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Development of groundnut (*Arachis hypogaea* L.) transgenics for resistance to *Aspergillus flavus*

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

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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CERTIFICATE

This is to certify that the thesis entitled **"Development of groundnut (*Arachis hypogaea* L.) transgenics for resistance to *Aspergillus flavus*"** submitted by **Mrs. Sowmini Sunkara** for the award of degree of **Doctor of Philosophy** in **Biotechnology** to Jawaharlal Nehru Technological University Hyderabad, Hyderabad-85 is the original research work carried out by her 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 **"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 joint supervision of Dr. Kiran K. Sharma, Principal Scientist, and Dr. Farid Waliyar, Director, West and Central Africa, ICRISAT, 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.

Sowmini Sunkara

TO WHOMSOEVER IT MAY CONCERN

I hereby declare that the dissertation entitled upon **"Development of groundnut (*Arachis hypogaea* L.) transgenics for resistance to *Aspergillus flavus*",** is an original and independent record of research work undertaken by **Ms Sowmini Sunkara** at Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, under the joint supervision of Dr. Kiran K. Sharma, Principal Scientist, Director, PTTC, ICRISAT and Dr. Farid Waliyar, Director, West and Central Africa, ICRISAT, except where specifically stated to the contrary, and it has not been submitted for any degree or diploma of any other University.

Dr. G Dileepkumar

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Date:

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Submitted: February, 2014

Abstract

Peanut (*Arachis hypogaea* L.) is an economically important oilseed crop which is grown widely in the semi-arid tropics, and India being the second largest producer in world with annual yield of 5.78 MT (FAOSTAT, 2012). Aflatoxin contamination of peanut has gained global significance due to the deleterious effects of these contaminants on human and livestock health, and the consequent importance in international trade. Although, aflatoxin contamination does not affect crop productivity, it makes the produce unfit for consumption while the higher aflatoxins load in the exportable commodities jeopardize the export earnings. Breeding efforts so far have not resulted in varieties with durable resistance to high levels of *A. flavus* infection and consequent aflatoxin production. Biotechnological applications involving the genetic engineering technology provides an attractive approach for developing transgenic events to circumvent this important problem. Plant lipoxygenases (LOXs) are hypothesized to play an important role in mediating host-pathogen interactions by initiating the octadecanoic branch in response to fungal attack, catalyzing the oxidation of polyunsaturated fatty acids such as linoleic acid (18:2) and α-linolenic acid (18:3) to produce unsaturated fatty acid hydroperoxides. Jasmonic acid (JA), a derivative of α-linolenic acid has been reported as a potent inhibitor of aflatoxin biosynthesis. At ICRISAT, work was initiated using the LOX gene approach

for addressing this recalcitrant problem in peanut. A highly efficient peanut transformation protocol using cotyledon explants was used to produce 25 marker-free transgenic events of peanut by incorporating *PnLOX3* gene under the control of constitutive and seed specific promoters. These transgenic events were characterized at molecular level using PCR, Southern hybridization and RT-PCR assays for the presence and expression of the transgene which were further evaluated under contained greenhouse conditions.

1. Introduction

Leguminosae is an important family of angiosperms consisting of many species related to human nutrition, pasture and fodder needs. In terms of human nutrition they are important protein and mineral rich seed bearing plants which rank next to cereals. They are mostly herbaceous, such as peas, lentils, beans which are collectively known as pulses and commonly referred to as 'poor man's meat' in certain cultures gaining due importance and quantitative significance as food additives. Although, there are several species and subspecies classified as food legumes, only few (15 to 20) genera are very important. Hundreds of cultivars within these genera are included in 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 the production and cultivated area for total food legume output. In order of importance, peanut, cowpea and beans form essential staple food in the diets of millions. Peanuts share approximately 10 percent among production of 286.7 million metric tons of world total oilseeds behind soybeans (53%), rapeseed (15 %) and cotton seeds (12 %) .

The decrease in peanut productivity is mainly contributed by various biotic, abiotic and economic factors. The economic status of the small and marginal farmers restricts them to use poor quality local seed in addition to minimum or no fertilizer applied during cultivation which is essential as peanut is mostly grown in marginal and poor soils of low fertility. Use of complex fertilizers may also lead to deficiencies of multi-nutrients such as calcium and sulfur affecting the yields. Non-adoption of seed treatment against seed-borne diseases results in decay and death of seed/seedling.

A more recalcitrant plant/microbe interaction is that of seed-infecting fungi of which a troublesome seed colonizer is the genus *Aspergillus* that infects oilseeds, especially peanut, maize and cottonseed, and contaminates them with aflatoxin or sterigmatocystin, two related carcinogenic mycotoxins

(Refs….). Aflatoxins are toxic, carcinogenic, teratogenic and immuno-suppressive substances, produced by *Aspergillus flavus* and *A. parasiticus*, are associated with both acute and chronic toxicity in humans and animals causing liver cirrhosis, acute liver damage, 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).

Peanut is one of the most susceptible legume crops which act as the host to *A. flavus* invasion and subsequently aflatoxin production. 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. The presence of deteriorative fungi, *Aspergillus flavus* and *A. parasiticus*, with ability to produce mycotoxin of type “aflatoxins” in peanuts represents a serious hazard for human and animal health, and it has been reported to grow in all countries around the world (Williams et al., 2004).

After identification of the peanut aflatoxin problem in 1963 (Bampton, 1963), many studies have been carried out in a number of peanut-producing countries. It is evident that peanut could be invaded by *A. flavus* and *A. parasiticus* and subsequently become contaminated with aflatoxin, before harvest and postharvest (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 occurs during postharvest storage (Swindale, 1987; Ahmed et al., 1989). Although adopting some cultural practices, curing and drying, and storage practices can minimize aflatoxin contamination, these may not be suited to small-scale farming in the developing countries, especially in tropical areas. Chemical control and removal of toxin have not yet been completely successful (Mehan et al., 1987). Use of peanut varieties that are resistant to

infection by *A. flavus*, or resistant to aflatoxin production if colonized by the fungus was suggested to be an effective solution to the problem (Mehan et al., 1987; Mixon, 1986; Petit et al., 1987).

The frequency of pre-harvest infection of peanut 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 peanut produced in all countries are prone to aflatoxin contamination, situation is particularly alarming in South Asia and Sub-Saharan Africa, as most of the peanut 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 peanut and peanut-products (van Egmond and Jonker, 2002). For instance, the USDA has set 20 μg kg^{-1} and European Union has set a limit of 2 to 4 μg kg^{-1}. Several other countries have set limits ranging from 10 to 30 μg kg^{1}.

Figure 1: Peanuts infected with *Aspergillus* fungi are the source of aflatoxin which is a primary problem for peanuts worldwide (Dely et al., 2005).

Domination of the cereals in the food sector resulted only in marginal increases in the overall yield of pulses (Sunkara, 2007). Recent concerns over the importance of these crops led to augmented efforts to improve the quality and quantity. Classical and modern breeding technologies resulted in limited success in interchange of the desirable characters in these crops. Recent advances in molecular biology techniques, plant tissue culture and genetic transformation have provided an impetus to these efforts. Biotechnological improvement with amalgamation of all the above strategies has emerged as a potential tool for crop improvement in these crops.

Extensive efforts in 1980's were carried for identification of resistance to *A. flavus/A. parasiticus* infection and aflatoxin contamination which 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 a 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 peanut 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 peanut are not easily available.

Better knowledge of biochemical mechanisms involved in response to the environmental change and host-plant interaction helps in identifying plant mechanisms which are responsible for aflatoxin synthesis, thereby increasing the possibility of usage of genetic engineering research in plant varieties for enhanced host-plant resistance. Various antifungal proteins and peptides have been isolated from a wide range of plants which have been already tested for their antifungal activity against *Aspergillus* spp. Aflatoxin resistant transgenic crops would not only control *A. flavus*, but

also other microbial [fungal, bacterial, and viral] diseases that cause significant economic losses in crop production. Hence, development of transgenic varieties with antifungal traits that confer resistance to aflatoxin-producing fungi will be extremely valuable and will be an aid to the breeding tools.

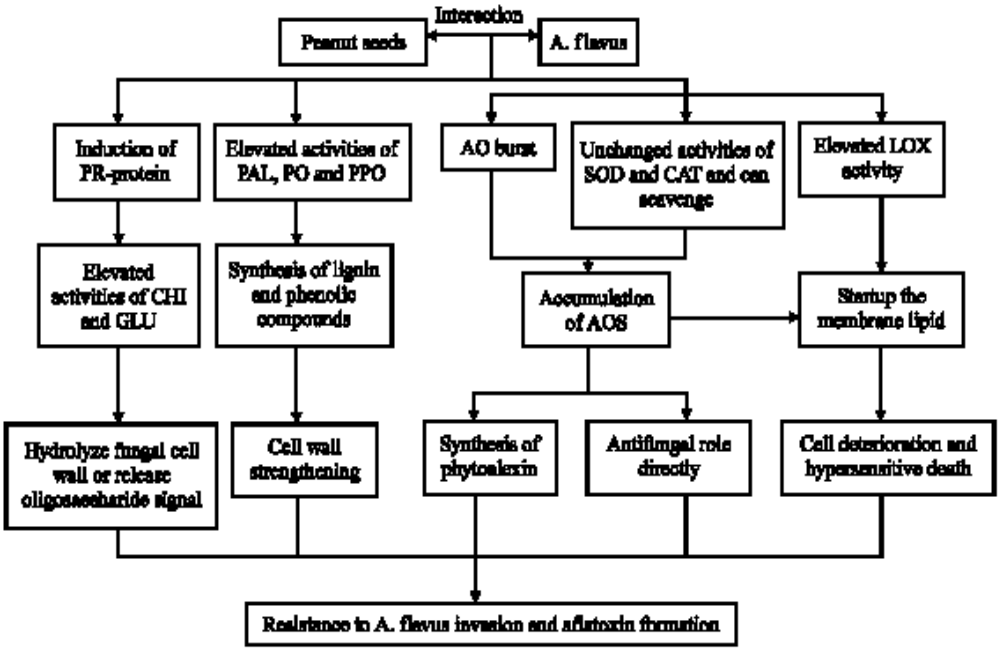


Figure 2: Schematic drawing of defense responses activated by the interaction of peanut-*Aspergillus flavus*; PR: pathogenesis-related protein, PAL: phenylalanine ammonia lyase, PO: polyphenol oxidase, AO: active oxygen, SOD: superoxide dismutase, CAT: catalase, LOX: lipoxygenase, CHI: chitinase, PPO: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

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 peanut (Refs.…). 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., 2013, Personal communication).

Initially many of the legume 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 successful genetic engineering of crop plants. Each species responds differently in tissue culture and different protocols were successfully used for genetic transformation (Ref to some recent reviews.. The recent developments and increase in using different approaches of transformation and regeneration of fertile plants will substantiate the value of this approach to be realistic and dependable.

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 peanut cultivars. Promising transgenic events can be selected and deployed for farmer cultivation and simultaneously, they also can be used as resistant donors to incorporate resistance into peanut cultivars through conventional breeding programmes. ICRISAT has successfully developed the techniques for efficient transformation and regeneration of peanut that has already resulted in the development of several transgenic peanut 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***' at DDPSC**,** '13S and 9S LOX ***(lipoxygenase)*** genes' (Burow et al., 1997, 2000; Tsitsigiannis et al., 2005), '***rice chitinase***' at KSU (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 peanut events.

Objectives

The objectives of the present study were aimed to develop groundnut varieties with very low to non-existent levels of aflatoxin contamination.

1) Sub-cloning of Lipoxygenase gene (*PnLOX3*) from pTMK 12.6 (Tsisigiannis et al., 2005) along with 35*S* promoter into binary vector.
2) Genetic transformation of groundnut genotypes using binary vector containing lipoxygenase gene through *Agrobacterium* mediated transformation.
3) Identification of transgenic plants showing high expression through molecular characterization by PCR, Southern blotting, RT-PCR, Western blotting, Northern blotting and ELISA techniques.
4) Confirmation of resistance through fungal bioassays.

2. Review of literature

Peanut

Peanut is one of the world's most popular oil and protein rich legume crops, cultivated universally in more than 100 countries. The geographical classification of peanut 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 peanut area and about 94% of total production (Freeman et al., 1999). On the global scale, India is a major producer of peanut 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.

Peanut-History, Origin, and Distribution

Peanut 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 L.* has the widest distribution of any *Arachis* species. It is a major crop in tropical and sub-tropical areas of the world. Species related with *A. hypogaea* are currently evaluated for farmer use (Stalker & Moss, 1987; Moss, 1985b) whereas *A. villosulicarpa* was majorly cultivated in the Brazilian state northwestern part of Mato Grosso by Indians (Gregory et al., 1973). *A. repens* and *A. glabrata* are grown in South America as ground covers in urban areas. Peanut 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).

Peanut is virtually unexplored at the genomic level because of the large genome size (2,800 Mb/1C) and complication. It is known to be originating from South America around the current borders of Brazil, North Eastern Paraguay, Bolivia and northern Argentina. The geographical distribution of peanut is delineated into six continents: the Americas, Africa, Asia, Near East, Europe, and Oceania (Gregory et al. 1980), grown in 25.2 million ha throughout the world in over 100 tropical and sub-tropical countries (FAOSTAT 2010) between the latitudes 40° N and 40° S with a total global production of 36.5 m t (FAO 2008). Both Krapovickas (1969, 1973) and Gregory et al. (1980) postulated a planalto profile from Corumba to Joazeiro, and confirmed Brazil, as the centre from which *Arachis* distribution occurred. The geocarpic habit of peanuts appear to be an unusual feature that has interesting consequences for dispersal by water

and population structure whose species distribution is associated to a large extent with the different main river systems (Gregory et al. 1973).

A better understanding of the taxonomic relationships between taxa and level of speciation of *Arachis* presents an important resource of novel alleles for cultivated peanut crop improvement which can be used as a prerequisite for its effective use as secondary gene pool in peanut breeding programs. *Arachis* is a genus of about 70 species of annual and perennial flowering plants in the pea family, with only 23 species being cultivated of which *A. hypogaea* and *A. villosulicarpa* are mostly cultivated for their nutritional qualities. *A. hypogaea* is widely grown across the globe as an important major food crop compared to others such as *A. villosulicarpa* which is only accepted by Indians. Other species in the genus such as *A. pintoi* are cultivated worldwide as forage since leaves act as a source of high-protein feed for grazing livestock and as a soil conditioner to plants providing nitrogen source in agroforestry and permaculture systems.

A. hypogaea is classified into two subspecies and six varieties (var. *hypogaea* and var. *hirusta* in subsp. *hypogaea*; var. *fastigiata*, var. *vulgaris*, var. *peruviana* and var. *aequatoriana* in subsp. *fastigiata*). It is highly likely to have originated through hybridization event between two diploid species (Kochert et al. 1996). The domesticated peanut (*A. hypogaea*) is an amphidiploid or allotetraploid (2n = 4x = 40) with two sets of chromosomes expected to be originated from an interspecific cross between two different species *A. duranensis*, an A-genome diploid, and *A. ipaensis*, a B-genome diploid. Peanut can readily cross with tetraploid *A. monticola*, a species which has a “weedy” conspecific relation to peanut (Hilu and Stalker 1995) or which might have evolved as a weedy phenotype from *A. hypogaea* (Stalker and Simpson 1995, Jung et al. 2003). All other species in the section are diploid (2n = 2x = 20) annual and perennial species and cross with *A. hypogaea* with varying degrees of difficulty. Several lines of evidence supporting this hypothesis include archeological data (Simpson et al. 2001), the frequency of common molecular markers (Kochert et al. 1991, 1996), cytological characteristics (Seijo et al. 2004) and gene sequence data (Jung

et al. 2003, Ramos et al. 2006),but recent molecular data identify other putative A-genome progenitor candidates (Milla et al. 2005). Even though the A- and B- genomes of peanut can be readily distinguished by molecular polymorphisms, the level of polymorphism among peanut cultivars and accessions is very low, suggesting a genetic bottleneck at the time peanut originated (Kochert et al. 1996).

Peanut crop grows best in sandy, well- drained soils in a wide range of field conditions from clays to sands and from acidic to alkaline soils. As compared to other oil seeds, peanut plants are relatively drought resistant, which makes them to play a significant impact in tropical and sub-tropical regions of Asia, Africa, and North and South America where precipitation exceeds evaporation for only 2-7 months per year (Bunting et al., 1985).

Thousands of peanut cultivars are grown which are distinguished by branch length and branching habit which are classified into two main growth forms, bunch type which grows upright, while runner types grow near the ground. Of these Spanish, Valencia, Runner, and Virginia along with Tennessee red and white groups are the major popular cultivar groups which are grown preferentially for their flavor, oil content, size, shape, and disease resistance. Most peanuts marketed with the shell are mostly Virginia type followed by Valencias. Mostly Runners and Spanish cultivars of peanuts are used for peanut butter whereas Spanish cultivars are also used for peanut candy, salted nuts. Introducing a new cultivar of peanut every year by breeding or any other modern methods introduces changes in the planting rate, adjusting the planter, dryer, harvester, sheller and cleaner thus affecting the methods of marketing.

Consumer's preference

Peanuts 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. Peanut 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. Major seed

proteins of peanut as well as of other leguminous crop species, are deficient in the essential sulphur containing amino acid methionine.

Peanuts are primarily utilized as food, feed and fodder. They can be eaten raw, roasted, boiled and used in recipes. Edible oil extracted from the nuts is important for human consumption and the meal is used for livestock feed. Salted peanuts, peanut brittle, peanut butter (primarily used for its long storage capacity and high protein content in the commercial manufacture of sandwiches, peanut candy bars, peanut butter cookies, and cups), and shelled nuts (plain/roasted) which form popular confections made from peanuts. Compared to Brazil nuts, cashews, walnuts, peanuts are less expensive and hence are often used as a major ingredient in mixed nuts. Recently, usage of bakery products of raw, unshelled green peanuts boiled in brine as a snack is been increasing in the United States along with use of fried peanut recipes - allowing both nut and shell as feeding source. Peanuts are also widely used in other areas, such as cosmetics, nitroglycerin, plastics, dyes, medicines, textile materials and paints.

Most peanuts 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 also play an important role 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 peanut is gaining importance as an income source in tree plantations, such as coconut, rubber, or banana. In Africa and Asia, many peanuts are intercropped between maize, sorghum and pulses.

Peanuts are considered to be nutritionally important by providing over 30 essential nutrients and phytonutrients. Peanuts are a good source of niacin, folate, fiber, magnesium, vitamin E, manganese and phosphorus. They also are naturally free of trans-fats and sodium, and contain about 25% protein. Peanuts are also used to help fight malnutrition. High protein, high nutrient and high energy based peanut- pastes developed so far are currently being used as therapeutic food to aid in famine relief for saving malnourished children in developing countries by organizations like the

World Health Organization, UNICEF, Project Peanut Butter and Doctors Without Borders which include Plumpy Nut, MANA Nutrition, and Medika Mamba.

Peanut Productivity constraints

India has the largest peanut growing area with 4.90 million ha (20.46 %) and stands second in the production at 5.78 million tons (15 %) with an average yield of 11794 Hg/Ha next to China which is second in area with 4.73 million ha (18.88 %) and leads in production at 16.87 million tons (41.71%) with an average yield of 35670 Hg/ha (FAOSTAT 2012). From USDA estimates (FAS 2000), peanuts ranked third in production among oilseeds and 90% of world peanut production was accounted by developing countries (ERS 2001) with 2.5% increase annually.

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 peanut crop that result in great economic losses annually. Though the world harvested area of peanut has changed very little since 1970s with an annual growth of only 0.1% (between 1972-1990) and 1.2% (between 1991-2000), the production has increased from 0.8 metric tons (during 1972) to 1.37 metric tons (during 2000) i.e., 1.9% increase per year (Revoredo and Fletcher 2002). It is generally accepted that the average yield of peanut is below its presumed potential, and efforts to improve the productivity of this crop by conventional breeding means have not been very effective. Since the mid-1970s edible peanuts have increased in both domestic consumption and export trade. In contrast, production in Africa has declined by 17 percent over the last two decades. Acreage, production and productivity of peanut in India has shown large amount of fluctuations since 1993-94 to 2006-07. The productivity of peanut in India suffers mainly since 80% of the crop is grown under rainfed conditions by resource poor farmers (Kaushik 1993). Lack of irrigation facilities to protect the crop from soil moisture deficit during breaks in rainfall in monsoon season affects germination. Rainfall pattern during the pre-sowing months and availability of substitute high-value oilseed crops

like soybean and sunflower with short durations requiring less water had significant negative impact on acreage allocation decisions of the farmers (Patil et al. 2009). Resource-poor farmers who obtain low yields of 500-800 kg ha^{-1} due to various biotic and abiotic constraints grow about 93.8 percent of the world's production of peanut. Moreover, a big gap exists between the realized yield and potential yield of peanut at both subsistence and commercial systems of production in Asia and Africa. Frequent aflatoxin contamination in peanut produced in developing countries has drastically reduced peanut exports for Asia and sub-Saharan Africa (Freeman et al., 1999; Ntare et al., 2005).

Peanut production process from planting to harvest is majorly affected by different types of biotic and abiotic stresses which cause annual yield losses of over US$ 3.2 billion (Dwivedi et al. 2003). The major abiotic factors affecting peanut production include drought, high temperature, low soil fertility, low soil pH and iron chlorosis. Among the biotic factors, fungal diseases, virus diseases, bacterial wilt disease, aflatoxin contamination, nematodes, foliar insect pests, and soil insect pests, pod borer (*Helicoverpa* spp.) play a significant role in yield reduction (Sharma and Oritz, 2000; Dwivedi et al. 2003). The plant disease management technologies are greatly influenced by environmental pollution, deleterious effects of chemicals on non-target organisms, resurgence of pesticide resistance among pathogens, outbreak of secondary pathogens.

The major reason behind this is the lack of sufficient and satisfactory levels of genetic variability within the germplasm of cultivated peanut. Many wild annual *Arachis* species, which possess a wealth of agronomically desirable genes, are sexually incompatible with the cultivated varieties. Several advanced research institutes or groups are working with ICRISAT and other partners to apply modern biotechnology to the problems of peanut improvement in developing countries.

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. 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 elsewhere. Tools of genetic engineering can be exploited as an additional method for introduction of agronomically useful traits into established cultivars.

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 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) which has made rapid progress, resulting in an increase in the understanding of how cells work at molecular, physiological and biochemical levels.

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).

Tools of plant biotechnology such as marker-assisted breeding, in vitro mutagenesis, tissue culture, embryo rescue and genetic transformation have contributed to provide solutions to reduce the constraints (Dita et al., 2006) which could result in yield increases achieved by development and use of cultivars addressing the abiotic and biotic stresses. Comprehensive

reviews on the history of molecular marker development in peanut were provided by Stalker and Mozingo (2001) and Dwivedi et al. (2003). The important bottleneck for molecular breeding is the lack of understanding the molecular genetic basis of stress resistance that regulate the expression of stress-related genes which is a fundamental issue in plant biology. This has been answered by the emergence of "omics" technologies and the establishment of model legume plants such as *Glycine max, Medicago truncatula* and *Lotus japonicus* (Cannon et al. 2009) which will be helpful for the legumes crop improvement (Bertioli et al. 2011).

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 two decades, worldwide cultivation of biotech crops has been increasing at a fast pace. During this seventeen-year period of commercialization from 1996 to 2012, global area of biotech crops has an unprecedented increase of about 100-fold from 1.7 million ha to 170.3 million ha with an increasing participation of developing countries representing an annual growth rate of 6% from 160 million ha in 2011 (James, 2012). Of the 28 countries that grow biotech crops which constitute more than half the world's population i.e., about ~60% or ~4 billion people, 20 were by developing countries which grew 52% of biotech crops and 8 were by industrial countries which grew 48% of biotech crops. The growth rate for biotech crops was three times faster and five times larger in developing countries when compared to industrial countries, 90% of which is being grown by resource poor farmers thus making biotech crops as the fasted adopted crop technology (ISAAA Brief No. 44-2012). Sudan and Cuba planted its first biotech crops Bt cotton and Bt maize respectively in 2012. The cumulative economic benefits of developing countries were high i.e., US$ 49.6 billion when compared to US$ 48.6 billion from industrial countries.

Biotech mega countries are those countries which grow 50,000 ha, or more, of transgenic crops. In 2012, there were 18 mega-countries which

reflects that adoption of biotech crops is being at a more balanced and stabilized way by a broader group of countries with 80% increase in the number of mega-countries from 10 in 2003. The 18 mega-countries were USA with 69.5 million ha, followed by Brazil with 36.6 million ha , Argentina with 23.9 million ha, Canada with 11.6 million ha, India with 10.8 million ha, China with 4.0 million ha, Paraguay with 3.4 million ha, South Africa 2.9 million ha, Pakistan with 2.8 million ha, Uruguay with 1.4 million ha, Bolivia with 1.0 million ha, Philippines with 0.8 million ha, Australia 0.7 million ha, Burkina Faso with 0.3 million ha, Myanmar with 0.3 million ha, Mexico 0.2 million ha, Spain 0.1 million ha, and the Chile with 0.1 million ha (James, 2012) which were rated in descending order of hectarage of biotech crops.

Globally, US continued to be the lead producer of biotech crops with 69.5 million hectares whereas Canada grew 8.4 million hectares of biotech canola, India cultivated 10.8 million hectares of Bt cotton with whilst China grew 4.0 million hectares of Bt cotton with record adoption rates of 97.5%, 93% and 80% respectively. China, India, Brazil, Argentina, and South Africa, collectively grew 78.2 million hectares (46% of global) which represent ~40% of the global population of 7 billion remains the five leading developing countries growing biotech crops. India also increased its farm income from biotech crops especially through Bt cotton alone by US$ 12.6 billion (2002 – 2011).

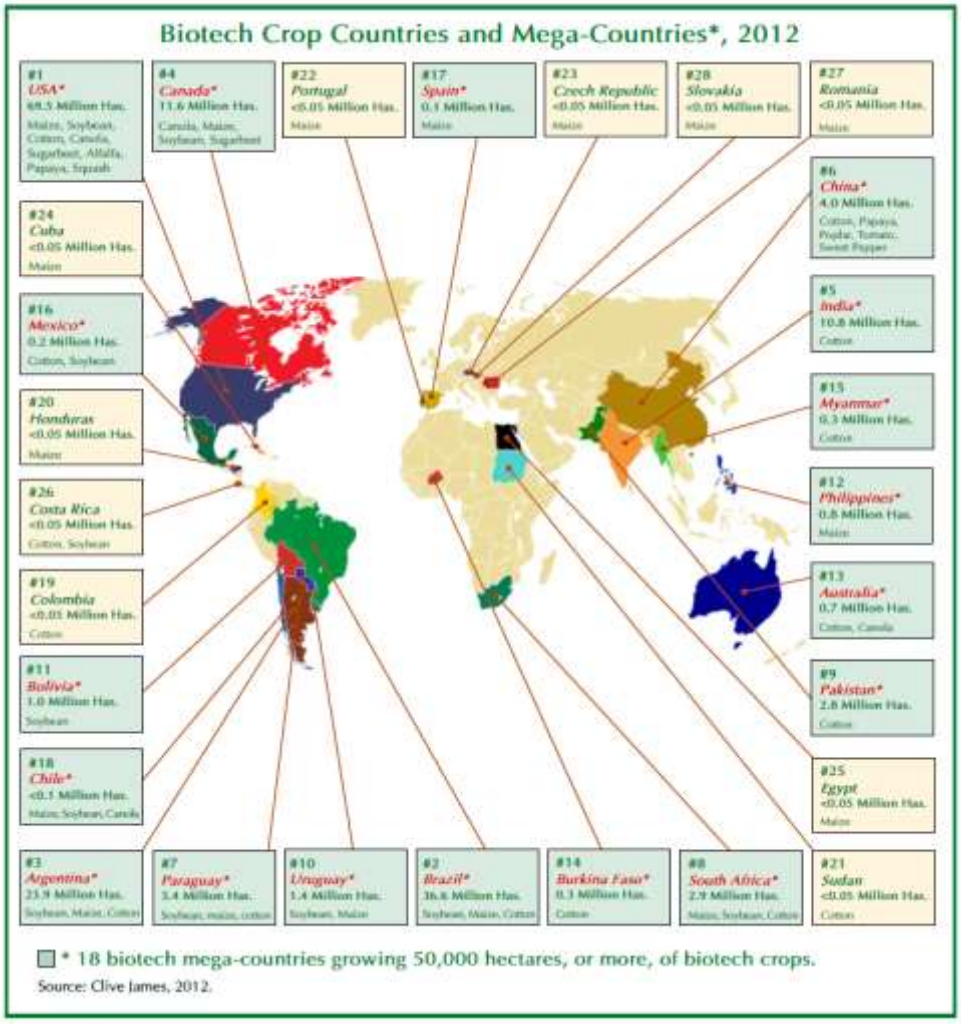


Figure 3: Global map of biotech crop countries and Mega countries* in 2012 (James, 2012)

Tools of genetic engineering and molecular biology have provided with unprecedented power to develop and manipulate novel genotypes thus resuting in 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 labor 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 use of transgenic technology have registered steady increase in area (170.3 m ha) and have slowly spread across 30 countries (James, 2012). The prerequisite for sustainable use of biotechnology for crop improvement is development and deployment of transgenic plants (Sharma et al., 2000). Advances in genetic transformation and gene expression have made rapid progress in genetic engineering during the last decade (Hilder and Boulter 1999; Sharma and Oritz, 2000). In addition, genetic engineering not only allows the use of several novel desirable genes into a single event thus widening the pool of useful genes but also reduces the time and effort taken for introgression of 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 programs.

Transgenic research has opened exciting opportunities in plant protection which result in prolonged benefit in sustainable agriculture with high degree of safety which is also an important part of second green revolution. The techniques of genetic modification will allow breeders to access new gene pools, particularly those of wild *Arachis* species, bringing valuable traits into the modern cultivated peanut that cannot be addressed by conventional means. Development of transgenic peanut therefore has a good potential for its improvement. Advances in biotechnology have provided

alternative pest control strategies that are based on natural biological processes. Tissue culture and genetic engineering have proven as important powerful tools in biotechnology that have been extensively used, either by taking advantage of naturally occurring defense mechanisms, which confer disease resistance of avoidance, or by modifying plant genome to develop pest resistance.

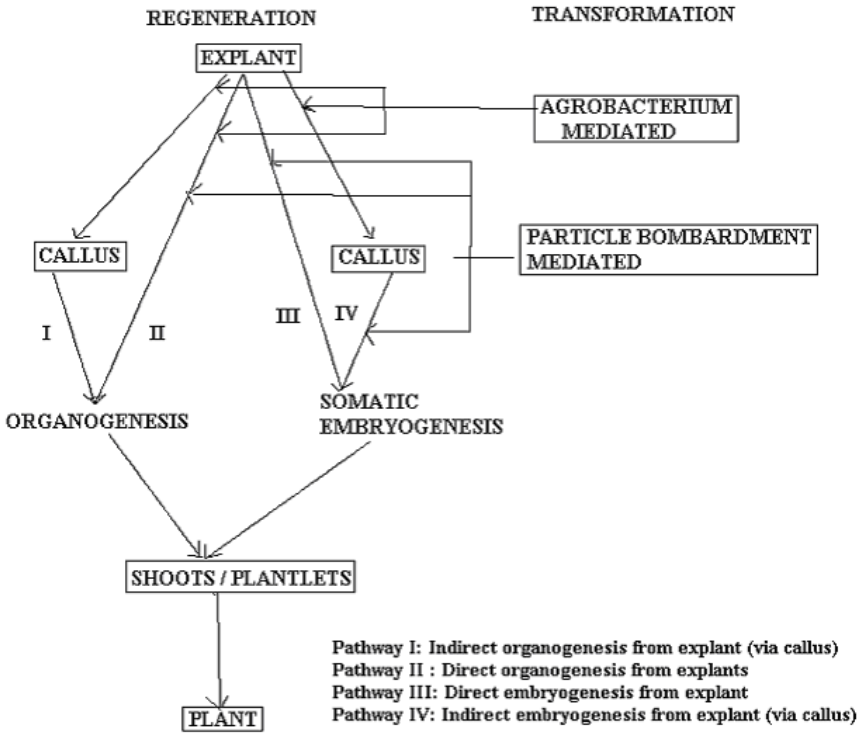


Figure 4: Flow chart showing different pathways of *in vitro* regeneration and their amenability to the two major methods of gene delivery (Atika et al., 2003).

Gene cloning and vector constructs

Genes for transformation can be broadly divided into those that will be used to overcome agronomic limitations (high yield potential, resistance to biotic and abiotic stresses) and ones that could be used to enhance value-added traits (Schnall and Weissinger, 1995). Although major emphasis is currently being placed on improving the primary constraints, the manipulation of value-added traits, such as flavor and nutrition will be of much concern for peanut improvement using transgenic technology. Transgenic technology could conceivably be used in peanut for the

introduction of disease and pest resistance as well as value-added traits such as improved vitamin, protein, and oil quality, enhancing the crop product value, quality, and safety. The genus *Arachis*, which itself is a repository for most of the valuable pest and disease resistance genes, could be used to transform cultivated peanut varieties (Bhatnagar-Mathur et al. 2008). Current efforts include incorporating immunity or very high resistance to several viral and fungal diseases through transformation of peanut cultivars that have very high demand for which no adapted resistant peanut genotypes are available. Improved crop protection through the transfer and expression of disease resistance genes will decrease or eliminate the usage of pesticides, which are costly to the grower and may be harmful to the environment.

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).

Gene transformation methods

Despite of significant advances over the past decade, development of efficient transformation methods still take many years of painstaking research (Sharma et al., 2005). Groundnut transformation like all other transformation system relies on the common key elements. Development of an efficient transformation system in crops for the introduction of genes also depends on the various factors such as development of reliable and reproducible tissue culture regeneration systems, selection and preparation of suitable gene constructs and vectors, molecular characterization of transgenic plants for confirming stable and efficient gene expression, recovery and multiplication of transgenic plants, phenotypic evaluation of transgenic plants for checking their effectiveness against the biotic and abiotic stresses in the field condition, transfer of genes by conventional breeding methods to elite cultivars, biosafety assessments including health, food, and environmental safety and deployment of genetically modified plants.

Developments in genetic transformation for incorporation of novel genes into the peanut gene pool have emboldened researchers with new opportunities for crop improvement in this important legume to pursue the development of transgenic peanut plants resistant to various diseases, insect pests, enhanced nutritional quality and abiotic stresses (Sharma and Anjaiah 2000; Rohini and Rao 2001). Transformation of plants involves the stable introduction of desirable DNA/gene sequences into the nuclear genome of cells, which are capable of giving rise to a whole transformed plant. Transformation and regeneration are interdependent and the totipotency (i.e., single cell capable of giving rise to a whole plant in vitro) of the somatic plant cells via organogenesis or somatic embryogenesis under appropriate hormonal and nutritional conditions (Skoog and Miller 1957) is the essential feature for development of an efficient tissue culture techniques. Totipotent cells give rise to adventitious shoots or somatic

embryos, which are both competent and accessible for gene transfer and will give rise directly to nonchimeric transformed plants.

A suitable system for selection of transgenic tissues and plants is one of the most important aspects of any transformation system. The utility of any particular gene construct as a transformation marker varies depending on the plant species and explant involved. 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 5. Promoters are essential to control expression of the gene and also provide valuable insights about the overexpression or silencing of any gene in response to external stimuli. The most commonly developed transgenic plants use either the constitutive promoters like 35S of the cauliflower mosaic virus (CaMV) or the maize ubiquitin or potato ubiquitin (Yang et. 2003; Joshi et al. 2005) to drive expression of the gene of interest in their gene constructs. These promoters being constitutive in nature sometimes results in expression of the downstream transgenes in all organs and at all the developmental stages, which can be metabolically expensive leading to undesirable pleiotropic effects (Bhatnagar-Mathur et al. 2008). Hence, use of inducible or tissue-specific promoters is increasing in recent years for enhancing targeted gene expression, which also safeguards against biosafety and regulatory concerns to certain extent. Use of these tissue-specific constructs is also important in RNAi technology to augment gene silencing strategies (Bhatnagar-Mathur et al. 2008).

Genetic transformation of plants is performed using a wide range of tools, the basic gene transfer techniques are grouped under two categories (Potrykus, 1985). Different methods of DNA transfer have been developed for the production of transgenic peanut over the last few years. The most commonly used means of DNA delivery or transferring novel genes into either organogenic or embryogenic cultures of plant cells/ peanut are either

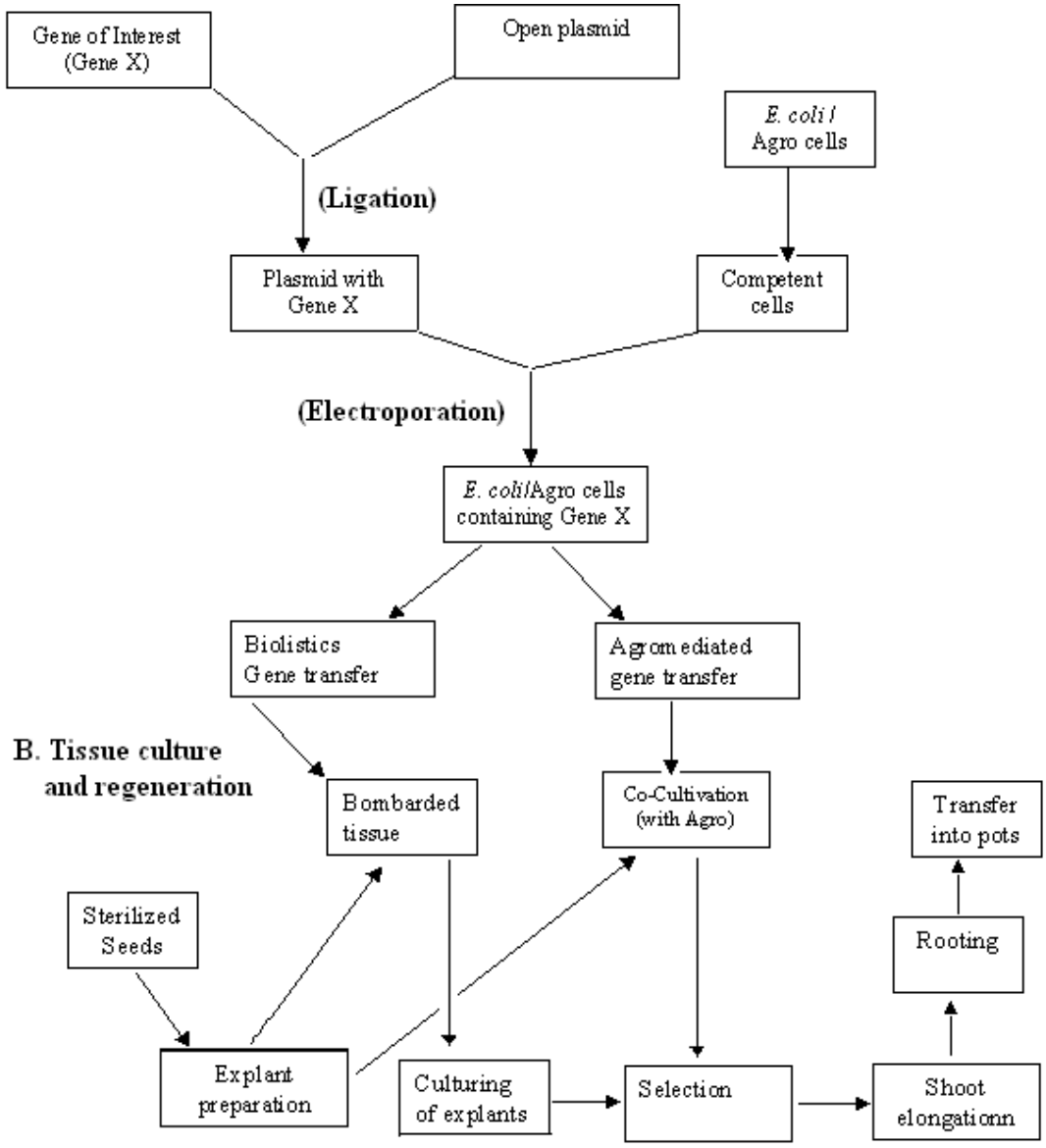


Figure 5: A schematic view of the various strategies for the genetic transformation of crop plant. A. Cloning and transformation; B. Tissue culture and transformation**.**

biologically by *Agrobacterium tumefaciens* or by direct gene transfer using microprojectile/ particle bombardment or by electroporation.

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. The choice between using microprojectile bombardment or *Agrobacterium* as the means by which to deliver DNA is determined by several factors including the laboratory facilities and technical skills available, the species and/or cultivar to be transformed (many monocots are still recalcitrant to transformation with *Agrobacterium*, although this is improving all the time), and the regeneration system.

***Agrobacterium tumefaciens* mediated DNA transfer**

Transformation of plants through *Agrobacterium*-mediated DNA transfer is currently the most commonly used means of vector mediated genetic transformation method 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., 1985; Rogers et al., 1986). Nucleic acid hybridization studies have shown that octopine and nopaline plasmids are closely related having a wide stretch of homology in the T-DNA region (Willmitzer et al., 1983).

The Ti-plasmid contains a well-defined T-DNA region encoding a series of genes responsible for the synthesis of auxins and cytokinins in transformed plant cells apart from genes (Akiyoshi et al., 1984; Inze et al., 1984), which induces over production of phytohormones that cause tumor proliferation. The genes on the Ti-plasmid and their functions were identified by transposon insertion and deletion mutagenesis methods. (Holsters et al., 1980; Garfinkel et al., 1980; Degreve et al., 1982).

The genetic elements encoded by *Agrobacterium*, which are essential for T-DNA transfer, are the T-DNA border sequences and the chromosomal virulence genes present on the Ti plasmid outside the T-DNA. The attachment of *Agrobacterium* to the plant cell is mediated by the chromosomal virulence genes (Douglas et al., 1985; Thomashow et al., 1987), which are constitutively expressed. The 25 base pair direct repeats flanking of the T-DNA (Yadav et al., 1982, Zambryski et al., 1982) are the only part of T-DNA important for transfer. The right border repeats is an essential cis acting element for transfer whereas the left border repeat is thought to merely signal where the transfer of DNA normally ends. Subsequent steps in the T-DNA transfer require the proteins encoded by the *vir* region (*vir a, vir b, vir c, vir d, vir e and vir g).*

Agrobacterium tumefaciens infects only wounded actively dividing plant cells (Hooykaas and Beijersbergen, 1994). The cells secrete wound specific compounds such as aceto-syringone and alpha hydroxy aceto-syringone. These phenolic compounds act as chemo attractants for *Agrobacterium* (Ashby et al., 1987) and inducers of the *vir* genes (Stachel et al., 1985). Both processes are proposed to be mediated by the gene products of *vir a* and *vir g* (Stachel and Zambryski, 1986). The constitutively expressed Vir a protein acts as a 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).

The naturally-evolved unique system of *Agrobacterium* transfers the foreign DNA sequences precisely into plant cells using Ti plasmids. *Agrobacterium*-mediated transformation is the preferred method over microprojectile bombardment for gene delivery as it results in higher frequency of stable transformation with single or fewer integrated transgene copies, thus reducing the risk of gene silencing and transgene rearrangements. Moreover, when compared to direct DNA delivery system, *A. tumefaciens* infections are less complex and *Agrobacterium*-mediated transformation is generally precise in transferring and integration into the plant genome as it delivers long stretches of T-DNA between the right and left borders.

***Agrobacterium*-mediated genetic transformation in groundnut**

The transformation and regeneration protocols for peanut are now well-established. Transformation techniques and plant regeneration from in vitro cultured tissues have been described for many species (Lindsey and Jones 1989; Dale et al. 1993; Birch 1997). 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.

Research is being carried out globally with single or multiple gene introductions to produce disease resistant, pest-resistant, healthier, and high-quality peanuts. 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). 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. A number of independently transformed groundnut plants with coat protein gene of IPCV were produced by this method. 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. A protocol was also standardized using immature embryonic leaf lets, which developed transgenic plants through *Agrobacterium*-mediated transformation (Daniel 2002). Recently, promoter tagged peanut transgenics using the cotyledonary nodes as explants and a promoter-less fusion gene *nptII:gus* were produced (Anuradha et al. 2006).

Importance of *Aspergillus flavus* 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, pistachio nuts, walnuts and pecans. 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.

The fungus *Aspergillus* is quite common with a typical yellow green appearance in nature which increases its population during hot dry weather. Thus, drought stress, extreme geocarposphere temperature or insect, nematode, and fertilizer stress during the latter part of the growing season compromise plants self-defense to fungal invasion and exacerbate aflatoxin formation in the seeds [Hill et al., 1983; Holbrook et al., 2000; Sanders et al., 1993; Guo et al., 2005] which also is reported to impair both plant growth and yield performance.

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). It is a saprophytic fungus that is capable of surviving on many organic nutrient sources like tree leaves, plant debris, cotton, decaying wood, , compost piles, animal fodder, dead insect and animal carcasses, outdoor and indoor air environment (air ventilation system), stored grains, and even human and

animal patients (Yu et al., 2010; 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. These mycelium congregates under adverse conditions such as dry and poor nutrition and form resistant structures called 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.

Mycotoxins are toxic metabolites produced by fungi, especially by saprophytic moulds growing on food stuffs or animal feeds. Several mycotoxins in agricultural products cause economical problem creating health hazards to people and animals. They are pathologically classified as hepatotoxins, nephrotoxins, vomitoxins and neuro-musculotoxin, some of which are potentially carcinogenic and mutagenic [Samuels, 1984; Stoloff, 1985]. Among various mycotoxins, aflatoxins have gained significance due to their deleterious effects on human beings, poultry and livestock. It was first recognized in 1960, where there was a severe outbreak of a disease referred as "Turkey 'X' Disease" in UK, in which over 100,000 turkey poults were died (Allcroft et al., 1961; Lancaster et al., 1961). Aflatoxin was named after *Aspergillus flavus* toxin. 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 potent toxic, mutagenic, carcinogenic, immunosuppressive agents depending on the level and duration of exposure. They are produced as secondary metabolites on variety of food products by the fungus *Aspergillus flavus* and *A. parasiticus* [Castegnaro & McGregor 1998, Pittet 1998]. These are a group of structurally related toxic

bisfuronocoumarin compounds, the ones most commonly produced by *A. flavus* are B_1 and B_2, while *A. parasiticus* produces two additional aflatoxins, G_1 and G_2. These toxins are largely associated with food commodities produced in the tropics and subtropics which include cereals such as maize, sorghum, pearl millet, rice, wheat; oilseeds such as groundnut, soybean, sunflower, cotton; spices such as chilli, black pepper, turmeric, coriander and ginger; nuts such as almond, brazil nuts, pistachio, walnut, coconut; milk and milk products. These toxins are completely heat stable, so neither cooking nor freezing destroys the toxin and make them remain on the food indefinitely. Aflatoxin B_1 being the most toxic metabolite, is reported as a potent carcinogen and has been associated with liver cancer. 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, β-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 metabolic product which is excreted in the milk and urine of dairy cattle and other mammalian species fed with aflatoxin-contaminated food or feed.

Nutrition and health effects of aflatoxins

Contamination of food, feed and agricultural commodities by aflatoxins impose an enormous economic concern and puts consumers at high-risk health hazards. Aflatoxin (especially aflatoxin B_1) has been reported as a potent carcinogen in animals and humans. The extent of carcinogenicity is largely dependent on the dose, the duration of exposure, and the animal involved which is been categorized into two types of illness.

Acute illness: Acute illness is as a result of consumption of foods contaminated with very high levels of aflatoxin. Williams *et al.*, 2004 has

reported that no animal species is resistant to acute toxic effects of aflatoxins. 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).

Chronic illnesses/ Cancers: Exposure of living beings to exceptionally low levels of toxin in traded commodities [US 10 ppb in grain; and 0 ppb in milk; EU 4 ppb and 0 ppb in milk] results in chronic illness which in turn is asscociated with cancers (specially liver cancers). This liver toxicity can produce a cumulative effect over time and lead to diseases like hepatic fibrosis, cirrhosis and fatty liver disease.

These metabolites are capable of binding to protein, DNA and RNA thus interfering with the normal cellular functions resulting in initiation of carcinogenesis, mutagenesis or necrosis of the liver which result in foetal mis-development and miscarriages. Aflatoxins are also been reported as potent immune suppressors in humans and animals due to their interference with activities of important cells that boost immunity in the body. They are also reported to cause growth reduction due to protein synthesis interference and micronutrient [vitamins A, B_{12}, C, D and E; minerals zinc, selenium, iron and calcium] deficiency and also to play an important role in slowing the recovery rate from protein malnutrition [kwashiorkor]. Hence, they are being strongly linked to HIV/AIDS, malaria and other several nutritional-related illnesses in humans.

Economic effects of aflatoxins

Aflatoxin contamination in all crops result in direct economic effects resulting in loss of produce or loss of market value including increased costs of veterinary and human health care services, indirect economic effects from loss of animals, costs for food-borne disease surveillance and food monitoring etc. Depending on the market, economic losses may reach 100%, when the entire produce/product is rejected by the market if aflatoxin levels are higher than acceptable standards. It is estimated that Africa loses over United States dollars 670 million annually due to requirements for

European Union aflatoxin standards for all food exports and world over, billions of dollars are lost by farmers and traders due to aflatoxin contamination (Otsuki *et al.*, 2001; Guo *et al.*, 2009). It is therefore, very essential that all parties involved in the process of producing and marketing groundnuts should ensure that contamination from aflatoxins is minimized as much as possible.

US Food and Drug Administration [FDA] has put regulations on levels of aflatoxin at 20 ppb [parts per billion] in food and feed and in milk of lactating animals fed with aflatoxin-contaminated feed at 0.5 ppb. These regulatory guidelines [within U.S. as well as those enforced internationally] have put a tremendous economic burden [~$932 million] on U.S. agriculture due to crop losses caused by mycotoxigenic fungi *A. flavus* which causes aspergillosis [a life-threatening human disease, ~30%] particularly in patients who are immunosuppressed or have chronic lung disease. Among all the aflatoxins AFB_1 has been the most toxic and potent carcinogen because of its association with hepatocellular carcinoma (liver cancer).

Table 1: Regulatory limits for aflatoxins in food and feed (US FDA) (Park and Troxell, 2002)

Food and Feed	Accepted aflatoxin levels [μg/kg.ppb]
Human foods [except milk]	20
Milk	0.5
Animal feeds [except as listed below]	20
Cottonseed meal [as a feed ingredient]	300
Corn and peanut products for breeding beef cattle, swine and mature poultry	100
Corn and peanut meal for finishing swine	200
Corn and Peanut meal for feed lot beef cattle	300
Corn for immature animals and dairy cattle	20

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.

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., 2005; 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. Various antifungal proteins and peptides have been isolated from a wide range of plants which have been already tested for their antifungal activity against *Aspergillus* spp. Aflatoxin resistant transgenic crops would not only control *A. flavus*, but also other microbial [fungal, bacterial, and viral] diseases that cause significant economic losses in crop production. Hence, development of transgenic varieties with antifungal traits that confer resistance to aflatoxin-producing fungi will be extremely valuable and will be an aid to the breeding tools.

Better knowledge of biochemical mechanisms involved in response to the fungal infection and environmental change helps in establishing the identity of plant mechanisms that inhibit aflatoxin formation thereby incorporating specific antifungal genes into plant varieties through genetic engineering research results in enhanced host-plant resistance. The

common procedure followed in studying the ability of the novel antifungal gene to control fungal growth and aflatoxin production is using the readily transformable model plant such as *Arabidopsis* or tobacco to test transgene expression and perform in vitro bioassays using extracts from transgenic tissues. The recent developments and increase in using different approaches of transformation and regeneration of fertile plants will substantiate the value of this approach to be realistic and dependable.

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).

Current research of developing plant disease resistance mechanism involves functional genomics technology to analyze expressed genes based on the available data on expressed sequence tags (EST's) and microarray which helps to identify pathways involved in the resistance mechanisms (Luo et al., 2005a; Luo et al., 2005). In groundnut, the resistance (Somerville et al., 1999; Sweigard et al., 2001; Keon et al., 2003) trait against *Aspergillus flavus* infection is both quantitative and also effected by environment factors such as drought stress (Widstrom et al., 2003).

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 thereby inhibiting the production of aflatoxin. 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.

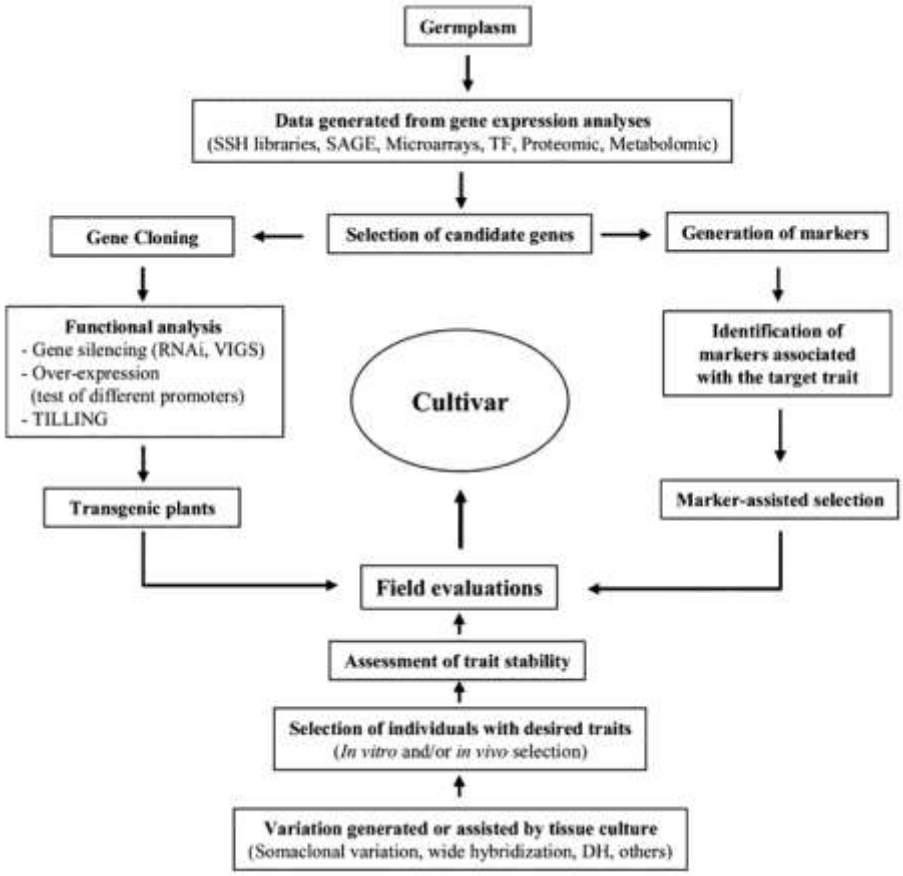


Figure 6: Outline of key steps involved in Integrated scheme for plant molecular breeding using biotechnology. DH: Double haploid; RNAi: RNA interference; SAGE: Serial analyses of gene expression; SSH: Suppression subtractive hybridization; TILLING: Target-induced lesion IN genome; TF: transcription factors; VIGS: Virus-induced gene silencing (Dita et al, 2006).

Transgenics with non-pathogen derived resistance

Genetic enhancement in peanut through conventional breeding and chemical control has yielded only limited success (Nigam et al. 2012) and the narrow genetic base of the cultivated peanut *Arachis hypogaea* L. hampers the development of improved varieties through conventional breeding leaving with the development of transgenics as the only option.

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).

Hence, necessity of the availability of transgenic varieties with antifungal traits is extremely valuable for using it as a breeding tool [Rajasekaran et al., 2006]. Several reviews has been reported so far on different antifungal enzymes/peptides and proteins used in genetic engineering of susceptible crop species to combat *A. flavus* infection and aflatoxin contamination [Shah et al., 1997; Rajasekaran et al., 2002; de Lucca et al., 2005; Broekert et al., 1997; Kalyani et al., 2012]. They include α-defensins, thionins, osmotins, plant nonspecific lipid transfer proteins

[ns-LTPs], knottins, impatiens antimicrobial peptides, ribosome inactivating proteins [RIP's], lectins and lectin-like peptides. Several industrial and academic laboratories have started to undertake transgenic approaches to prevent invasion by *Aspergillus* fungi or to prevent biosynthesis of aflatoxin because of the availability of efficient modern biotechnological tools which help them in evaluation of plant-pathogen protein interactions, genomics and field ecology of the fungus.

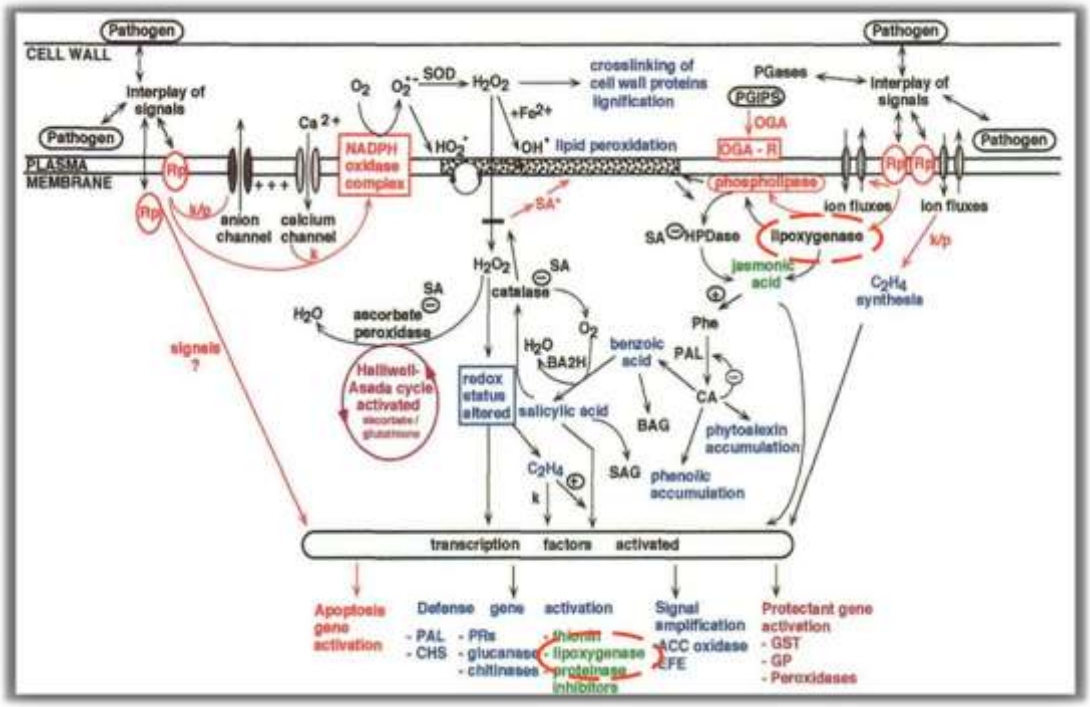


Figure 7: Complexity of signaling events controlling activation of defense responses (Hammond-Kosack & Jones, 1996).

Update on genetic transformation of peanut against *A. flavus*

Peanuts are susceptible to aflatoxin contaminations which are toxic, carcinogenic substances produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Since conventional breeding methods for controlling aflatoxin are only partially effective, novel biotechnological methods for enhancing host plant resistance to preharvest *A. flavus* invasion and aflatoxin contamination is considered to be the most cost-effective control measure. Besides, a complete knowledge of the resistance associated proteins/genes and their contribution to host plant resistance (comparative

proteomics) is critical to harness their cumulative or complementary benefits in peanut for *A. flavus* infection and aflatoxin contamination.

Peanut produces stilbene phytoalexins in response to fungal infection. Organ-specific expression of multiple copies of a gene for stilbene synthesis (Stilbene synthase) has proven to inhibit fungal growth and spore germination of *Aspergillus* species and aflatoxin contamination. Hydrolytic enzymes such as chitinases and glucanases, which degrade the fungal cell wall, also pose as attractive candidates for development of disease-resistant peanut plants (Eapen 2003). Similarly, glucanase gene from tobacco introduced into peanut (PR protein from heterologous source) showed enhanced disease resistance to in vitro seed colonization (IVSC) and no accumulating aflatoxin (detected by HPLC) (Sundaresha et al. 2010). Maize and peanut transgenic expressing synthetic version of maize ribosome inhibiting protein gene, *mod1*, showed enhanced resistance to *A. flavus* and reduced aflatoxin contamination (Weissinger et al. 2003).

The aflatoxin biosynthetic pathway in vitro has been shown to be suppressed by enzyme encoded by soybean *loxl* gene that catalyzes the formation of a specific lipoxygenase metabolite of linoleic acid, (13S)-hydroperoxyoctadecadienoic acid ((13S)-HPODE). Transgenic peanut expressing soybean *loxl* gene under the control of carrot embryo specific promoter (DC3) (Ozias-Akins et al. 2000) resulted in reduction in the aflatoxin content. Efforts are being carried out at ICRISAT for generation of peanut transgenics with the rice chitinase gene (Prasad et al. 2012). Work is also being progressed at ICRISAT in developing construct for use in RNAi approach to suppress 9-hydroperoxide fatty acid producing lipoxygenases since incorporation of plant antisense genes for the 9-hydroperoxide fatty acid producing lipoxygenases also reduces mycotoxin contamination. Other antifungal genes such as D5C (Weissinger et al. 1999), tomato anionic peroxidase (tap 1), and synthetic peptide D4E (Ozias-Akins et al. 2000) are transformed into peanut and evaluated for antifungal activity against *A. flavus*. However, pure D5C showed strong activity against *A. flavus* in vitro, due to phytotoxicity of D5C, transgenic peanut callus showed poor recovery

of plants. Expression of *cry1A(c)* (Ozias-Akins et al. 2002) in transgenic peanut lines could also be an effective means of inhibiting *A. flavus* infection by reducing the damage into peanut pods by lesser cornstalk borer (LCB) *Elasmopalpus lignosellus*, since it has been clearly reported that aflatoxin contamination can increase with insect damage (Lynch and Wilson 1991). Similarly, Ozias-Akins et al. (2003) reported 60-70% reduction in *A. flavus* colony growth in transgenic peanut lines expressing the bacterial chloroperoxidase gene (Rajasekaran et al. 2000). Niu et al. 2009 reported antifungal activity in transgenic peanut by transforming with a non-heme chloroperoxidase gene from *Pseudomonas pyrrocinia.*

Several laboratories experimented with potential antifungal gene constructs that offer resistance in vitro, in situ, or *in planta* to *A. flavus* often stacked with insect-resistant genes. For example, it is often speculated that bollworm or insect injury to cotton bolls serves as an entry point for *A. flavus* spores, although concrete evidence is not available yet (Zipf and Rajasekaran, 2003). Studies have shown that aflatoxin contamination is not directly correlated with pink bollworm damage and contamination may occur in the absence of damage (Henneberry et al., 1978; Russell, 1980; Bock and Cotty, 1999). However, aflatoxin contamination in peanuts (Lynch and Wilson, 1991) or in tree nuts (Gradziel et al., 1995) is positively correlated with insect damage.

Lipoxygenases

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 from 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 from linolenic acid) and 13*S*-HPODE (13*S*-HPOTE from 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-C_{12} 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). 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). 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 utilized in various physiological processes such as seed germination, growth and development, 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). 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.

Lipoxygenase pathway activation generates a series of diverse, antifungal, volatile aldehydes that affect the growth of *Aspergillus flavus* and

indirectly has an effect on aflatoxin production. 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).

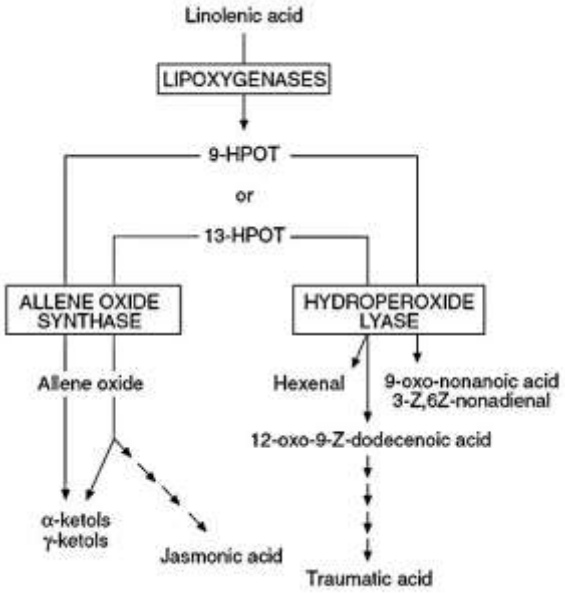


Figure 8: Overview of lipoxygenase pathway (Loiseau et al, 2001)

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). Invitro observations suggested that exogenous 9*S*-HPODE extended the time of aflatoxin gene transcription whereas 13*S*-HPODE and 13*S*-HPOPTE inhibited aflatoxin gene transcription.

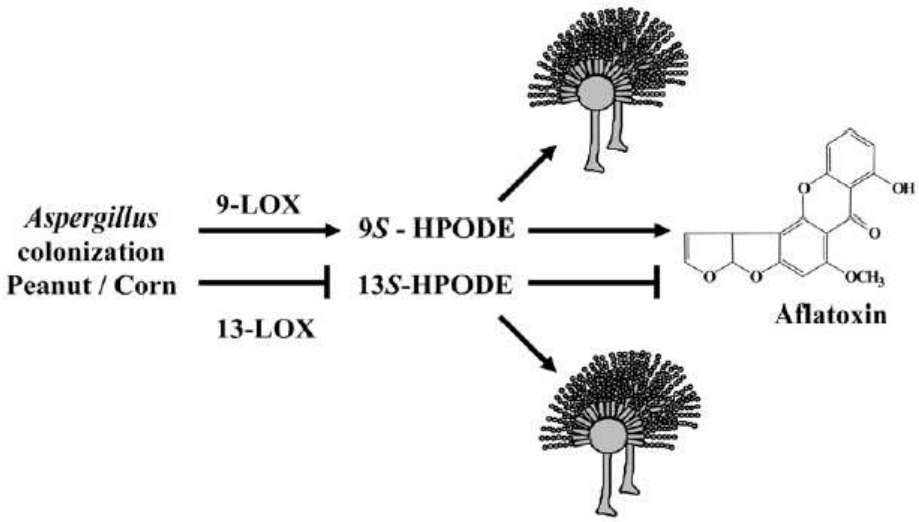


Figure 9: Model depicting the role of *Aspergillus* seed colonization in regulation of 13 and 9 lipoxygenases and their derivatives as molecules modulating mycotoxin biosynthesis and fungal sporulation (Tsitsigiannis et al., 2005).

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). Similarly, other LOX pathway like methyl jasmonate and aldehyde products of 13*S*-HPODE and 13*S*-HPOTE 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) which are specifically expressed in seed with high levels of expression in mature

embryo and immature cotyledons. In contrast to *PnLOX1*, the amount of 13 S-HPODE formed was 78 and 80% for *PnLOX2* and *PnLOX3* respectively. 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 from 70 to 80% identity with legume LOXs and 55 to 60% with potato, tomato, and *Arabidopsis* spp. From all the above studies (Tsitsigiannis et al.,2005) suggested that in *Aspergillus* seed–aflatoxin interactions, 9*S*-HPODE act as putative susceptibility factor wheras 13*S*-HPODE molecules act as resistance factors.

3. Materials and Methods

Plasmid Constructs used

Escherichia coli DH5α strain was used for plasmid cloning and propagation. Similarly, *Agrobacterium tumefaciens* disarmed C58C1Rif^R (Van Larebeke et al., 1974; Simoens et al., 1986) strain was used for plant transformation. *E. coli* and *Agrobacterium* were grown in Luria Bertani (LB) and Yeast extract broth (YEB) media at 37 ^{0}C and 28 ^{0}C, respectively (Sambrook et al. 1989), with appropriate antibiotics. The plasmids used for the construction of binary vector include pRT 103 (Messing et al., 1985), pGEM-T Easy (Promega, Cat no: A1360), pTMK 12.6 (Tsitsigiannis et al., 2005) and pPZP200 (Gateway vectors).

Preparation of *Escherichia coli* competent cells

A single colony of *E. coli* strain DH5α was inoculated in 5 mL of LB broth (Annexure 1.1) and incubated overnight at 37°C in an orbital shaker set at 200 rpm. Using 1% of this overnight grown culture as inoculum, 100 mL of LB broth was inoculated for preparing competent cells. The culture was grown at 37°C with continuous shaking until the optical density (O.D) at 600nm reached 0.4. The cell density is arrested by placing the flask on

ice for 10 min. After incubation, the culture was transferred into sterile 50 mL tubes and centrifuged at 4°C for 10 min at 5000 rpm. The pellet from all tubes was pooled into single tube after washing with ice cold sterile water and centrifuged at above given conditions. The resulting pellet was then re-suspended into 1/10th volume of ice-cold TSB buffer (Annexure 2.2) and incubated on ice for 10 min. 100 µL of this cell suspension was then aliquoted into sterile 600 µL vials and were immediately frozen in liquid nitrogen (-196°C). These competent cells were then stored in -80°C until further use.

***Escherichia coli* transformation**

A 50 µL mixture containing 30 µL KCM solution (5X; Annexure 2.1), 1 µL of plasmid DNA (100 ng) and 19 µL of sterile distilled water was added to 100 µL of competent cells (*E. coli* DH5α strain) and incubated for 20 min on ice followed by a room temperature incubation for 10 min. 850 µL of LB broth was immediately added to the above mixture which was then incubated at 37°C for 1 hr in a rotary shaker to facilitated growth of the transformed cells. The cells were spun down at 6,000 rpm for 2 min at room temperature and the pellet was suspended in 200 µL of fresh LB broth before being spread on LB plates (Annexure 1.2) containing the appropriate antibiotic. After 16 hr incubation at 37°C, the transformed DH5α colonies were screened by colony PCR and confirmed by restriction digestion analysis.

Isolation of plasmid DNA from *E. coli*

A single isolated colony of bacterial culture was selected and was inoculated in about 10-20ml liquid LB medium with appropriate selection overnight at 37^{0}C. 1.5 ml of bacterial culture was taken in an eppendorf tube and centrifuged at 5000 rpm for 3-4 min at room temp. The pellet was resuspended in 100 µl GTE buffer (Annexure 3.1) and was incubated on ice for 5 min. 200 µl of lysis buffer (Annexure 3.2) was added, tube was inverted several times to mix the contents and was incubated for 5 min on ice. To the suspension, 150 µl 5M potassium acetate (Annexure 3.5) was added, the mixture was inverted several times to mix and was incubated on ice for 5

min. This suspension was centrifuged at 10000 rpm for 10 min and the supernatant was transferred into a fresh tube (care was taken not to carry over the precipitate or floating material). Later equal volumes of phenol: chloroform (1:1) mixture was added to the supernatant and centrifuged at 10000 rpm for 10 min. The supernatant was collected into fresh tube and the plasmid DNA was precipitated by addition of 0.1 volume of 3M sodium acetate and 0.8 volume of isopropanol. The mixture was allowed to stand at room temp for 2 min and centrifuged at 10000rpm for 10 min. The resulting pellet was subjected to a brief wash with ice cold 70% ethanol (Annexure 3.6) and was centrifuged at 5000rpm for 5 min. The pellet was air/ vacuum dried and was dissolved in 50µl TE buffer treated with RNAse. The concentration and quality of the plasmid DNA was checked by resolving in 0.8% agarose gel.

Construction of binary vector pPZP200>35S:*PnLOX*3:poly A

Restriction endonucleases and T4 DNA ligase, obtained from New England Biolabs, were used for cloning as recommended by the supplier. The strategy followed for the construction of binary vector containing peanut lipoxygenase gene (*PnLOX3*) is as follows:

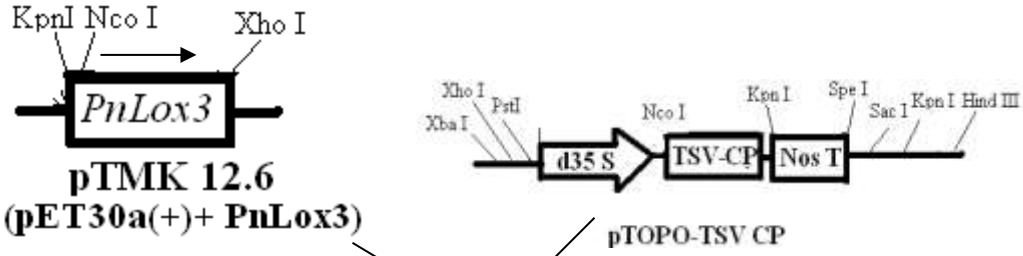
1. The plasmid pRT 103 (Messing et al., 1985) was digested with *Pst*I enzyme and the 649bp fragment containing CaMV 35S promoter, multiple cloning site and Poly A signal was separated on 1% agarose gel and purified using Qiagen® gel extraction kit. This fragment was then ligated into the dephosphorylated *Pst*I site of the vector pGEM-T Easy (Promega, Cat no: A1360). Products of the ligation were then introduced into DH5α cells through KCM method as described above and selected recombinants on LB media (Annexure 1.2) 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 *Pst*I, *Sph*I+*Sal*I and *Sac*I and electrophoresis on 1% agarose gel. The new plasmid was designated as pGEMT>35S: polyA.

2. The cDNA fragment coding for lipoxygenase gene (*PnLOX3*) was subcloned from pTMK 12.6 (Tsitsigiannis et al., 2005) by restricting pTMK 12.6 with *Kpn*I– *Xho*I. This 2700 bp fragment was then ligated into the *Kpn*I– *Xho*I site of the vector pTOPO>TSV-CP. Products of the ligation were then introduced into DH5α cells through KCM method and selected recombinants on LB media containing kanamycin. Colonies carrying recombinant plasmid containing the lipoxygenase gene in desired orientation were identified by restriction with *Kpn*I– *Xho*I and electrophoresis on 1% agarose gel. The new plasmid was designated as pTOPO>*PnLOX3*.

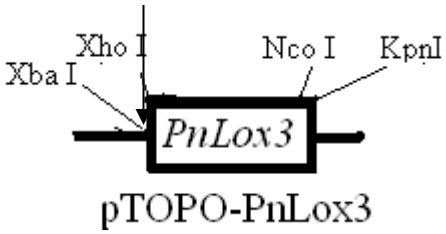
3. The plasmid pTOPO>*PnLOX3* was then restricted with *Kpn*I– *Xba*I to release lipoxygenase gene fragment and this 2706bp fragment is then ligated to *Kpn*I– *Xba*I site of pGEMT>35S:poly A. Products of the ligation were then introduced into DH5α cells through KCM method and the recombinants were selected on LB media containing ampicillin. Colonies carrying recombinant plasmid containing the lipoxygenase gene in desired orientation were confirmed by restriction with *Kpn*I– *Xba*I and electrophoresis on 1% agarose gel. The new plasmid was designated as pGEMT>35S:*PnLOX3*:poly A.

4. The plasmid pGEMT>35S:*PnLOX3*:poly A was 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 3375 bp fragment was then ligated to *Spe*I- *Sal*I site of pPZP200 carrying PBNV gene. Products of the ligation were then introduced into DH5α cells through KCM method and selected recombinants on LB media containing spectinomycin. Colonies carrying recombinant plasmid containing the lipoxygenase gene driven by CaMV 35S promoter and poly A signal in desired orientation were confirmed by restriction with *Spe*I–*Sal*I and electrophoresis on 1% agarose gel. The new plasmid is designated as pPZP>35S:*PnLOX3*:poly A.

Step I:



Restrict with *Kpn*I- *Xho*I and clone into *Kpn*I-*Xho*I site of pTOPO-TSV CP



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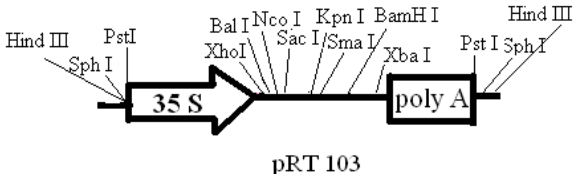
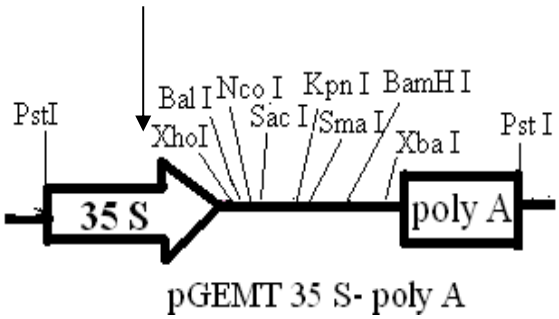
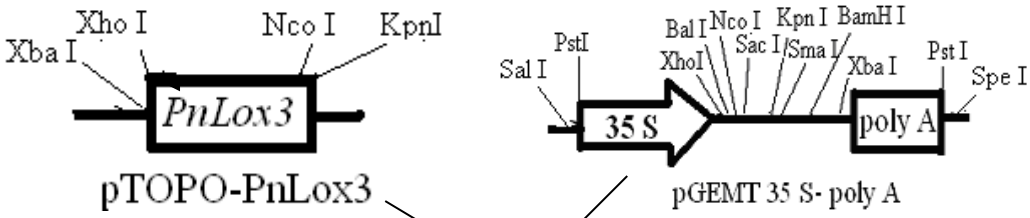


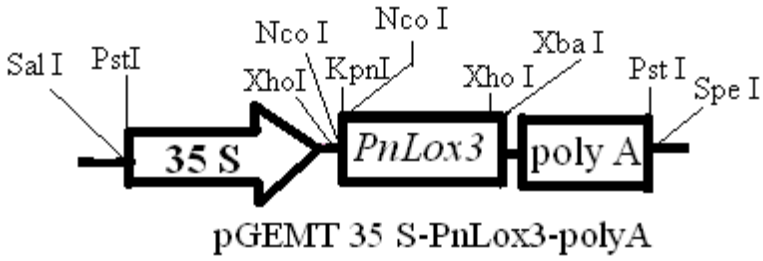
Pst I fragment cloned into *Pst*I site of pGEMT-easy



Step 3:

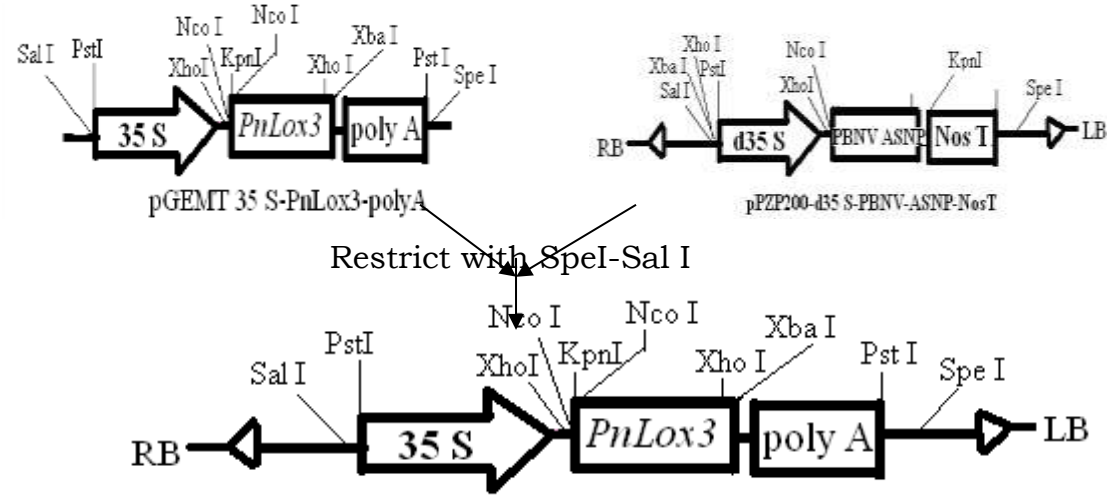


Restrict with Kpn I-XbaI and clone into Kpn I-Xba I site of pGEMT 35S-polyA



pGEMT 35 S-PnLox3-polyA

Step 4:



pPZP200-35 S-PnLox3-polyA

Figure 10: Summary of strategy followed for sub-cloning peanut lipoxygenase gene (*PnLOX3*) into binary vector pPZP200

Preparation of *Agrobacterium tumefaciens* competent cells

Single colony of *A. tumefaciens* strain was inoculated in 5 mL of YEB broth (Annexure 1.3) and incubated overnight at 28°C in an orbital shaker set at 200 rpm. Using 1 mL of the overnight grown culture as inoculum, *A. tumefaciens* was cultured in 100 mL YEB broth at 28°C with continuous shaking. The absorbance of the culture was measured at 600 nm and the cells were arrested at 0.4 O.D (optical density) by placing the flask on ice for 30 min. The cells were then transferred into ice-cold sterile 50 mL tubes and centrifuged at 5000 rpm for 10 min at 4°C. The pellet was suspended in ice-cold 10% glycerol (Annexure 2.3) and the volume was made up to 50% of the initial volume. After 20 min incubation on ice, the cell suspension was centrifuged at 4°C for 10 min at 5,000 rpm. The pellet was suspended in ice-cold 10% glycerol and the volume was made up to 40% of the initial volume.

After 20 min incubation on ice, the cell suspension was spun down at 5,000 rpm for 10 min at 4°C and the pellet was re-suspended in 3 mL of ice-cold 10% glycerol. The cell suspension was then distributed into aliquots of 50 µL into sterile 600 µL capacity tubes, immediately frozen in liquid nitrogen (-196°C) and stored thereafter in -80°C until further use.

Agrobacterium tumefaciens bacterial transformation

A 50 µL competent cells (*A. tumefaciens* C58C1Rif^R strain) were mixed with 1 µL of plasmid DNA (100 ng). Transformation was carried out in electroporator (Bio-Rad®, USA) by loading the above mixture into a pre-cooled cuvette and electroporated at field strength of 6.25-12 kVcm^{-1} for 4-8 ms as described by the manufacturer. 1 mL of YEB broth was added immediately to prevent the cells from shock and cell suspension was incubated at 28°C for 1 hr in a rotary shaker to facilitate growth of the transformed cells. The cells were spun down at 6,000 rpm for 2 min at room temperature and suspended in 200 µL of fresh YEB broth before being spread on YEB plates (Annexure 2.2) containing the appropriate antibiotic. After 48 hr incubation at 28°C, the transformed *Agrobacterium* colonies were screened by colony PCR and restriction digestion.

Two of the clones of the binary construct pPZP>35S:*PnLOX3*:poly A were confirmed by sequencing and the clone which had no mismatches and mispairing was introduced into disarmed *Agrobacterium tumefaciens* strain C58C1Rif^R through electroporation (as described above) and grown on YEB selection medium (Annexure 1.4) containing 100 mg/l spectinomycin for use in *Agrobacterium*-mediated genetic transformation studies. Simultaneously glycerol stocks of both *E. coli* and *Agrobacterium* bacterial cultures harboring new constructs were made and maintained at -80 ^{0}C for further use.

Isolation of plasmid DNA from _Agrobacterium tumefaciens_

Agrobacterium strain was grown on YEB agar plates containing 100µg/ml spectinomycin. Single isolated colonies were grown in 25ml YEB at 28°C on an orbital shaker overnight and 10 ml bacterial suspension was

pelleted by centrifuging for 10 min at 6000 rpm. The pellet was suspended in 1 ml GTE. 30 µl of lysozyme was added to this suspension and incubated for 5 min at room temperature. To the re-suspended bacterial culture, 2 ml of freshly prepared lysis buffer was added and the samples were placed on ice for 5 min. 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. After 5 min incubation the solution was centrifuged for 15 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. Equal volumes of phenol-chloroform: iso-amyl alcohol (24:1) was added and a brief spin at 10000 rpm for 15 min was given. The aqueous phase was collected into a fresh tube and to it equal amounts of chloroform: isoamylalcohol (24:1) was added and the sample solution was centrifuged for 15 min at 10000 rpm. The aqueous phase was then 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. The sample was later centrifuged for 15 min at 10000 rpm and the pellet was washed with 80% ethanol and air-dried. The dried pellet was dissolved in 30 µl of TE.

Agrobacterium culture preparation for co-cultivation

Primary culture of *Agrobacterium* was prepared by inoculating single colony of *Agrobacterium* in 20 ml YEB medium (with 50 mg/l each of spectinomycin and rifampicin) and grown overnight at 28° C at 200 rpm. 5 ml of this overnight grown culture was centrifuged at 5000 rpm for 15 min and the pellet was re-suspended in the regeneration medium (0.5 X MS; Annexure 4.9) so as to dilute it to an $O.D_{600}$ ~ 0.5. This suspension was used for co-cultivation for tobacco leaf discs and groundnut cotyledon explants using *Agrobacterium* mediated transformation.

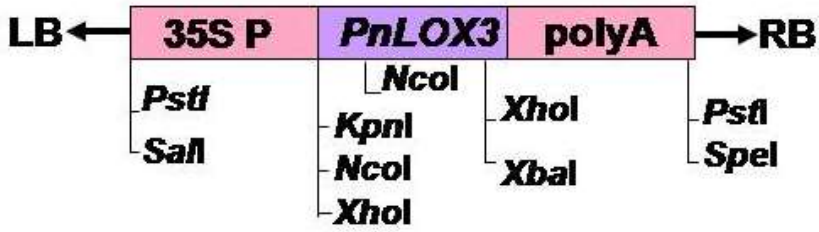


Figure 11: Schematic representation of T-DNA region of the binary vector construct pPZP200>35S:*PnLOX3*:polyA used in this study.

Plant transformation and regeneration system

***Agrobacterium*-mediated genetic transformation in tobacco**

The regeneration and transformation efficiency of tobacco plants is higher as compared to other crops. Simple media composition and easy regeneration protocols help in using tobacco as a model system for basic transformation studies. The prime quality of the introduced gene in transgenics is that it should not interfere adversely with the plant biology and physiology, i.e., the transgenic plant should not show phenotypic differences as compared to the control plants.

Based on this, to check the effects of our marker-free construct on plants, we transformed tobacco variety *Xanthi* with pPZP>35S:*PnLOX3*:polyA using *Agrobacterium*-mediated transformation technique employing leaf discs.

Explant preparation

Tobacco (*Nicotiana tabacum* L., Var. Xanthi) seedlings were grown in Magenta boxes on 0.5X MS media under sterile controlled- environment condition for two weeks. Transformation of tobacco was done with some modifications in standard leaf-disc method (Horsch et al., 1988). Leaf-discs were taken from aseptic plants grown in the light at 22^{o}c in MS medium supplemented with 2% sucrose (Murashige and Skoog, 1962). The fully expanded leaves were surface sterilized by two-three sequential treatments with 70 % ethanol for 30 sec followed by wiping with sterile tissue paper.

These were further washed with 15 % clorox solution for 10 min, and then washed thrice with sterile water.

Regeneration and transformation

The fully expanded leaves were removed which were then cut into 1 cm^2 pieces approximately, with a sterile leaf disc borer and cultured in 9 cm diameter plastic petri dishes containing ~20 ml of MS4 medium (Annexure 4.9) that contained MS medium supplemented with 10 µM BAP, 0.5 µM NAA, 30 g/l sucrose and 8 g/l Bacto-agar (HiMedia Laboratories Pvt. Ltd., India) at pH 5.8. The leaf discs were dipped in *Agrobacterium* inoculum which facilitated the bacterial adhesion to the cut ends of the leaves and then the leaf discs were transferred to the same media with their abaxial side in contact with the media. Plating density was maintained to ten co-cultivated leaf disc explants which were later sealed with parafilm and incubated in a 16:8 light/dark regime at 26 ± 1^0 C under continuous light of 100 $\mu Em^{-2}s^{-1}$ for 72 h in a 16:8 light/dark regime under white fluorescent light. At the end of this period, explants were transferred onto MS4C medium (MS4 medium supplemented with 250 mg/l cefotaxime). Explants were sub-cultured onto fresh MS4C medium at two week intervals for shoot regeneration which were then transferred onto MSC medium for 35- 40 days with two week intervals in between each subculture for shoot elongation.

Rooting and transplantation

The elongated shoots were sub-cultured into fresh MSC medium at two week intervals until roots appear. These rooted shoots were transferred to the pots containing autoclaved sand and soil (1:1) mixture maintained in the pre field evaluation (P_2) glass house until flowering and seed set.

***Agrobacterium*-mediated genetic transformation in groundnut**

Seeds of popular peanut (*Arachis hypogaea* L.) cultivar JL 24 (Spanish type) were obtained from the Peanut Breeding Unit of ICRISAT, Patancheru, India which was chosen for its good ability for transformation and desirable agronomic characteristics. Groundnut regeneration and transformation protocols standardized at ICRISAT *via* direct organogenesis for JL 24 variety

(Sharma and Anjaiah, 2000) has been used for producing the transgenic plants using the marker-free construct pPZP>35S:*PnLOX3*:polyA.

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-soaked mature seeds via co-cultivation with the *Agrobacterium* strain C58 harboring the binary vector pPZP>35S:*PnLOX3*:polyA. All the tissue culture and transformation works were carried out under the laminar air flow in absolute asceptical conditions.

Seed sterilization, explant preparation

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.). 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. 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.

Regeneration and transformation

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; Annexure 4.9) 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 was adjusted to 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 µg ml^{-1}). Care was taken to 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 µg ml^{-1} cefotaxime in which plating density was maintained at 5 explants per plate. The organogenic tissues starts differentiating into shoot buds at this stage which was continued for two more 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 (25 x 150 mm) containing shoot elongation medium (SEM). SEM consists of MMS with 2 µM BA and called as MMS 36-2 (Annexure 4.9). 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.

Rooting and transplantation

The elongated shoots (5-6 cm) regenerated through *Agrobacterium*-transformation systems were transferred to root induction medium (RIM; Appendix 3.9) comprising of MMS supplemented with 5 µM α-naphthaleneacetic acid (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.

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

Young leaves from 5 leaf staged greenhouse 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.

Genomic DNA isolation from tobacco

Based on the protocol defined by Dellaporta et al. (1983), genomic DNA was extracted from putative tobacco plants with few modifications. Tobacco leaf tissue (50- 100 mg) was collected in 1.7 mL vial, which was pulverized into powder using liquid nitrogen. This ground tissue was homogenized with 700 µL of modified Dellaporta extraction buffer (Annexure 5.6) containing RNase (Annexure 5.5). After incubating the samples on ice for 5 min, cell lysis was initiated with the addition of 70 µL of 10% SDS (Annexure 5.8). The samples were mixed by inverting followed by addition of 230 µL of 5M potassium acetate (pH 5.2; Annexure 3.4), incubated for 5 min at on ice and centrifuged at 10,000 rpm for 10 min at 4ºC. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and thoroughly mixed. The mixture was centrifuged at 10,000 rpm for 10 min at room temperature and 0.6 volume of iso-propanol was added to the supernatant for precipitating DNA. After thorough mixing and centrifugation, the pellet was washed with 1 mL of 70% ethanol and air/vacuum dried. The pellet was finally suspended in 30 µL of TE buffer (Annexure 5.4) and stored at 4ºC. The DNA samples were visualized by resolving on 0.8% agarose gel and quantified using spectrophotometry and NanoVue (Eppendorf, Germany).

Mini preparation of isolation of genomic DNA from groundnut

Leaf material of glass house grown putative transformants was collected in 1.7 ml eppendorf tubes. Mini preparation of genomic DNA from putative groundnut plants was carried out using the above mentioned modified Dellaporta method. Finally DNA was dissolved in TE buffer and the samples were stored at 4ºC whose concentration was quantified by resolving in 0.8 % agarose gel and NanoVue.

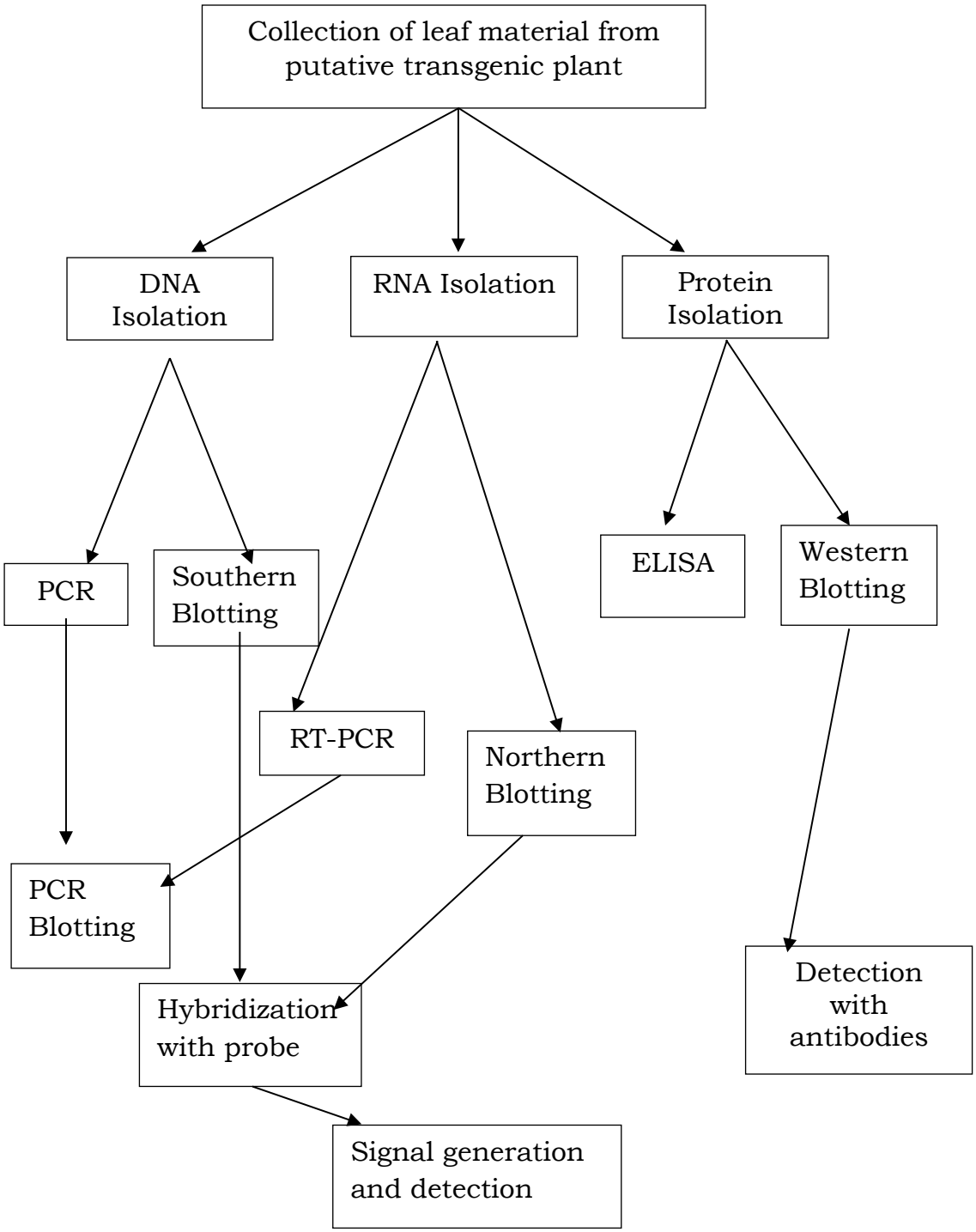


Figure 12: A schematic view of various techniques used for the molecular characterization and analysis of putative transgenic plants.

Maxi preparation of isolation of genomic DNA from groundnut

The genomic DNA from the leaf tissue of one- month old putative groundnut transgenics was extracted according to the protocol suggested by Porebski et al. (1997). The principle behind isolation DNA by cetyl trimethyl ammonium bromide (CTAB) extraction buffer (Annexure 5.7) is that CTAB forms an insoluble complex with nucleic acids. Leaf tissue (~500 mg-1 g) was ground in liquid nitrogen into fine powder with a mortar and pestle. To the powdered leaf tissue, 10 ml of CTAB 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. 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. Then 0.5 volumes of 5 M NaCl, double the volume of 95% chilled ethanol was added and the contents were mixed by inverting the tubes very gently followed by incubation for 30 minutes at -20°C and centrifugation at 10,000 rpm for 10 minutes. The pellet was then washed with ice-cold 70% ethanol followed by brief centrifugation for 3-4 minutes. Then pellet was air dried and dissolved in 500 µl of 10X TE buffer (pH 8.0).

Purification and Quantification of genomic DNA

10µl of RNase was added to the above TE dissolved DNA to degrade RNA which was then incubated for 30 min at 37°C. Later equal volumes of phenol: chloroform (1:1) was added to the DNA solution and was inverted slowly for three to four times. After centrifugation for 15 min at 10,000rpm the aqueous phase was collected in fresh tube to which equal volumes of chloroform: iso-amyl alcohol (24:1) was added and centrifuged at 10000 rpm for 15 minutes. Later, 0.8 volumes of isopropanol was added to the aqueous phase and incubated at -20^{0}C for 1 hour and centrifuged at 11000 rpm for 20 min. The pellet was washed with 70%ethanol and centrifuged at 10000 rpm for 5min. The pellet was then dried and dissolved in TE buffer. Later DNA samples were

resolved on 0.8% agarose gel using 1X TAE running buffer to check the quality of DNA.

The concentration of DNA samples was determined spectro-photometrically by measuring the absorbance at 260 nm. This is based on the principle that one unit of absorbance at 260nm is equivalent to 50 µg/ml of ds DNA. Concentration of DNA is calculated according to the following formula

DNA (µg/ml) = O.D at 260nm x dilution factor x 50 µg/ml

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

Isolation of RNA (TRIzol Method)

The total RNA was extracted from leaves of transgenic and nontransgenic tobacco and groundnut plants using TRIzol® reagent (Ambion Inc. USA) according to manufacturer's protocol. Leaf samples (!50-100 mg) were collected from PCR positive transgenics in DEPC treated eppendorf tubes. Tissue was ground into fine powder in presence of liquid nitrogen and was homogenized with 1ml TRIzol and incubated for 5min at 15-30 ^{0}C. To this 200µl of chloroform per 1ml of TRIzol reagent was added and the mixture was shaken vigorously for 15 sec and incubated for 2-3 min at 15- 30 ^{0}C. It was centrifuged at 10000 rpm for 15 min at 2-8 ^{0} C. The aqueous phase (~ 600µl) was transferred into a fresh tube to which 0.5 ml isopropyl alcohol was added and gently mixed. This mixture was incubated for 10min at 15- 30^0 C centrifuged at 10000 rpm for 10 min at 2-8 ^{0}C. Later supernatant was removed and the pellet was washed with 1ml of 75% DEPC treated ethanol and entrifuged at 6500 rpm for 5min at 2-8 ^{0} C. Finally the pellet was air dried and the RNA was dissolved in 20 µl DEPC treated water. RNA was quantified by UV spectrophotometry at 260 and 280 nm (A_{260} /A_{280} ~ 2.0; A_{260} = 40 µg RNA/ml) and quality assessed by electrophoresis in 1.5% non-denaturing agarose gels. Formula used for calculation the RNA concentration was

RNA (μg/ml) = O.D at 260nm x dilution factor x 40 μg/ml

Molecular confirmation of transgenic plants

PCR analysis of putative transgenic tobacco plants

Genomic DNA isolated from the leaves of untransformed and putative transformed tobacco plants using modified Dellaporta method was used as a template for performing PCR. PCR was performed in a total volume of 25 μl containing 200ng of template DNA, 2.5 μl of 10 X PCR buffer (10X PCR buffer: 200 mM Tris HCl, 500 mM KCl), 1 μl of 50 mM $MgCl_2$, 0.2 μM each of forward and reverse primer, 200 μM of each dNTP, and 1.0 U of Taq DNA polymerase (Invitrogen BioServices India Pvt. Ltd). The total volume was made upto 25 μl with sterile distilled water. PCR was performed in a programmable thermal cycler (Eppendorf) with initial with lipoxygenase gene specific primers LOX IntFP 5'- CCC CGC ATT TTC TTC TCC AAC -3' LOX IntRP 5'- CTC CAC TGC CAT TCC TCT CTT -3' (Table 1) under identical PCR conditions of initial denaturation at 95^0C for 5 min amplification was performed in 40 cycles of 1 min at 95^0C, 90 sec at 58.8^0C and 90 sec at 72^0C followed by final extension at 72^0C for another 10 min amplifying 1356 bp amplicon. For further confirmation PCR was also carried with junction primers JLox3 FP 5' - CAA TCC CAC TAT CCT TCG CA- 3' JLoX3 RP 5' - CCC CTT TTC CAT CAC CTC TT- 3' amplifying 714 bp. After initial denaturation at 95^0C for 5 min, amplification was performed for 38 cycles of 1 min at 95^0C, 1 min at 59.3^0C and 1 min at 72^0C followed by final extension at 72^0C for another 10 min. PCR products were fractionated on 1% agarose gel and documented.

PCR analysis of putative transgenic groundnut plants

Genomic DNA isolated from the leaves of untransformed and transformed groundnut plants using CTAB method was used as a template for performing PCR. PCR was performed in a total volume of 25 μl containing 10-20 μg of template DNA, with lipoxygenase gene specific primers and junction primers under identical PCR conditions as described earlier. PCR products were fractionated on 1% agarose gel and documented.

Reverse-transcriptase PCR (RT-PCR)

cDNA synthesis was carried out from the above RNA using Protoscript® First Strand cDNA synthesis kit (New England BioLabs Inc., MA, UK).

Total RNA (~1 ng–2 µg) isolated as per above mentioned protocol was taken in a fresh DEPC treated 0.5 ml tube. To it 2 µl of dT23VN primer (50 µM), 4 µl of dNTP Mix (2.5 mM each)was added and made up to the final volume of 16 µl nuclease-free H_20. This mixture was incubated at 70°C for 5 minutes and briefly centrifuged and promptly put on ice. To this 16 µl mix (RNA/primer/dNTP, 2 µl of 10X RT buffer, 1 µl RNase Inhibitor (10 U/µl), 1 µl M-MuLV Reverse Transcriptase (25 U/µl) was added and made up to the final volume to 20 µl with nuclease-free H_20. This mixture was incubated at 42°C for one hour followed by incubation at 95°C for 5 minutes to inactivate the enzyme. To this mixture 1 µl of RNase H (2U/ µl) was added to degrade the RNA and was incubated at 37°C for 20 minutes which was followed by incubation for 5 minutes at 95°C to inactivate the enzyme. The cDNA thus synthesized was diluted to 50 µL with DEPC-treated H_2O and used for PCR amplification of specific genes. The cDNA product was stored at -20°C until further use.

RT-PCR was performed using 2 µl freshly isolated cDNA under conditions explained earlier using lipoxygenase gene specific primers amplifying 1356bp product which was then verified by resolving on 1% agarose gel.

Southern blot Analysis

Genomic DNA (~49 µg) of groundnut isolated from CTAB method was used for digestion with *Hind*III enzyme to detect the copy number of the transgenics in T_3 generation. DNA blotting and transfer of the DNA to positively charged nylon membrane was performed according to Southern (1975) and Sambrook et al., 1989. Labelling of probe and detection was done

The process of the southern blotting analysis of the DNA consists of restriction digestion of the genomic and plasmid DNA samples, agarose electrophoresis of the digested DNA,transfer of DNA onto a solid support (positively charged nylon membrane) by either vacuum transfer or capillary transfer, preparation of labeled probe and hybridization of the membrane with the labelled probe,post hybridization stringency washes, signal generation and detection.

About 40 µl genomic DNA (~40µg) was digested with 5 µl *Hind*III restriction enzyme (NEB; 20 U/µl). The reaction was set up in presence of 10 µl 10X restriction enzyme buffer and the reaction volume was adjusted to a final volume of 100 µl with nuclease free water. The reaction was incubated at 37^0C for overnight followed by heat inactivation for 10 min at 65^0C to inactivate the enzyme. The digested DNA was resolved on 0.8% agarose gel at 40V for overnight which was later visualized under UV transilluminator.

Before transferring the digested DNA onto the nylon membrane the gel was washed with distilled water for 10 min followed by treatment with depurination buffer (Annexure 6.1) under gentle agitation at room temperature for 15 minutes for depurinating the DNA. Later the gel was treated with sufficient volume of denaturation buffer (Annexure 6.2) by agitating at room temperature for 45 minutes to denature the DNA. Then the gel was washed for 15 min with sufficient volume of neutralization solution (Annexure 6.3). Then DNA from the agarose gel was transferred onto the solid support such as positively charged nylon membrane (Amersham Pharmacia-Biotech ®) by capillary transfer method (Sambrook and Russel, 2001) using 20X SSC buffer (Annexure 6.4) for overnight. Later the blot was dried using a clean tissue paper and the DNA was cross linked to the positively charged membrane by placing the blot in UV crosslinker (Thomas Scientific, USA) at 1200 $Jsec^{-2}$ for 1 min.

Southern hybridization was performed using Non-radiolabeled AlkPhos direct labeling and detection system (Amersham®, GE healthcare, UK). The cross-linked membrane was rolled with DNA side facing inside and placed carefully in a glass hybridization bottle. As suggested by the manufacturer, the membrane was incubated in hybridization buffer (Annexure 6.6) pre-warmed to 55°C at 0.125 mL cm^{-2} of membrane. The membrane was prehybridized for 4 hr in hybridization oven (Thomas Scientific, USA) set at 55°C with constant rotations (40 cycles min^{-1}).

The PCR purified junction fragment between 35S promoter and *PnLOX3* fragment (714 bp) amplified from pPZP>35S:*PnLOX3*:polyA binary construct using junction primers was used for preparing the probe for the detection of copy number. Using the AlkPhos® direct labeling kit, 100 ng of probe was labeled as instructed by the manufacturer (Amersham®, GE healthcare, UK). Ten µL of gel eluted PCR amplified junction product fragment (10 ng μL^{-1}) was denatured by heating in boiling water bath for 10 min and snap chilled on ice. This 10µL denatured probe was mixed with 10 µL of diluted cross linker (2 µL cross linker was diluted to 10 µL with sterile water), 10 µL of reaction buffer, and 2 µL of labeling reagent. These contents were mixed gently, briefly spun and were incubated at 37°C for 1 hr. This labeled probe was later used for hybridization reaction.

After 3- 4 hr of pre-hybridization, the labelled probe was added into the hybridization bottle (care was taken to prevent the addition of probe onto the blot directly) containing the membrane and incubated at 55°C in a hybridization oven with continuous rotations (40 cycles min^{-1}) for overnight.

After 16-20 hr of hybridization, the membrane was removed from the hybridization solution and washed with pre-warmed (55°C) primary wash buffer (3 mL cm^{-2} of membrane; Annexure 6.7). Following two washes of 10 min each, the membrane was washed in 1X secondary wash buffer (3 mL cm^{-2} of membrane; Annexure 6.9) at room temperature with gentle agitation for 5 min. After repeating the secondary wash for another 5 min with fresh secondary

wash buffer, the membrane was drained off excess buffer and placed on non-absorbant surface of Saran-wrap®.

For detecting the labeled probe bound genomic DNA fragment, chemi-luminescent detection substrate CDP-Star™ (Amersham Biosciences, UK) was used. This substrate utilizes the probe bound alkaline phosphatase protein and emits photons in the form of signals that can be captured on an X-ray film. The CDP-Star™ substrate reagent was pipetted onto the DNA side of the membrane and incubated for 5 min reaction at room temperature. Later the substrate was drained off and the membrane was carefully wrapped in Saran-wrap®. Care was taken to remove any air bubbles trapped between the membrane and the plastic film and the covered membrane was fixed in the X-ray cassette.

After 4-16 hr exposure of the membrane to X-ray film signal detection was performed in dark room. The exposed X-ray film was developed in a tray containing the X-ray Kodak GBX developer (Annexure 6.10) for 30 sec, washed with water for 30 sec and treated with Kodak GBX fixer (Annexure 6.11) for 30 to 45 sec. The film was further rinsed with water for 2 min and air-dried.

Fungal bioassays for phenotypic evaluation of transgenics

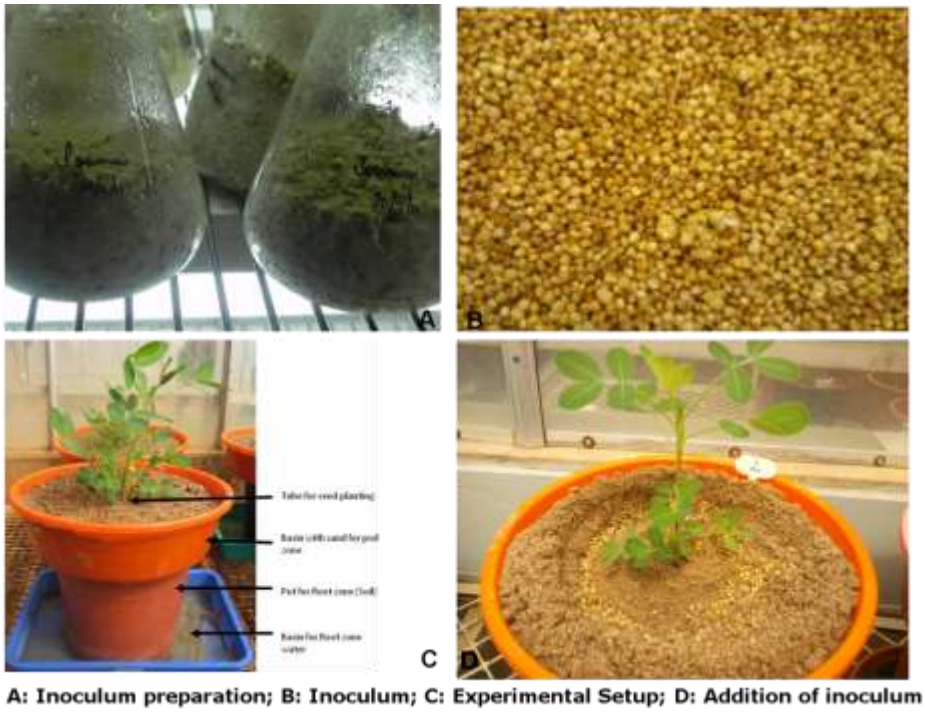
Aflatoxin contamination in peanut is an extremely variable characteristic that primarily occurs under heat and drought stress (Wilson and Stansell, 1983; Cole et al., 1995). Groundnut transgenics harboring pPZP>35S:*PnLOX3*:polyA along with untransformed control were screened for resistance to *A. flavus* seed infection by in vitro seed colonization (IVSC), and subsequent contamination by aflatoxin by Indirect competitive ELISA.

Methodology

Glasshouse Experiment 1 (February, 2011- June, 2011)

Six transgenic events of groundnut var. JL24 harboring the marker free binary construct pPZP200>*35S: PnLOX3:*polyA raised at the Genetic Transformation Laboratory, ICRISAT and characterized at molecular level were used for screening under glasshouse conditions. These events were advanced to

T_3 generation and are being used for our studies to assess their resistance to *A. flavus* and aflatoxin contamination.



A: Inoculum preparation; B: Inoculum; C: Experimental Setup; D: Addition of inoculum

Figure 13: Experimental setup used for performing fungal bioassay under glasshouse conditions

The experiment was conducted in P2 greenhouse facility of ICRISAT. The transgenic plants along with untransformed plants of JL24 variety as susceptible control and J11 variety as resistant control were sown in 9-inch pot which is filled with sand: compost (90:10) above which is placed the basin filled with sand. Pods and roots are physically separated by planting seed individually in tubes, which have been inserted through the center of a basin. Pod set is restricted to the soil filled basin while the roots grow underneath the basin into a pot. Necessary precautions were taken to irrigate only at root zone from 60-75 days (~2 months) after sowing and also to induce drought at pod zone after 45 days (~ 1 ½ month) before harvesting.

AF11-4 strain of *A. flavus* taken from Mycotoxin Diagnostics and Virology Laboratory (MDVL), ICRISAT, was used in the present study. Conidia were

harvested from *A. flavus* infected groundnut seeds by stirring them in sterile distilled water containing 1-2 drops of Tween-20 (Annexure 7.4). The conidial suspension was adjusted to 4 x 10^6 conidia per ml with haemocytometer. Inoculum (organic-matrix) was prepared by growing *A. flavus* on autoclaved sorghum seeds at 28^{o}C for 5 days. After sporulation, inoculum is either used in experiments or stored at 4^0C for further use.

For inoculations, 25 g of the organic-matrix treatments per pot (*A. flavus* sporulated sorghum seed) was applied and raked into the soil surface within 5-10 cm surrounding the planting tube. Total no. of five *A. flavus* inoculations was done as follow: I^{s}t inoculation at the time of sowing, IInd inoculation after 3rd week of sowing, IIIrd inoculation after 5th week of sowing, IVth inoculation after 7th week of sowing and Vth inoculation after 9th week of sowing.

Glasshouse Experiment 2 (January, 2013- May, 2013)

Two transgenic events of groundnut var. JL24 harboring the binary construct pPZP200>*35S: PnLOX3:*polyA were used for screening under glasshouse conditions in this experiment. These events were advanced to T_4 generation. After final inoculation, at 65-70 days of planting, thermocouples are installed to a depth of 5 cm for recording soil temperature. Stress is induced at this stage by maintaining the pod zone under dry condition and root zone is watered until harvesting. After harvesting, pods are collected and dried. The plants were screened by PCR for confirming the presence of the gene (using junction and internal primers).

After harvesting fresh pod weight was recorded and dried under sunlight in glass house conditions for about 1-2 weeks. Subsequently dried pod wt. was recorded and the visual scoring was recorded for *A. flavus* damage in pod. Later, the pods were shelled and scored visually for *A. flavus* damage in the seed. In vitro seed colonization for *A. flavus* infection and ELISA was carried out to estimate aflatoxin contamination

Aspergillus flavus population studies

To validate *A. flavus* group population densities, soil samples were collected before planting, at flowering and at harvest. Soil samples were air-dried, sieved (2 mm) and 10 g of soil sample was mixed with 100 ml water. A 1 ml aliquot of 1:1000 dilution was spread on AFPA medium (Annexure 7.1), a medium selective for *A. flavus*/*A. parasiticus* group fungi. Plates were incubated for 5 days at 28 °C, followed by recording the counts of *A. flavus* group colonies. Results were recorded as colony-forming units of *A. flavus* group fungi/g soil.

Aspergillus flavus colonization studies (Post harvest seed infection)

Seeds were surface sterilized in 0.1% Mercuric chloride for 2 min and subsequently rinsed with three changes of sterile distilled water. Seeds were then placed in petriplates and sterile water was added to adjust to 30% seed moisture (seed weight basis) which were incubated for 7 days at 28°C in humid atmosphere (seed germinator, 98 ± 2% relative humidity). Percentage rating of seed with observed fungal growth was recorded. Infection in these studies was the visual development of conidiophores resulting after fungal penetration of the seed coats and cotyledons. Seeds were observed under 20 to 50 X dissecting microscope while taking observations. Severity of the fungal growth (colonization) on the kernels was rated on a scale of 1-5 (ref). The severity was rated according to the percent of the kernel surface covered by visible mycelia growth, where; 1 = none, 2 = 1 to 20%, 3 = 21 to 50%, 4 = 51 to 70%, and 5 = 71 to 100%. After scoring for severity of colonization, samples that had been infected with *A. flavus* were oven dried at a temperature of 50-60 ^{0}C for 3-5 days. Seeds were further analyzed for aflatoxin content by ELISA.

Biochemical Studies: Aflatoxin analysis by ELISA

After taking observation on pre-harvest aflatoxin screening, each sample was extracted in a fume hood with methanol-water (70: 30 @ 5 ml/g of tissue; Annexure 7.2) by homogenization in a Waring Blender for one minute. The

homogenate was filtered through Whatman No. 41 filter paper (Catalog No 1441 150). Duplicate aliquots of 2-3 ml each were collected for the estimation of aflatoxin AFB_1 by Indirect ELISA. Commercially available, AlfaB_1-oxime-Bovine serum albumin (AFB_1-BSA) was used to produce antiserum in rabbits (MDVL, ICRISAT). The same hapten coupled with Alkaline phosphatase (ALP) (hapten-BSA-ALP) was used in ELISA studies.

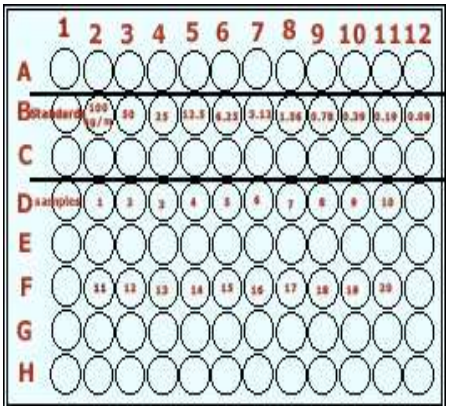
Estimation of aflatoxin AFB_1 by Indirect ELISA

ELISA plate wells were coated with 150 µl (100 ng/ml) of aflatoxin B_1-BSA prepared in sodium carbonate buffer, (Annexure 7.6) and incubated overnight at 4°C. They were then washed in PBS-T, added with BSA (Bovine serum albumin) (0.2%) and allowed to stand at 37°C for 1 hr. ELISA plates were again washed with PBS-T (Annexure 7.3) and aflatoxin B1 (100 µl) standards were added ranging from 25 ng to 10 pg/ml. Pre-incubation was carried out with 50 µl antiserum diluted in PBST-BSA (1:6000) and kept for 45 min at 37°C. Sample extract prepared earlier with methanol were added to wells at 1:10 dilution in PBS-T BSA ((Annexure 7.5). Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase were used at 1:4000 dilution to detect rabbit antibodies attached to aflatoxin B1-BSA. *p*-Nitro phenyl phosphate was used as substrate at 0.5 mg/ml. Absorbance was recorded at 405 nm with an ELISA plate reader (Labsystem, 352) check after incubation at 28°C in dark for 45 min to 1hr. Standard curves were obtained by plotting log 10 values of aflatoxin B_1 dilutions at A_{405}. Aflatoxin B_1 (ng/ml) in sample was determined from the standard curves as {aflatoxin B_1 µg/kg of groundnut = (aflatoxin (ng/ml) in sample x buffer (ml) x extraction solvent (ml) / sample weight (g).

AFB_1-BSA conjugate was prepared in carbonate coating buffer at 1:10,000 dilution (15 ml of coating buffer+1.5 µl of AFB1-BSA conjugate) and 150 µl of diluted toxin-BSA was dispense to each well of ELISA plate which was incubated for 1h at 37°C or overnight at 4°C. The plate was washed (3-times) with PBS-T. Blocking was carried over by adding 200 µl of 0.2% BSA prepared

in PBS-Tween per each well of ELISA plate and incubated at 37^0C for 1h. Cross absorption was performed by diluting antiserum (1 µl of AFB antisera in 6 ml BSA) in a tube which was then incubated for 45 min at 37^0C. Plates were washed thrice with PBS-T. Healthy groundnut extract from previous experiment was used as a control. It was diluted to 1: 10 (500 µl of extract + 4.5 ml of BSA), from which 1 ml was taken and added to 2.6 µl of AFB_1 standard in sterile eppendorf or antisera tube. 100 µl of the above diluted healthy groundnut extract in BSA was first coated in all standard wells leaving 1^{st} two rows in which 100 µl of AFB_1 standard was taken directly in duplicate. Serial dilutions were carried out from the second row leaving the first row (both wells) and healthy control in the last. These cover upper two rows of the plate.

10 µl samples were taken in duplicates for estimation to which 90 µl BSA was added (1:10 dilution; see Fig.). 50 µl of antiserum was added to each of the dilutions of aflatoxin standards (100 µl), samples and groundnut seed extract (100 µl) intended for analysis and the plate was incubated for 1h at 37°C or overnight at 4°C to facilitate reaction between toxin present in the sample with antibody. After the incubation these plates were washed with PBS-T thrice. Then 150 µl of 1 in 5000 dilution (2.5 µl in 12 ml) of goat anti-rabbit IgG, labeled with alkaline phosphatase which was prepared in PBS-Tween containing 0.2% BSA was added to each well and incubated for 1h at 37°C. These plates were later washed thrice with PBS-T. Then 150 µl of ALP substrate (1 mg/ml or 0.5 mg/ml p-nitrophenyl phosphate prepared in 10% diethanolamine buffer, Annexure 7.7) was added and the plate was incubated in dark for 1 h at room temperature or for short intervals for color development. ELISA plate readings were recorded at 405 nm in ELISA reader (BioRad).



Toxin standards show gradual increase from no colour to pale yellow to deep yellow thus indicating the presence of high toxin in samples with no color development and no toxin presence in the samples with deep yellow color

Calculations:

Using the OD values obtained for AFB_1 standards, a standard curve was drawn, taking AFB_1 concentrations on the X-axis and OD values on the Y-axis. Using this standard curve, the aflatoxin concentration present in samples was calculated according to the formula.

$$AFB_1\ (\mu g/kg): (A \times D \times E)/G$$

A= AFB_1 concentration in sample extract (ng/ml)

D= Times dilution with buffer

E= Extraction solvent volume used (ml)

G= Sample weight (g)

Statistical Analysis

Percentage seed infection and aflatoxin analysis

The percentage of *A. flavus* infection and aflatoxin content was subjected to analysis of variance (ANOVA) and mean values in each treatment was compared using LSD at the 5% level of significance (P=0.05) using GenStat version 12.1. Correlation analysis was done using Pearson correlation coefficient at 5% level of significance among the transgenics and untransformed control plants for *A. flavus* infection and aflatoxin content.

Chi square Analysis

Gene segregation pattern was calculated in groundnut transgenics transformed with marker free binary construct pPZP>35S:*PnLOX3*:polyA, using a chi-square test based on PCR results obtained in T_0, T_1, T_2, T_3 & T_4 generations according to the following formula

Observed positives (Obs +ve) = number of positives obtained after screening

Expected positives (Exp +ve) = Total No of seeds used for multiplication/experimentation x 0.75

Observed negatives (Obs –ve) = number of negatives obtained after screening

Expected negatives (Exp –ve) = Total No of seeds used for multiplication/experimentation x 0.25

If $\frac{(\text{Obs +ve} - \text{Exp +ve})^2}{\text{Exp+ve}} + \frac{(\text{Obs–ve} - \text{Exp-ve})^2}{\text{Exp-ve}}$ is less than 3.84 then it follows Mendalian ratio

If $\frac{(\text{Obs +ve} - \text{Exp +ve})^2}{\text{Exp+ve}} + \frac{(\text{Obs–ve} - \text{Exp-ve})^2}{\text{Exp-ve}}$ is greater than 3.84 then it follows Non-Mendalian ratio

pET Expression studies and IPTG induction

Single colony of *E. coli* $BL21_{DE3}$pLysS cells transformed with pTMK12.6 (Tsitsigiannis et al., 2005; pET40a+ vector containing the peanut lipoxygenase gene) was inoculated into 5ml of LB media with the 50mg/l kanamycin and incubated at 37°C overnight in an orbital incubator. From this overnight culture 1% culture was inoculated to a required volume of LB media with appropriate antibiotic and incubated at 37°C in the shaker until A_{600} reaches 0.5- 06.7 (~2 to 4 hours). 1ml of cells was centrifuged for 10 min in a microfuge at maximum speed as was saved as an un-induced culture. Supernatant was

removed and mixed with SDS PAGE sample buffer which was stored at -20^{0}C until SDS-PAGE analysis. 0.1mM IPTG (Annexure 8.1) was added to the final concentration and continued to incubate at 28^{0}C for 4h or overnight. Then the cells from both induced and un-induced cultures were harvested at 10000 rpm for 3 min at 4^{0}C and the pellet was re-suspended in 1/10th volume of cell lysis buffer (Annexure 8.2). Lysozyme (10mg/ml) was added to the final concentration of 1mg/ml and then incubated on ice for 30-45 min. After the incubation the cells were lysed with probe sonicator in presence of ice. Then these cells were harvested at 10000rpm for 10 min at 4^{0}C. The soluble fraction (filtered through the 0.22 micrometer filter) and the pellet were analyzed with SDS PAGE (Annexure 8.7; 8.8) analysis for presence of protein. Since the cell lysate did not have the protein fraction, the pellet was used for further expression studies.

Protein quantification

Bradford's assay was used to estimate the concentration of the protein in the eluted fractions. Protein standards were prepared using BSA (Bovine serum albumin, Sigma) and the standards, ranging from 9 μg to 1 μg were added to the wells of the microtiter ELISA plates (Tarson). Ten μl of the eluted/crude protein fractions were added in duplicates to the wells of ELISA containing 190 μl of Bradford's solution (1:5 diluted) and the colour development was measured at $A_{595\ nm,}$ 5 min after incubation at room temperature. Standard graph was plotted between the absorbance and the standard protein concentration on the Y-axis and X-axis respectively.

Pepsin digestibility test

20 ul (~80μg) protein from crude fraction was taken was mixed with 400 μl simulated gastric fluid (SGF buffer; Annexure 8.3) containing 0.3% pepsin and was incubated at 37 ^{0}C. 22 μl of this sample was collected into eppendorf tube at 5 sec, 10 sec, 20 sec, 30 sec, 1 min, 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 1 hr, 1.30 hr & 2 hr intervals. Reaction was stopped with 8 μl

chilled 0.16M Na_2CO_3. 30 µl 2X sample dye (Annexure 8.13) was added to the samples. Samples were boiled for 5 min prior to loading of which 30 µl was loaded onto 10 % SDS PAGE gel. The gel was processed with coomassie brilliant blue staining (Annexure 8.9) method for checking the digestibility of LOX protein. SGF without pepsin was used as a control, where no digestion observed when analyzed on 10% SDS-PAGE. Similarly 1ug and 100ng of BSA (without SGF treatment) was loaded for comparison.

Promoter isolation and characterization studies

Two novel promoters have been cloned for seed specific expression as seed is the ultimate target for *A. flavus* infection and aflatoxin contamination.

Groundnut seed specific promoter isolation

Oligonucleotide gene-specific primers for *8A4R19G1* gene (GenBank accession no. DQ450071) were synthesized using the Primer 3 software (Rozen and Skaletsky 2000), and used for amplifying the gene from the genomic DNA isolated from peanut and sequenced. Upon confirmation of the sequence obtained using BLAST analysis, a 523 bp of 5-flank upstream sequences of the gene was isolated using Genome Walker Universal Kit (DSS TaKaRa Bio India Pvt. Ltd.) from the peanut genomic DNA and sequenced. Based on the obtained sequence, oligonucleotide primers GSP FP 5'-AAC CGG ATC CAG CTT TAA TAG CAA CTA GGC-3' and GSP RP 5'- AACC GGA TCC GGG AAA CAG CAA CTG CTA-3' were synthesized and used to amplify the putative promoter region (GSP) using polymerase chain reaction (PCR). The PCR reactions were carried out in a total volume of 25 µl that contained 200 ng of template DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 µM each of forward and reverse primer, 200 µM of each dNTP, and 1.0 U of Taq DNA polymerase (Invitrogen BioServices India Pvt. Ltd). PCR was performed in a programmable thermal cycler (Eppendorf) with initial denaturation at 95 ^{0}C for 5 min followed by 35 cycles of denaturation for 1 min at 95 ^{0}C, annealing for 1 min at 59.1 ^{0}C

and extension for 1 min at 72 ^{0}C, with a final extension for 10 min at 72 ^{0}C. The amplified product (~523 bp) was fractionated on 1 % agarose gel and purified using the Nucleospin Gel elution kit (Bioserve Biotechnologies, India Pvt. Ltd.). Eluted bands of the PCR product were ligated into pCR-Blunt-II-TOPO vector by using Zero Blunt® TOPO® PCR Cloning Kit **(**Invitrogen BioServices India Pvt. Ltd**.**) followed by blue-white selection (Sambrook et al., 1989). Plasmids isolated from the white colonies were confirmed by restriction digestion analysis using *Eco*RI and/or *Bam*HI followed by sequencing. Orientation of the promoter fragment was confirmed by restriction digestion with *Sph*I and *Hinc*II. The sequence has been submitted with NCBI GenBank as HM215006.

Chickpea lectin promoter isolation

Oligonucleotide primers were designed based on 5' galactose binding lectin sequence of peanut lectin (CPFP 5' GTGTGTGTGGCACAGCAATA 3'and CPRP 5' TTGAATTTGCATGCATCAGG 3') using the Primer3 software. About 500 mg of fresh young chickpea leaves were used to extract the genomic DNA by the CTAB method. Promoter regions of the chickpea lectin genes (CPLP) were amplified by PCR by denaturation at 94 ^{0}C for 5 min followed by 30 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 52 ^{0}C for 45 seconds, extension at 72 ^{0}C for 1.5 min, followed by a final extension at 72 ^{0}C for 10 min. The amplified products were verified on 1 % agarose gels prior to elution and purification using Nucleospin Gel elution kit (Bioserve Biotechnologies India Pvt. Ltd.). Eluted bands of the PCR product were ligated in the pGEM-T easy vector using TA cloning protocols (Promega Pte Ltd., Singapore) and subsequently transformed to *E. coli* by KCM method followed by blue-white screening. Plasmids from the white colonies were isolated, verified on 0.8 % agarose gel and subjected to restriction digestion analysis by *Eco*RI and or *Not*I followed by sequencing.

The chickpea lectin promoter was re-amplified with *Bam*HI site-carrying primers (FPLP-5'-GGA TCC GTG TGT GTG GCA CAG CAA TA-3', RPLP-5'-GGA

TCC TTG AAT TTG CAT GCA TCA GG -3'). PCR conditions included an initial denaturation at 95 ^{0}C for 5 min followed by 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 65 ^{0}C for 1 min, extension at 72 ^{0}C for 1 min, and a final 10 min extension at 72 ^{0}C. The amplified products were verified on 1 % agarose gels, eluted and purified from agarose by using a gel elution kit. The eluted bands were ligated into PCR-Blunt-II-TOPO using TOPO T-A cloning protocols (Invitrogen Bioservices India Pvt. Ltd.). Using the KCM method, the ligation mixture was transformed into the competent cells of *E. coli* followed by their blue/ white screening. Plasmids from the white colonies were isolated, verified on 0.8 % agarose gel and subjected to restriction digestion analysis by *Eco*RI and or *Bam*HI followed by sequencing. The sequence has been submitted with NCBI GenBank as EU560424

Sequence analysis

Nucleotide sequences obtained after sequencing were analysed using NCBI BLAST analysis (Altschul et al. 1997) and 'gene tool' softwares such as Gibb's sampling (Lawrence et al. 1993), Melina software (Poluliakh et al. 2003) and MEME (Multiple Expectation Maximisation for Motif Elicitation (Bailey and Elkan 1994; Bailey et al. 2006) for presence of the promoter motifs. The GSP and CPLP promoter sequences were also analyzed using various database search programs such as PlantCARE database (Lescot et al. 2002; Rombauts et al. 2002) and Genomatix- MatInspector softwares based on PLACE database (Higo et al. 1999).

Electro-mobility shift Assay (EMSA)

Nuclear proteins were isolated from the seeds of peanut and chickpea using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India). PCR amplified products of promoter fragments of GSP and CPLP were end-labelled with biotin 3' End DNA Labeling Kit (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) and used as a probe. In vitro DNA-protein binding assay was carried out as described by Light

Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) by combining solutions of freshly isolated nuclear proteins (~5-10 μg) and 3' biotin labelled nucleic acid fragments. The resulting binding mixtures were fractionated by electrophoresis on 6 % native (non-denaturing) polyacrylamide gel for CPLP fragment and by electrophoresis on 0.8% agarose gel for the GSP fragment. These were then transferred to Hybond-N+ nylon membrane (GE Healthcare, New Jersey, USA) and developed according to manufacturer's instructions.

Construction of Plant expression vectors

In order to confirm promoter activity in the plant system, a binary vectors were constructed by replacing the single CaMV 35S promoter in pPZP200>35S:GUS (Fig. 14a) by the CPLP promoter of chickpea at the *Bam*HI site to produce pPZP200>CPLP:GUS (Fig. 1b). Similarly, the plasmid pPZP200>GSP:GUS was constructed by replacing the single CaMV 35S promoter of pPZP200>35S:GUS with the GSP promoter of peanut at the *Bam*HI site. Subsequently, the complete cassette containing *uid*A gene driven by GSP promoter was subcloned into binary vector pCAMBIA2300 using *EcoR*I and *Hind*III restriction sites, thereafter referred to as pCAMBIA2300>GSP:GUS (Fig. 14c). Restriction with *Sph*I enzyme was done to ensure that the promoters were cloned in correct orientation upstream of the *uid*A gene. The recombinant binary vector plasmids, pPZP200>CPLP:GUS (Fig. 14b) and pCAMBIA2300>GSP:GUS (Fig. 14c) were mobilized into *A. tumefaciens* strain C58 after confirmation with restriction analyses, and glycerol stocks stored at -80 °C until further use.

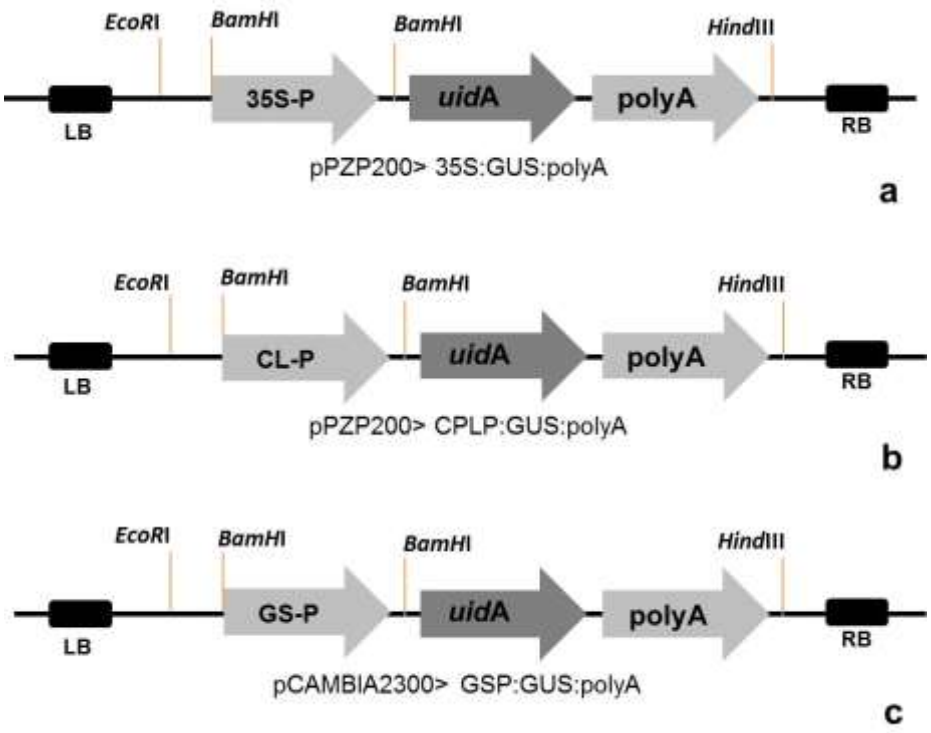


Figure 14: Schematic representation of T-DNA region of the binary vector constructs used in this study (a) pPZP200>35S:*uidA*:polyA, (b) pPZP200>CPLP:*uidA*:polyA, (c) pCAMBIA2300>GSP: *uidA*:polyA.

Preparation of the bacterial culture for *Agro*-infection

Primary culture of *A. tumefaciens* strain C58 harboring the binary plasmids was prepared by inoculating single colony of *Agrobacterium* in 20 ml YEB medium (with 50 mg/l each of kanamycin and rifampicin for pCAMBIA2300>GSP:GUS and 50 mg/l spectinomycin for pPZP200>CPLP:GUS and pPZP200>35S:GUS), and grown overnight at 28 ^{0}C at 200 rpm. For floral dip transformation of *Arabidopsis,* the overnight culture (~10 %) was added to 20 ml of fresh medium with the same antibiotic and grown to the stationary phase (O.D$_{600}$~2.0). Cells were harvested by centrifuging at 5500 g for 20 min and the pellet was re-suspended in 0.5X MS (Murashige and Skoog 1962), 5 % sucrose and 0.05-0.1 % teepol to obtain the desired density (O.D$_{600}$~ 2.0). For tobacco transformation, 5 ml of the overnight-grown culture was pelleted at 5500 g for 10 min, the supernatant discarded, and the pellet was re-suspended

in 0.5X MS so as to dilute it to an $O.D_{600}$ ~ 0.5. This suspension was used for the co-cultivation of tobacco leaf discs using *Agrobacterium*-mediated transformation.

Agrobacterium-mediated gene transformation in *Arabidopsis*

Seeds of *Arabidopsis thaliana* (Col-1) were sown in sand: soil (1:1) mixture in 4 cm pots and kept in the culture room until germination. Plants at the 4-leaf stage were transferred to the greenhouse and irrigated every 4 days until inflorescences appeared (Fig. 3a). Plants with inflorescences of about 5 cm were transformed with suspension cultures of *A. tumefaciens* harboring the binary plasmids carrying GSP and CPLP promoter fragments, using floral dip protocol of Clough and Bent (1998). Plants were inoculated by direct drop-by-drop inoculation to every flower by using a micropipette (Trujillo et al. 2004) and covered with plastic bags and incubated in dark for 10-24 h (Fig. 3b). Inoculation with the *Agrobacterium* was repeated twice at 3 d intervals, and the seeds collected when all the siliques dried.

Agrobacterium-mediated genetic transformation in tobacco

Agrobacterium-mediated transformation of tobacco was carried out using standard leaf-disc method (Horsch et al. 1988) with some modifications as described earlier. The explants containing regenerated shoot buds with the plasmid pCAMBIA2300>GSP:GUS construct (containing the *npt*II gene) were subjected to selection with 50 mg/l kanamycin. These explants were further sub-cultured onto fresh MS medium for 35- 40 d at 2 wk intervals for shoot elongation and rooting. The rooted shoots were transferred to the pots containing autoclaved sand and soil (1:1) mixture and maintained in a containment glasshouse until flowering and seed set.

Molecular characterization of putative tobacco transgenics

Genomic DNA was isolated from the leaves of T_0 and T_1 generation transgenic plants of tobacco by using the modified CTAB method (Porebski et al. 1997). PCR was set-up in a total volume of 25 µl containing 10-20 µg of

template DNA for amplification of the 1213 bp *uid*A gene fragment using primers GusFp 5'-TGA TCA GCG TTG GTG GGA AAG-3' GusRp 5'-TTT ACG CGT TGC TTC CGC CAG-3'. The PCR conditions included initial denaturation for 5 min at 95 ^{0}C, followed by 35 cycles of denaturation for 1 min at 95 ^{0}C, annealing for 90 sec at 58.8 ^{0}C and extension at 72 ^{0}C for 90 sec followed by final extension for 10 min at 72 ^{0}C. PCR products were fractionated on 1% agarose gel. Similarly, RT-PCR analysis was carried out to confirm integration of the *uid*A gene using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA, USA) on total leaf RNA isolated using the TRIzol® reagent (Invitrogen, USA) and from seed tissues using RNA isolation kit (MACHEREY-NAGEL, Germany). The primer sequences for the GUS transcripts were same as those described for the PCR analysis.

Histochemical and Fluorometric Analysis

The harvested seeds of tobacco and *Arabidopsis* were subjected to GUS assays using X-gluc (Annexure 9.3) as the substrate with overnight incubation at 37 ^{0}C (Jefferson 1987). To confirm the β-glucuronidase enzyme specific activity in *Arabidopsis* and tobacco transformants, GUS assay was carried out in different tissues like seeds, maternal tissues, cotyledons, stem, root, leaves and flower. For the GUS assay, tissue samples were collected in 1.5 ml eppendorf tubes and treated with 500 µl GUS assay solution followed by incubation for 16-24 h at 37 ^{0}C. While the tissues were cleared with serial transfers in 70% alcohol, the final samples showing blue coloration were photographed.

The fluorometric assay for specific GUS enzyme activity was quantified by measuring the hydrolysis rate of the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (MUG) (HiMedia Laboratories Pvt. Ltd.) as described by Jefferson (1987). Standards were prepared with different concentrations, i.e., 1 mM, 1 µM and 100 nM of 4-methylumbelliferone sodium

salt (4-MU; HiMedia Laboratories Pvt. Ltd.) in 0.2 M sodium carbonate. 2 mM of 4-MUG was added to each sample as the substrate.

Seed extracts prepared with GUS extraction buffer (Annexure 9.9) were used for histochemical analysis. Plant materials (seeds, flower and leaf) were vigorously ground to a finely pulverized powder with a pestle and mortar under liquid nitrogen in 500 µL GUS extraction buffer. The extract thus obtained was centrifuged at 8,000 g for 5 min at 4 ^{0}C. The supernatant was recovered and, 50 µL of extract was added to 950 ml of 4-MUG assay buffer (2 mM; Annexure 9.10), to initiate the reaction. The reaction was stopped by adding 200 µl of the reaction to 1.8 ml of 0.2 M Na_2CO_3 stop buffer (Annexure 9.6) at intervals of 0, 30, 60 min, and overnight, and fluorescence measured using a DyNA Quant 200™ fluorometer (Hoefer Scientific Instruments, San Francisco, CA), following the manufacturer's instructions. The protein concentrations of each sample were determined (Bradford, 1976) with a spectrophotometer (Shimadzu, UV-1650PC) at OD_{595} using Quick Start Bradford Protein Assay kit (BioRad), and the GUS enzyme activity was expressed as pmoles of 4-methylumbelliferone (MU) produced per mg protein per min.

Construction of binary vectors pPZP200>GSP:*PnLOX*3-poly A and pPZP200>CPLP:*PnLOX*3:poly A

CaMV 35S promoter was released from pPZP200>35S:*PnLOX*3:poly A and was replaced by seed specific promoters of groundnut (GSP) (Sowmini et al., 2013) and chickpea (CPLP) (Sowmini et al., unpublished) with *Sal*I and *Sac*I restriction enzymes. The new binary constructs were designated as pPZP200>GSP:*PnLOX*3:poly A and pPZP200>CPLP:*PnLOX*3:poly A respectively. These binary constructs were subsequently mobilized into disarmed *Agrobacterium* strain C58C1Rif^R through electroporation and recombinants were selected on YEB media containing spectinomycin for use in *Agrobacterium*-mediated genetic transformation studies. Further work is in progress with developing transgenics with lipoxygenase gene under control of these two novel promoters and their molecular characterization studies.

Results

4.1 Vector construction

The 2726 bp fragment containing the peanut lipoxygenase gene (*PnLOX3*) sequence was sub-cloned from plasmid pTMK12.6 (8058 bp) into the pTOPO>TSVcp vector (5079 bp). This plasmid designated as pTOPO>*PnLOX3* (6147 bp) contains kanamycin as selection marker in *Escherichia coli* strain DH5α. Construction and orientation of this vector was confirmed by plasmid restriction analysis with *Kpn*I-*Xho*I, *Hind*III, *EcoR*V, *BamH*I, *Sma*I, *Spe*I-*Sal*I. The restriction analysis of this vector showed linearized fragment of size 6147 bp with restriction with *EcoR*V and *BamH*I. The restricted pattern also showed two fragments of 2172 bp and 3975 bp sizes in digestion with *Nco*I and 2726 bp and 3421 bp fragments with *Kpn*I-*Xho*I restriction respectively (Fig. 14). Similarly restriction analysis with *Kpn*I-*Xba*I double digestion showed two fragments of 2735 bp and 3412 bp sizes (Fig. 15).

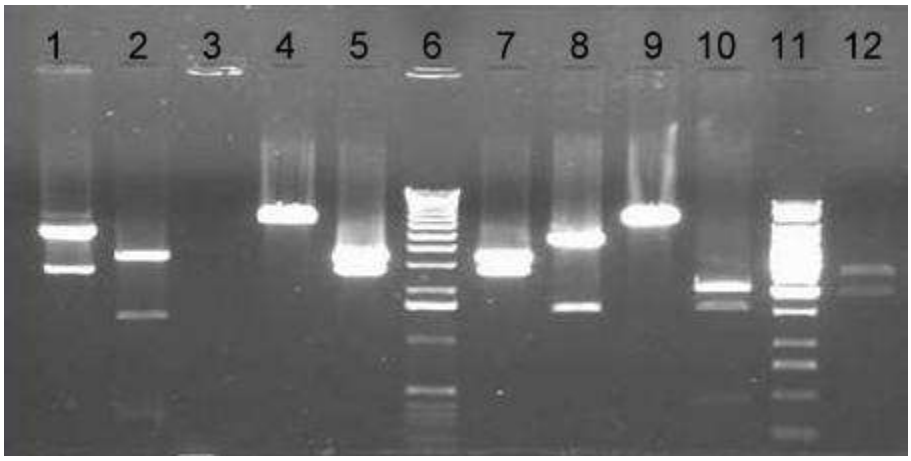


Figure 15: Restriction digestion analysis for confirmation of the clone pTOPO>*PnLOX3*. Lane 1: pTMK 12.6 restricted with *Kpn*I-*Xho*I; Lane 2: pTOPO>TSVCP restricted with *Kpn*I-*Xho*I; Lane 4: pTOPO>*PnLOX3* restricted with *BamH*I; Lane 5: pTOPO>*PnLOX3* restricted with *Kpn*I-*Xho*I; Lane 6: 1 KB ladder; Lane 7: pTOPO>*PnLOX3* restricted with *Kpn*I-*Xba*I; Lane 8: pTOPO>*PnLOX3* restricted with *Hind*III; Lane 9: pTOPO>*PnLOX3* restricted with *EcoR*V; Lane 10: pTOPO>*PnLOX3* restricted with *Nsi*I; Lane 11: 100bp ladder; Lane 12: pTOPO>TSVCP restricted with *Nco*I.

Figure 16: Restriction digestion analysis for confirmation of the clone pTOPO>*PnLOX3*. Lane 1: pTMK 12.6 restricted with *Xho*I-*Kpn*I; Lane 2: NEB 1kb ladder; Lane 3: pTOPO>*PnLOX3* restricted with *Kpn*I-*Xba*I.

The 649 bp fragment containing the 35S promoter and polyadenylation (poly A) terminator sequences was sub-cloned from plasmid pRT 103 (3343 bp) into the pGEM-T Easy vector (3015 bp). This plasmid designated as pGEMT>35S:polyA (3664 bp) contains ampicillin as selection marker in *E. 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 also showed two fragments of 3015 bp and 649 bp sizes with *Pst*I digestion and two fragments of 2989 bp and 675 bp sizes in double digestion with *Spe*I+ *Sal*I, respectively (Fig. 16).

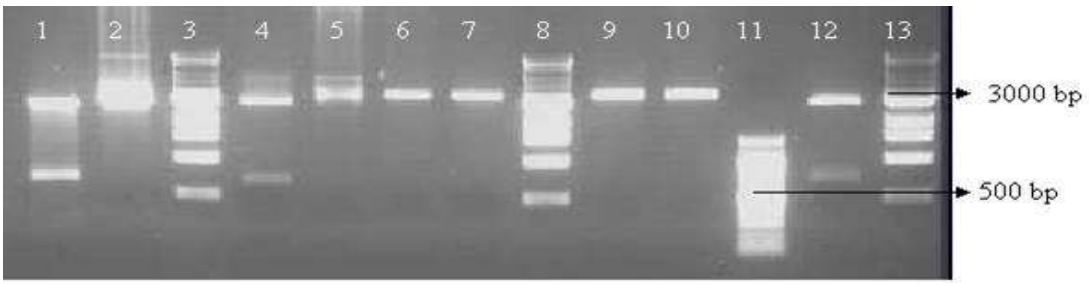


Figure 17: Restriction digestion analysis for confirmation of the clone pGEMT>35S:polyA. Lane 1: pRT 103 restricted with *Pst*I; Lane 2: pGEM-T Easy restricted with *Pst*I; Lane 3, 8, 13: 1 Kb ladder; Lane 4: pGEMT>35S:polyA restricted with *Pst*I; Lane 5: pGEMT>35S:polyA plasmid; Lane 6: pGEMT>35S:polyA restricted with *Kpn*I; Lane 7: pGEMT>35S:polyA restricted with *Spe*I; Lane 9: pGEMT>35S:polyA restricted with *Sal*I; Lane 10: pGEMT>35S:polyA restricted with *Sma*I; Lane 11: 100 bp ladder; Lane 12: pGEMT>35S:polyA restricted with *Spe*I + *Sal*I.

The 2735 bp fragment containing the peanut lipoxygenase gene sequence was then sub-cloned from plasmid pTOPO>*PnLOX3* (6147 bp) into the pGEMT>35S:polyA (3664 bp). This plasmid designated as pGEMT>35S:*PnLOX3*:polyA (6399 bp) contains ampicillin as selection marker in *E. coli* strain DH5α. Construction and orientation of this vector was confirmed by plasmid restriction analysis with *Kpn*I-*Xba*I, *Xba*I, *Sal*I, *Hind*III, *Eco*RV, *Spe*I-*Nsi*I. The restriction analysis of this vector showed linearized fragment of size 6339 bp with restriction with *Xba*I, *Sal*I and *Hind*III. The restricted pattern also showed two fragments of 2735 bp and 3663 bp sizes with *Kpn*I-*Xba*I double digestion (Fig. 17).

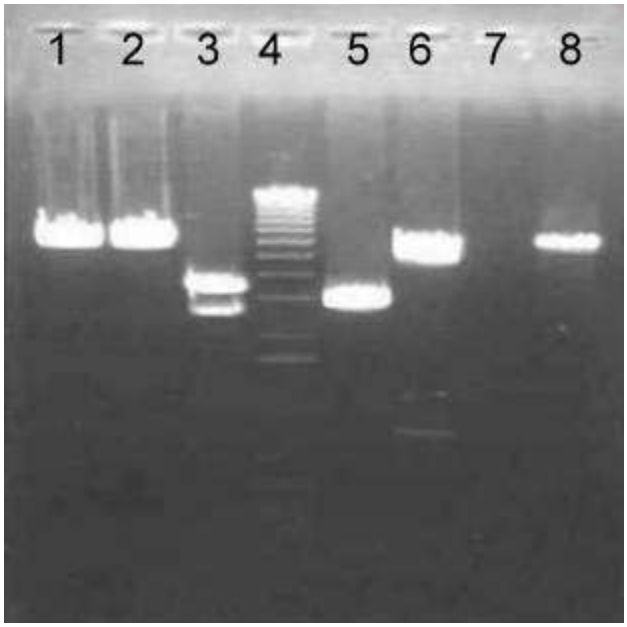


Figure 18: Restriction digestion analysis for confirmation of the clone pGEMT>35S:*PnLOX3*:polyA. Lane 1: pGEMT>35S:*PnLOX3*:polyA restricted

with *Hind*III; Lane 2: pGEMT>35S:*PnLOX3*:polyA restricted with *Sal*I; Lane 3: pGEMT>35S:*PnLOX3*:polyA restricted with *Kpn*I-*Xba*I; Lane 4: 1 Kb ladder; Lane 5: pGEMT>35S:*PnLOX3*:polyA restricted with *Spe*I-*Nsi*I; Lane 6: pGEMT>35S:*PnLOX3*:polyA restricted with *EcoR*V; Lane 8: pGEMT>35S:*PnLOX3*:polyA restricted with *Xba*I.

The 3412 bp fragment containing peanut lipoxygenase gene under the control of CaMV35S promoter and poly A terminator sequences was sub-cloned from pGEMT>35S:*PnLOX3*:polyA (6399 bp) into the pPZP200>d35S:PBNV (8432 bp). This plasmid designated as pPZP200>35S:*PnLOX3*:polyA (10247 bp) contains spectinomycin as selection marker in *E. coli* strain DH5α. Construction and orientation of this vector was confirmed by plasmid restriction analysis with *EcoR*V, *Nsi*I, *Spe*I, *Sal*I, *Xho*I, *Hind*III, *Kpn*I, *Bam*HI and *Spe*I-*Sal*I. The restriction analysis of this vector showed linearized fragment of size 10247 bp with restriction with *Kpn*I, *Spe*I, *Sal*I, *Sma*I. The restricted pattern showed two fragments of 3412 bp and 6835 bp sizes with *Spe*I+ *Sal*I double digestion and two fragments of 2746 bp and 7501 bp sizes with *Xho*I digestion respectively (Fig. 18).

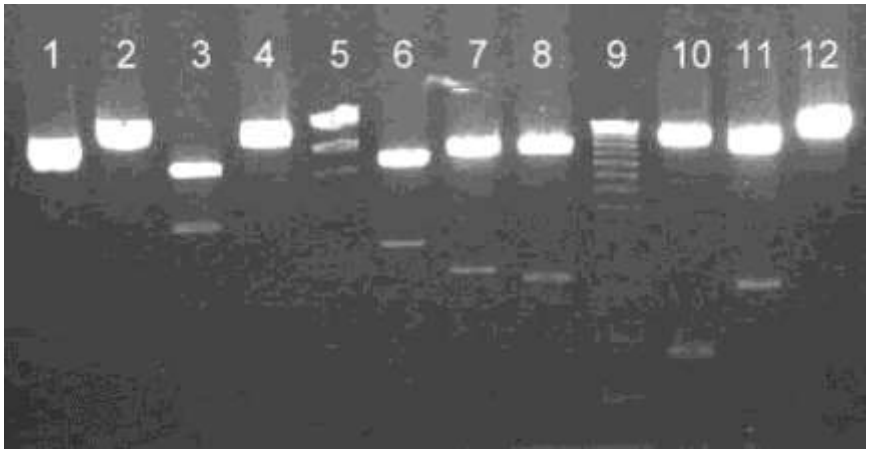
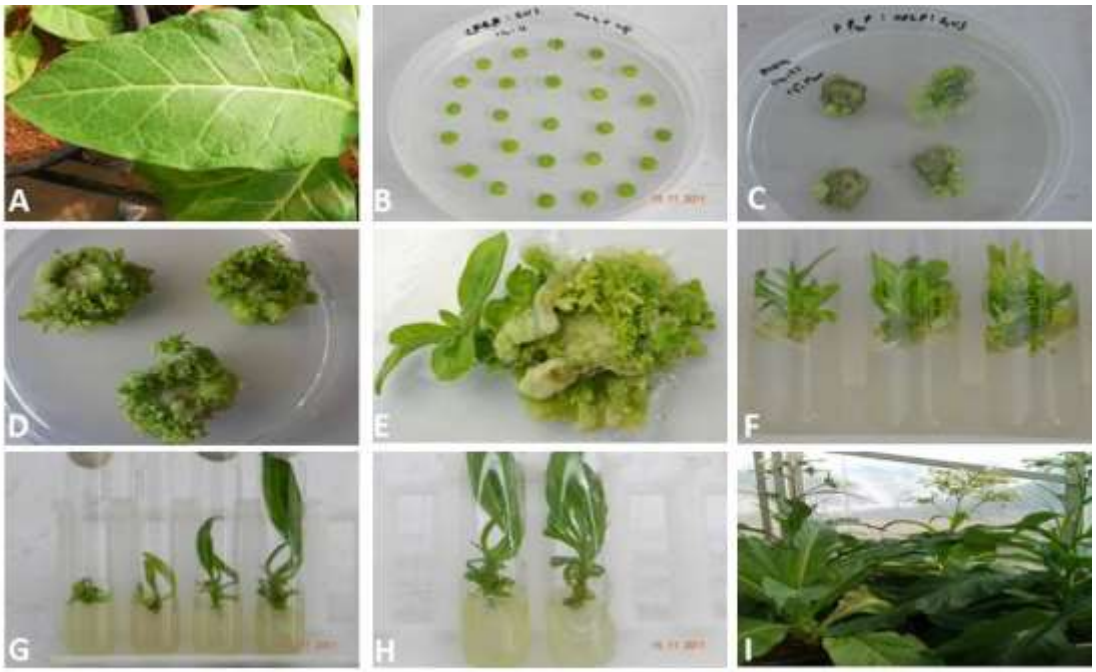


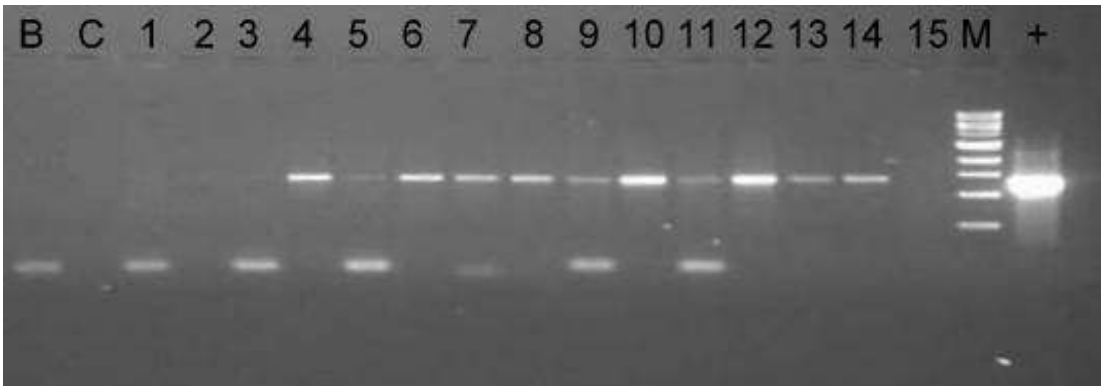
Figure 19: Restriction digestion analysis for confirmation of the clone pPZP200>35S:*PnLOX3*:polyA. Lane 1: Plasmid; Lane 2: Restriction with *Spe*I; Lane 3: Restriction with *Spe*I-*Sal*I; Lane 4: Restriction with *Sal*I; Lane 5: Lambda *Hind*III ladder; Lane 6: Restriction with *Xho*I; Lane 7: Restriction with *Hind*III; Lane 8: Restriction with *Nsi*I; Lane 9: NEB 1 Kb ladder; Lane 10: Restriction with *EcoR*V; Lane 11: Restriction with *Bam*HI; Lane 12: Restriction with *Kpn*I.

4.2 Regeneration and transformation studies

4.2.1 Tobacco transformation

Of the 25 putative transgenic plants, 23 were positive for the presence of *PnLOX3* gene. This was confirmed by PCR and by RT-PCR using internal lipoxygenase gene specific primers (*PnLOX3* gene sequence) amplifying 1356 bp. The T_0 plants were harvested and the seeds were stored for further molecular and physiological analysis, if required to be performed.





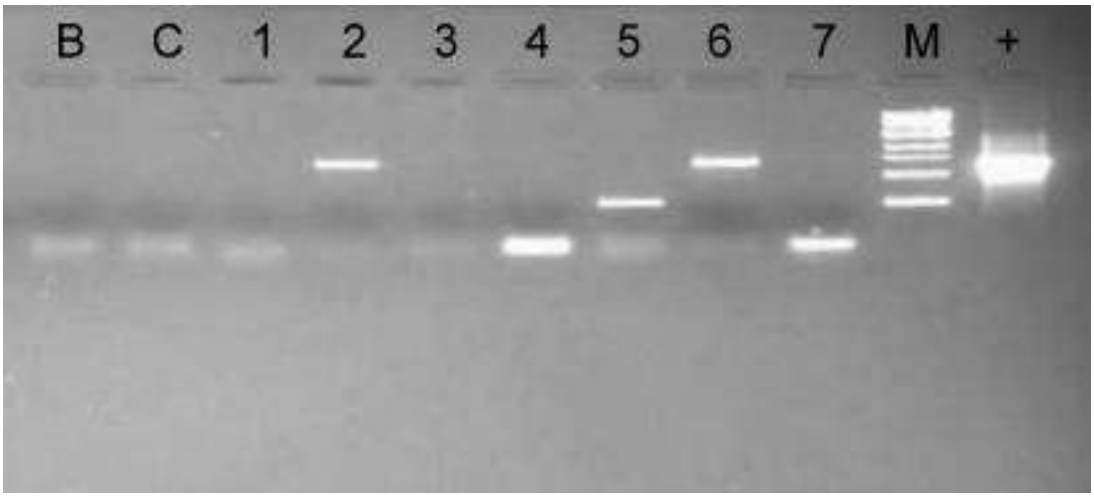


Figure 20: Tobacco transformation and molecular characterization. A-I: Tobacco leaf disc method of transformation using *Agrobacterium tumefaciens* (Horsch et al., 1988) with binary construct harboring pPZP200>35S:*PnLOX3*:polyA. J: PCR analysis of putative transgenics using internal lipoxygenase primers amplifying 1356 bp. K. RT-PCR analysis using internal primers.

4.2.2 Transformation of groundnut

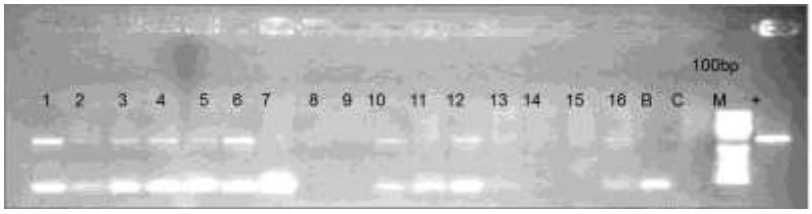
The putative transgenic plants resulted from transformation of groundnut with pPZP200>35S:*PnLOX3*:polyA appeared phenotypically normal without any morphological abnormalities in any form. In general, it was observed that the explants response and shoot bud proliferation were higher in untransformed 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. Phenotypic observations showed that putative transgenic events exhibited delay in elongation, acute growth retardation in *invitro* and a very low survival percentage in greenhouse conditions. This may be due to constitutive expression of the lipoxygenase gene which is also involved in ABA biosynthesis.

Figure 21: Groundnut transformation protocol (Sharma & Anjaiah, 2005). A. Mature JL24 seeds; B-D. Decoated, de-embryonated and split cotyledonary explants; for *Agrobacterium*-mediated transformation kept on shoot induction medium (SIM) containing MS supplemented with 20 µM BA and 10 µM 2,4-D; E-F. Explants turned green and enlarged kept for Induction of adventitious multiple shoot buds on SIM with 250 µg ml^{-1} cefotaxime; G-I. Multiple shoots elongated on shoot elongation medium, MS supplemented with 2 µM BA; J. Rooting of the elongated shoots on root induction medium, MS supplemented with 5 µM NAA; K-L. Initial transfer into jiffy pots containing sterile sand for the establishment of the roots covered in perforated poly bag and incubated in growth chamber for 4 days; M-N. Fully established healthy plants transferred into bigger pots containing sand: soil mixture and maintained in the P2 greenhouse.

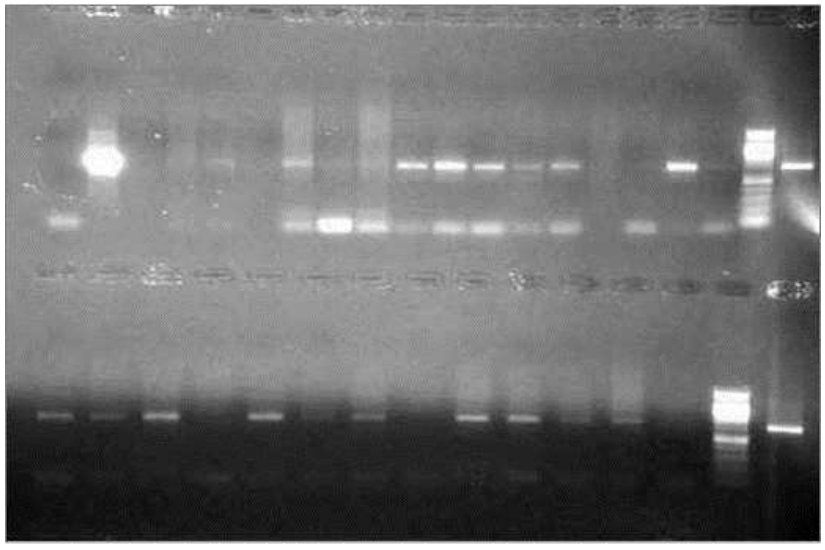
4.3 Molecular characterization of transgenics

4.3.1 PCR

Of the 25 putative transgenic plants produced using marker-free construct pPZP200>35S:*PnLOX3*:polyA, 14 events were confirmed positive by PCR with internal primers amplifying 1356 bp amplicon. These PCR positive plants were re-confirmed by PCR using junction primers (between CaMV 35S promoter and *PnLOX3* gene sequence) with the desired amplicon size of 714 bp. These T_0 plants were harvested and the seeds were stored for further molecular and physiological analysis under cold conditions of which 6 events have been advanced to T_4 generation. RT-PCR was performed with PCR positive events using internal primers amplifying 1356 bp product.



T_0



T_1

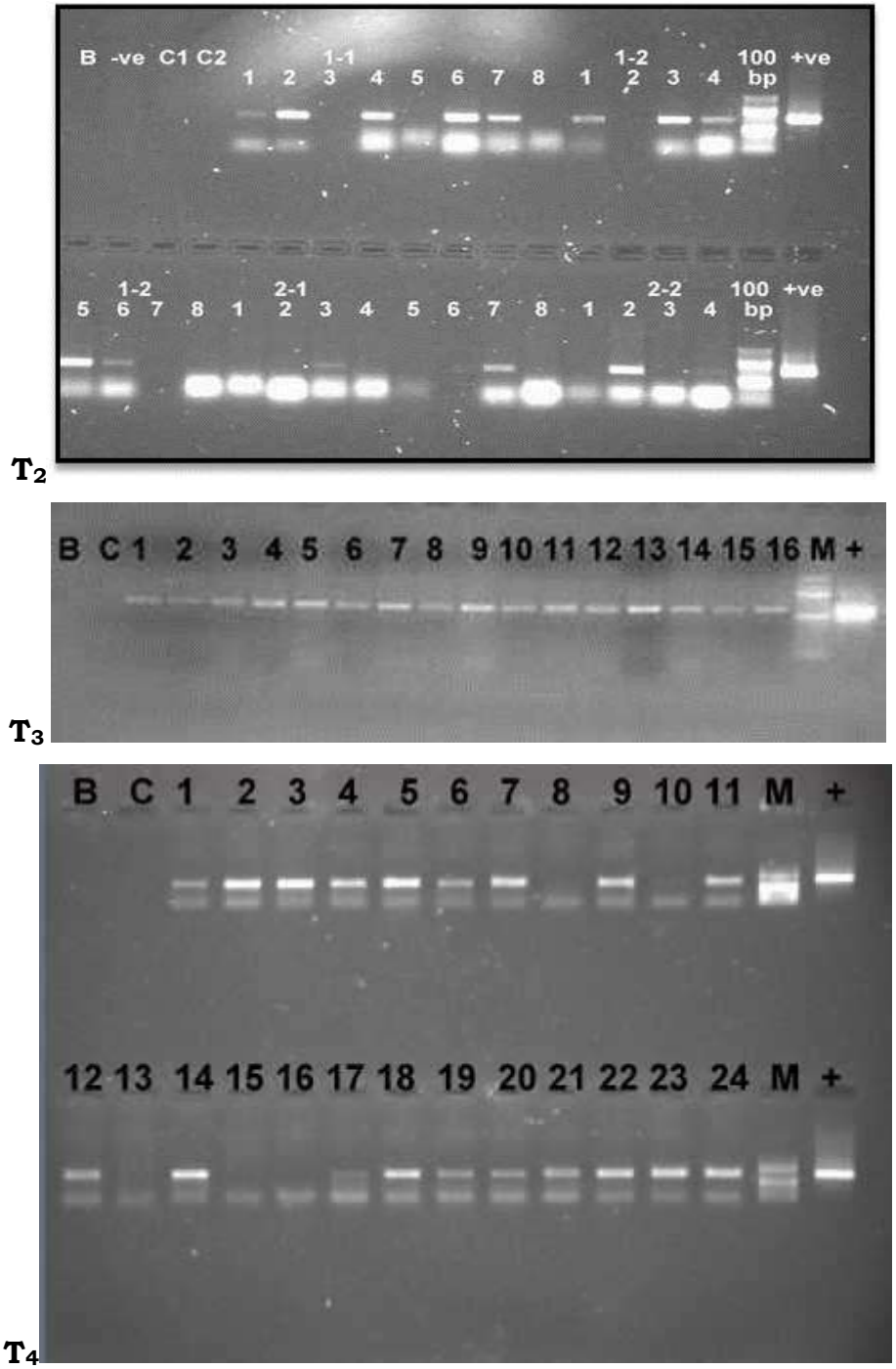


Figure 22: PCR amplification of *PnLOX3* in transgenic peanut plants with junction primers amplifying 714 bp.

4.3.2 RT-PCR studies

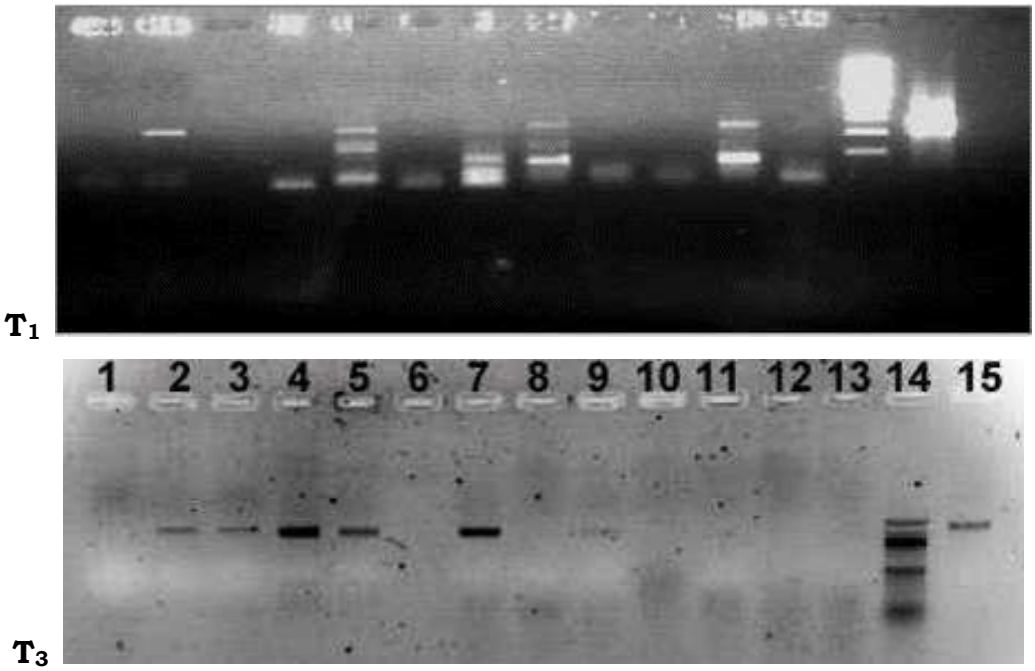


Figure 23: RT- PCR amplification of *PnLOX3* in transgenic peanut plants with internal primers amplifying 1356 bp.

4.3.3 Southern blotting

Southern blotting was performed to confirm the presence of *PnLOX3* transgene in T3 generation transgenics carrying pPZP200>35S:*PnLOX3:*polyA. Restriction analysis of plasmid DNA with *Hind*III restriction enzyme resulted in linearization. This was used for the copy number estimation in the transgenics which resulted in presence of single copy gene as shown in figure 23.

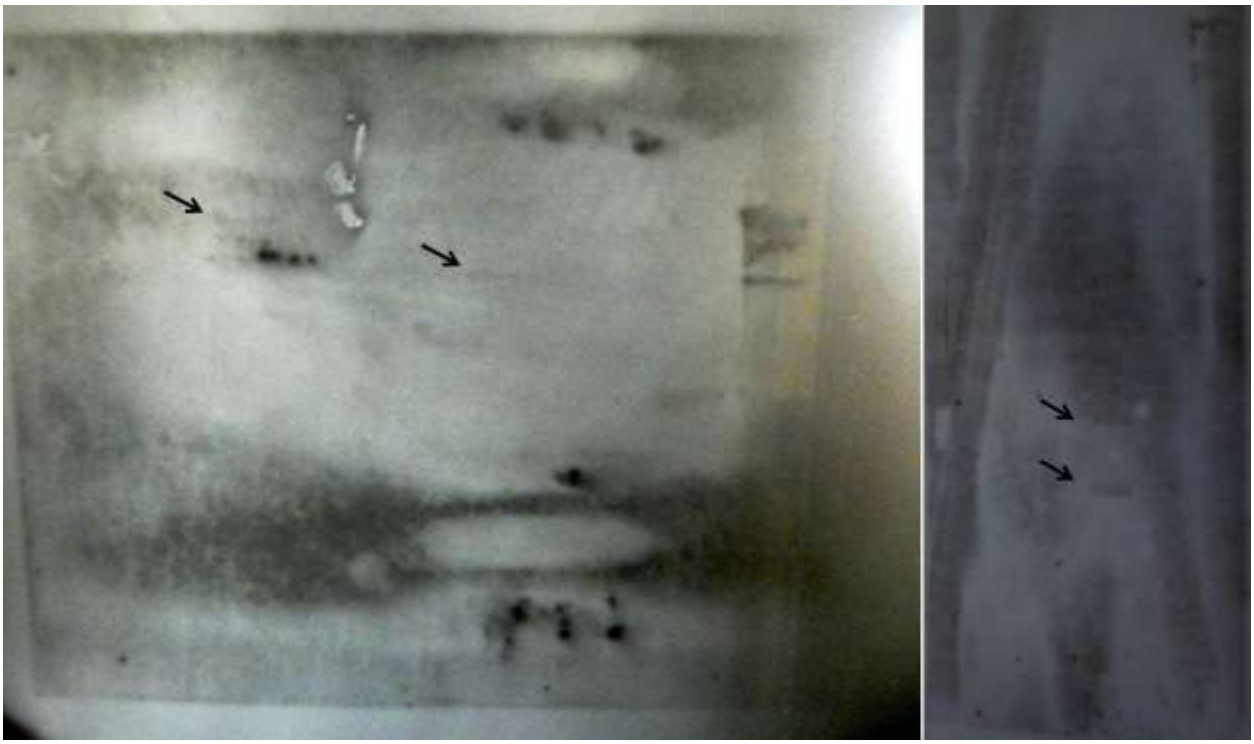


Figure 24: Southern blotting analysis of genomic DNA restriction of T_3 groundnut transgenics carrying binary construct pPZP200>35S:*PnLOX3*:polyA with *Hind*III enzyme. A: Lane 1: Sample 2-1-1-10; Lane 2: Sample 2-1-5-5; Lane 3: Sample 3-1-1-6; Lane 4: Sample 5-2-1-34; Lane 5: Sample 5-2-2-6; Lane 6: Sample 6-4-1-25; Lane 7: Blank; Lane 8 JL24 control DNA; Lane 9: Plasmid pPZP200>35S: *PnLOX3*: polyA .B. Lane 1: Sample 2-2-1-1; Lane 2: Sample 3-1-1-6; Lane 3: Sample 5-2-2-6 ; Lane 4: Control JL24; Lane 5: Plasmid pPZP200>35S: *PnLOX3*: polyA.

4.4 Fungal bioassays

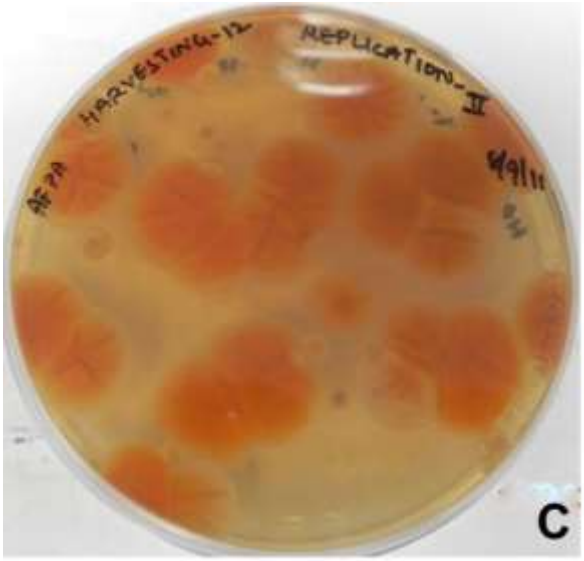
In order to identify the underlying mechanism of resistance in these genotypes, the harvested pods were dried, hand-shelled and seeds were separated into infected and healthy based on visual *A. flavus* infection. The healthy seeds in the respective genotypes were further plated to check the latent *A. flavus* infection. The results of the visual scoring of infection in the harvested seeds indicated no significant differences among the tested genotypes. However upon plating while JL 24 had 5% infection while no preharvest infection was recorded in the rest of genotypes. Intriguingly, based

on initial visual scoring transgenic event #5 & #6 apparently had *A. flavus* infection similar to untransformed control.

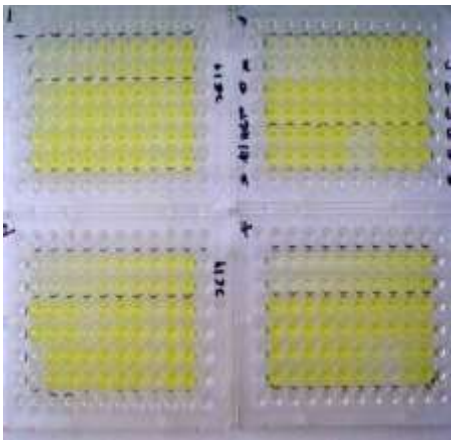
Random seed samples from individual genotypes were analyzed using ELISA for determining aflatoxin contamination. Of the two transgenic events tested, while event # 6 had *A. flavus* infection comparable to its untransformed counterpart JL 24, it accumulated significantly lower aflatoxin content (17.9 ppb) than JL 24 (78.6 ppb) and resistant check J 11 (58.7ppb), thus explaining that different mechanisms of resistances are responsible for A. flavus infection and aflatoxin contamination.

***A. flavus* population studies**

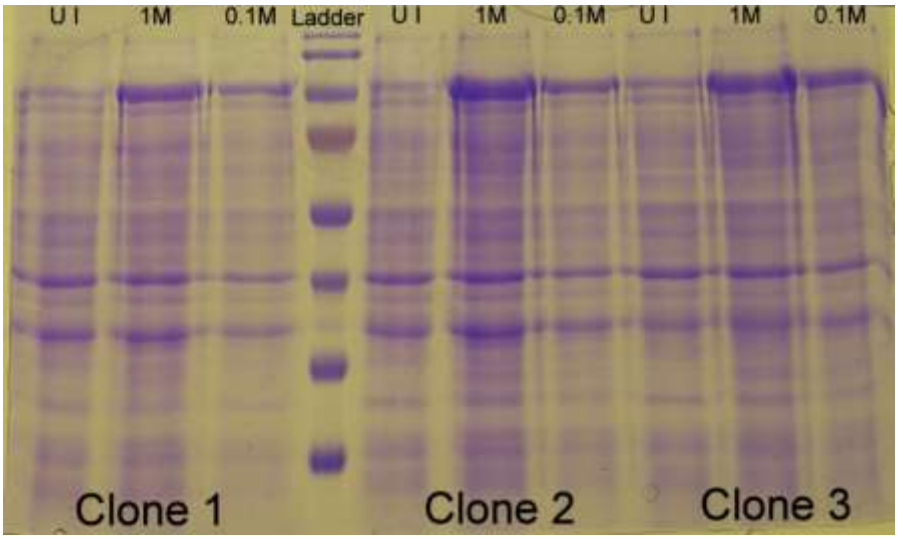
A. flavus population studies was done by plating 10^{-2} and 10^{-3} dilutions of soil samples on *Aspergillus flavus/parasiticus* agar (AFPA) medium which were collected before sowing, during flowering and after harvest which resulted sowing- 0 CFU/gm, flowering–8000 CFU/gm, harvest –20-25k CFU/gm.



ELISA Analysis

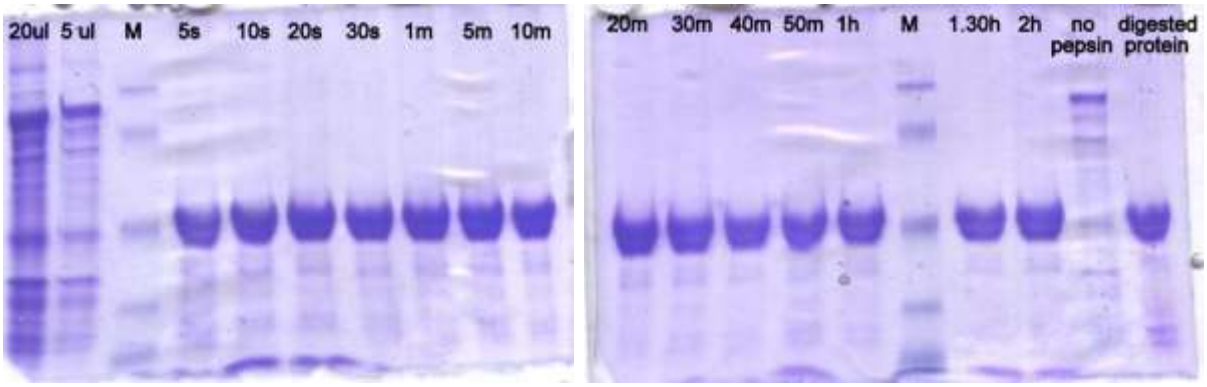


4.5 Protein expression studies



Pepsin digestibility test

Pepsin digestion test resulted in digestion of lipoxygenase protein starting at 5 sec interval only as shown in below figure.



4.6 Statistical Analysis

4.6.1 Inheritance of *PnLOX3* gene in groundnut transgenic progenies

PCR screening of *PnLOX3* gene in T_1, T_2 and T_3 transgenic plants was used to study the inheritance pattern of transgene. Gene segregation pattern was derived using a chi-square test. Transformation of groundnut plants with pPZP200>35S: *PnLOX3*: polyA construct showed Mendelian pattern of inheritance and segregation of the *PnLOX3* gene with 3:1 Mendelian ratio at p = 0.05 for almost all T_0 events and randomly selected T_1 and T_2 plants (Table below).

Event #	No of plants tested	PCR positives	Negatives	Chi square ($\chi^{2)}$	Mendalian ration
1	2	2	0	0.666667	Yes
2	4	3	1	0	Yes
3	5	2	3	3.266667	Yes
4	6	2	4	5.555556	No
5	6	5	1	0.222222	Yes
6	6	3	3	2	Yes
8	2	1	1	0.666667	Yes
9	2	1	1	0.666667	Yes

* χ^2 value at 0.05% probability at 1 df is 3.84. Calculated values above 3.84 were non-significant, and the samples did not fit for 3:1 segregation ratio.

Event #	No of plants tested	PCR positives	Negatives	Chi square (χ^2)	Mendalian ration
1-1	14	10	4	0.095238	Yes
1-2	14	8	6	2.380952	Yes
2-1	14	9	5	0.857143	Yes
2-2	14	11	3	0.095238	Yes
2-4	7	5	2	0.047619	Yes
4-1	14	8	6	2.380952	Yes
1-1-1	6	3	3	2	Yes
1-1-2	6	4	2	0.222222	Yes
1-1-3	6	2	4	5.555556	No
1-1-4	6	4	2	0.222222	Yes
1-1-5	6	5	1	0.222222	Yes

2-2-1	3	2	1	0.111111	Yes
3-1-1	6	5	1	0.222222	Yes

* χ^2 value at 0.05% probability at 1 df is 3.84. Calculated values above 3.84 were non-significant, and the samples did not fit for 3:1 segregation ratio.

4.7 Promoter characterization studies

EMSA studies

To examine binding of the nuclear proteins to the regulatory elements of the chickpea lectin (CPLP) promoters, gel mobility shift assays were carried out using nuclear extracts from different plant tissues. The biotin labeled PCR amplicon CPLP was used as DNA probes. The EMSA of CPLP fragment with the chickpea seed nuclear extracts revealed a stronger binding affinity and distinctly shifted bands (Fig.) when compared to the chickpea lectin promoter DNA probe.

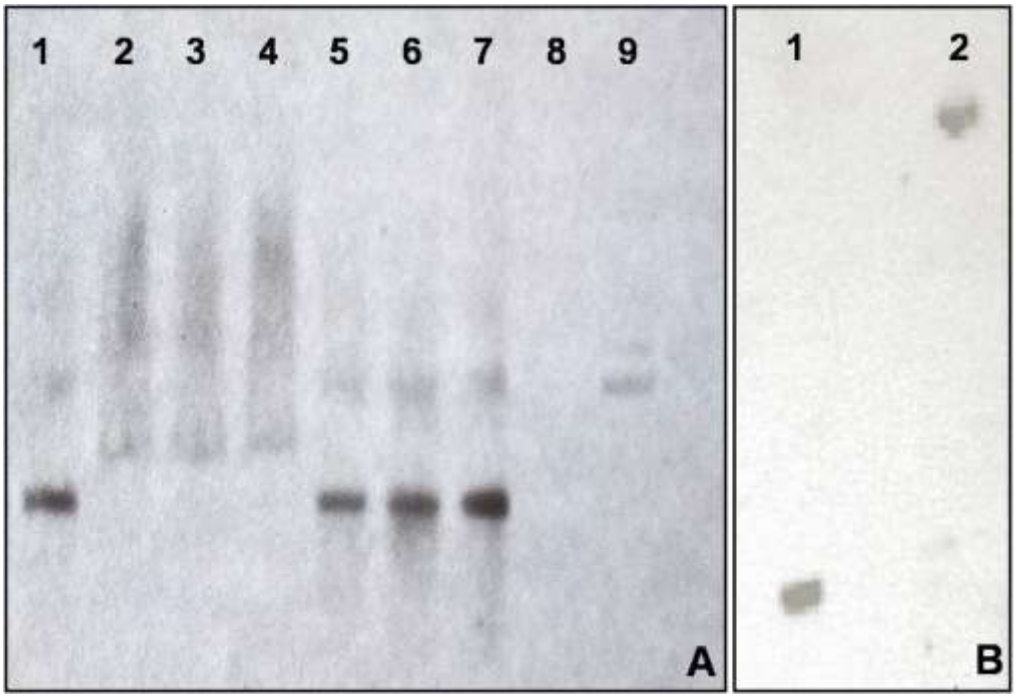
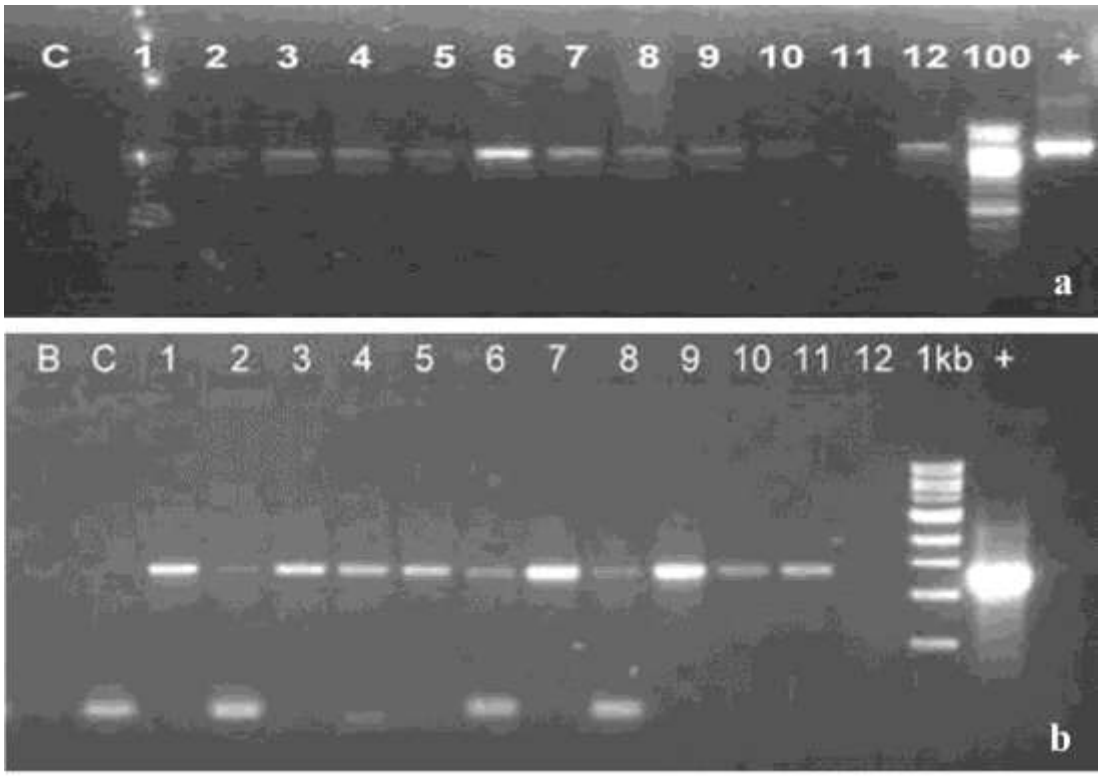


Figure 25: Electro Mobility Shift Assay (EMSA) for the confirmation of promoter regions from peanut and chickpea. (A) Peanut seed-specific promoter (GSP) binding assay on 0.8% agarose gel. Lane 1 contains unbound GSP fragment, Lanes 2-4 carry GSP fragment bound with peanut seed nuclear extracts in presence of EDTA and KCl, Lanes 5-7 carry GSP fragment bound with leaf, immature seed, and testa extracts, respectively, Lane 8 is blank and the Lane 9 carries the 100 bp ladder; **(B)** Chickpea lectin promoter (CPLP)

binding assay on 6% native PAGE. Lane 1 contains the unbound CPLP fragment, Lane 2 carries CPLP fragment bound with chickpea seed nuclear extracts.

Molecular characterization studies in tobacco

Agrobacterium tumefaciens (C58) mediated transformation was carried out using modified floral dip transformation in *Arabidopsis* and in tobacco using modified leaf disc transformation and over 30 transgenic events were produced transforming the binary vectors containing *uidA* gene driven by GSP promoter and CPLP promoters. Stable integration of *uidA* gene in tobacco putative transgenics was confirmed by PCR and RT-PCR using *uidA* gene specific oligonucleotide primers amplifying 1213 bp.



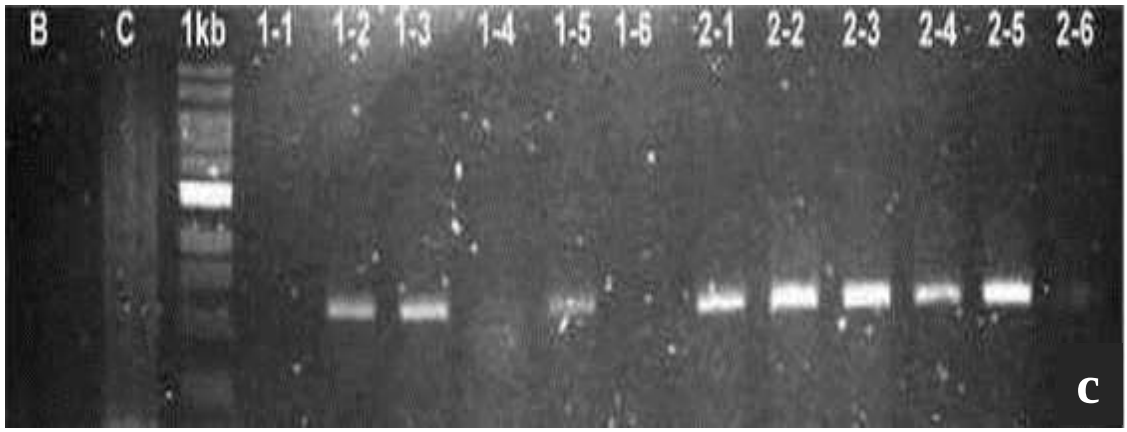
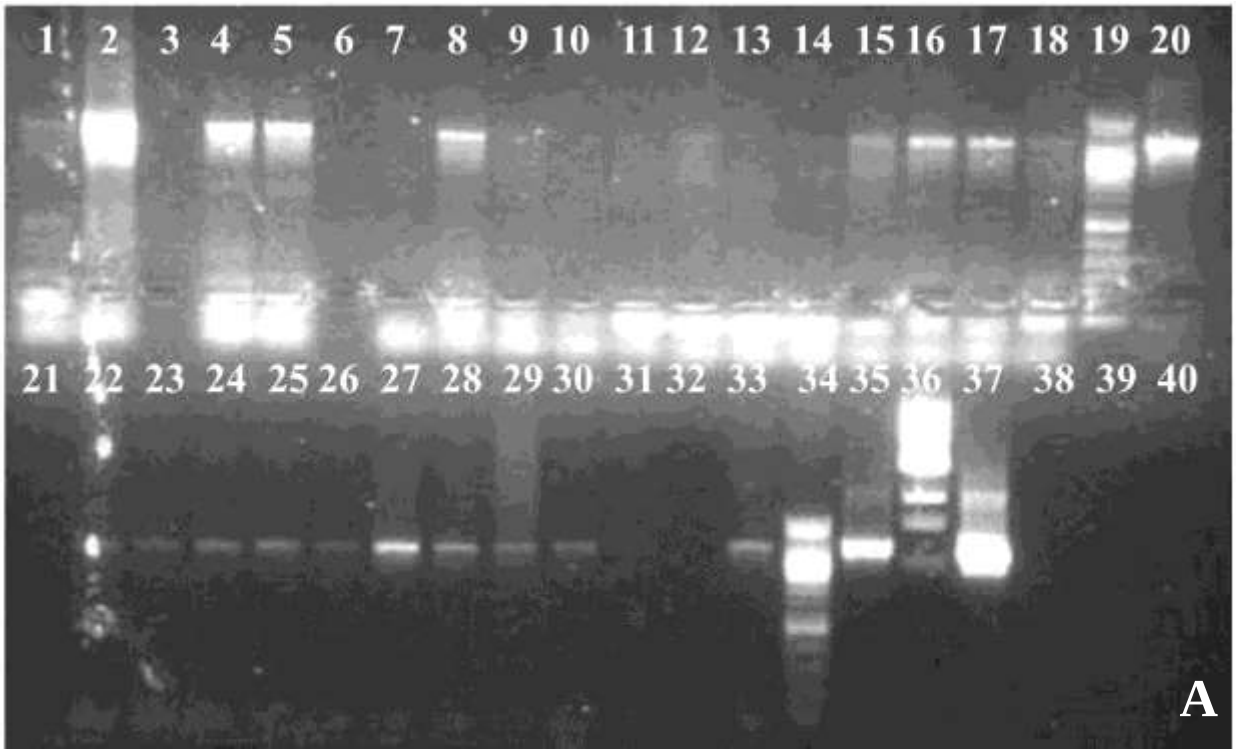


Figure 26: PCR analysis of tobacco transgenics transformed with the binary constructs containing GUS gene under control of seed specific promoters. a-b: T_0 and T_1 generation tobacco transgenics transformed with pPZP200>CPLP:*uidA*:polyA. c: T_1 tobacco transgenics transformed with pCAMBIA2300>GSP:*uidA*:polyA.



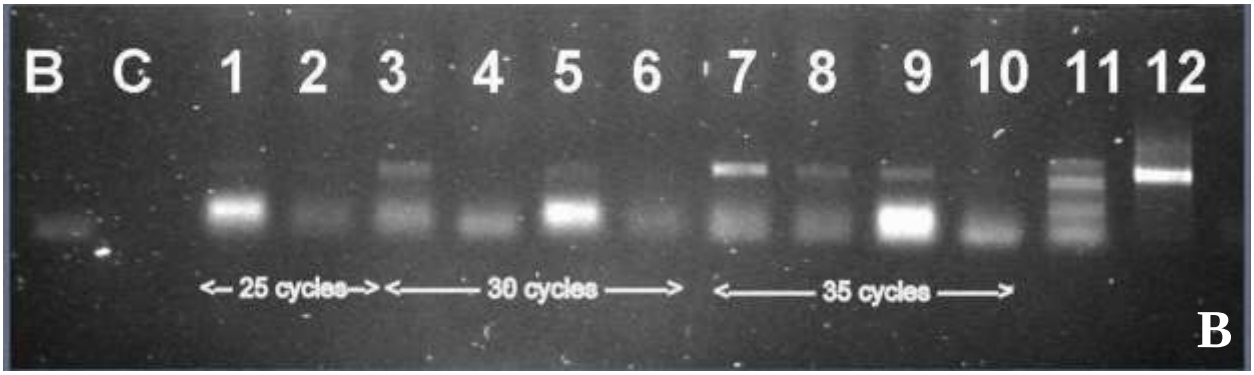


Figure 27: RT-PCR analysis of T_1 generation tobacco transgenics transformed with the binary constructs containing GUS gene under control of seed specific promoters. A: tobacco transgenics transformed with pPZP200>CPLP:*uidA*:polyA. B: T_1 tobacco transgenics transformed with pCAMBIA2300>GSP:*uidA*:polyA.

Histochemical analysis

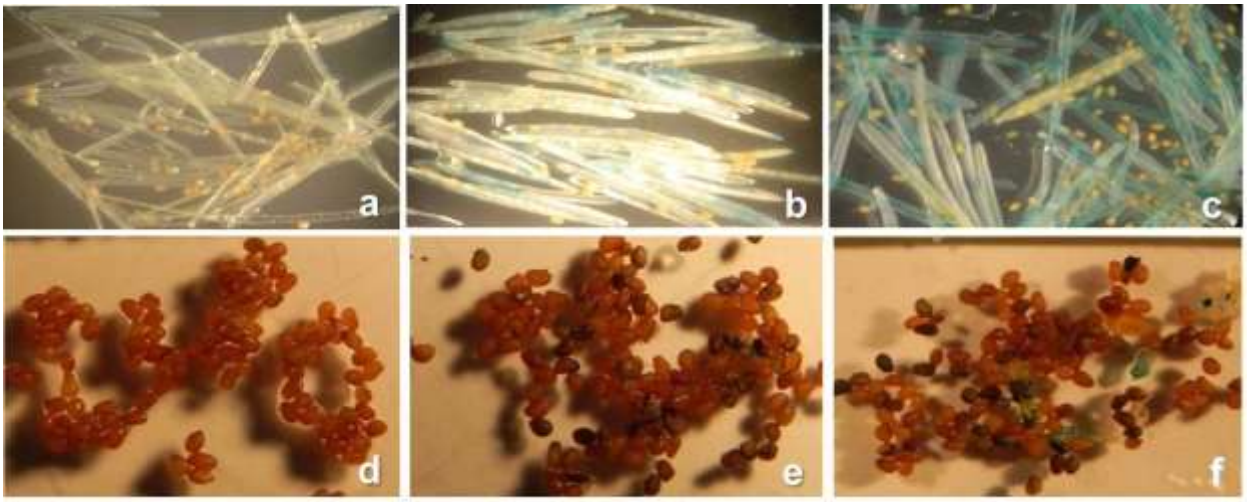


Figure 28: Validation of seed-specific promoters in transgenic *Arabidopsis* and tobacco (a,d Histochemical GUS assay in untransformed control showing negative; (**b,e)** GUS expression in transgenic seeds carrying the plasmid pCAMBIA2300>GSP:*uidA*:polyA **(c,f)** GUS expression in transgenic seeds carrying the plasmid pPZP200>CPLP:*uidA*: polyA

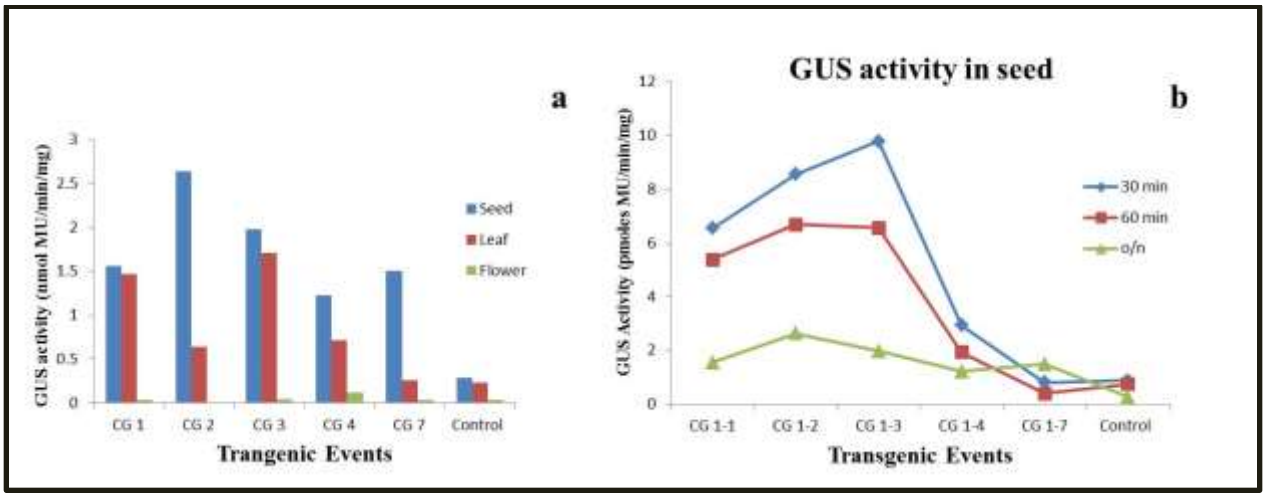


Figure 29: Expression patterns of CPLP seed-specific promoters based on GUS activity using MUG assay (**a)** Comparative expression patterns in seed, leaf and flower tissues (**b)** GUS activity measured at different time intervals (30 min, 60 min and overnight assay) with five replicates in the seeds of independent transformants with promoter construct (CG=CPLP).

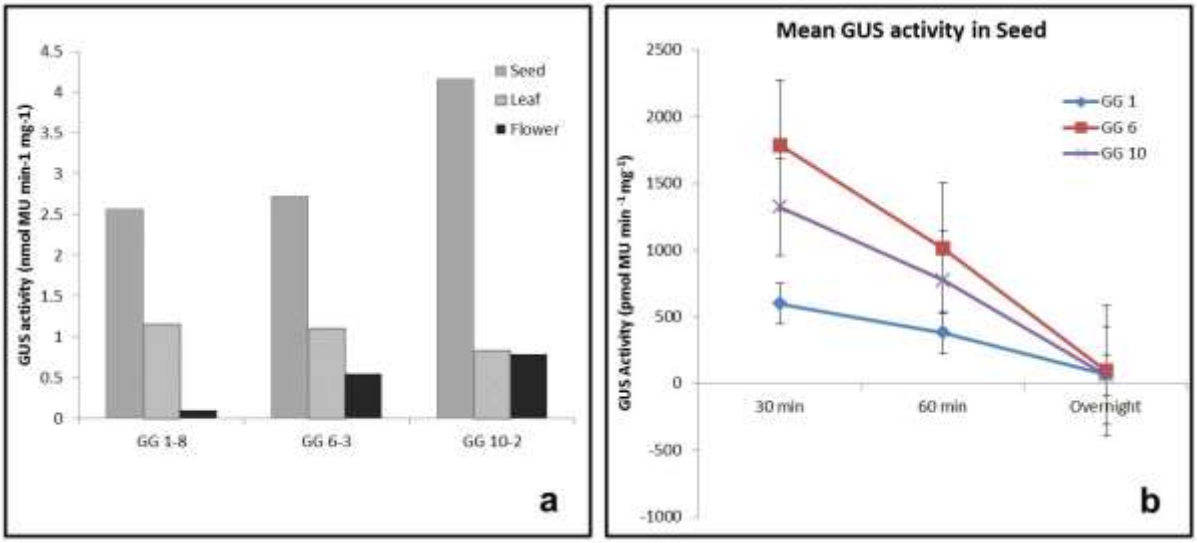


Figure 30: Expression patterns of GSP seed-specific promoter based on GUS activity using MUG assay (**a)** Comparative expression patterns in seed, leaf and flower tissues; (**b)** Mean GUS activity ± SE measured at different time intervals (30 min, 60 min and overnight assay) with five replicates in the seeds of independent transformants with promoter construct (GG=GSP).

Summary

- Binary vector construction by sub-cloning peanut lipoxygenase gene (*PnLOX3*) under constitutive and tissue (seed) specific promoters.
- Developed 25 marker free groundnut transgenics carrying peanut lipoxygenase gene.
- Molecular confirmation of presence and integration of transgene over generations (T_0-$T_{4)}$ by PCR, RT-PCR & Southern blotting.
- Novel protocol (mimicking micro-sick plots/field conditions) of fungal bioassay used for green house evaluation of transgenics to understand *A. flavus*-drought interactions.
- Isolated and validated two novel seed specific promoters from groundnut and chickpea.

Appendix

1. Media for bacterial culture maintenance

1.1 Luria Bertoni (LB) medium pH -7

Bacto –peptone	10 g
Yeast Extract	5 g
Sodium chloride	10 g

Dissolve all the components in 1000ml distilled water and autoclave before use

1.2 LB Agar medium pH -7

Bacto –peptone	10 g
Yeast Extract	5 g
Sodium chloride	10 g
Agar	15 g

Dissolve all the components in 1000ml distilled water and autoclave before use

1.3 Yeast Extract Broth Medium pH- 7

Bacto-Peptone	5 g
Yeast extract	1 g
Beef extract	5 g
Sucrose	5 g
Magnesium sulphate ($MgSO_4$ $7H_2O$)	0.5 g

Dissolve all the components in 1000ml distilled water and autoclave before use

1.4 YEB Agar Medium pH- 7

Bacto-Peptone	5 g
Yeast extract	1 g
Beef extract	5 g
Sucrose	5 g
Magnesium sulphate ($MgSO_4$ $7H_2O$)	0.5 g
Agar	15 g

Dissolve all the components in 1000ml distilled water and autoclave before use

2. Reagents for competent cell preparation

2.1 5X KCM

1M KCl	5 ml
1M $CaCl_2$	1.5 ml
1M $MgCl_2$	2.5 ml
Distilled H_2O	1 ml

Mix all the above components. Filter sterilize and store at 4^0C until use

2.2 Transformation Storage Buffer (TSB)

LB medium (pH-6.1-autoclaved)	5 ml
PEG	1 g
DMSO	500 µl
1M $MgCl_2$	100 µl
1M $MgSO_4$	100 µl
Glycerol	1 ml

Mix the components and filter sterilize and make the final volume to 10ml.

2.3 10% Glycerol

Dissolve 100 ml glycerol in 1 litre water. Autoclave before use.

3. Reagents used for plasmid isolation

3.1 GTE

1 M Glucose	5 ml
1 M Tris-Cl, pH 8.0	2.5 ml
0.5 M EDTA, pH 8.0	2 ml
Distilled water	90.5 ml

Autoclave before use

3.2 Lysis solution

10 N NaOH	200 µl
Distilled water	8.8 ml
10% SDS	1 ml

Note: Prepare fresh from the stocks

3.3. Neutralizing solution

Potassium acetate	29.2 g
Glacial acetic acid	11.2 ml
Distilled water to	100 ml

3.4 5 M Potassium acetate

Potassium acetate	29.4 g
Glacial acetic acid	11.2 ml

Dissolved potassium acetate in distilled water and adjust the pH to 5.2 with glacial acetic acid and make up the volume to 100 ml with distilled water. Autoclave before use.

3.5 3 M Sodium acetate

Dissolve in 24.61 g sodium acetate in 80 ml of distilled water. Adjust the pH to 4.8 with acetic acid and autoclave before use.

3.6 70% Ethanol

Absolute alcohol	70 ml
Distilled water	30 ml

4. Stock solutions composition for MS (Murashige and Skoog, 1962)

4.1 BAP

Dissolve the 22.53mg hormone in few ml of NaOH and make up the volume to 100ml with distilled water.

4.2 2, 4 -D

Dissolve the 22.1mg hormone in few ml of ethanol and make up the volume to 100ml with distilled water.

4.3 NAA

Dissolve the 18.66mg hormone in few ml of NaOH and make up the volume to 100ml with distilled water.

4.4 Major components

MS NH_4NO_3	66 g/400 ml	166 g/ lt
MS KNO_3	38 g/400 ml	95 g/ lt
MS $MgSO_4\ 7H_2O$	14.8 g/400 ml	37 g/lt
MS KH_2PO_4	6.8 g/400 ml	17 g/lt
MS $CaCl_2$	17.60 g/400 ml	44 g/lt

Dissolve all the above components in 1000ml distilled water.

4.5 MS Minor

Potassium iodide(KI)	83 mg
Boric acid (H_3BO_3)	620 mg
Manganous sulphate ($MnSO_4$)	2230 mg
Zinc sulphate ($ZnSO_4\ 7H_2O$)	860 mg
Sodium molybdate ($Na_2MoO_4\ 2H_2O$)	25 mg
Copper sulphate ($CuSO_4\ 5H_2O$)	2.5 mg
Cobaltous chloride ($CoCl_2\ 6H_2O$)	2.5 mg

Dissolve all the above components in 1000 ml distilled water.

4.6 MS Organics

Nicotinic acid	50 mg
Pyridoxine HCl	50 mg
Thiamine HCl	10 mg
Glycine	200 mg

Dissolve all the above components in 1000 ml distilled water.

4.7 B5 Organics

Nicotinic acid	50 mg
Pyridoine monohydrochloride	50 mg
Thiamine hydrochloride	500 mg

Dissolve all the above components in 250ml distilled water.

4.8 Fe-EDTA

EDTA $2H_2O$	3.73 g
$FeSO_4$ $7H_2O$	2.78 g

Dissolve all the above components in 1000ml distilled hot water.

4.9 Media composition used in tobacco and groundnut tissue culture

Component	½ MS	Tobacco tissue culture		Groundnut tissue culture		
		MS	MS4	SIM (36)	SEM (36-2)	RIM (NAA5)
NH_4NO_3	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
KNO_3	10 ml	20 ml	20 ml	20 ml	20 ml	20 ml
$MgSO_4.7H_20$	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
KH_2PO_4	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
$CaCl_2$	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
MS-Minor	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
MS -Organics	5 ml	10ml	10ml	__	__	__
B_5-Organics	__	__	__	10 ml	10 ml	10 ml
MS Fe-EDTA	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Myo-Inositol	5 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Sucrose	20 g	30 g	30 g	30 g	30 g	30 g

Agar	__	7 g	7 g	7 g	7 g	7 g
pH	5.8	5.8	5.8	5.8	5.8	5.8
BA	__	__	10 ml	20 ml	2 ml	__
2,4-D	__	__	__	10 ml	__	__
NAA	__	__	5 ml	__	__	5 ml

SIM-Shoot Initiation Medium; **SEM**-Shoot Elongation Medium; **RIM**-Root Initiation Medium.

5. Reagents for plant DNA Isolation

5.1 1 M Tris

Tris-HCL 12.1 g
Dissolve in 100 ml of SDW

Adjust the pH to 8 with concentrated HCl.

5.2 5M NaCl

Dissolve 29.22 g NaCl in 100 ml of distilled water and autoclave before use.

5.3 0.5 M EDTA

Dissolve 18.61 g EDTA in 100 ml of distilled water. Adjust the pH to 8 with NaOH pellets and autoclave before use.

5.4 10X TE buffer

1M Tris (pH 8.0) 1 ml
0.5 M EDTA (pH 8.0) 200 µl

Make up the final volume to 100ml with distilled water and autoclave before use.

5.5 RNAse preparation (10mg/ml)

RNAse 100 mg
1 M Tris HCl (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^{0}C.

5.6 Modified Dellaporta's DNA extraction buffer

1M Tris (pH 8.0)	10 ml
5M NaCl	10 ml
0.5 M EDTA (pH 8.0)	10 ml

Make upto final volume 100ml with distilled water and autoclave before use. Add 200 µl/100 ml β-mercaptoethanol just before use in the buffer. Add ~30µl/ml of RNAse to the extraction buffer just before use.

5.7 CTAB DNA extraction buffer (Porebski et al., 1997)

1M Tris (pH 8.0)	20 ml
0.5 M EDTA (pH 8.0)	4 ml
5M NaCl	28ml
Distilled water	48 ml

Add 2% CTAB (w/v) and autoclave before use. Add 1% PVP and 0.3% β-mercaptoethanol just before use. Make upto final volume 100ml with distilled water and autoclave before use

5.8 10% Sodium Dodecyl Sulphate (SDS)

Dissolve 10 g of SDS in 100 ml of SDW. This should be stored at room temperature.

5.9 DEPC treated water

DEPC	1 ml
Distilled water	1 L

Incubate at 37°C for 24 h and autoclave.

6. Reagents for Southern blotting

6.1 Depurination solution

37% HCl (0.2 N)	4.5 ml

Make up the volume to 250 ml with distilled water.

6.2 Denaturation solution

5M NaCl	40 ml
5 M NaOH	50 ml

Dissolve in 500 ml distilled water and autoclave.

6.3 Neutralization solution

NaCl (3 M)	87.7 g
Tris (1.5 M)	90.8 g

Dissolve in 400 ml distilled water and adjust the pH to 7.0 and make up the volume to 500 ml.

6.4 20x SSC

Sodium Citrate (0.3 M)	88.2 g
NaCl (3 M)	175.3 g

Dissolve in 800 ml of distilled water. Adjust pH to 7.0 with 1 N HCl and make up to 1 L. Sterilize by autoclaving.

6.5 0.5 M Sodium phosphate

Dissolve 6.9 g Sodium phosphate in 100 ml of SDW and adjust the pH to 7 with 5 M HCl.

6.6 Hybridisation buffer

Hybridisation Buffer	100 ml
Blocking reagent	4 g
NaCl	1.46 g

Note: Blocking reagent should be added very slowly and care should be taken to prevent formation of lumps.

6.7 Primary Wash Buffer

Urea	60 g
SDS	0.5 g
0.5 M $NaPO_4$ (pH 7)	50 ml
NaCl	4.35 g
1M $MgCl_2$	0.5 ml
Blocking reagent	1 g

Dissolve in autoclaved distilled water and make up the volume to 500 ml. This buffer can be stored for one week at 4^0C and should be avoided re-heating.

6.8 20 X secondary wash buffer

Tris base	121 g
NaCl	112 g

Adjust pH to 10. Make up the volume to 1 litre. This can be stored for 4 months at 4^0C.

6.9 1 X secondary wash buffer

Dilute 50 ml of 20 X secondary wash buffer in 1 litre autoclaved distilled water and add 2 ml of 1 M $MgCl_2$ per litre. This should be made fresh just before use.

6.10 Kodak developer

D-19 (big pack)520C	148 g
D-19 (Small pack) 10g	

Dissolve in 1 litre of sterile distilled water. Filter the solution through kim wipes

6.11 Fixer

Dissolve 264 g of powder in 1000 ml sterile distilled water.Filter the solution through kim wipes

7. Buffers and reagents used in Aflatoxin and ELISA analysis

7.1 *Aspergillus flavus/parasiticus* Agar medium (AFPA)

Bacteriological peptone	10 g
Yeast Extract	20 g
Ferric ammonium citrate	0.5 g
Agar	15 g

Dissolve the above chemicals in 1 lt distilled water and autoclave before use. Add 0.2 g chloramphenicol and 2 mg of dichloran just before use.

7.2 Aflatoxin Extraction buffer

Prepare methanol: water in 70: 30 ratio. Add KCl at 0.5% concentaration before use

7.3 Phosphate buffered saline Tween (PBS-T)

Na_2HPO_4	2.38 g
KH_2PO_4	0.4 g
KCl	0.4 g
NaCl	16 g
Tween 20	1 ml

Make up the final volume to 2 litres.

7.4 Distilled water-Tween

Dissolve 2 ml Tween 20 in 2 ml distilled water.

7.5 PBS-T BSA

Dissolve 200 mg BSA in 100 ml PBS-T.

7.6 Coating Buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Dissolve the above chemicals in 1 litre distilled water and adjust the pH to 9.6.

7.7 10% Diethanolamine

Dissolve 10 ml diethanolamine in 100 ml distilled water. Adjust the pH to 9.8. Make sure that the solution does not turn yellow.

8. Reagents used in protein/ PAGE analysis

8.1 100mM IPTG

Dissolve 0.2383 g of IPTG in 10ml of water. Filter sterilize and store at –20^{0}C.

8.2 Lysis Buffer

Tris base	1.21 g
NaH_2PO_4	13.8 g
Urea	480.5 g

Dissolve in 600 ml of distilled water. Adjust the pH to 8.0 with 1 N NaOH and make up the volume to 1 L. Just before use add 0.1% β-mercaptoethanol and lysozyme (20 μg/ml).

8.3 Simulated Gastric Fluid (SGF) reaction buffer

SGF reaction buffer preparation-Add 122.8 mg of NaCl to 59.2 ml distilled water and pH was adjusted to 1.2 with 1N HCl. Pepsin was added to final concentration of 0.3% (w/v) in 1 ml volume of SGF mixture

8.4 30% acrylamide stock solution

Acrylamide	29% (w/v)
Bis-acrylamide	1%(w/v)

Filter the prepared stock solution and Store at 4^{o}C. It is light sensitive and store it in an amber colored bottle.

8.5 10% Ammonium per sulphate (APS)

0.1 g of APS (10% (w/v)) stock solution is prepared in 10 ml deionized water and stored at 4 degrees. APS decompose slowly, and fresh solution should be prepared.

8.6 Native PAGE (6%)

30% Acrylamide	2 ml
0.5X TBE	1 ml
10% APS	100 μl
Water	6.89 ml
TEMED	10μl

8.7 SDS-PAGE Resolving gels Composition

Composition	**10%**	**12%**	**15%**
H_2O	4 ml	3.3 ml	2.3 ml
30% Acrylamide	3.3 ml	4 ml	5 ml
1.5M Tris (pH 8.8)	2.5 ml	2.5 ml	2.5 ml

10% SDS	100 µl	100 µl	100 µl
10% APS	100 µl	100 µl	100 µl
TEMED	4 µl	4 µl	4 µl

8.8 SDS-PAGE 5% Stacking Gels Composition

Solution Components	**6ml (2 gels)**
H2O	4.766
30%Acrylamide	1.0
1.0MTris(PH- 6.8)	750 µl
10%SDS	60 µl
10%APS	60 µl
TEMED	4 µl

8.9 Staining solution

Coomassie Brilliant Blue-R (R-250)	200 mg
Methanol	40 ml
Glacial Acetic acid	7 ml
MilliQ water	53 ml

8.10 Destaining solution

Methanol	20 ml
Glacial Acetic acid	7 ml
MilliQ water	73 ml

8.11 10X SDS PAGE Electrode Buffer

Tris base	30 g
Glycine	144 g
SDS	10g

Dissolve the above chemicals in distilled water and adjust the pH to 8.3 and make up the final volume to 1 litre.

8.12 1X SDS PAGE Buffer

Dilute 100 ml of 10X SDS PAGE buffer into 1 litre distilled water.

8.13 Laemmli buffer (2x SDS sample loading dye)

Stacking gel buffer	2.5 ml
Glycerol	2 ml
10% SDS	2 ml
2-mercaptoethanol	500 µl
Bromophenolblue	1 mg
Distilled water to	10 ml

9. Reagents used in GUS Analysis

9.1 X-Glucuronide (100mM)

Dissolve 26 mg of X-Gluc in 500 µl DMSO. Store in dark until use.

9.2 10% Triton X-100

Dilute 0.2 ml Triton X-100 with 1.8 ml distilled water.

9.3 GUS staining solution

0.5 M EDTA (pH 8)	200µl
0.1 M Na2PO4 buffer	5ml
10% Triton X-100	100 µl
50mM Potassium Ferrocyanide	200 µl
50mM Potassium Ferricyanide	200 µl
Methanol	2ml
100mM X-Gluc	200 µl
Sterile water	1.2 ml

9.4 4-Methylumbelliferone (MU) stock solution (1mM)

Dissolve 1.762 mg of 4-MU in 10 ml autoclaved distilled water. This solution should be stored in dark at 4^0C.

9.5 4 MU stock solution (1µM)

Dilute 10 µl of 1 mM 4-MU solution with 10 ml of autoclaved distilled water. This solution should be stored in dark at 4^0C.

9.6 Carbonate Stop buffer (0.2M)

Dissolve 21.2 g Na_2CO_3 in 1 litre water.

9.7 4- Methylumbelliferone standard (50nM 4-MU)

Dilute 100 µl of 1 µM 4-MU solution with 1.9 ml of carbonate stop buffer. This solution should be prepared fresh just before use.

9.8 30% Sarcosyl

Dissolve 0.3 g sodium lauryl sarcosine in 1 ml distilled water.

9.9 GUS Extraction Buffer

0.1 M $NaHPO_4$ (pH 7)	50 ml
β-mercaptoethanol	70 µl
0.5 M EDTA (pH 8)	2 ml
30% Sarcosyl	330 µl
10% Triton X-100	1 ml

Make up the volume to 100 ml with distilled water.

9.10 MUG Assay Buffer

Dissolve 25 mg 4- Methylumbelliferyl β-D- glucuronide in 25 ml of GUS Extraction buffer. This buffer can be stored at 4 ^{0}C for two weeks.

10. Buffers used in electrophoresis

10.1 50X TAE

Tris Base (Trizma base)	242 g
Glacial acetic acid	57.1ml
EDTA	37.2g

Dissolve EDTA in 600 ml water. Then add Tris base and glacial acetic acid and make up the final volume to 1000 ml with distilled water and adjust pH to.5.

10.2 Preparation of 1X TAE for 5 L

Dilute 100 ml of 50X TAE and make up the volume to 5 litres with distilled water.

10.3 10x TBE buffer, pH 8.3

Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 ml
Distilled water	1 L

Note: No need to adjust pH

10.4 6X Loading Dye

Bromophenol blue	30 mg
Xylene cyanol FF	30 mg
Glycerol	60 ml
0.5M EDTA (pH 7.6)	20 ml
Sterile distilled water	20 ml

Store at room temperature or at +4°C for periods up to 12 months.

10.5 5x Sample buffer (Gel loading buffer)

Bromophenol blue (0.25%)	5mg
Xylene cyanol FF (0.25%)	5mg
Glycerol (30%)	3ml
Sterile distilled water	10ml

10.6 SYBR Green dye

Dilute commercially available SYBR green dye in 5times DMSO and store at room temperature. Use 2 µl of this dilute dye for 100 ml agarose gel.