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Transgenic approaches for improving fungal disease resistance in groundnut

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Abstract

Fungal diseases in groundnut are the most significant limiting factor causing more than 50% yield losses throughout the world. Genetic enhancement in groundnut through conventional breeding and chemical control has yielded only limited success. More recently, genetic transformation has led to possibility of transforming crops for increased resistance to fungal diseases. This review summarizes the advances of genetic engineering applied for improvement of groundnut disease resistance against fungal pathogens. Fungal resistant transgene of plant, bacterial or fungal origin can be introduced into groundnut for enhanced disease resistance. Progress in engineering fungal disease resistance in transgenic ground nut has been accomplished through expression of PR proteins, antifungal proteins, antimicrobial proteins, ribosome-inactivating proteins (RIP) and phytoalexins.

Keywords: transgenic, groundnut, fungal diseases, genetic engineering, antifungal, resistance

Introduction

Groundnut *(Arachis hypogaea* L.) is the world's most important oilseed crop, and a major food legume, cultivated in over 100 tropical and subtropical countries of the world. Annual economic losses of over US$ 3.2 billion[1] are caused by biotic and abiotic stresses in groundnut crop. Among biotic stresses, fungal pathogens are the most severe constraint to groundnut production. Early leaf spot caused by *Cercospora arachidicola* S. Hori *(Mycosphaerella arachidis* Deighton), late leaf spot caused by *Phaeoisariopsis personata* Berk. & MA Curtis (M. *berkeley/),* rust *(Puccinia arachidis),* crown rot *(Aspergillus niger* Teigh.), collar rot caused by *Aspergillus spp.,* root rot caused by *Macrophomina phaseolina,* stem rot caused by *Sclerotium rolfsii* and Yellow mold *(Aspergillus flavus* and *A. parasiticus)* causing aflatoxin contamination are the major fungal diseases affecting groundnut crop. These diseases cause severe loss of yield worldwide[2]. In addition to yield loss leaf spot and rust adversely influence seed quality and *Sclerotium rolfsii* cause indirect losses such as reduction in both dry weight and oil content of groundnut kernels. Late leaf spot and rust caused annual economic losses of over US$ 599 million and US$ 467 million, respectively1. Aflatoxin contamination in top groundnut-producing states of the USA caused average annual losses of US$ 26 million to its southeastern groundnut industry[3]. Although, chemical fungicides have been widely used for fungal disease control in groundnut, but they are costly and environmentally undesirable. The use of disease resistant groundnut cultivars is the only means of controlling fungal diseases in groundnut. Wild relatives of groundnut possess resistance to foliar diseases to the level of even immunitt,5, But the interspecific hybridization has not been highly successful in introgression of the desirable traits where desired due to complexity of

inheritance and several inherent breeding barriers[6,7]. Though a moderate degree of resistance against aflatoxigenic fungi is available in the cultivated gene pool, success in breeding has been almost non-existent. Relatively little efforts have been made so far in breeding for resistance to the soil-borne pathogens. In addition, the breeders have been using a very limited stock of the primary gene pools, To add to this problem, isozyme and RFLP have shown that variability at the molecular level is low in cultivated groundnut[9]. This narrow genetic basis of the cultivated groundnut *Arachis hypogaea* L. hampers the development of improved varieties through conventional breeding. The identification, isolation and cloning of antifungal genes facilitate the genetic enhancement of groundnut by allowing insertion of exogenous antimicrobial genes from different species into groundnut.

Engineering resistance against fungal diseases in groundnut

The different approaches to engineer enhanced fungal disease resistance in transgenic plants can be classified into five groups.

1. Hydrolytic enzymes (chitinases, glucanases), antifungal proteins (osmotin, thaumatin-like), peptides (thionins, defensins, lectin), or antimicrobial compounds that are directly toxic to pathogens or that reduce their growth in situ.
2. Gene products that directly inhibit pathogen virulence products or enhance plant structural defense genes,
3. Gene products that directly or indirectly activate general plant defense responses.
4. Resistance genes involved in the hypersensitive response.
5. Resistance genes involved in the interactions with avirulence factors.

Fig. 1. Transgenic plants with enhanced disease resistance have been engineered to express gene products to counterattack fungal virulence products (from hypha on left), enhanced expression of plant-derived gene products (inside of cell) or gene products from nonplant sources (outside of cell). The results from these experiments are summarized in Table 1.

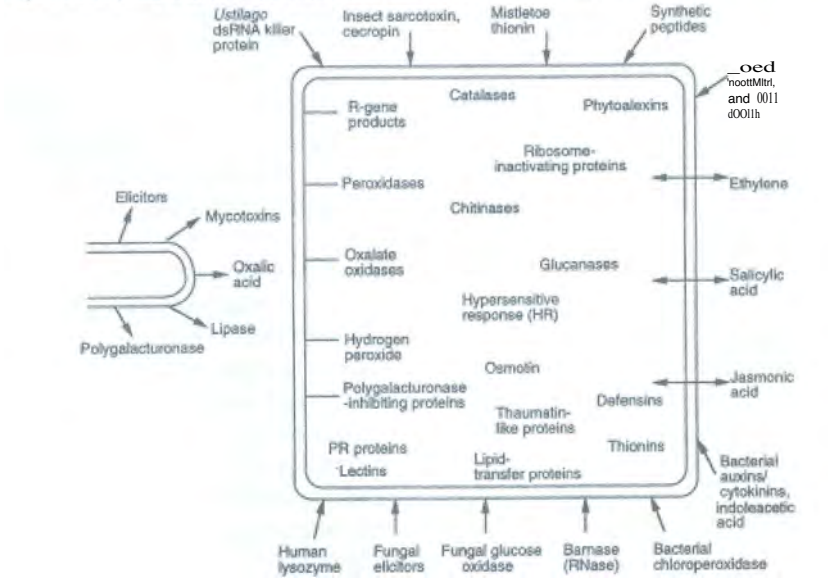

Fig. 1. Transgenic plants with enhanced disease resistance have been engineered to express gene products to counterattack fungal virulence products (from hypha on left), enhanced expression of plant-derived gene products (inside of cell) or gene products from nonplant sources (outside of cell)12.

For a non-conventional gene transfer, the basic requirement is standard and easily reproducible in vitro regeneration and recombination techniques. The transformation protocols for groundnut are now well established and development of groundnut transgenics for fungal disease resistance is in various stages of characterization under containment and/or controlled field conditions. Different tissues of groundnut including leaflet, somatic embryo, embryo axis, cotyledon, and hypocotyl have been successfully used as explants in generating transgenic lines. Most of the antifungal genes used to control early and late leaf spot *(C. arachidicola* and C. *persona tum* respectively), S. *minor,* white mold *(S. rolfsil),* and *Aspergillus f/avus/parasiticus* in groundnut act in some part of the inducible or constitutive host resistance pathway. None of these are classical genes that act in a gene-for gene resistance scenario [10].

The following tested proteins are potentially useful against fungal pathogens of groundnut.

Chitinase and glucanase

Chitinase (EC 3.2.1.14) are poly (1,4-(N-acetyl-a-D-glucosaminide) belong to the PR-3 family of pathogenesis-related proteins. It inhibits the fungal growth directly by attacking chitin, a major component of fungal cell walls and indirectly by releasing elicitors which induce various defense responses in plants[11]. Several lines of evidence indicate that overexpression of chitinase gene in transgenic plants can enhance fungal disease resistance in several crops. However, chitinase expression is effective against *Botrytis cinerea* and *Rhizoctonia solanP2* but ineffective against other pathogens, such as *Cercospora nicotianae, Colletotrichum lagenarium* and *Pythium* spp. indicating that differences exist in sensitivity of fungi to chitinase. Transgenic groundnut expressing a tobacco chitinase gene[13] and rice chitinase and an alfalfa glucanase gene [14] was shown to possess enhanced resistance to the late leaf spot and *Sclerotinia* blight respectively. Rice chitinase gene introduced into groundnut varieties through *Agrobacterium*-mediated transformation evidenced the enhanced resistance of groundnut transgenics against *A. f/avus,* late leaf spot and rust (Kalyani et al., unpublished).

a-1,3-glucanases (E.C.3.2.1.39) belong to the PR-2 family of pathogenesis-related proteins. These enzymes catalyze the cleavage of a-1,3-glucosidic bonds of a-1,3-glucan, another constituent of the fungal cell wall. Release of oligosaccharide elicitors is another mode by which plant glucanases could elicit other defense responses[15]. Synergistic effect of chitinase and glucanase has been exploited for inhibition of fungal infection in transgenic carrot, tomato and tobacc0[16,17]. Glucanase gene from tobacco introduced into groundnut (PR protein from heterologus source) showed enhanced disease resistance against *Cercospora arachidicola* and *Aspergillus f/avus*[18].

Osmotin

Osmotin is a basic 24-kDa, PR-5 protein that induce fungal cell leakiness, presumably through a specific interaction with the plasma membrane that results in the formation of transmembrane pores[19]. Osmotin exhibit antifungal activity in vitr0[20,21,22] and showed enhanced lytic activity in combination with chitinase and b-1,3-glucanase[23]. Transgenic potato overexpressing osmotin gene show delayed expression of disease symptoms caused by *Phytophthora infestans24.* In groundnut, Vasavirama and Kirti[25] overexpressed SniOLP (osmotin like protein cloned from *Solanum nigrum)* and Rs-AFP2 (defensin gene from Radish *(Raphanus sativus))* in double construct to produce groundnut transgenics for resistance against *Cercospora arachidicola* Hori. and *Phaeoisariopsis personata.*

Antimicrobial proteins or cysteine rich proteins

Plants and other organisms may contain antimicrobial proteins (low molecular mass compounds around 5 kDa) that are not necessarily associated with induced defense response, but the presence of these proteins exhibit resistance to pathogens. These are cysteine rich proteins such as defensins, thionins, lipid transfer proteins, hevin and knottin type etc.

Defensins

Defensins were classiued as PR-12 family members[26]. Plant defensins are small, cysteine-rich, defense-related antimicrobial peptides (-5 kDa in size) and are present in most

plant species studied[27],2s. Interaction of plant defensins with specific, high-affinity binding sites on fungal cells, results in membrane permeabilization and eventual cell death[29],30. Constitutive expression of radish defensin gene in transgenic tobacco plants that resulted in enhanced resistance to *Alternaria longipes* urst demonstrated the potential role of defensin in plant defense[31]. Transgenic plants of groundnut with mustard defensin gene have shown increased disease resistance to *Cercospora arachidicola* Hori. and *Phaeoisariopsis personata*[32].

Phytoalexins

Phytoalexins are antimicrobial low-molecular-weight substances synthesized by plants in response to infection[33]. Introduction of genes encoding certain phytoalexins, such as *trans-resveratrol* and medicarpin, in transgenics showed enhanced resistance or delayed disease symptom against fungal pathogens[34]. Overexpression of gene encoding phytoalexin such as resveratrol in transgenic groundnut could provide protection from microbial infections through resveratrol synthesis[34].

Ribosome-inactivating proteins (RIPs)

The plant defensive ribosome-inactivating proteins (RIPs) act at the ribosome and inhibit protein elongation by N-glycosidic cleavage which release speciuc adenine base from the sugar phosphate backbone of 28S rRNA. RIPs differ signiucantly in their substrate speciucity, but do not inactivate self-ribosomes[35]. Transgenic tobacco plants that express a barley RIP gene exhibited increased protection to *R. solani* infection[36]. In vitro studies showed synergism in antifungal activity between RIP and class I chitinase when applied to *Trichoderma reesei* and *Fusarium sporotrichioide~7.* Transgenic tobacco plants simultaneously expressing the barley RIP gene and a gene encoding a barley class II chitinase showed improved protection to *R. solani* attack as compared to growth inhibition by a single protein gene product[38]. The authors suggest that the hydrolytic activity of chitinase enables an increased uptake of RIP into the fungal cells and therefore enhances inhibition of fungal growth. Maize and groundnut transgenic expressing synthetic version of maize ribosome inhibiting protein gene, mod1, showed enhanced resistance to A. *flavu~9.*

Oxalate oxidase

Oxalic acid is required for effective pathogenesis by *Sclerotinia sclerotiorum* and many other fungal pathogens[40],41. Oxalic acid favors fungal infection, by acidification that facilitates cell wall-degrading enzyme activity, through pH mediated tissue damage, or via sequestration of Ca^{2}_{+} ions[42]. Oxalate oxidase belongs to the germin family of proteins and catalyzes the degradation of oxalic acid to produce carbon dioxide and hydrogen peroxide (HP2)43. Over-expression of barley oxalate oxidase gene in transgenic groundnut, showed enhanced resistance to oxalic acid producing fungi, *Sclerotinia minor4*[4].

Lipoxygenase

Lipoxygenases (LOXs; EC 1.13.11 .12) are nonheme iron-containing enzymes found in plants, animals and fungi. It catalyzes dioxygenation of pOlyunsaturated fatty acids containing a (Z,Z)-1,4-pentadiene system to produce an unsaturated fatty acid hydroperoxide called oxylipins. In plants, linolenic and linoleic acids are the most common substrates for LOX that leads to two possible products, the 9- and 13-hydroperoxy fatty acids[45]. This two products and lipoxygenase enzymes (LOX), could playa role in the *Aspergillus/seed* interaction, exhibiting sporogenic effects on *Aspergillus* Spp.46 and differentially modulate aflatoxin pathway gene transcription[47]. Reducing A. *flavus* infection could not completely control the aflatoxin contamination; hence possibility exists to control the induction of aflatoxin biosynthesis by manipulating host factors that signal stress. Introducing a plant gene in groundnut encoding for lipoxygenase pathway enzyme, inhibit the production of aflatoxin produced by *Aspergillus flavus* in groundnut. Further incorporation of plant antisense genes for the 9-hydroperoxide fatty acid producing lipoxygenases in groundnut transgenic also reduces mycotoxin contamination. The aflatoxin biosynthetic pathway *in vitro* has been shown to suppress by enzyme encoded by soybean *loxl* gene that catalyses the formation of a specific lipoxygenase metabolite of linoleic acid, 13(S)-hydroperoxyoctadecadienoic acid

(HpODE)4S. Ozias-Akins et al.[4s] introduced soybean *loxl* gene into groundnut under the control of carrot embryo specific promoter (DC3) for suppressing the aflatoxin biosynthetic pathway. Since DC3 promoter found to be inactive in groundnut, they are planning to carry out future work with potato ubiquitin promoter which confers high level of expression in young leaves, pod walls, seed coat, immature and mature cotyledons.

Other anti-fungal genes

Invitro bioassay using crude protein extract from transgenic groundnut expressing non-heme choloroperoxidase (CPO) gene from *Pseudomonas pyrrocinia,* showed antifungal activity against *A. f/avus4[9].* Other antifungal genes such as D5C50, tomato anionic peroxidase (tap 1), and synthetic peptide D4E1[4s] are transformed into groundnut and evaluated for antifungal activity against *A. f/avus.* However, pure D5C showed strong activity against *A. flavus in vitro,* transgenic groundnut callus showed poor recovery of plants, due to phytotoxicity of D5C.

Integration with functional genomics

Although the application of transgenic technologies has enormous potential for enhancing fungal disease resistance in groundnut, the critical component needed for groundnut cultivar development is identification of novel antifungal genes that can be used for transgenic research. Groundnut is virtually unexplored at the genomic level because of the large genome size (2,800 Mb/1 C) and the complication. Therefore, in groundnut Expressed Sequenced Tags (EST) would be quick and economical approach to identify important groundnut genes involved in defense response against fungal infections and also provide data on gene expression and regulation[51,52].

After cluster analysis of 1825 EST's, gene chip with 400 unigenes produced, and used for identifying genes for disease resistance and drought tolerance in groundnut[53,54]. The 10 specific genes identified by microarray were further validated by real time PCR analysis[54]. Similar efforts have also been made by Luo et al. 2005[55].Recently efforts have been made to identify and characterize the peanut EST regulated during interaction with the fungus *Cercosporidium personatum* (causing late leaf spot) using suppressive subtractive hybridization (SSH) to prepare the subtracted cDNA libraries[56]. Utilizing genomic and proteomic tools genes and proteins associated with *A. parasiticus* and drought stress were identified[57,5s,59]Identified genes could be used for enhanced fungal disease resistance in groundnut through marker-assisted selection in breeding or by direct up or down regulation of the target gene using genetic engineering. Identification of novel promoter and enhancer elements will also be critical to achieving efficacious expression of antifungal/anti-mycotoxin genes. In addition to nuclear transformation, development of plastid transformation protocols for groundnut will enable high-level expression of multiple resistance genes in the transgenic crop as well as reduce the chance of outcrossing of the transgene.

Control of transgene expression

Existing constitutive promoters (the CaM V 35S[60]) or potato ubiquitin 3 promoter[61] presently used in transgenics are active in whole plant and do not provide the control necessary to get rid of potentially harmful byproducts from the edible portions of plants. Developmental, tissue specific and inducible promoter could be used for regulated control of targeted gene expression. This approach should reduce the constant exposure of the pest to toxic gene products and might reduce the probability of pest to develop resistance. In addition targeted gene expression reduces the increased energy demands on the host plant imparted by constitutive promoters hence lessening the chances for reduced plant growth and yield. Seed specific promoters such as cottonseed a-globulin B gene promoter[62], the barley lemma *(lem1)* gene promoter[63] and soybean vegetative storage protein gene *(VSp)*[64] are useful for seed specific expression of antifungal gene that provide greatest level of protection against mycotoxigenic fungi that infect seeds. Pathogen/wound-inducible promoters such as the maize proteinase inhibitor *(mp/)* gene promoter[65] and the poplar *win3. 12T* gene promoter[66] respond to mechanical and insect damage to plant tissues and also to fungal infection. These types of inducible promoters

would provide activation of antifungal gene expression at a very early stage of fungal invasion and only at the site of wounding/infection thus reducing the chances of any deleterious effects on plant growth and development. In groundnut, where mechanical damage of seeds by boring insects facilitates entry of *A. flavus* and subsequent aflatoxin contamination, expression of antifungal gene under control of inducible promoter might be useful. Expression of *Bt crylA©* gene controlled by *vspB* promoter in groundnut potentially could provide high levels of the gene product in young pods, which suffers the most damage from lesser cornstalk borer (LCB) *(Elasmopalpus lignosellus)* feeding. It is a possible means of inhibiting *A. flavus* infection in peanut pods by reducing LCB damage since several reports revealed that aflatoxin contamination is positively correlated with insect damage[67].

Conclusions

Genetic engineering of groundnut with antifungal gene offers alterantive to traditional breeding and fungicide application, for managing fungal diseases in groundnut. It allows introgression of genes not available in *Arachis* genus or that have pleiotropic effects on yield potential or quality profiles. Several reports have confirmed that constitutive production of PR proteins (chitinase, oxalate oxidase, glucanase,) in groundnut transgenic plants results in increased fungal disease resistance to late leaf spot, *Sclerotinia* blight and *A. flavus*[13,14,15] but much work remains if high levels of resistance are to be achieved. The gene-pyramiding approach may prove to be an effective way of enhancing disease resistance of groundnut germplasm to various fungal diseases because different cellular components of the pathogen are the target for PR proteins. Transgenic lines of groundnut expressing two different antifungal genes rice chitinase and glucanase stably inherited and expressed the transgene over 3 generations showed enhanced resistance to *Sclerotinia* blight[15]. Antifungal proteins like trypsin inhibitor gene and lipid transfer protein (against *A. flavus)* showing strong bioactivity against the fungal pathogens and other additional useful genes are expected to be cloned in near future that could be used for genetic transformation in groundnut for enhanced fungal disease resistance. Using RNAi gene silencing Chen et al.[65] identified resistance associated proteins (RAPs) for aflatoxin resistance in Maize. Similar strategy may be useful in groundnut. Enhancing the expression of these proteins can be an effective approach to control aflatoxin contamination in groundnut[65] Transgenic groundnut carrying genes for resistan'ce to several fungal diseases are in various stages of evaluation and will be available to groundnut researchers for introgression into the target groundnut cultivars. Functional gene isolation and promoter identification and isolation are also very important. In most cases, control of temporal and spatial expression of exogenous gene is crucial. In groundnut, several promoters with the expression only in seeds have been cloned and their functional analysis is underway (Huang, pers. comm.). In conclusion, enhanced fungal disease resistance in transgenic ground nut can be achieved by increased gene expression for high levels of resistance in transgenic plants, testing new gene combinations, isolating new genes and better targeting of product from single genes.

As genetic engineering approaches typically target single gene to develop groundnut genotypes with fungal disease resistance. This approach could not provide high level of resistance against pathogens. Recently using microarray it is possible to study changes in expression pattern of thousand genes simultaneously that might induce series of defense reaction in groundnut plants. Thus in groundnut ESTs (expressed sequence tags) from disease resistant genotypes may be a boon to discover native defense/resistance genes. In addition, the accessibility of cDNA sequences has further intensified the molecular characterization of genes of interest and provided sequence information for marker development, microarray construction, and genome annotation. The availability of this resource may enable the identification and analysis of complex biological interactions between plant and pathogens[69]. Thus application of genomic tools and transgenic technology together greatly facilitate the genetic enhancement of cultivated groundnut.

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