertions by *Agrobacterium* mediated transfer are inherited in mendelian fashion (Budar et al., 1986; Feldmann and Marks, 1987) where as direct gene transfer methods often result in large direct repeated arrays of introduced DNA (DeJonge and Bootsma, 1984; Potrykus et al., 1985). The T-DNA copy number varies considerably in transformed plants (Thomashow et al., 1980, Zambryski et al., 1982) and two unlinked T-DNA elements can integrate into the same plant cell (Depicker et al., 1985; Petit et al., 1986). T-DNA junctions can vary from tandem inverted repeats, direct repeats or single copy insertions (Zambryski et al., 1982; Spielmann and Simpson, 1986). The expression of transgenes can vary considerably between independently transformed plants (Hobbs et al., 1993; Jefferson et a.l, 1990; Blundy et al., 1991). In some instances there is a positive correlation with copy number and other studies have shown no association or even negative correlation (Hobbs et al., 1993). Transgene expression may some times be unstable or may decline over generations (Vaucheret et al., 1998).

2.6.2. Inheritance of transgenes

Transgenes are expected to behave as dominant genes due to their hemizygous state in recipient genome and thus segregate as dominant loci in a typical 3:1 Mendelian ratio (Barro et al., 1998; Cambell et al., 2000). 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. Ulian et al. (1994) reported the variability in the inheritance pattern based on the detection of the transgene expression. Skewed segregation of the introduced genes during meiosis leading to non-mendelian inheritance may be caused due to various reasons such as linkage to a recessive lethal gene, mutational effect of T-DNA insertion and chromosomal re arrangement.

2.6.3. Expression of transgenes

Gene silencing (GS) is the phenomenon of non-or minimal expression of a transgene or a homologous gene in a transgenic plant (Hammond et el., 2001). Gene silencing may occur at transcriptional (TGS) and post transcriptional (PTGS) levels. TGS involves inhibition of transcription and association with methylation of promoter region. In cases of PTGS, though genes are transcribed, their mRNA is degraded. PTGS is associated with methylation of the coding region of the transgenes. TGS has been found to be associated with multiple copies of transgenes and inverted repeats (Irs) in transgenes, which can act in *cis* as well as in *trans* (Stam et al., 1997; Wang and Waterhouse, 2001), suggesting a homology dependent mechanism of gene silencing. PTGS has been associated with high copy number of transgenes, strength of the promoter or the stability of transcripts. Viruses are known to infect plants and accumulate their transcripts at very high level. PTGS may be a mechanism to stop transcription of viral genes. Recent studies indicate that some plants use PTGS to recover from viral infection and to stay free from future infection. As organisms compete with each other for their survival, some plant viruses have evolved mechanisms to counter or suppress PTGS (Ratcliff et al., 1997).

Since gene silencing is an unpredictable phenomenon, researchers have tended to discard plants showing silencing of the desired phenotype and use those plants which do not show silencing. Attempts to over express endogenous have often resulted in silencing of transgenes as well as endogenous genes, a phenomenon known as co-suppression. To avoid transgene-silencing care must be taken while designing transgene by following guidelines i.e. i) Avoid the use of potential methylation acceptor sites. ii) DNA repeats to be avoided by using different promoters and termination signals to drive expression of different genes in the vector. iii) Vectors can be designed to incorporate MARs or insulators and even the genes for suppression of gene silencing.

2.7. Regeneration and genetic transformation system in groundnut

There are numerous reports of tissue culture and transformation of groundnut from various explants (Kartha et al., 1981; Sastri and Moss, 1982; Kanyand et al., 1994). Regeneration of groundnut in vitro occurs through either organogenesis or embryogenesis. Regeneration by organogenesis in groundnut occurs by the development of shoots directly on the surface of cultured explants (McKently et al., 1991; Hazara et al., 1989). Shoot organogenesis and plants were also successfully obtained using immature leaflets (McKently et al., 1991; Daniel, 2002). Regeneration via somatic embryogenesis has been reported and used for transformation studies in groundnut (Ozias-Akins et al., 1989; Sellars et al., 1990; Chengalrayan et al., 1994 and 1997; Baker, 1995). However, conversion of somatic embryos into plants remains inefficient and limits the application of somatic embryogenesis in many systems including genetic transformation (Wetzstein and Baker, 1993). Direct regeneration systems have advantages, due to the rapidity of morphogenesis and no requirement of frequent subculture. Besides, *de novo* production of shoot primordial is extremely

rapid and initially synchronous with the period of cellular differentiation. Such a regeneration system favors easy accessibility for *Agrobacterium*-mediated genetic transformation. Though there are numerous reports of tissue culture and regeneration of groundnut from diverse explants not much success with genetic transformation of *Arachis* species was achieved until recently due to the lack of efficient protocols to regenerate whole plants from the transformed tissues. Sharma and Anjaiah (2000) successfully obtained high frequency direct shoot regeneration from cotyledons in various groundnut genotypes. The protocol published by Sharma and Anjaiah (2000) for the genetic transformation in groundnut have emboldened researchers to pursue the development of transgenic groundnut plants capable of producing resistant to various diseases, insect- pests and abiotic stresses. Several methods for DNA transfer are used for transformation of groundnut. Several workers reported the transfer of novel genes into actively growing groundnut cells using biologically based *Agrobacterium*–mediated transformation or the direct DNA delivery through micro projectile /bombardment methods. Groundnut tissue is susceptible to infection by wild type strains of *A. tumefaciens* (Lacorte et al., 1991). Some workers adopted non-tissue culture based approaches that do not depend on the regeneration of adventitious shoot buds for generating transgenic plants of groundnut (Rohini and Rao, 2000).

2.7.1. *Agrobacterium tumefaciens* mediated gene transfer

The earliest evidence for A*grobacterium tumefaciens* mediated transformation for gene transfer in groundnut using hypocotyl explants was reported for the first time by Dong et al., (1990) followed by Lacorte et al., (1991). Eapen and George, (1994) reported *Agrobacterium* mediated transformation using leaf explants of groundnut with a transformation frequency of 2%. Immature embryonic axis has also been employed as explant for *Agrobacterium* mediated transformation in groundnut.

Mckently et al. (1995) developed a procedure where embryonic axis from mature seeds of groundnut co-cultivated with *A. tumefaciens* was stably transformed. Similarly, a very low frequency of transformation was obtained with leaflet explants on regeneration medium, MS medium with BA (3 mg/L) and NAA (1 mg/L) supplemented with 100 mg/L kanamycin for selection. Cheng et al., (1997) obtained fertile transgenic plants with 0.3% frequency using leaf segments. Li and co-workers (1997) introduced the nucleocapsid gene of tomato spotted wilt virus along with the *uid* A and *npt*II marker genes in a sense orientation into groundnut using *Agrobacterium*-mediated transformation. Venkatachalam, (1998 and 2000) reported pre-cultured groundnut cotyledons co-cultivated for 2 days with *Agrobacterium* strain LBA 4404, harboring pBI 121 containing *uid* A and *npt*II genes, followed by transfer on an embryo induction medium containing NAA, BAP, kanamycin and cefotaxime resulted in transformed embryos, which efficiently gave rise to shoots (47%) on MS medium containing BAP and kanamycin. A non-tissue culture based transformation method involving direct co-cultivation of cotyledon attached embryo axis with *Agrobacterium* treated with wounded tobacco leaf extract resulted in a stable 3% transformation frequency (Rohini and Rao, 2000). An efficient system with high transformation frequency (>55%) based on cotyledon explants forming adventitious shoot buds (>90%) have been developed by Sharma and Anjaiah (2000). A number of independently transformed groundnut plants with coat protein gene of IPCV were produced by this method. A protocol was also standardized using immature embryonic leaf lets, which developed transgenic plants through *Agrobacterium*-mediated transformation (Daniel 2002).

2.7.2.Biolistics mediated gene transfer

Microprojectile bombardment or particle gun bombardment has been found as an attractive alternative method for DNA delivery in crop plants and has been demonstrated as a practical means of introducing a number of important genes. Clemente et al. (1992) reported the transient expression and stable transformation has been observed in callus lines from immature groundnut leaflet tissue bombarded with micro carrier particles carrying plasmid DNA. From 875 leaflets of the cultivar UPL PN 4 bombarded, 202 kanamycin resistant calli were recovered but only 1 untransformed shoot was produced. Similar observations were reported by Schnall and Weissinger (1995), where regenerated plants form slow growing brown callus as well as green clusters formed by bombarding leaflets did not show any stable transformation. However, bombardment of 1-2 year old embryogenic callus derived from immature embryos followed by stepwise selection for resistance to hygromycin B in solid and liquid media produced transgenic shoots at a frequency of 1% (Ozias-Akins et al., 1993). By using apical and lateral meristematic tissues of mature embryonic axis transgenic groundnut plants were produced which had a relatively low transformation frequency of 0.9-1% (Brar and Cohen, 1994).

Transgenic groundnut plants expressing the *cryIAc* gene for resistance towards Corn stalk borer (*Elasmopalpus lignosellus*) have been reported (Singsit et al., 1997) using somatic embryos form immature cotyledons of groundnut bombarded with vectors containing codon modified *Bacillus thuringiensis cryIAc* gene and *hpt* gene for selection with a efficiency of 0.85 to 2.3 transgenic lines per bombardment. In biolistics the transient gene expression as assayed by GUS assay has been found to be affected by both particle size and amount of DNA used for coating and was found to be positively correlated with gene copy number (Lacorte et al., 1997). Livingstone

and Brich (1999) efficiently transformed both Spanish and Virginia types of groundnut by particle bombardment into embryogenic callus derived from mature seeds, followed by single step selection for hygromycin B resistance resulting in 3-6 independent transformants per bombardment of 10 cm^2 embryogenic calluses with copy number ranging from one to twenty with a mean of four copies. Recent reports show further increased transformation efficiencies, ranging from 2.6±3.5 to 19.8±18.5 the hygromycin B resistant lines per bombardment (5 cm^2) with fertility rates of 32% (Wang et al., 1998). A high frequency transformation and regeneration of somatic embryos via micro projectile bombardment has been achieved with constructs containing *hpt* gene and nucleocapsid protein (N) gene of the lettuce isolate of tomato spotted wilt tospovirus (Yang et al., 1998). Yang et al. (1998) observed the primary transformants containing a single copy of the transgene expressed the N protein and multiple gene integration transformants indicated gene-silencing mechanism.

Bombardment of embryogenic cultures and selection on hygromycin appears to be the most widely used at least three different groups to transform multiple cultivars including runner, Virginia and Spanish market types (Livingstone and Brich.1999; Magbabua et al., 2000; Ozias-Akins et al., 1993; Wang et al., 1998; and Yang et al., 1998) Higgins et al. (2004) and Dietzgen et al. (2004) were obtained groundnut lines that, exhibiting high levels of resistance to groundnut stripe virus (PStV) following co-bombardment of embryogenic callus derived from mature seeds of the commercial cultivars Gajah and NC7 with the hygromycin resistance and one of two forms of the PStV coat protein (CP) gene. Padua, (2000) employed electroporation method for direct gene transfer into intact embryonic leaflets of groundnut in a modified electroporation buffer (EPRm) supplemented with 75 µM

NaCl. A positive effect on the number of shoots and regeneration efficiency was observed using electric strengths of 500-625 v/cm.

2.8. Importance of fungus (*Aspergillus*) in groundnut

The genus *Aspergillus*, a member of the phylum Ascomycota, includes over 185 known species. To date, around 20 of them have been reported to cause harmful infections in humans and animals of which, the most infamous species in this genus is *Aspergillus flavus*. Next to *Aspergillus fumigatus*, it is the second most common cause of invasive and non-invasive aspergillosis in humans and animals (Denning, 1998; Denning et al., 1991; Denning et al., 2003) and in some geographic areas it is the leading causative agent for aspergillosis. *A. flavus* produces many secondary metabolites including aflatoxins; the most toxic and most potent carcinogenic natural compounds that cause aflatoxicosis and induce cancers in mammals. *A. flavus* causes diseases of many agricultural crops such as maize (corn), cotton, groundnuts (groundnuts), as well as tree nuts such as Brazil nuts, pecans, pistachio nuts, and walnuts. Its ability to attack seeds of both monocots and dicots, and to infect seeds produced both above and below the ground, demonstrates that this fungus has evolved a battery of mechanisms to breach the resistance of host.

These fungi are ubiquitous, being found virtually everywhere in the world. This ubiquitous mold not only reduces yield of agricultural crops but also decreases the quality of the harvested grains. Due to *A. flavus* infection to the crops and aflatoxin contamination in grains, hundreds of millions dollars are lost to the U.S. and world economy annually. They are soil borne, but prefer to grow on high-nutrient media (e.g., seed). In nature, *A. flavus* is one of the most abundant and widely distributed soil-borne molds and can be found anywhere on earth. It is a saprophytic

fungus that is capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, animal fodder, cotton, compost piles, dead insect and animal carcasses, outdoor and indoor air environment (air ventilation system), stored grains, and even human and animal patients (Klich, 1998). Its optimal range for growth is at 28 - 37 °C and can grow in a wide range of temperatures form 12 to 48 °C. The heat tolerance nature contributes to its pathogenicity on humans and other warm-blooded animals. The fungus mostly exists in the form of mycelium or asexual conidia spores. Under adverse conditions such as dry and poor nutrition, the mycelium congregates to form resistant structures called sclerotia. The fungus over-winters either as spores or as sclerotia. The sclerotia germinate to form new colonies when growth conditions are favorable (Bennett et al., 1986; Cotty, 1988). Because of its ability to grow at low water activity, *A. flavus* is also capable of colonizing seeds of grains and oil crops. In general, high ambient temperature and plant stress are the two environmental parameters most closely correlated with *A. flavus* infections in plants.

***Aspergillus flavus* is the predominant species that produces aflatoxins**

Infection of groundnut (*Arachis hypogaea* L.) seed by *Aspergillus flavus* and *A. parasiticus* is a serious problem. This infection can result in the contamination of the seed with aflatoxins, which are toxic fungal metabolites. Aflatoxins are a group of structurally related toxic polyketide-derived secondary metabolites produced mainly by certain strains of *A. flavus* and *A. parasiticus*. Their structures are composed of bis-furan-containing dihydrofuranofuran and tetrahydrofuran moieties (rings) fused with a substituted coumarin. The aflatoxins, B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) are the major four toxins among at least 16 structurally related toxins (Goldblatt, 1969). *A. flavus* produces aflatoxins B_1 and B_2. Other toxic compounds

produced by *A. flavus* are cyclopiazonic acid, kojic acid, ß-nitropropionic acid, aspertoxin, aflatrem and aspergillic acid. *A. parasiticus* produces aflatoxin G_1 and G_2 in addition to B_1 and B_2, but not cyclopiazonic acid (Bennett et al., 2003; Yu, 2004; Yu et al., 2004). Aflatoxin B_1 is predominant, the most toxic and most potent hepatocarcinogenic natural compound ever characterized (Squire, 1989). Aflatoxin M_1 is a major metabolic product of aflatoxin B_1 in animals and is usually excreted in the milk and urine of dairy cattle and other mammalian species that have consumed aflatoxin-contaminated food or feed.

***Aspergillus flavus* is the leading cause of aflatoxicosis**

The identification of aflatoxin as a food poison originated form the incidence of a mysterious "Turkey-X" disease in 1960 when approximately 100,000 turkey poults in England died (Allcroft et al., 1961; Lancaster et al., 1961). The culprit was later identified as aflatoxin produced by *A. flavus* in groundnut-meal feed. Aflatoxin was named after *Aspergillus flavus* toxin. Aflatoxins produced by *A. flavus* have both hepatotoxic and carcinogenic actions, depending on the level and duration of exposure. The ingestion of aflatoxins in contaminated food or feed causes a disease called aflatoxicosis. Acute aflatoxicosis is produced when moderate to high levels of aflatoxins are consumed. Symptoms include acute liver damage, acute necrosis, cirrhosis, or in severe cases, acute liver failure and death (Fung et al., 2004; Lewis et al., 2005). Aflatoxins in liver irreversibly bind to protein and DNA to form adducts such as aflatoxin B_1-lysine in albumin (Skipper et al., 1990). Disruption of the proteins and DNA bases in hepatocytes causes liver toxicity (Azziz-Baumgartner et al., 2005; Tandon et al., 1978). In humans, patients experience high fever, rapid progressive jaundice, edema of the limbs, pain, vomiting, alteration in digestion, absorption and/or metabolism of nutrients and swollen livers. Outbreaks of acute

aflatoxicosis from contaminated food in humans have been documented in Kenya, India (Ngindu et al., 1982), Malaysia, and Thailand (CAST, 2003; Lye et al., 1985). One of the first major documented reports of aflatoxicosis in humans occurred in western India in 1974 where 397 persons were affected and 108 persons died. More than 150 villages were involved (Krishnamachari et al., 1975). As recently as July 2004, an incident of aflatoxin poisoning in Kenya had occurred involving 317 cases and 125 deaths due to consumption of aflatoxin contaminated maize (corn), the largest and most severe outbreaks of acute aflatoxicosis documented worldwide (CDC, 2004; Lewis et al., 2005). Chronic dietary exposure to aflatoxins is a major risk of hepatocellular carcinoma, particularly in areas where hepatitis B virus infection is endemic (Bressac et al., 1991; Fung et al., 2004; Hsu et al., 1991; Wogan et al., 1992).

Aflatoxin B_1 is a very potent carcinogen in humans and animals including nonhuman primates, birds, fish, and rodents. Liver is the primary target organ of acute and chronic injury. Aflatoxin B_1 is modified into a more toxic and carcinogenic by-product during etoxification by a cytochrome P_{450} monooxygenase in liver. The epoxide form of aflatoxin binds to guanine residues in DNA; forms guanyl-N7 adducts, and induces mutations. One mutation, a G to T transversion (Baertschi et al, 1989; Bressac et al., 1991) in codon 249 of the p53 tumor suppressor gene is generally believed to be the mechanism for initiating formation of hepatocarcinomas (Coursaget et al., 1993; Hsu et al., 1991; Ozturk, 1991). Aflatoxin B_1 is also a potential immunosuppressive agent. Continuous low-level exposure of aflatoxin to growing vertebrates may enhance their susceptibility to infection and tumorigenesis (Raisuddin et al., 1993). In the developed countries, aflatoxin contamination to agricultural crops is monitored and aflatoxin levels are strictly regulated. A guideline

of 20 parts aflatoxin per billion parts of food or feed substrate (ppb) is the maximum allowable limit imposed by the U.S. Food and Drug Administration for interstate shipment. European countries have established more stringent guidelines to a much lower level (3-5 ppb). Crops are destroyed or decontaminated if the content exceeds the official regulatory levels, resulting yearly in billion dollar losses worldwide. In developing countries where detection and monitoring are non-existent and there are regular food shortages, food safety is the major issue. In summary, aflatoxin contamination of agricultural commodities poses a potential risk to livestock and human health (Bennett, 1987; Bennett et al., 2005; Bhatnagar et al., 2002; Cleveland et al., 1992; Cotty, 1997; Eaton et al., 1994; Hall et al., 1994; Jelinek et al., 1989; Lancaster et al., 1961; Richard et al., 2003). It is not only a serious food safety concern, but has significant economic implications for the agriculture industry worldwide.

2.9. Strategies for developing resistance to *Aspergillus flavus*

The development of host-plant resistance would be an effective approach to eliminate aflatoxin accumulation in groundnut (Guo et al., Holbrook et al., 2000; Mixon, 1986). More understanding of host resistance mechanisms should speed the development of resistant cultivars. Progress has been made in an attempt to prevent aflatoxin contamination in crops (Robens et al., 2003) through crop management and handling, microbial ecology and bio-competitive microbes, and crop resistance through genetic engineering and conventional breeding (Guo et al; Guo et al). In the past decade, studies have identified some groundnut germplasm with resistance to *A. flavus* infection and aflatoxin production.

Molecular studies have provided useful information towards a better understanding of complex host pathogen interactions for a number of important crop species. These studies are starting to identify genes and gene products that determine resistance or susceptibility to a pathogen. In particular, studies on gene expression during induction of the host defense response have facilitated a clearer picture of the possible roles of various gene products in the plant-microbe interaction (Farmer and Ryan, 1992). Functional genomics technology based on expressed sequence tags (EST's) and microarray has been used widely in the research of plant disease resistance mechanism in recent years. In groundnut, the resistance (Somerville et al., 1999; Sweigard et al., 2001; Keon et al., 2003) trait against *Aspergillus flavus* infection is quantitative trait and also effected by environment factors such as (Widstrom et al., 2003) drought stress.

To further illustrate the resistance mechanism in groundnut against *A. flavus* and identify resistance genes, functional genomics technologies have been used to analyze expressed genes and to identify pathways involved in the resistance mechanisms (Luo et al., 2005a; Luo et al., 2005). Functional genomics technology has advantages compared with traditional techniques especially in the interaction between plant, pathogen and environment. By using this technology, genome-wise global view of gene expression could be acquired. The comparison of gene expression in different cultivars and treatments could be revealed in one hybridization reaction and the expression data from different treatments can be compared for specific gene functions (Luo et al 2005).

Because of the complexity of the *Aspergillus*-plant interactions, better understanding of the genetic mechanisms of resistance will be needed using both conventional and molecular breeding for crop improvement and control of preharvest

aflatoxin contamination (Guo et al., 2005). This technology has been applied to characterize transcript abundance, complexity and stability and to identify novel target genes and pathways that are associated with biological process, to determine the regulatory elements of genes and pathways, to characterize gene functions by profiling genetic mutants and to profile gene expression pattern as a marker to predict biological performance. The lack of genomic information for groundnut has hindered the progress in developing genetic and genomic tools and information for breeding and genetic enhancement.

Depending on the source of the genes used, there are two approaches for development of genetically engineered fungal resistance in plants. The former approach is based on the concept of pathogen-derived resistance (PDR) (Hamilton, 1980; Sanford and Johnson, 1985). In PDR a part or a complete fungal gene is introduced into the plant, which subsequently, interferes with one or more essential steps in the life cycle of the fungus. Non -pathogen-derived resistance, on the other hand, is based on utilizing host resistance genes and other genes responsible for adaptive host processes elicited in response to pathogen attack, to obtain transgenics resistance to fungus.

2.9.1. Transgenics with non-pathogen derived resistance

Invasion of preharvest host plants, corn, cotton, groundnut and tree nuts in the field by *A. flavus*, is a complicated process involving multiple genetic and biological factors (Brown et al., 2001; Cleveland et al., 2005; D'Souza et al., 2001; Shimizu et al., 2001). A few pathogenicity factors have been reported in *A. flavus*. Hydrolytic activity of *A. flavus* plays an important role in absorbing nutrients from host plants for fungal growth. Hydrolytic enzymes such as cellulases, glucanases, chitinases, amylases, pectinases, could be pathogenicity factors during fungal invasion of crops.

The genes responsible for such biological processes are very difficult to identify through conventional molecular cloning methods. However, some of the genes encoding for hydrolytic enzymes including amylase, cellulase, pectinases, proteases, chitinase, chitosanases, pectin methylesterases, endoglucanase C precursor, glucoamylase S1/S2 precursors, β-1,3-glucanase precursor, β-1,4-D-glucan cellobiohydrolase A precursor, glycogen debranching enzyme and xyloglucan-specific endo-β- 1, 4-glucanase precursor, have been identified from the *A. flavus* EST (Yu et al., 2004) and genome sequence databases.

There is limited information known about crop fungus interaction. Several compounds have been isolated that are inhibitory to fungal growth, including a chitinase, amylase and trypsin inhibitors (Brown et al., 2001; Chen et al., 1999; Cleveland et al., 2005; Fakhoury et al., 2001), and ribosome inactivating proteins (Nielsen et al., 2001). Fatty acid peroxides, known as oxylipins, affected aflatoxin formation (Wilson et al., 2001). With the availability of *A. flavus* whole genome microarray, it is much easier to identify genes expressed during fungal invasion of crops. Genes involved in such process could be targeted for inhibiting fungal growth and/or aflatoxin formation. Knowledge on crop-fungus interaction could help plant breeders to develop resistant commercial crops against fungal infection (Cleveland et al., 2005; Guo et al., 2003).

2.9.1.1. Lipoxygenases

The filamentous fungi *Aspergillus flavus* and *A. parasiticus* colonize oil seed (e.g., corn, groundnut, cotton, and nut) and cause tremendous yield and economic loss through tissue maceration, as well as a significant health problem by the contamination of the seed with the mycotoxin aflatoxin, the most potent natural

carcinogen known (Bennett and Klich 2003; Cleveland et al., 2003). Oilseed crops are frequently subject to contamination by mycotoxins produced by *Aspergillus* spp., particularly aflatoxin (AF) and to a lesser extent sterigmatocystin (ST). Several studies have suggested that metabolites generated from the plant lipoxygenase (LOX) pathway may either decrease or increase mycotoxin production by *Aspergillus* spp. The family of genes encoding lipoxygenases (LOXs) has been of particular interest due to potentially significant role(s) in plant-microbe interactions.

Lipoxygenases (EC 1.13.11.12) are enzymes found in most eukaryotes that catalyze the dioxygenation of *cis, cis*-1, 4-pentadiene moieties of fatty acids (Hildebrand, 1989; Siedow, 1991; Gardner, 1995). Lipoxygenases (LOXs, linoleate:oxygen oxidoreductases) catalyze the formation of hydroperoxy derivatives of polyunsaturated fatty acids and thus the first step in the synthesis of fatty acid metabolism in plants. Seed oxylipins (oxygenated polyenoic fatty acids) may be produced by plant lipoxygenases (LOXs), a functionally diverse class of non heme dioxygenases implicated in physiological processes such as growth and development, seed germination, senescence, formation of flavor and aroma compounds, and stress- and pest-related responses (Farmer et al., 2003; Hildebrand et al., 1998; Howe and Schilmiller 2002; Porta and Rocha-Sosa 2002; Wasternack and Hause 2002). Oxylipins recently have been implicated as signaling molecules for cross-kingdom communication in plant–pathogen interactions. A developing case for cross-kingdom communication in plant– pathogen interactions occurs in the interaction between seed and mycotoxigenic *Aspergillus* spp. Metabolites of the LOX-pathway have been identified as compounds with antimicrobial activity, growth regulators, flavors and odours as well as signal molecules (Rosahl 1996; Feussner and Wasternack 2002). Based on these effects and on the correlation between increases in LOX content and

the onset of specific processes, LOX has been proposed to be involved in the plant response to wound stress.

Distinct plant LOX isozymes preferentially introduce molecular oxygen into linoleic and linolenic acids either at C-9 (9- LOX) or at C-13 (13-LOX) of the hydrocarbon backbone of the fatty acid to produce *cis-trans* 9*S*- or 13*S*-hydroperoxy linoleic acid (9*S*- or 13*S*-HPODE) or 9*S*- or 13*S*-hydroperoxy linolenic acid (9*S*- or 13*S*-HPOTE). The primary products of LOXs, 9*S* and 13*S* fatty acid hydroperoxides, are proposed to have regulatory roles in plant and animal metabolism (Hildebrand, 1989; Gardner, 1995).

Linoleic acid and its two plant lipoxygenase (LOX) oxylipin products 9- and 13-hydroperoxy fatty acids (9*S*- and 13*S*-HPODE) have been shown to have a significant effect on differentiation processes in the mycotoxigenic seed pathogens *Aspergillus* spp. i.e., in *A. nidulans, A. flavus,* and *A. parasiticus*. Whereas all of the 18 C polyunsaturated fatty acids could promote sporulation in all three species, 9*S*-HPODE stimulated and 13*S*-HPODE inhibited mycotoxin production, presumably by structurally mimicking endogenous *Aspergillus* sporogenic factors: *oxylipins* derived from oleic, linoleic, and linolenic acid (Burow et al., 1997; Calvo et al., 1999; Tsitsigiannis et al., 2004a and b, 2005). The role of *Aspergillus* seed colonization in regulation of 13 and 9 lipoxygenases and their derivatives as molecules modulating mycotoxin biosynthesis and fungal sporulation is illustrated in figure below.

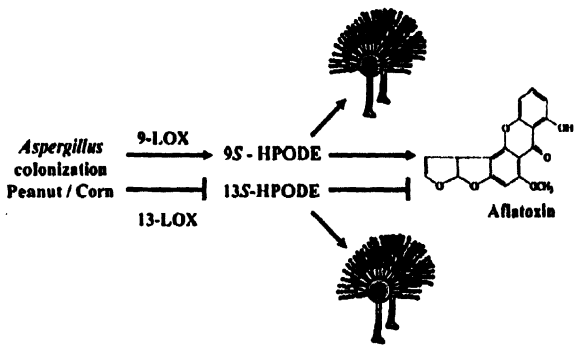

Figure 5: Model depicting the role of Aspergillus seed colonization in regulation of 13 and 9 lipoxygenases and their derivatives as molecules modulating mycotoxin biosynthesis and fungal sporulation (Tsitsigiannis et al., 2005).

Here we report on the cloning and characterization of a groundnut lipoxygenase gene expressed during *Aspergillus* colonization. The gene is inhibited by the *Aspergillus* infection process and the active protein produces a mixture of 9*S*- and 13*S*-hydroperoxides. This is the first description of an *Aspergillus* inducible plant gene whose products, in turn, have been shown to induce *Aspergillus* development (Calvo et al., 1999) and modulate *Aspergillus* mycotoxin biosynthesis (Burow et al., 1997).

In plants, 13*S* hydroperoxides are intermediates in the pathway for the production of traumatin, traumatic acid and methyl jasmonate * Gardner, 1995, 1998). The 13-monohydroperoxides are precursors of biologically active compounds such as traumatin, jasmonic acid, and methyl jasmonate, which have hormone- like regulatory and defense-related roles in plants (Blee 2002; Feussner and Wasternack 2002). Jasmonate products of the LOX pathway can serve as signals that act to induce

expression of genes for defense response in plants (Farmer and Ryan, 1992). Both 9*S* and 13*S* hydroperoxides can also serve as intermediates for the production of oxo-fatty acids, volatile alcohols, aldehydes and ketols (Gardner, 1995; Gardner et al., 1996). Due to their free-radical nature, fatty acid hydroperoxides can be quite active by themselves and are capable of producing membrane damage and promoting cell death (Ricker and Bostock, 1993). 13*S*-hydroperoxy fatty acids directly or indirectly repress AF and ST biosynthesis and provide in vitro evidence that specific seed lipoxygenase activity could provide resistance to mycotoxin contamination by *Aspergillus* spp. Other LOX pathway metabolites (e.g., methyl jasmonate and aldehyde products of 13*S*-HPODE and 13*S*-HPOTE) also have been reported to inhibit or stimulate fungal development and aflatoxin production (Doehlert et al., 1993; Goodrich-Tanrikulu et al., 1995; Vergopoulou et al., 2001; Zeringue, 1996).

Studies of *Aspergillus*/seed interaction have also implicated a role for LOX metabolites in this plant/microbe interaction (Doehlert et al., 1993; Goodrich-Tanrikulu et al., 1995; Zeringue et al., 1996; Burow et al., 1997; Gardner et al., 1998). This interaction is quite complex, as an additional factor, the production of the mycotoxin aflatoxin by *Aspergillus* spp., must be considered. Several studies have shown that *Aspergillus* development and aflatoxin production can be separately affected by LOX products (Goodrich- Tanrikulu et al., 1995; Burow et al., 1997; Gardner et al., 1998; Calvo et al., 1999). Both 13*S*- and 9*S*HPODE, which are chemically similar to endogenous hydroxy linoleic sporulation factors produced by *Aspergillus* (Chanpe and el-Zayat, 1989), induce sporulation in *A. nidulans*, *A. flavus* and *A. parasiticus* (Calvo et al., 1999). Furthermore, studies have shown that 13*S* hydroperoxides (Burow et al., 1997) and possibly methyl jasmonate (Goodrich-Tanrikulu et al., 1995) decrease mycotoxin production by *Aspergillus*. On the other

hand, 9*S*-HPODE was shown to extend the expression of the genes in the aflatoxin pathway and possibly promotes mycotoxin biosynthesis in this manner (Burow et al., 1997). These observations may partially explain why the level of *Aspergillus* infestation is not necessarily indicative of the level of aflatoxin contamination (Lee et al., 1980).

In groundnut seed, *Aspergillus* infections induced expression of *PnLOX1* encoding a mixed-function LOX producing approximately 21% 9*S*-HPODE and 59% 13*S*-HPODE (Burow et al., 2000). However, biochemical analysis of the infected seed—in contrast to non-infected seed—showed a steady increase in 9*S*-HPODE content of the seed during the course of *Aspergillus* colonization (Burow et al., 2000). These results led to the conclusion that additional groundnut seed *LOX*s, both 9 and 13-LOX, actively participate in the seed–*Aspergillus* interaction where 9-*LOX* gene expression would be induced and 13-*LOX* expression possibly repressed during fungal infection.

PnLOX2 and PnLOX3 are both 13*S*-HPODE producers (13-LOX) and are specifically expressed in seed and expression is highest in rnature embryo and immature cotyledons.In contrast to PnLOX1, the amount of 13 S-HPODE formed was 78 and 80% for PnLOX2 and PnLOX3 respectively. These studies further support a case for 9-*LOX* as susceptibility factors and 13-*LOX* as resistance factors in mycotoxin contamination in seed crops. Invitro observations suggested that exogenous 9*S*-HPODE extended the time of aflatoxin gene transcription whereas 13*S*-HPODE and 13*S*-HPOTE inhibited aflatoxin gene transcription. The deduced amino acid sequence of PnLOX2 and PnLOX3 (we will refer to both of them as PnLOX2-3) showed 99% identity to each other and 91 and 92% identity, respectively, to PnLOX1. PnLOX2-3 had significant structural identity with several other plant LOXs, ranging

form 70 to 80% identity with legume LOXs and 55 to 60% with potato, tomato, and *Arabidopsis* spp. The results of these studies suggest that 9*S*-HPODE and 13*S*-HPODE molecules act as *putative susceptibility and resistance factors* respectively, in *Aspergillus* seed–aflatoxin interactions.

2.9.1.2. Chitinase & β-1, 3-glucanase

Intensified use of fungicides has resulted in the accumulation of toxic compounds potentially hazardous to humans and environment (Cook et al., 1983) and also in the buildup of resistance of the pathogens (Dekker et al., 1982). In order to tackle these national and global problems, effective alternatives to chemical control are being investigated and the use of antagonistic microbes seems to be one of the promising approaches (Cook, 1985). Antagonism Antagonism may be accomplished by competition, parasitism, antibiotics, or by a combination of these modes of action (Deacon et al., 1992; Whipps, 1992). Parasitism involves the production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi (Elad et al., 1985). Chitinases and β-1, 3-glucanases are important components of plant defense in response to attack by pathogens.

Many defense mechanisms are triggered in plants in response to infection by plant pathogens. Infection of plant with pathogens induces the accumulation of a group of proteins collective known as pathogenesis-related proteins (PR-proteins). Chitinases and β-1, 3-glucanases are included in the subclass of defense response genes, which encode PR proteins. The PR-proteins have certain characteristic properties; such as they are selectively extractable at low pH and highly resistant to proteolytic enzymes. Among the PR-proteins, chitinases belonging to the PR-3 group appear to be potential candidates for management of fungal diseases. Among them,

the production of pathogenesis-related (PR) proteins is well documented (Datta et al., 1999). The expression of PR-proteins in plants is correlated with resistance in a number of plant–pathogen interactions (Van loon, 1997). Hence, several attempts have been made to exploit these PR-proteins to develop disease-resistant transgenic plants (Broglie et al., 1991; Grison et al., 1996; Lin et al., 1995; Liu et al., 1994).

Parasitism of pathogenic fungi, facilitated by the production of hydrolytic enzymes, is involved in biological control of fungal diseases. Among the hydrolytic enzymes, chitinases are of prime importance since chitin is a major cell wall constituent in the majority of phytopathogenic fungi. Chitinases inhibit fungal spore germination and germ tube elongation (Manjula et al., 2004), and lyse hyphal tips (29). Purified chitinases of *Trichoderma harzianum* (El-Katatny et al., 2001), *Gliocladium virens* (Dipetro et al., 1993), *Serratia marcescens* (29), *Serratia plymuthica* (Frankowski et al., 2001), and *Streptomyces* sp. (Gomes et al., 2001) were highly antifungal. The importance of chitinolysis in biological control of fungal diseases of plants was supported by several observations, namely increased disease control by chitin-supplemented application of chitinolytic biocontrol agents (Manjula et al., 2001; Zhang et al., 2000), and greater field efficiency of chitinase preparations in disease control (Shternshis et al., 2002). Biocontrol strains that over express chitinase had increased antifungal activity (Limon et al., 1999), and mutant strains, deficient in chitinase production, exhibit reduced antifungal activity and disease control ability (Zhang et al., 2000). Biocontrol efficacy of non-chitinolytic *Pseudomonas fluorescens* was enhanced by expression of the *Chi*A gene form *S. marcescens* (Downing et al., 2000).

Chitinase and β-1, 3-glucanase are the important PR-proteins in defending the plant against pathogens. They can protect the plant from fungal infection by their

direct lytic action on fungal cell wall or by releasing oligosaccharide signal molecules that can activate a variety of plant defenses. Activities of chitinase (Boller, 1985; Nasser et al., 1990) and β-1, 3-glucanase have been reported to exist and inhibit the growth of *A. flavus* in mature corn kernels and several isoforms of two hydrolytic (Neucere et al., 1995; Lozovaya et al., 1998) enzymes appear in response to *A. flavus* infection (Ji et al., 2000). Most PR proteins have isoforms with different isoelectric points, *i.e.* acidic and basic types. Generally, basic PR proteins are intracellularly targeted to the vacuole, and the acidic proteins are located extracellularly (Meins et al., 1992). However, limited information has been reported on the activities and isoforms of these two hydrolytic enzymes in groundnut kernels. Liang et al., 2005 reported the identification and characterization of β-1, 3-glucanase in cultivated groundnut.

Chitinases inhibit fungal growth both directly and indirectly. They hydrolyze fungal cell walls, which contain chitin, the substrate for the enzyme, and by this direct action fungal hyphal lysis and inhibition of fungal growth occur (Roberts et al., 1988; Schlumbaum et al., 1986). The chitinases can release elicitors from the fungal cell walls by their enzymatic action and these elicitors induce various defense responses in plants (Ren et al., 1992). It has been demonstrated that constitutive, high-level expression of chitinases in transgenic plants can enhance resistance to a variety of pathogens (Broglie et al., 1991; Lin et al., 1995; Marchant et al., 1998; Tabei et al., 1997). When a pathogen lands on a host surface, it activates the defense mechanism of the host probably by releasing elicitors from its cell walls (Lamb et al., 1989). Elicitor-inducible chitinases may be more useful as their activity will increase several-fold upon a pathogen's invasion and suppress the pathogen. Some of the chitinases are not elicitor-inducible. In barley, the 34-kDa chitinase is not induced in aleurone

protoplasts upon treatment with elicitor (Sheba et al., 1994). In cucumber, among the three acidic class III chitinase genes, Chi 1 and Chi 3 genes are not induced by the elicitors whereas the Chi 2 gene is induced by pathogens as well as by abiotic elicitors (Lawton et al., 1994). Similarly, in rice suspension-cultured cells, basic chitinase transcripts were induced upon elicitor treatment, whereas acidic chitinase genes showed very weak induction (Xu et al., 1996).

Not all chitinases show antifungal action against pathogens of the host from which the chitinases have been isolated (Broekaert et al., 1988; Mauch et al., 1988; Verburg et al., 1991) and only specific chitinases exhibit antifungal activity (Sela-Buurlage et al., 1993). Chitinases purified from pea pods did not inhibit the test pathogens, *Fusarium solani* f.sp. *pisi* or *F. solani* f.sp. *phaseoli* (Mauch et al., 1988). Antifungal action of a chitinase isolated from suspension-cultured cells has not been reported from any dicotyledonous or monocotyledonous plants and this is the first time a chitinase from cultured cells has been found to evince antifungal action. Chitinases purified from thorn apple, tobacco and wheat inhibited growth of saprophytic fungi but did not inhibit growth of *Botrytis cinerea* (Broekaert et al., 1988). Similarly, the purified *Arabidopsis* chitinase inhibited growth of *Trichoderma reesei*, but growth of several pathogenic fungi including *Alternaria solani, Fusarium oxysporum, Sclerotinia sclerotiorum, Gaeumannomyces graminis* and *Phytophthora megasperma* was not inhibited (Verburg et al., 1991). Thus, sensitivity of different pathogens to different chitinases may vary widely (Rokem et al., 1986). Proper selection of chitinase genes is very important for the development of transgenic plants with enhanced disease resistance. Elicitor-inducible, antifungal chitinase may have great potential for effective protection against pathogens. Such chitinase has not yet been purified or characterized in rice. Rapid increase in chitinase activity in rice cells

due to elicitor treatment and the antifungal property of the purified 35 kDa chitinase suggest that chitinase plays a role in disease resistance of rice. Hence, attempts were made to assess whether any chitinase is induced in rice suspension-cultured cells by fungal elicitor. This chitinase shows high toxicity to rice sheath blight pathogen. Such a chitinase gene will be highly useful for developing transgenic disease-resistant plants.

Chitinase [poly{1,4-(N-acetyl-β-D-glucosaminide)} glycanohydrolase], (EC 3.2.1.14) catalyses the hydrolysis of chitin, a polymer of unbranched chains of β-1, 4-linked 2-acetamido-2-deoxy-D-glucose (GlcNAc; N-acetylglucosamine) (Jeuniaux, 1966; Mauch and Staehelin, 1989). This enzymatic degradation of chitin to produce GlcNAc is performed by a chitinolytic system, which has been found in micro-organisms, plants, and animals (Flach et al., 1992). The chitinolytic enzymes are traditionally divided into two main classes: (1) endochitinases and (2) N-acetyl glucosaminidases (sometimes termed chitibiase, EC 3.2.1.30). The existence of a third class of enzyme, exochitinase, has been suggested (Robbins et al., 1988). Endochitinases randomly hydrolyse GlcNAc polymers, eventually giving diacetylchitobiose as the major product. N-acetyl glucosaminidases preferentially act on a dimer (Gooday, 1990). The exochitinase also catalyses progressive release of diacetylchitobiose units form the non-reducing ends of chitin chains.The molecular masses of plant chitinases and lysozymes are generally in the range 24-36 kDa; the enzymes occur as monomers and are basic or acidic, i.e., have high or low isoelectric points (Boller, 1988; Collinge et al., 1993; Boller,1993).

Chitinases catalyze the hydrolytic cleavage of chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine (GlcNAc) that constitutes major parts of the cell walls of some, but not all, fungal pathogens and of the exoskeleton of

arthropods. Several purified plant chitinases have been shown to inhibit the growth of some fungal pathogens, particularly in combination with β-1,3-glucanases (Mauch et al., 1988; Sela-Buurlage et al., 1993; Graham and Sticklen, 1994; Kombrink and Somssich, 1997). Furthermore, constitutive co-expression of genes encoding chitinases and other PR proteins (*e. g.*, β-1,3-glucanase) yielded transgenic plants with enhanced disease resistance (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995; Kombrink and Somssich, 1997). However, contrary data also exist (Neuhaus et al., 1991a; Nielsen et al., 1993). At any rate, one major role of chitinases seems to be a protective one, owing either directly to their anti-microbial activity or indirectly to the release of elicitor-active signal molecules from pathogen surface structures that trigger the activation of defense responses. However, apart from being induced in response to infection, chitinases are also expressed in an organ- and cell type-specific manner during development of uninfected plants, suggesting that they might have additional, as yet unknown functions. Recently, certain chitinases were shown to be constitutively expressed only in certain organs or cell types, such as flowers or epidermal cells (Wemmer et al., 1994; Ancillo et al., 1999). Little is known about their endogenous functions, and a plant-derived substrate has not been identified. Even for a chitinase that has been demonstrated to rescue the carrot cell mutant ts11 defective in embryo development, the mode of action or the endogenous substrate(s) are unknown (de Jong et al., 1992, 1993; Kragh et al., 1996).

Chitinase isoenzymes differ in their substrate specificities with respect to oligomeric substrates and usually need a chain length of at least three β-1,4-linked *N*-acetyl-D- glucosamine residues. Some chitinases release the *N*-acetyl- D-glucosamine monomer after digestion of chitin; others release chitobiose as the smallest end product. Many plant chitinases also possess some lysozyme activity, hydrolyzing β-1,

4 linkages between *N*-acetyl muramic acid and *N*-acetyl-D-glucosamine in the bacterial peptidoglycans.

Chitinase and β-glucanase genes exist as families: seven classes of chitinases and at least three classes of glucanases have been recognized (Collinge et al., 1993; Meins et al., 1992, 1994; Neuhaus, 1999; Leubner-Metzger and Meins, 1999). Individual members within each class can reside at different chromosomal locations (Li et al., 1999), and some exhibit differential expression depending on the pathogen. In addition to the pathogen-inducible PR proteins, other PR proteins that are developmentally regulated have also been identified in almost all plant species (Van Loon, 1999). In polyploid species such as hexaploid wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD), which carry paralogous sets of PR genes on homoeologous chromosomes, there might be more opportunities for structural and functional evolution of PR genes with respect to pathogen induction, developmental regulation and recruitment for new function. To analyze the role of specific PR proteins in the defense process, it is desirable to induce their expression by a given pathogen, identify the specific chitinase and glucanase genes induced and determine the positions of the expressed genes in the plant genome.

3. MATERIALS AND METHODS

Materials and methods

3.1. Plasmid Constructs used

The plasmids used for the construction of binary vector include pRT 103 (Messing et al., 1985), pGEM-T Easy (Promega, Cat no: A1360), pGEM-7Zf (+) (Promega, Cat no: P2251), pTMK 12.6 (Tsitsigiannis et al., 2005) and pCAMBIA 2300. Plasmid vectors used in this study and their sub-cloning strategy is illustrated in Figure 6.

3.1.1 Construction of binary vector pCAMBIA 2300:lox3

Binary plasmid pCAMBIA 2300 (-*npt*II)(Fig.6H) (Madhurima Bhatnagar, 2005, Unpublished) was used for the construction of vector containing coding sequence of the lipoxygenase gene (Tsitsigiannis et al., 2005). The plasmid pCAMBIA 2300 carries the selectable marker gene *Neomycin phosphotransferase* II (*npt*II) under the control of CaMV 35S promoter and poly-A terminator. The *npt*II fragment was released from the plasmid by restricting with *Xho*1 and re-ligating it back. The resulting plasmid was designated as pCAMBIA 2300 *(-npt*II).

Restriction endonucleases and T4 DNA ligase, obtained from New England Biolabs, were used for cloning as recommended by the supplier. The strategy of the construction of binary vector containing lipoxygenase gene was as follows:

1. The plasmid pRT 103 (Fig. 6 A) was digested with *Pst*I enzyme and the 649bp fragment containing CaMV 35S promoter, multiple cloning site and Poly A signal was separated on 1% agarose gel and purified using Qiagen® gel extraction kit. This fragment was then ligated into the dephosphorylated *Pst*I site of the vector pGEM-T Easy (Fig. 6B). Products of the ligation were then introduced into DH5α

cells through electroporation and selected recombinants on LB media containing ampicillin, IPTG and X- gal. The bacterial colonies turned into blue were rejected whereas white colonies assumed as transformed with the desired fragment were selected. Colonies carrying recombinant plasmid containing the CaMV 35S promoter and Poly A signal in desired orientation were identified by restriction with *Ps* I, *Sph*I + *Sal*I and *Sac*I and electrophoresis on 1% agarose gel. The new plasmid was designated as pGEMT-35S (Fig. 6C).

2. The cDNA fragment coding for lipoxygenase gene (lox) of *Pnlox 3* was subcloned from TOPO vector into *Nco*I– *Xho*I site of pET 30a forming pTMK 12.6. Restricting pTMK 12.6 (Fig. 6D) with *Kpn*I– *Xho*I isolated the fragment containing lipoxygenase gene. This 2700bp fragment should be then ligated into the *Kpn*I– *Xho*I site of the vector pGEM-7Zf (+) (Fig. 6E). Products of the ligation should be then introduced into DH5α cells through electroporation and selected recombinants on LB media containing ampicillin, IPTG and X-gal. The bacterial colonies turned into blue should be rejected whereas white colonies assumed as transformed with the desired fragment should be selected. Colonies carrying recombinant plasmid containing the lipoxygenase gene in desired orientation are identified by restriction with *Kpn*I– *Xho*I and electrophoresis on 1% agarose gel. The new plasmid was designated as pGEM7Z-Lox 3 (Fig. 6F).
3. The plasmid pGEM7Z-Lox 3 should be restricted with *Kpn*I– *Xba*I to release lipoxygenase gene fragment and this 2706bp fragment should be then ligated to *Kpn*I– *Xba*I site of pGEMT-35S. Products of the ligation should be then introduced into DH5α cells through electroporation and the recombinants were selected on LB media containing ampicillin. Colonies carrying recombinant plasmid containing the lipoxygenase gene in desired orientation should be

identified by restriction with *Kpn*I– *Xba*I and electrophoresis on 1% agarose gel. The new plasmid was designated as pGEMT 35S-Lox 3 (Fig. 6G).

4. The plasmid pGEMT 35S-Lox 3 should be restricted with *Spe*I– *Sal*I to release the fragment containing lipoxygenase gene under the control of CaMV 35S promoter and Poly A signal. This 3375bp fragment should be ligated to *Xba*I- *Sal*I site of pCAMBIA 2300 *(-nptII)* (Fig. 6H). Since *Xba*I and *Spe*I are iso-schizomers they can be re-ligated to each other. Products of the ligation should be then introduced into DH5α cells through electroporation and selected recombinants on LB media containing kanamycin. Colonies carrying recombinant plasmid containing the lipoxygenase gene driven by CaMV 35S promoter and Poly A signal in desired orientation should be identified by restriction with *Spe*I– *Xba*I and electrophoresis on 1% agarose gel. The new plasmid is designated as pCAMBIA 2300 *(-nptII)*-Lox 3 (Fig. 6 I).

Subsequently, it is mobilized into *Agrobacterium* strain C58 through electroporation and recombinants were selected on YEB media containing kanamycin for use in *Agrobacterium*-mediated genetic transformation studies.

Cloning Strategy:

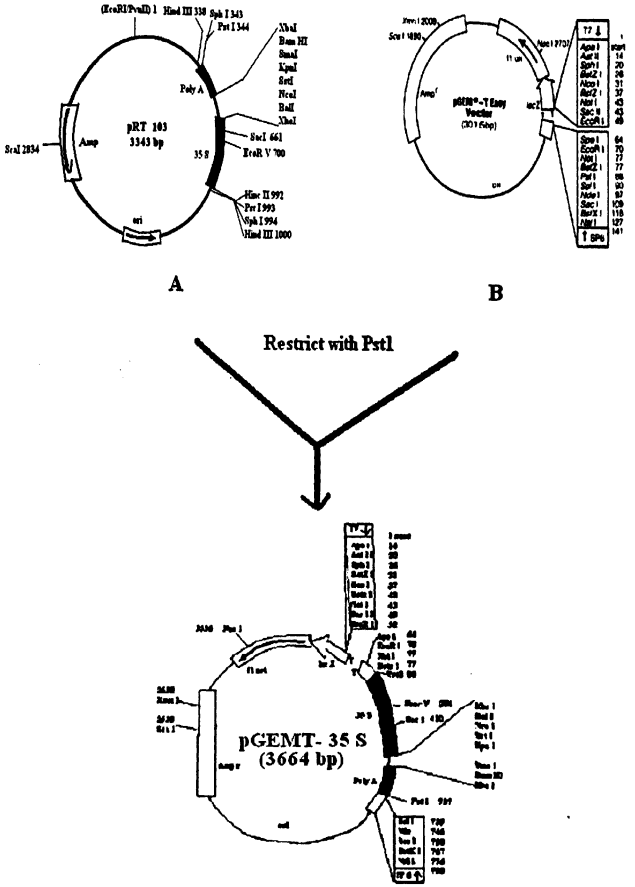

Figure 6: A-C. Plasmid vectors used in this study and their sub-cloning strategy:

A. pRT 103; B. pGEM-T Easy; C. pGEMT- 35S.

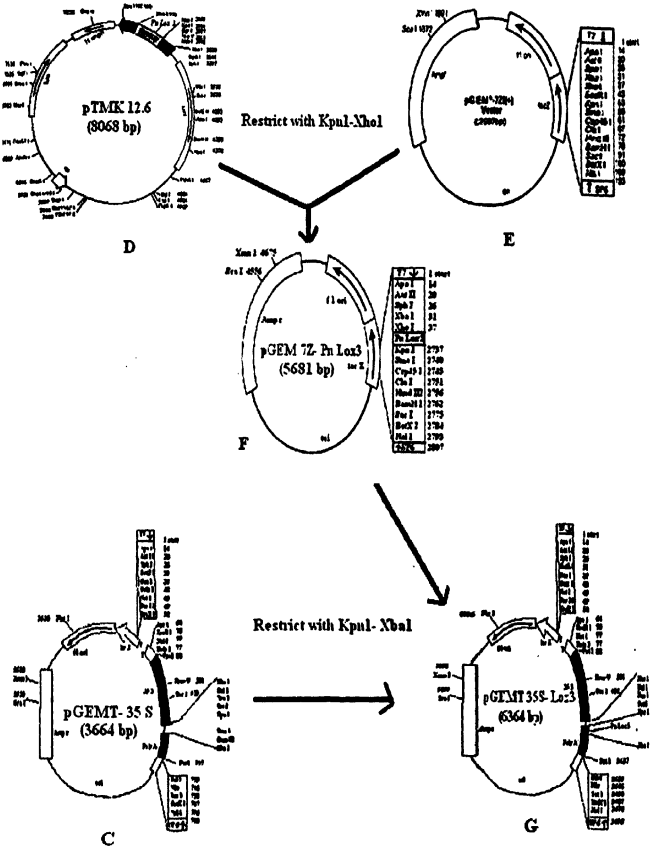

Figure 6: C-G. Plasmid vectors used in this study and their sub-cloning strategy:
C. pGEMT-35S; **D.** pTMK 12.6; **E.** pGEM-7Zf(+); **F.** pGEM 7Z-Lox3;
G. pGEMT 35S-Lox3.

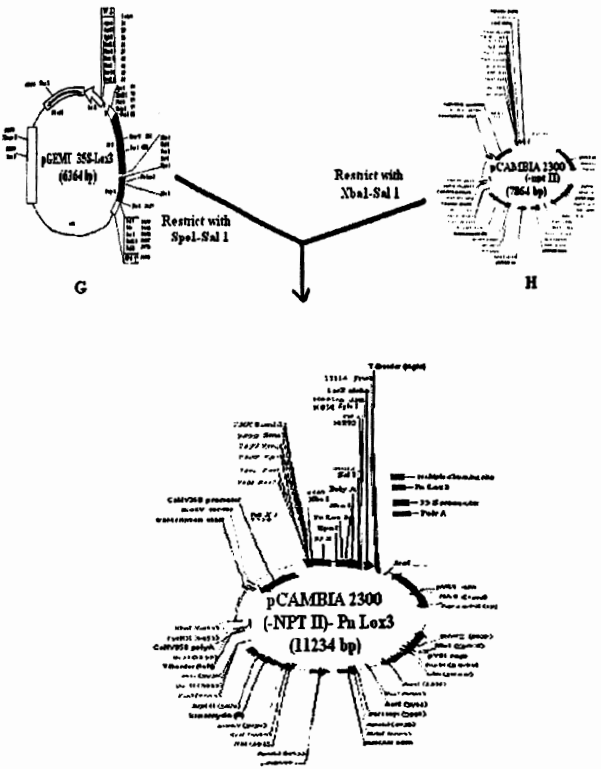

Figure 6: G-I. Plasmid vectors used in this study and their sub-cloning strategy: G. pGEMT 35S-Lox3; H. pCAMBIA 2300 (-*npt*II); I. Binary vector construct pCAMBIA 2300 (-*npt*II): Lox 3.

3.2. Transformation and regeneration systems

The groundnut transformation protocol by using *Agrobacterium*-mediated gene transfer reported earlier (Sharma and Anjaiah, 2000) was followed for the development of transgenic groundnut plants for fungal resistance against *Aspergillus flavus*. Genetic transformation of groundnut was carried out by using the cotyledon explants from pre-saoaked mature seeds via cocultivation with the *Agrobacterium* stain C 58 harboring the binary vector pCAMBIA2300 (-*npt*II): RCH (Kalyani, 2005, Unpublished). Mature seeds of popular groundnut cultivar JL 24 (Spanish type) were used in all the experiments reported here. All the tissue culture and transformation works were carried out under the laminar air flow in absolute asceptical conditions.

3.2.1 *Agrobacterium*-mediated gene transfer

3.2.1.1. Seed sterilization, explant preparation and culture conditions

Mature and well-dried groundnut pods were selected and the shelled seeds were surface sterilized by rinsing in 70% ethanol for 1 min followed by treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min. and then washed thoroughly four to six times. The seeds were soaked in sterile distilled water for 2 h in sterile water before use (Fig. 11 A). After removing the seed coat from the sterilized seeds, the embryo axis was removed surgically and each cotyledon was cut into two vertical halves to obtain the cotyledon explants (Fig.11 B-D.) The *Agrobacterium* suspension was poured in a petriplate so as to make a thin film (2-3 mm) at the base of petriplate. Freshly excised cotyledon explants were taken and the proximal cut ends were immersed into bacterial suspension for few seconds and placed on shoot induction medium.

3.2.1.2. Bacterial culture

A. tumefaciens strain C58 harboring the plasmid pCAMBIA2300 (*-nptII*): RCH was maintained on YEB agar plates containing 50 μg ml^{-1} kanamycin was used for transformation experiments. A single bacterial colony was grown in 20 ml of YEB (Sambrook et al., 1989) supplemented with 25 μl kanamycin overnight at 28 ^{0}C. 5 ml of the overnight grown bacterial culture was taken and centrifuged at 5000 rpm at 4 ^{0}C for 10 minutes and the pellet obtained was re-suspended in sterile 30 ml of half strength liquid MS (Murashige and Skoog, 1962) medium containing 3% sucrose (1:6 dilution). Care should be taken that O.D of the culture should be 0.8- 1 at 650 nm. This suspension was stored at 4 ^{0}C for 2 h and used for co-cultivation.

3.2.1.3. Plant regeneration and selection of stable transformants

The cotyledonary explants were co-cultivated with *Agrobacterium* cells and immediately implanted on shoot induction medium (SIM) with the proximal cut ends embedded in the medium. The SIM standardized earlier (Sharma and Anjaiah, 2000) called as modified MS medium-36 (MMS-36; *Appendix-II*) consists of MS inorganic salts, organic constituents (Gamborg et al., 1968), and 3% sucrose. The medium was supplemented with 20 μM BA and 10 μM 2,4-D. The pH of the medium should be 5.8 (adjusted before autoclaving). The media were solidified with 0.8 % (w/v) Hi-Media® Bacto agar and autoclaved at 121 ^{0}C. After cooling, the medium was dispensed into 90 x 16 mm sterile disposable petriplates.

The inoculated explants were plated at a density of 5 cotyledons per Petri Plate that was sealed with Parafilm® and incubated at 26 ± 1^0 C under continuous light of 100 μEm $^{-2}$ S^{-1} irradiance provided by cool daylight fluorescent lamps. The cotyledon explants co-cultivated with *Agrobacterium* were incubated for 72 h and transferred to

the fresh SIM supplemented with filter-sterilized cefotaxime (250 $\mu g\ ml^{-1}$). Make sure that the cut end of the explant (or the region from where shoots are expected) is in close contact with the medium. Plating density was maintained at five explants per plate. After two weeks, multiple shoot buds appeared on the explants, while shoot buds continue to form. At this stage the explants bearing shoot buds were transferred again to fresh SIM containing 250 $\mu g\ ml^{-1}$ cefotaxime. Maintain the plating density at 5 explants per plate. The organogenic tissues starts differentiated into shoot buds and continued for two weeks. During this period, the explants that are turned into pale and bleached appearance were considered as untransformed ones and they were discarded and calculated percent of stable transformants. Subsequently, the proximal parts of the explants were excised and transferred to culture tubes (25x150 mm) containing shoot elongation medium (SEM). SEM consists of MMS with 2 μM BA and called as MMS 36-2 (*Appendix II*). The shoots were sub-cultured for 2-3 times in SEM for an interval of 2-3 weeks each which has helped in the development and elongation of adventitious shoot buds .The elongated shoots were transferred to root induction medium (RIM; *Appendix I*) comprising of MMS containing 5 μM α-naphthaleneacetic acid (NAA) and maintained for 28 days.

3.2.1.4. Rooting and transplantation of putative transgenic plants

The elongated shoots (5-6 cm) regenerated through *Agrobacterium*-transformation systems were transferred to root induction medium (RIM) comprising of MMS with 5 μM NAA and maintained for about 4 weeks. After sufficient roots were formed, the plants were removed from the culture tubes and thoroughly washed with distilled water and transplanted to 3-inch (dia) pots containing autoclaved sand and soil (1:1) mixture with little amount of Thiram (fungicide). The pots were maintained in a growth chamber at 25 ^{0}C under high humidity (RH 65%) by covering

with perforated polythene tube for 5-6 d. In the initial phase of the acclimatization, small slits were made on the corners of the polythene cover. Later, they were shifted to P2-level greenhouse. After 1 wk the polythene cover was removed and plants were transferred to bigger pots (13 inch dia) which consisted of autoclaved sand and red soil in 1:1 ratio supplemented with small amount of manure and di-ammonium phosphate (DAP). The primary transformants in the containment glasshouse were named as T_0 generation. Upon flowering (2 month after transplantation) and pod formation (about 4 mo), the mature seeds were collected (progeny of T_1 generation) and used for advancement of next generations as T_1, T_2 and so on.

3.3. Molecular characterization of transgenic plants

Various techniques were followed for the molecular characterization of putative transgenic plants. The plant is tested at transcriptional, translational and at the gene expression level to test the presence of transgenes. Preliminary screenings for the presence of transgene in putative transformants were carried by polymerase chain reaction (PCR; Mullis, 1990). Stable integration and number of copies of the inserted DNA are confirmed by Southern hybridization while gene expressions (mRNA) were analyzed by RT-PCR (Reverse Transcriptase RNA dependendent DNA polymerase), Northern hybridization and protein synthesis by Western blotting (Sambrook et al., 1989).

1) Analysis of transgenic Plants at DNA level

2) Analysis of transgenic Plants at RNA level

3) Analysis of transgenic Plants at protein level

A schematic view of various techniques used for the molecular characterization and analysis of transgenic plants is illustrated in Figure 15. Young leaves from 5 leaf staged green house growing transgenic plants were collected and fixed in liquid nitrogen for isolating DNA, RNA and proteins to confirm the transformation and identify transgenic through molecular analysis.

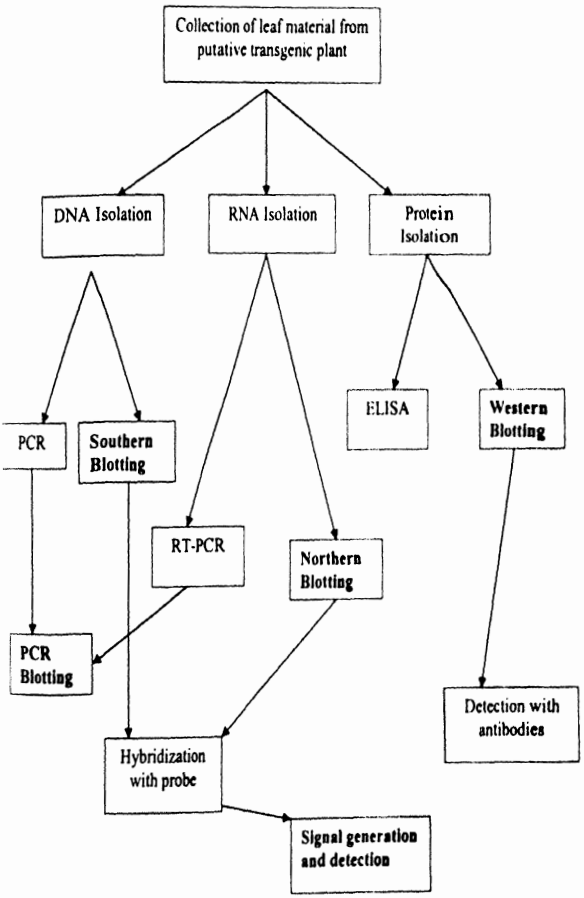

Figure 15: A schematic view of various techniques used for the molecular characterization and analysis of putative transgenic plants.

Analysis of Transgenic Plants at DNA level

3.3.1. Genomic DNA isolation:

The genomic DNA from the putative transgenics was extracted according to the protocol suggested by Porebski et al. (1997). The principle behind isolation DNA by CTAB extraction buffer is that CTAB forms an insoluble complex with nucleic acids. It is highly versatile as even milligram amounts of tissue can be used when sample size is limiting.

Procedure

1. Genomic DNA was isolated from leaf material of glass house grown putative transformants. Leaf tissue (500 mg) was ground in liquid nitrogen in to fine powder with a mortar and pestle.
2. To the powdered leaf tissue, 10 ml of PVP mixed extraction buffer (65°C) was added and transferred to 30 ml centrifuge tubes. The contents were mixed well buy inverting the tubes 3 to 5 times and incubated at 65°C for 45 min.
3. Then 10 ml of chloroform: octanol solution (24:1 ratio) was added and centrifuged at 6,500 rpm for 20 minutes. The supernatant aqueous phase was pipetted out with a blunt end pipette tips very carefully into the fresh centrifuge tubes.
4. Then 0.5 volumes of 5 M NaCl, double the volume of 95% chilled ethanol was added and the contents were mixed by vortexing the tubes very gently followed by incubation at -20°C for 30 minutes and centrifugation at 10,000 rpm for 10 minutes.

5. The pellet was washed with ice-cold 70% ethanol by brief centrifugation for 3-4 minutes.
6. The pellet was air dried and dissolved in 500µl of 10 mM Tris EDTA (pH 8.0).

3.3.2. Purification of genomic DNA:

1. To the dissolved pellet 10µl of RNase (10 mg/mL) was added to degrade RNA by incubating at 37°C in water bath for another 30 min.
2. Later equal volumes of phenol: chloroform (1:1) was added to the TE dissolved DNA solution. The vial consisting of the solution was inverted slowly for three to four times and centrifuged for 10 to 15 min at 10,000rpm.
3. To the aqueous phase equal volumes of chloroform:iso-amyl alcohol (24:1) was added and centrifuged at 12500 rpm for 15 minutes and the aqueous phase was collected.
4. Later, 0.8 volumes of isopropanol was added and incubated at -20^{0}C for 1 hour and centrifuged at 11000 rpm for 20 min.
5. 70%ethanol was added for washing the pellet and centrifuged at 10000 rpm for 5min.
6. The pellet was dried and TE buffer was added to the dry pellet.

To test the quality of DNA, samples are run on 0.8% agarose gel using 1X TAE running buffer.

3.3.3. Quantification of genomic DNA

The concentration of DNA samples was determined spectro-photometrically by measuring the absorbance at 260 nm. One 260 unit is equivalent to 50 µg/ml of ds DNA.

The absorbance ratio at 260 and 280 nm was used as an indication of purity of the nucleic acids (not less than 1.8) of the samples.

3.3.4. Isolation of plasmid DNA:

Procedure:

1. *Agrobacterium* strain was grown on YEB agar plates containing 20μg/ml kanamycin. Single isolated colonies were grown on 25ml agar at 28°C on a shaker overnight and 10 ml bacterial suspension was pelleted by centrifuging for 10 min at 6000 rpm.
2. The pellet was suspended in 1 ml GTE and 30 μl of lysozyme and incubated for 5 min at room temperature in order to maintain the osmoticum.
3. To the resuspended bacterial culture, 2 ml of freshly prepared lysis buffer was added and the samples were placed on ice for 5 min.
4. After 5 min, 1.5 ml of 5M potassium acetate was added to the bacterial lysate and the samples were mixed well by inverting the tubes slowly and the mixture was placed back in ice for 5 min.
5. The solution was centrifuged for 20 min at 10000 rpm and the supernatant was transferred to fresh tubes to which 3 μl of RNase was added to remove RNA and incubated at 37 °C for 30 min.
6. Equal volumes of phenol-chloroform: iso-amyl alcohol (24:1) was added and a brief spin at 10000 rpm for 20 min was given.
7. To the aqueous phase equal amounts chloroform: iso amyl alcohol (24:1) was added and the sample solution was centrifuged for 20 min at 10000rpm.

8. The aqueous phase was collected into fresh tubes and 0.8 volumes of isopropanol or 2 to 3 volumes of ice chilled ethanol was added to precipitate nucleic acids and kept in -80 C for 30 min.
9. The sample was centrifuged for 15 min at 10000rpm and the pellet was washed with 80% ethanol and air-dried. The dried pellet was dissolved in 30 µl of TE.

4. RESULTS

Results

4.1. Vector construction

The 649 bp fragment driven by the 35S promoter and 35S polyadenylation (Poly A) terminator sequences was sub-cloned from plasmid pRT 103 (3343 bp) into the pGEM-T Easy vector (3015 bp). This plasmid (3664 bp size; Fig. 6C) contains ampicillin as selection marker in Escherichia coli strain DH5α. Construction and orientation of this vector was confirmed by plasmid restriction analysis with *Pst*I, *Kpn*I, *Spe*I, *Sal*I, *Sma*I, *Spe*I+ *Sal*I. The restriction analysis of this vector showed linearized fragment of size 3664 bp with restriction with *Kpn*I, *Spe*I, *Sal*I, *Sma*I. The restricted pattern showed two fragments of 3015 bp and 649 bp sizes with digestion with *Pst*I and two fragments of 2989 bp and 675 bp sizes with double digestion with *Spe*I+ *Sal*I, respectively (Fig. 9).

4.2. Regeneration and genetic transformation of groundnut by using *Agrobacterium*-mediated gene transfer

A total of 120 groundnut cotyledonary explants in two batches were used for co-cultivation and the results obtained at various stages were analyzed and summarized in Table 1. Untransformed controls of 20 explants were also maintained simultaneously without inoculation. The cotyledonary explants were initially cultured on shoot induction medium (SIM) for co-cultivation (Fig. 11D). Three days after the explants were transferred to SIM containing 250 μg ml^{-1} cefotaxime and cultured for 2 week. On SIM, over 83.3% of responded explants were turned into green and enlargement in size (Fig. 11E-F). During this period, initiation of multiple shoot buds differentiated at proximal cut ends of the explants and continued to form, which was observed in 92% of the initially

responded explants. The explants that were not responded became pale white, brownish and shrinked in size, which were discarded during next sub-culture. The explants bearing shoot buds were transferred to SIM containing 250 μg ml^{-1} cefotaxime and sub cultured for 2 week (Fig. 12 A-D). No selection procedure was followed since the construct pCAMBIA 2300: RCH (-*npt*II) lacks the marker gene that codes for kanamycin. Later, the shoot buds were cut into 2-3 pieces and transferred to shoot elongation medium (SEM) for 2-3 passages of 3 wk each (Fig. 12E). At this stage, most of the explants with shoot buds gave rise to 3-4 shoots from each. In some explants, the shoot buds were not elongated, remained static with abundant callus growth around it. Such explants were discarded and only elongated shoots (obtained over 93% from multiple shoot bud explants) were advanced for further sub culturing. The elongated individual shoots were cut at the base and the trimmed were transferred into root induction medium (RIM). The adventitious roots appeared within 2 weeks (%) and further developed for four weeks (Fig. 12F). The rooted shoots (Fig. 13A) were transplanted to small plastic pots containing autoclaved sand: soil mixture and maintained at RH 70% for a week and later into bigger pots (Fig. 13B-E). A total of 73 plants were established and maintained in P2 level greenhouse for further advancement (Fig. 14A-D).

These putative transgenic plants appeared phenotypically normal without any morphological abnormalities in any form. In general, it was observed that the explants response and shoot bud proliferation were higher in untransformed compared to the transformed explants. This indicates that during the process of transformation the induction of shoot buds might have been affected due to interaction between the transgene and host cells.

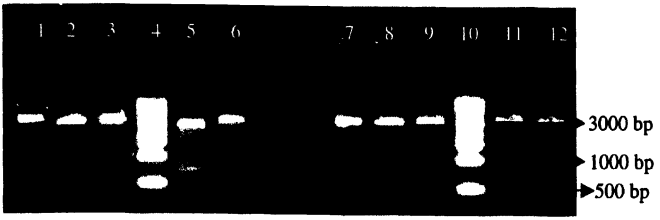

Figure 7: Restriction digestion of plasmids pRT 103 and pGEM-T Easy vector- Lane 1: pRT 103 plasmid; Lane 2: pRT 103 restricted with *Sma*I; Lane 3: pRT 103 restricted with *Kpn*I; Lane 4, 10: 1 Kb ladder (NEB); Lane 5: pRT 103 restricted with *Pst*I; Lane 6: pRT 103 restricted with *Sac*I; Lane 7: pGEM-T Easy plasmid; Lane 8: pGEM-T Easy restricted with *Nco*I; Lane 9: pGEM-T Easy restricted with *Pst*I; Lane 11: pGEM-T Easy restricted with *Sal*I; Lane 12: pGEM-T Easy restricted with *Spe*I.

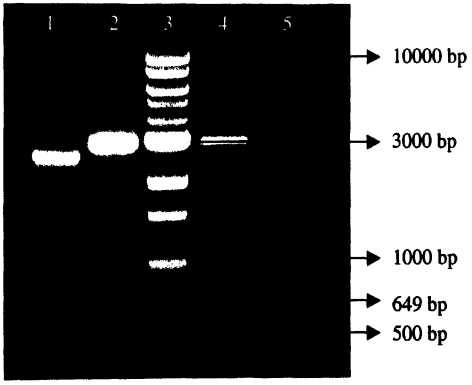

Figure 8: Restriction Digestion pattern for checking the clone pGEMT-35S- Lane 1: pRT103 restricted with *Pst*I; Lane 2: pGEM-T Easy restricted with *Pst*I; Lane 3: 1 Kb ladder; Lane 4: Clone restricted with *Pst*I; Lane 5: Clone plasmid

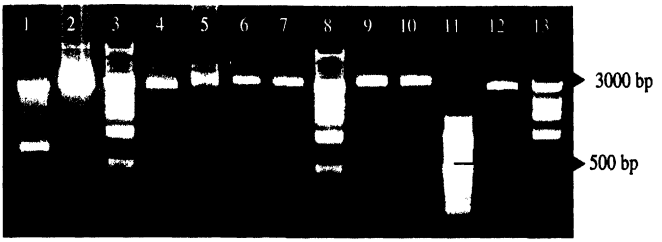

Figure 9: Restriction digestion analysis for confirmation of the clone pGEMT-35S- Lane 1: pRT 103 restricted with *Pst*I; Lane 2: pGEM-T Easy restricted with *Pst*I; Lane 3, 8, 13: 1 Kb ladder; Lane 4: pGEMT-35S (clone) restricted with *Pst*I; Lane 5: pGEMT-35S plasmid; Lane 6: pGEMT-35S restricted with *Kpn*I; Lane 7: pGEMT-35S restricted with *Spe*I; Lane 9: pGEMT-35S restricted with *Sal*I; Lane 10: pGEMT-35S restricted with *Sma*I; Lane 11: 100 bp ladder; Lane 13: pGEMT-35S restricted with *Spe*I + *Sal*I.

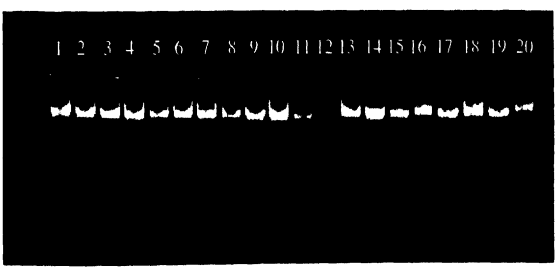

Figure 10: Genomic DNA samples of putative transgenics transformed with pCAMBIA 2300:RCH (-*npt*II).

Table 1: Groundnut regeneration (R) percent/ ratio during various stages of *Agrobacterium*-mediated transformation in cotyledon explants.

S.No	Responded explants during plant development	Nos.	Percent /ratio compared at various stages during regeneration
1	Total number of explants	120	
2	Explants responded	100	83.3
3	Explants with multiple shoot bud	92	76.6
4	Elongated shoots	112	93.3
5	Rooted shoots	99	82.5
6	Fully established plants	73	60.8

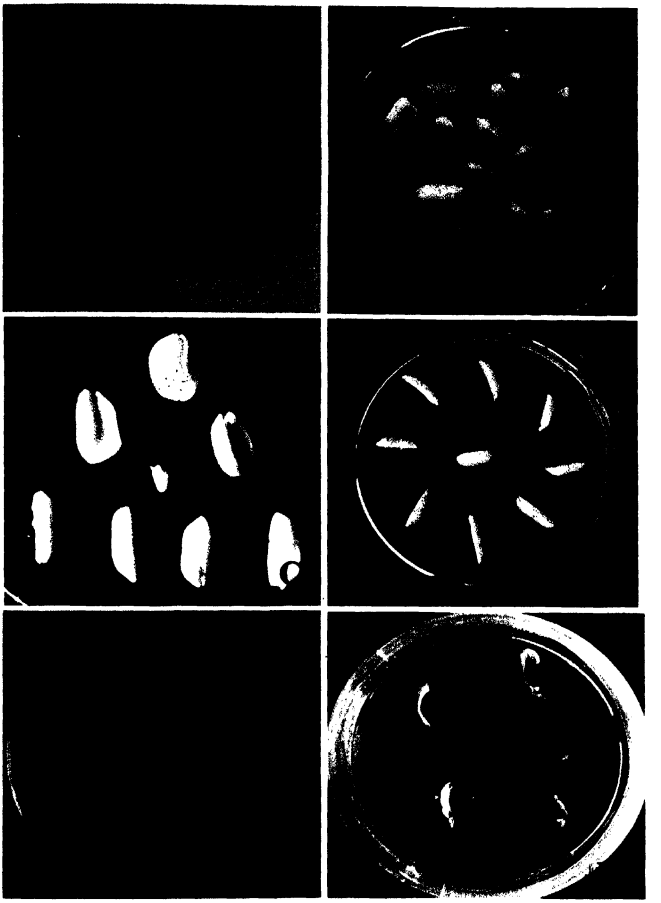

Figure 11. A-F. Preparation of cotyledon explants for the genetic transformation of peanut. **A.** Mature JL 24 seeds; **B.** De-coated seeds after the removal of seed coat from the pre-soaked seeds; **C.**De embryonated and split cotyledonary explants ; **D.** Vertically cut half cotyledon explants for *Agrobacterium*-mediated transformation; The cotyledon explants were incubated on shoot induction medium (SIM) containing MS supplemented with 20 μM BA and 10 μM 2,4-D after co-cultivation with *Agrobacterium*; **E-F.** Explants turned green and enlarged within 3 days prior to transfer to SIM with 250 μg ml^{-1} cefotaxime.

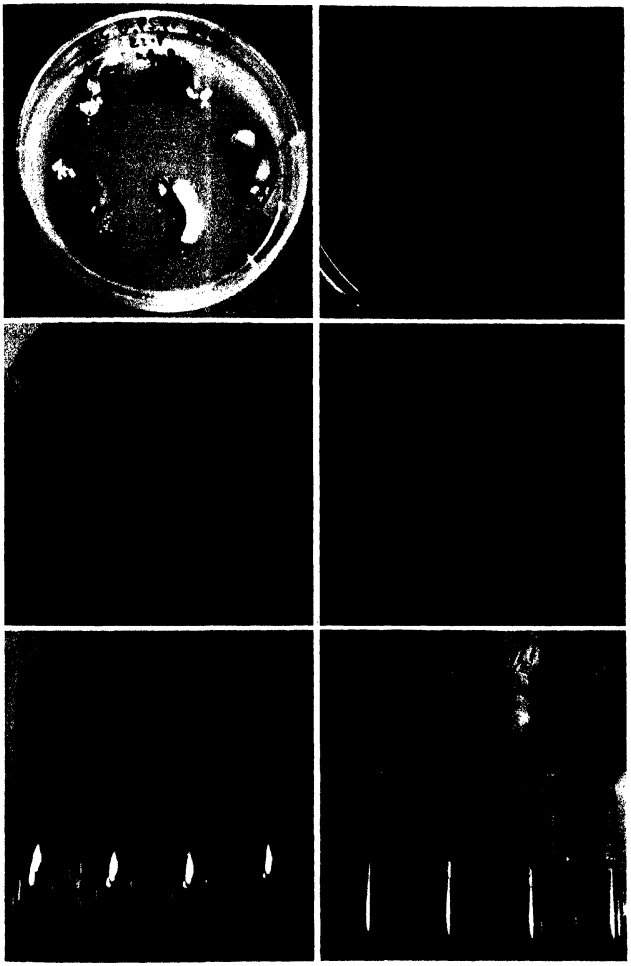

Figure 12. A-F. Regeneration and transformation of groundnut by using cotyledon explants through *Agrobacterium*-mediated gene transfer. **A-D.** Stages in the induction of adventitious shoot buds from cotyledon explants after 14 days of culture on SIM showing the formation of the multiple buds; **E.** Multiple shoots elongated on shoot elongation medium, MS supplemented with 2 μM BA; **F.** Rooting of the elongated shoots on root induction medium, MS supplemented with 5 μM NAA.

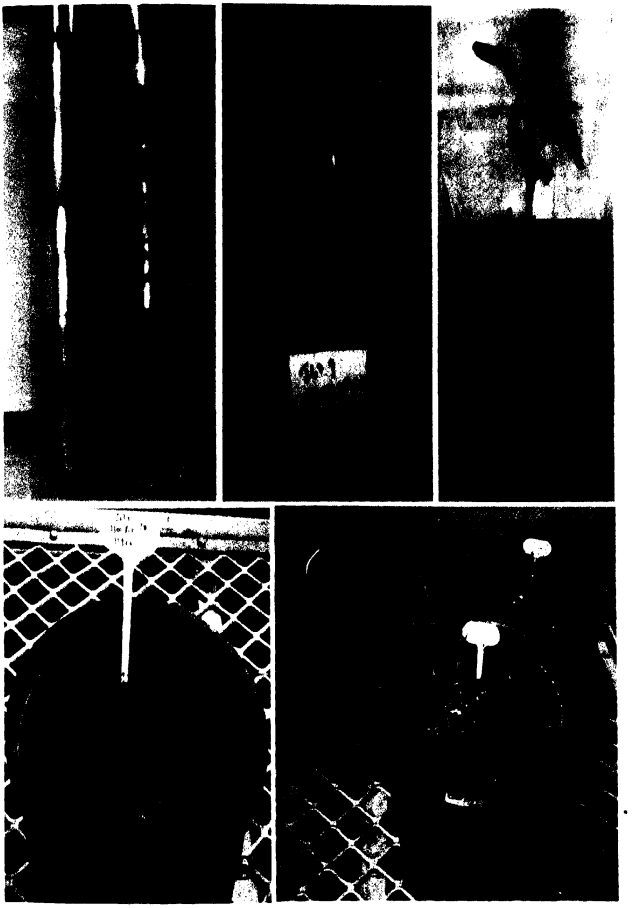

Figure 13. A-E. Rooting of the in vitro-formed shoots and their transplantation to the greenhouse. **A.** Well developed roots on an in vitro formed shoot after 28 days of culture on RIM showing the normal adventitious roots for transplantation; **B-C.** Initial transfer into jiffy pots containing sterile sand for the establishment of the roots and the seedlings covered in perforated poly bag and incubated in growth chamber for 4 days; **D-E.** Fully established healthy seedlings transferred into bigger pots containing sand: soil mixture and maintained in the greenhouse.

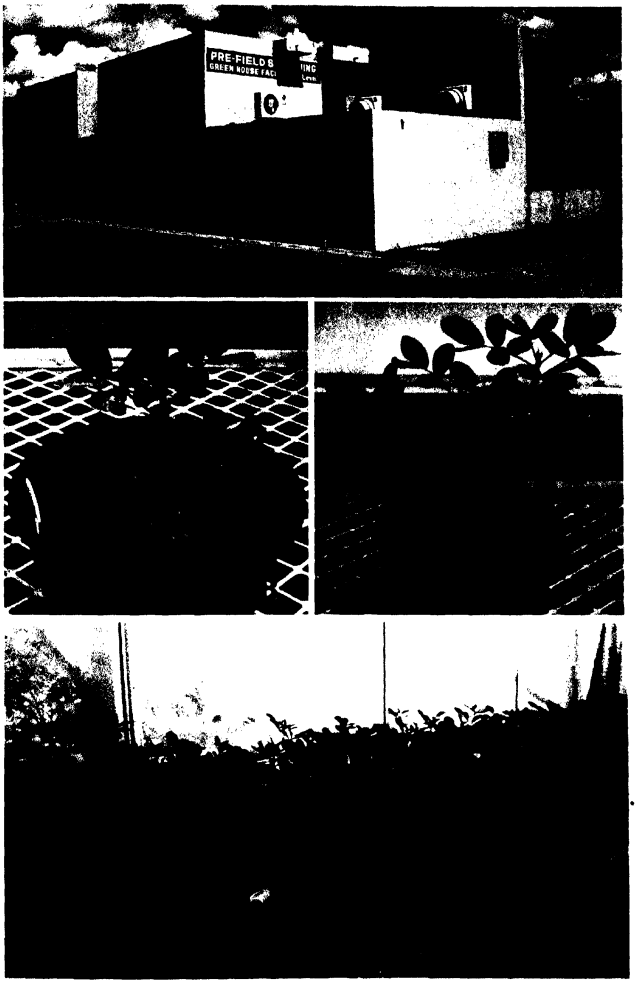

Figure 14. A-D. Transfer of the primary transformants of groundnut to the P2 level containment greenhouse and advancement of generations. **A.** P2 greenhouse facility at ICRISAT; **B-D.** Well established transgenic plants growing in the P2 greenhouse where the plants were phenotypically and morphologically normal.

5. DISCUSSION

Discussion

Infection of groundnut (*Arachis hypogaea* L.) seed by *Aspergillus flavus* Link:Fr. and *A. parasiticus* is a serious problem. This infection can result in the contamination of the seed with aflatoxins, which are toxic fungal metabolites. Progress has been made in an attempt to prevent aflatoxin contamination in crops (Robens et al., 2003) through crop management and handling, microbial ecology and bio-competitive microbes, and crop resistance through genetic engineering and conventional breeding (Guo et al; Guo et al).

Production of groundnuts with low or no aflatoxin immensely benefits subsistence farmers who grow groundnuts for their own consumption and sell surplus in local markets. The outputs of this project will provide genetically enhanced groundnut germplasm with resistance to the fungus and aflatoxin production, which can be deployed for cultivation by farmers, and also can be used by plant breeders for developing groundnut varieties with improved adaptation and resistance to *A. flavus* and aflatoxin production, and contribute to consumer health and improve the trade.

The present study was carried out to develop groundnut transgenic plants for durable resistance against the fungus *Aspergillus* by introducing the Rice chitinase and lipoxygenase gene. Genetic engineering of plants involves the stable integration of foreign DNA sequence usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. There are numerous reports on the in vitro regeneration and genetic transformation of groundnut by using various types of explants and transformation systems. Each transformation system has its own advantages and limitations. Hence, the published literature on groundnut regeneration and transformation was thoroughly reviewed to adopt the most widely used

transformation systems that allow high frequency regeneration following the protocols utilizing the cotyledon explants for *Agrobacterium*-mediated transformation (Sharma and Anjaiah, 2000). During the course of this study, about 73 putative transgenic plants carrying the marker free Rice chitinase gene were generated by the research group at GTLab, ICRISAT.

Development of transgenic groundnut plants carrying the *Rice Chitinase* gene

One of the most important elements in recombinant DNA (rDNA) technology is the cloning of desired genes and their sub-cloning in suitable vectors for plant transformation. The construction of chimeric genes allows the expression of any coding sequence under the control of 5' and 3' non-coding regions (promoter and terminator signals) of genes in plant (Herrera-Estrella et al., 1983). For *Agrobacterium*-mediated gene transfer, binary vector pCAMBIA 2300: RCH (-*npt*II) was used.

The development of an efficient regeneration system is a pre-requisite for genetic transformation of plants and the transformation frequency depends on the regeneration efficiency of the explants (Birch, 1997). Direct regeneration systems have advantages due to the rapidity of morphogenesis and no requirement of frequent subcultures. Besides, *de novo* production of shoots is extremely rapid and synchronous with the period of cellular differentiation (Hazara et al., 1989; McKently et al., 1991). The regeneration in groundnut by using various explants has been reported by several workers including, immature embryos, (Singh et al., 1980; Ozias-Akins et al., 1989; Baker and Wetzstein, 1992: Kanyand et al., 1994), embryo axis (Atreya et al., 1984, Hazra et al., 1989, Baker and Wetzstein, 1992, Ozaias-Akins et al., 1993), hypocotyls (Narsimhulu and Reddy. 1983; Dong et al., 1990; McKently et al., 1991), epicotyls (Cheng et al., 1997; Little et al., 2000), meristematic tissues

(Kartha et al., 1981), immature cotyledons (Ozaias-Akins et al., 1992), mature cotyledons (Sharma and Anjaiah 2000; Venkatachalam et al., 2000), and leaf segments (Pittman et al., 1983; Eapen and George, 1994; McKently et al., 1991; Baker and Wetzstein, 1992; Chengalrayan et al., 1995; Livingstone et al., 1995; Cheng et al., 1997). However, most of these studies reported low efficiency of regeneration, thereby resulting in low transformation frequencies ranging from 0.1-2.5%. Venkatachalam (1998, 2000) reported that the groundnut cotyledons pre-cultured on embryo induction MS medium containing NAA, BAP, resulted in transformed embryos, which efficiently gave rise to shoots (47%). The putative transgenic plants developed in vitro were transferred to pots and maintained in the containment P2 level greenhouse for advancement of generations.

Due to the lack of efficient protocols to regenerate whole plants from the transformed tissues, not much success with genetic transformation of *Arachis* species was achieved until recently. Sharma and Anjaiah (2000) successfully obtained high frequency direct shoot regeneration (92%) from cotyledon explants from mature pre-soaked seeds in several groundnut genotypes belonging to both Spanish and Virginia types. In the preset study this protocol was followed to obtain high regeneration frequency of up to 83.3% in cotyledon explants.

Analysis of a large collection of genetically modified plants generated in recent years has tremendously expanded our knowledge of physiological processes and gene regulation mechanisms. However, transgenes do not always behave as expected (Matzke, 1995; Stam et al., 1997). There is considerable variation in the expression of transgenes in individual transformants, which is not due to the copy number of inserted gene(s). Thus, gene activity is not exclusively determined by the strength of the promoter which controls transcription, rather the epigenetic effects also

influence gene expression levels. This sometimes leads to gene inactivation either by blocking transcription or by inhibiting mRNA accumulation. Although all the events are expected to possess the transgene, only some transgenic events exhibit resistance, while others show susceptibility or low resistance in greenhouse and field evaluations. While this may be due to the silencing of transgene, it remains unresolved whether this is due to methylation, or is a response to a change in chromatin structure and functions epigenetically to maintain and transmit the silent state.

The earliest evidence for *Agrobacterium*-mediated gene transfer in groundnut by using hypocotyl explants was reported by Dong et al. (1990) and Lacorte et al. (1991). *Agrobacterium*-mediated transformation by using leaf explants of groundnut resulted in a transformation frequency of 2% (Eapen and George, 1994) while Cheng et al. (1997) obtained fertile transgenic plants with 0.3% frequency. Venkatachalam (1998, 2000) reported that the pre-cultured groundnut cotyledons co-cultivated with *Agrobacterium* strain LBA 4404, harboring plasmid pBI 121 containing *uid*A and *npt*II genes, for 2 days prior to transfer on an embryo induction medium resulted in transformed embryos which efficiently gave rise to shoots (47%) on MS medium containing BAP and kanamycin. Similarly, Daniel (2002) reported an *Agrobacterium*-mediated transformation protocol by using immature embryonic leaves with transformation efficiency of 45% for the development of transgenic plants of groundnut for resistance to GRAV. An efficient *Agrobacterium*-mediated transformation system with high transformation frequency (>55%) based on cotyledon explants forming adventitious shoot buds (>90%) was reported by Sharma and Anjaiah (2000) for the development of groundnut transgenic plants carrying the coat protein gene of IPCV. Transformation efficiency is yet to be determined by using the binary vector pCAMBIA 2300: RCH (-*npt*II).

6. CONCLUSIONS

Conclusions

This study demonstrated the possibilities of obtaining a large number of transgenic events in groundnut with high frequencies through both *Agrobacterium* mediated genetic engineering. The study also was successful in preliminary regeneration of putative transgenic groundnut events carrying the pCAMBIA 2300: RCH (-*npt*II) under both invitro conditions and contained greenhouse. Future activities could involve detailed evaluation of the selected events to ascertain the threshold level of fungal resistance under contained and natural disease conditions. Salient features of this study are as follows:

- A cloning vector, pGEMT-35S was constructed by sub-cloning a *Pst* I fragment (649 bp) from plasmid pRT 103 carrying CaMV 35S promoter and a CaMV 35S polyadenylation sequence into plasmid pGEM-T Easy vector and the resulting construct pGEMT-35S was mobilized into *E. coli* strain DH5α by electroporation.
- A high frequency of shoot bud induction and regeneration (>70%) was obtained from cotyledons. Seventy-three independent putative groundnut transgenic events carrying pCAMBIA 2300: RCH (-*npt*II) were developed through *Agrobacterium tumefaciens* mediated genetic transformation methods, which are at various stages of development.

In conclusion, the regeneration system used in this study proved to be very efficient in terms of the regeneration of multiple adventitious multiple shoot and the protocol gains significance owing to its person and lab independent reproducibility. This regeneration system resulted in a comparatively high frequency of genetic transformation and recovery of valuable transgenic plants. The transgenic plants with

the incorporated *RCH* gene will be very useful in forthcoming future and can considerably help in the biotechnological improvement of groundnut resistant to fungus *Aspergillus*.

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APPENDICES

Appendix I

Composition of chemicals and stock solutions for MMS (Modified Murashige and Skoogs, 1962) medium for tissue culture of groundnut

Chemical	Concentration (grams per litre)	Stock
MS-Major Salts (50 X)		
*NH_4NO_3	1650	66.0 g/400 ml
*KNO_3	1900	38.0 g/400 ml
*$MgSO_4.7H_20$	370	17.6 g/400 ml
*KH_2PO_4	170	14.8 g/400 ml
*$CaCl_2$	440	6.8 g/400 ml
***MS-Minor Salts** (100X)		* in 1000 ml
KI	0.83	83 mg
H_3BO_3	6.2	620 mg
$MnSO_4.4H_2O$	22.3	2250 mg
$ZnSO_4.7H_2O$	8.6	860 mg
$Na_2MoO_4.2H_2O$	0.25	25 mg
$CuSO_4.5H_2O$	0.025	2.5 mg
$CoCl_2.6H_2O$	0.025	2.5 mg
***B5 Organics** (100X)		* in 1000 ml
Nicotinic acid	2.0	200 mg
Pyridoxine mono Hcl	2.0	200 mg
Thiamine Hcl	25.0	2500 mg
***MS- Organics** (100X)		* in 1000 ml
Glycin	2.0	
Nicotinic acid	0.5	200 mg
Pyridoxine mono Hcl	0.1	50 mg
Thiamine Hcl	0.5	50 mg
		100 mg
***MS Fe-EDTA (100 X)**		* in 1000 ml
EDTA.$2H_2O$	37.3	3.73 g/l
$FeSO_4.7H_2O$	27.8	2.78 g/l
MS-Inositol (100X)		
*Myo-inositol	100.0	10.0 g/l
Harmonoes		
*BA	2.25	22.53 mg/ 100 ml
*2,4-D	2.20	22.1 mg / 100 ml
*NAA	1.86	18.6 mg /100 ml
*IAA		

Appendix II

MMS media used at various stages of shoot regeneration from the cotyledon explants * (*Appendix I*) (Amounts are for 1 litre)

Component	MMS for cotyledonary explants		
	Shoot Induction Medium (36)	**Shoot Elongation Medium (36-2)**	**Root Induction Medium (NAA)**
Major			
*NH_4NO_3	10 ml	10 ml	10 ml
*KNO_3	20 ml	20 ml	20 ml
*$MgSO_4.7H_20$	10 ml	10 ml	10 ml
*KH_2PO_4	10 ml	10 ml	10 ml
*$CaCl_2$	10 ml	10 ml	10 ml
*MS-Minor	10 ml	10 ml	10 ml
*B5-Organics	10 ml	10 ml	10 ml
*MS Fe-EDTA	10 ml	10 ml	10 ml
*MS-Inositol	10 ml	10 ml	10 ml
Sucrose	30 g	30 g	30 g
Agar	8 g	8 g	7.5 g
pH	5.8	5.8	5.8
BA	20 ml	2.0 ml	---
2,4-D	10 ml	---	---
*NAA			5.0 ml

Appendix III

Preparation of chemicals and buffers useful in genetic transformation studies

Composition of DNA extraction buffer (Porebski et al., 1997)

Component	Stock Conc	Working conc for 100ml
Tris	1 M	20 ml
Nacl	5 M	56 ml
EDTA (pH 8.0)	100 mM	40 ml
CTAB	10 %	40 ml
β- mercaptoethanol	0.3 %	300 µl (added just before use)
H_2O		40 ml

2 N NaOH

NaOH 8g

Dissolve in 100 ml of SDW

5 M Potassium acetate

Potassium acetate 49.07g

Dissolve in 100 ml SDW

Make up the pH to 5.2 with acetic acid.

5 M NaCl,

NaCl 29.22g

Dissolve in 100 ml SDW

0.5 M Sodium phosphate

Sodium phosphate 6.9g

Dissolve in 100 ml of SDW

Adjust the pH to 7 with 5 M HCl.

1 M Tris

Tris-HCL 12.1g

Dissolve in 100 ml of SDW

Adjust the pH to 8 with 1N NaOH.

100 mM EDTA

EDTA 3.72g

Dissolve in 100 ml of SDW

Adjust the pH to 8 with 1N NaOH.

CTAB 10%

Dissolve 10 gm of CTAB in 100 ml of SDW.

10% SDS

Dissolve 10 gm of SDS in 100 ml of SDW.

2.5 M $CaCl_2$

$CaCl_2$ 36.75g

Dissolve in 100 ml of SDW

3 M Sodium acetate

Sodium acetate 24.61g

Dissolve in 100 ml of SDW

Adjust the pH to 4.8 with acetic acid.

50X TAE.

Tris-HCL Na_2	242g
EDTA.$2H_2O$	372.2g
Acetic acid	57.1 ml

Adjust the pH to 8.5; make up the volume to 1L with SDW.

Preparation of 1X TAE for 5 L

50X TAE	100 ml

Make up the volume to 5 L with SDW.

10x Electrophoresis buffer (TBE buffer, pH 8.3)

Tris base (0.45M)	54g
Boric acid (0.45M)	27.5g
0.5M EDTA, pH 8 (0.01M)	20ml

Distilled water to 1 liter

5x Sample buffer (Gel loading buffer)

Bromophenol blue (0.25%)	5mg
Xylene cyanol FF (0.25%)	5mg
Glycerol (30%)	3ml

Sterile distilled water to 10ml

1% Ethidium bromide solution

Ethidium bromide	100mg
Distilled water	10ml

Store in a dark coloured bottle at 4°C

LB medium/L (Sambrook et al., 1989)

Bacto–peptone	10
Yeast extract	5
Sodium chloride (NaCl)	10
Agar	15
PH	7

100mM IPTG

Dissolve 0.2383 gm of IPTG in 10ml of water. Filter sterilize and store at -20^0C.

RNase preparation (10mg/ml)

RNase	100 mg
1 M Tris HCl (10mM) pH 7.5	100 µl
5 M NaCl (15mM NaCl)	30 µl

Make up the volume to 10 ml with sterile water. Heat in boiling water for 15-20 min and allow cooling slowly to room temperature. Dispense into aliquots and store at -20^0C.