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REVIEW ARTICLE

Aflatoxin control through transgenic approaches

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Abstract
Control of preharvest aflatoxin contamination of susceptible crops such as corn, cotton, peanut, and tree nuts is possible through time-consuming and expensive agronomic practices. Breeding for disease-resistant crops is also very time consuming and does not lend itself readily to combat the evolution of new virulent fungal races. Moreover, availability of known genotypes with natural resistance to mycotoxin-producing fungi is a prerequisite for the successful breeding program. While it is possible to identify a few genotypes of corn or peanuts that are naturally resistant to *Aspergillus*, we do not know whether these antifungal factors are specific to *Aspergillus flavus*. In crops like cotton, there are no known varieties naturally resistant to *Aspergillus* spp. So far, the best options available to us are through biocontrol and/or genetic engineering. Availability of transgenic varieties with antifungal traits is extremely valuable as a breeding tool. Use of fungicides or chemicals can add to the cost of production. Moreover, the growing concerns regarding environmental safety and groundwater quality demand less dependence on agrochemicals. Disease-resistant transgenic crops would not only control mycotoxin-producing organisms such as *A. flavus, A. parasiticus* and *Fusarium* spp. but also other microbial (fungal, bacterial, and viral) diseases that cause significant economic losses in crop production. Above all, transgenic crops resistant to aflatoxin-producing fungi offer the promise of negating the adverse effects caused by the toxin on immunocompromised humans and animals. This review explores recent advances regarding genetic engineering approaches towards the control of aflatoxin contamination using native and heterologous genes.

Keywords: *Aflatoxin;* Aspergillus*; biotechnology; genetic engineering; mycotoxin; transgenic crops*

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Introduction

The saprophytic soil-borne fungus *Aspergillus* spp. produces the carcinogenic aflatoxins in lipid-rich seeds of corn, peanuts, and tree nuts. Conventional breeding has delivered much-improved cultivated crops by enhancing their agronomic traits such as disease resistance caused by other microbial pathogens. These successes have been possible because of the availability of desired resistance genes in the germplasm (Council for Agricultural and Science Technology, 2003; Bhatnagar et al., 2007; Bhatnagar et al., 2008). Resistance to mycotoxin-producing, saprophytic fungal pathogens such as *Aspergilli* is either not available in crops like cotton or very difficult to incorporate into breeding programs because it is a polygenic trait, as in the case of corn (White et al., 1999; Paul et al., 2003). In addition, breeding for disease-resistant crops is very time consuming, especially in perennial crops such as tree nut crops, and does not readily lend itself to combat the evolution of new virulent fungal races. For these reasons, transgenic varieties with antifungal traits that confer resistance to mycotoxin-producing fungi will be extremely valuable. Disease-resistant transgenic crops would not only control mycotoxin-producing organisms such as *A. flavus*, *A. parasiticus*, and *Fusarium* spp., but also several other microbial (fungal, bacterial, and viral) diseases that cause significant economic losses in crop production. Above all, transgenic crops resistant to aflatoxin-producing fungi offer the promise of negating the adverse effects caused by the toxin on immunocompromised humans and animals, and also provide

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environmentally safe alternatives to the current practices of using chemicals and pesticides. Success in this area will depend in large part on: (1) identification of resistance genes, native or foreign, that express inhibitory activity against the mycotoxigenic fungi; (2) development of efficient transformation methods that can be readily adapted to agronomically important crop varieties; (3) selection of gene promoters that will provide the desired spatial and temporal regulation of antifungal transgenes and result in efficacious levels of production of the antifungal protein/peptide; and (4) consumer acceptance of transgenic crops. This review focuses on recent advances in the first three issues as applied to crops susceptible to aflatoxin contamination. The last issue regarding consumer acceptance is beyond the scope of this review and has been addressed elsewhere (McHughen, 2000; Nelson, 2001; Letourneau and Burrows, 2002).

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Antifungal peptides and proteins of plant origin

To date, a large number of antifungal proteins and peptides have been isolated and studied from a wide range of plants. Some of these proteins have been assayed for their antifungal activity against *Aspergillus* spp. (Table 1). To differentiate between the two protein types, peptides are considered as proteins having a molecular mass below 10 kDa and less than 100 amino acid residues, while the term "protein" refers to any such molecule having a greater than 10 kDa molecular mass with more than 100 amino acid residues. Some of the groups have many members. Below are brief discussions of each group.

α-defensins

Defensins are produced by mammals, insects, and plants as part of their innate immune response to infection. The α-defensins are found in plants and seeds. They are small (approximately 5 kDa), cysteine-rich molecules with complex structures and are divided into two major classes based on the structure of their precursor proteins (Lay et al., 2003). Their structures are stabilized by four disulfide-linked cysteines present in a triple-stranded antiparallel β-sheet with only one α-helix (Terras et al., 1992; Terras et al., 1993; Osborn et al., 1995; Terras et al., 1995) and are homologous to those produced by human and rabbit neutrophils (Fant et al., 1997; Fant et al., 1998). Defensins from mammals and insects electrostatically bind to membranes, causing the development of pores through which essential minerals and metabolites leak from the cell (Patterson-Delafield et al., 1981; Lehrer et al., 1985; Lehrer et al., 1989). In contrast, plant α-defensins interact with specific, high-affinity binding sites on fungal cells, resulting in membrane permeabilization and eventual cell death (Thevissen et al., 1997; Thevissen et al., 2000). The particular mechanism that produces plasma membrane ion fluxes is not known at this time (Spelbrink et al., 2004).

Thionins

Thionins are similar in size to the α-defensins, with 45 to 47 amino acid residues per molecule. They are usually basic molecules, all having complex structures with two antiparallel α-helices and an antiparallel double-stranded β-sheet with six or eight cysteines forming disulfide bridges (Bruix, Gonzales, et al., 1993; Bruix, Jimenez, et al., 1993; Bruix et al.,1995; Fant et al., 1997). Thionins are present in seeds, stems, roots, and leaves of various plant species and are separated into five groups according to the number of cysteines present. Thionins bind via electrostatic forces to the negatively charged phospholipids of fungal membranes. This interaction results in pore formation and cell death (Florack and Stiekma, 1994; Thevissen et al., 1996).

Table 1. Examples of Plant Proteins Having Antifungal Activity against *Aspergillus flavus*.

Protein family	Protein	Mode of action	Reference
defensin	Ib-AMP$_3$	lethal	De Lucca et al., 1999
unknown	snakin-1	inhibits growth	Segura et al., 1999
ribosome-inhibiting protein	RIP-1	inhibits hyphal tip growth	Nielsen et al., 2001
PR-5	zeamatin	inhibits hyphal tip growth	Guo et al., 1997
lectin	AILP	inhibits germination and hyphal growth	Fakhoury and Woloshuk, 2001
PR-10	ZmPR-10	RNAse activity	Chen et al., 2006
Thionin	Purothionin hordothionin	lytic	Rajasekaran (unpublished)
	Trypsin inhibitor	amylase/trypsin inhibition	Chen et al., 1998; Rajasekaran, Cary, Chen, et al., 2008

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Plant nonspecific lipid transfer proteins (ns-LTPs)

These basic peptides, 9-10 kDa in size, are larger than the thionins and defensins (about twice the size). These peptides also contain eight conserved cysteines that form four disulfide bridges and a hydrophobic "tunnel" (Gomar et al., 1996; Da Silva et al., 2005; Lin et al., 2005). These tunnels allow the transfer of lipids across plant membranes (Kader, 1996). Though the mechanism of action is unknown, it is believed that ns-LTPs insert into fungal membranes and form pores that allow the cytoplasm and its constituents to exit the cell (Selitrennikoff 2001; Van Loon and van Strein, 1999). These peptides have been shown effective *in vitro* against a number of fungal pathogens (Ge et al., 2002, 2003).

Knottins

The knottins are small (36–37 amino acids in size) peptides having three disulfide bridges. They are present in *Mirabilis jalapa* seeds (Cammue et al., 1992). They have toxic, inhibitory, antimicrobial, cytotoxic, and insecticidal properties (Chiche et al., 2004). They have a knotted topography (hence the name) with one of the three disulfide bridges penetrating through a macrocycle formed by two other disulfides and interconnecting peptide backbones (Gracy et al., 2008).

The knottin PAFPs, produced by *Phytolacca americana*, has significant sequence similarities to the *M. jalapa* knottins, Mj-AMP$_1$ and Mj-AMP$_2$ (Shao et al., 1999). The latter peptides are known to inhibit several phytopathogenic fungi (Cammue et al., 1992). Several hydrophilic and positively charged residues surround the hydrophobic surface of PAFP-S, giving the molecule an amphiphilic character that is believed to be the main structural basis of its specificity to the fungal cell (Gao et al., 2001).

Impatiens antimicrobial peptides

Impatiens balsamina plant seeds contain a family of antifungal peptides (Ib-AMP$_1$ through Ib-AMP$_4$) that comprise about 0.5% of the total seed protein (Tailor et al., 1997). They are small, basic peptides, 20 amino acids long, that contain four cysteines residues forming two intramolecular disulfide bonds. These peptides have no significant homology with any other peptide or protein and are nontoxic to human, plant, and insect cell lines (Broekaert et al., 1997; Tailor et al., 1997). They inhibit the growth of several phytopathogens and are lethal to the germinating conidia of *Aspergillus flavus* and *F. moniliforme* (Tailor et al., 1997; De Lucca et al., 1999). A synthetic variant of Ib-AMP$_1$ was oxidized to the bicyclic native conformation and found to retain the antifungal properties of the native form (Thevissen et al., 2005).

Pathogen-related proteins

Pathogen-related (PR) proteins are genetically coded for by the host plant, but they are produced only when induced by infection by a specific pathogen (Van Loon et al., 1994; Walton, 1997; Van Loon and van Strien, 1999). They comprise a large family (PR1-PR17) of peptides based on their primary structure, serological relatedness, and enzymatic and biological activities (Linthorst, 1991; Stintzi et al., 1993; Van Loon et al., 1994, Van Loon and van Strien, 1999; Christensen et al., 2002). PR proteins are widely distributed in healthy plants in trace amounts but are produced in much higher concentrations following elicitation caused by infection. They have typical physicochemical properties that enable them to resist acidic pH and proteolytic cleavage, allowing them to survive the harsh environments of vascular compartment or cell wall or intracellular spaces (Stintzi et al., 1993). PR proteins have strong antifungal and other antimicrobial activity. Some inhibit conidial release and germination, while others are associated with strengthening the host cell wall and papillae. Certain PR proteins, such as β-1,3-glucanase (PR-2) and chitinases (PR-3,-4,-8, and -11), diffuse toward the invading pathogen and break down chitin-supported cell walls in certain fungi. Most PR groups can be subdivided into two classes. Generally, class I proteins are localized in the vacuole of the plant cell, whereas class II proteins are extracellular. The class I and II proteins are related both structurally and immunologically but differ in their induction patterns (Brederode et al., 1991; Ward et al., 1991).

Ribosome inactivating proteins

Ribosome inactivating proteins (RIPs) are widely distributed among higher plants (Stirpe and Barbieri, 1986; Mehta and Boston, 1998) and inhibit protein synthesis as a result of their *N*-glycosidase activity (Endo and Tsurugi, 1988). *In planta*, RIPs may act synergistically with chitinases and β-1,3-glucanases against invasive fungi (Kombrick et al., 1988; Mauch et al., 1988; Broekaert et al., 1989). There are three types of RIPs. Types 1 (single chain) and 2 (double chain) are 30 and 60 kDa, respectively, while the small RIPs are approximately 10 kDa in weight. Type 2 RIPs have two subunits, an "A" chain with enzymatic

properties and a "B" chain having lectin properties. Ricin, an extremely toxic molecule due to its lectin subunit, is a type 2 RIP. In contrast, type 1 RIPs have only an "A" chain present and, as a result, are much less cytotoxic due to the lack of the lectin chain. A few antifungal RIPs have been reported in plants. Tobacco leaves produce a type 1 RIP, TRIP, which is active against *Trichoderma reesei* (Sharma et al., 2004), while an antifungal RIP is present in asparagus (*Asparagus officinalis*) with activity against *B. cinerea* (Wang and Ng, 2001). Luffacylin is a small RIP from sponge gourd seeds (*Luffa cylindrica*), 7.8 kDa in weight with activity against *F. oxysporum* and *Mycospaerella arachidicola* (Parkash et al., 2002). Luffacylin inhibits translation in a rabbit reticulocyte lysate system with an IC_{50} of 140 pM and reacted positively in the N-glycosidase assay for RIPs.

Lectins

A number of lectins have antifungal properties. The stinging nettle (*Urticola dioica*) produces an agglutinin - UDA, a small, single-chain fungistatic, a small, single-chain fungistatic lectin that is resistant to heat and acid (Broekaert et al., 1989). UGA consists of two cysteine-rich chitin-binding domains that inhibit hyphal growth of several phytopathogenic fungi and is several times more active than hevein (Broekaert et al., 1989; Van Parijs et al., 1991). These peptides also differ in that the C terminal of hevein shows similarity to chitinases whereas UGA shows homology to tobacco PR-4 chitinases (Broekaert et al., 1990, Linthorst, 1991).

The orchid, *Gastrdia elata*, and tobacco, *Nicotiana tabacum*, produce the gastrodianins, a family of mannose-binding lectins (Wang et al., 2001). Gastrodianins are much more abundantly expressed in the fully opened flowers than the underground corms and may play a role in defense against insects and phytopathogens (Wang et al., 2007). In the orchid, the gastrodianins exist in two isoforms, both having a monomeric structure (Liu et al., 2005). The gastrodianins inhibit conidial germination and hyphal elongation and are induced upon fungal infection of the orchid and are highly active against several phytopathogenic fungi (Xu et al., 1998).

Lectin-like peptides

The hevein family of peptides are small, cysteine-rich peptides that bind to chitin, the same mode of action as lectins such as wheat germ agglutinin and stinging nettle lectin (Van Parijs et al., 1991). Hevein, a major allergen, is present in rubber tree latex (Archer, 1960; Walujono et al., 1975; Reyes-López et al., 2004). The hevein family, due to their small size and compact dimensions, may be able to enter the fungal cell wall and interact with the plasma membrane (Van den Bergh et al., 2004). Data suggests that the heveins are similar to the thionins and plant defensins in that all three groups cause polarization and concomitant permeabilization and leakage (Van den Bergh et al., 2004).

Genetic engineering of food crops

Gene constructs for plant transformation

Well-characterized antifungal/antimicrobial proteins from different sources, as indicated above, or synthetic proteins can be transferred to other food and feed crops lacking in those proteins to enhance host plant resistance to phytopathogens. Antifungal genes coding for such proteins can be transferred to other crops by any one of the available transformation methods (Potrykus and Spangenberg, 1995; Curtis, 2004). The most widely used nuclear transformation method employs a soil-borne *Agrobacterium* (Tzfira and Citovsky, 2008), which itself has been transformed to include a binary vector containing the gene of interest (antifungal genes) and selectable markers (Figure 1A). Alternatively, plasmids containing the gene of interest can be delivered into the plant cell nucleus or organelles (e.g., chloroplasts) by a biolistic device or "gene gun" (Christou, 1996). The later method has become the method of choice in transforming chloroplasts (Figure 1B) and other organelles. Other methods of transformation (e.g., electroporation, microinjection, somatic hybridization, and others) are rarely employed outside laboratory conditions and, as such, are not discussed in detail here.

When engineering a plant transformation vector the investigator has a number of different classes of promoters to choose from depending on which tissues and at what developmental stage expression of the transgene is desired (reviewed in Gurr and Rushton, 2005). Often the gene is placed under the control of a constitutive promoter such as the CaMV 35S (Schardl et al., 1987) or potato ubiquitin 3 promoter (Garbarino and Belknap, 1994). Placing the gene under control of a constitutive promoter usually ensures that all tissues will harbor the antifungal protein/peptide, though there is some difference in levels of transgene expression in different tissues and at different developmental stages. In many cases it is desirable to place expression of the antifungal

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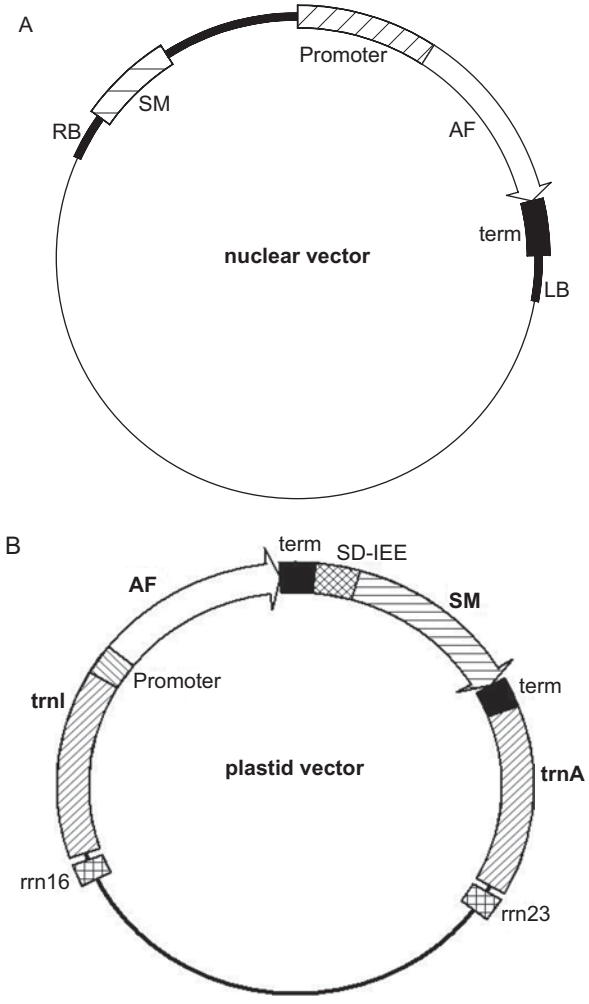

Figure 1. Schematic diagram of generic plasmid vectors used for nuclear and plastid transformation of plant tissues. [A] Binary vector for nuclear transformation—the main elements for these vectors: (1) Left and right T-DNA border sequences (LB and RB) required for integration of the transgenes into the plant genome; (2) SM, selectable marker gene; examples of SMs include antibiotic resistance genes such as *npt*II (kanamycin resistance), *hpt*II (hygromycin resistance), or resistance genes to various herbicides such as glyphosate, sulfonylurea, imidazolinones, and phosphonothricin; (3) promoter: these may include constitutive promoters such as enhanced CaMV 35S or ubiquitin control regions and wound or pathogen inducible promoters such as PINII or *mpi*; (4) transcriptional terminator (term) such as nopaline synthase (*nos*) terminator sequence; AF, antifungal gene of interest as listed in the text. [B] Plastid vector: The main elements in a plastid transformation vector usually include (1) Regions of homologous plastid DNA flanking the transgenes; the plastid rrn16-trnI and rrn23-trn23 gene regions are often used as flanking DNA for integration of the transgenes into the *trn*I-*trn*A intergenic region of the plastid genome via homologous recombination; (2) plastid promoter: the choice of plastid promoter can vary, but two commonly used sequences are the *Prrn* ribosomal RNA operon promoter, which drives gene expression in both green and non-green tissues, or the *psb*A photosystem II D1 gene promoter, which drives high-level gene expression under light conditions; (3) 3' UTR and transcriptional terminators (term); (4) SD-IEE region: sequence that provides a Shine-Dalgarno region and intercistronic expression element that allow for the generation of stable, translatable monocistronic mRNAs, thus facilitating transgene stacking in operons (for a description of vectors for plastid transformation, see Lutz et al., 2007); (5) SM, selectable marker gene: this is usually the *aad*A encoding spectinomycin resistance or the *npt*II gene encoding kanamycin resistance; AF, antifungal gene of interest as listed in the text.

gene under a tightly controlled promoter that will allow inducible or tissue-specific expression of the transgene, thus reducing the metabolic load on the host plant imparted by constitutive promoters, hence decreasing the chances for reduced plant growth and yield. Examples of tissue-specific promoters include the cottonseed α-globulin B gene promoter (Sunilkumar et al., 2002) and the barley lemma (*lem1*) gene promoter (Skadsen et al., 2002) that demonstrate seed-specific expression. Seed is often the target of infection of mycotoxigenic fungi and therefore seed-specific expression of the antifungal gene should provide the greatest levels of protection. There are a number of classes of inducible promoters, including the pathogen/wound-inducible promoters, such as the maize proteinase inhibitor (*mpi*) gene promoter (Tamayo et al., 2000) and the poplar *win3.12T* gene promoter (Yevtushenko and Misra, 2007). These

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promoters respond to mechanical and insect damage to plant tissues and also to fungal infection. In the case of aflatoxin contamination in crops such as peanut, maize, tree nut, and cottonseed, entry of the aflatoxigenic fungus *Aspergillus flavus* to the seed is usually facilitated by boring insects, so 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.

Often, despite the plethora of available promoters and transformation vectors, there is still the problem of lack of significant levels of expression of the transgene and hence lack of enough production of the antifungal protein/peptide to be efficacious in inhibiting the growth of the mycotoxigenic fungus. This can often be traced to events such as gene silencing due to multiple integrations of the transgene or position effects due to integration of the transgene within regions of the genome that are transcriptionally inactive. These events are common to nuclear transformation. An alternative to nuclear transformation is the transformation of the plastid genome in plants (Maliga, 2004; Verma and Daniell, 2007). Plastids are present in both green and non-green plant tissues. Plastid transformation eliminates many of the drawbacks associated with nuclear transformation (Table 2). Gene silencing does not occur in plastids, nor is there any position effect as integration is site-specific due to homologous recombination of flanking plastid DNA sequences that are present in the vector (Verma and Daniell, 2007). Another advantage of plastid transformation is the ability to generate significant levels of the transgene product due to high numbers of plastids and hence plastid genomes present in each cell, resulting in a very high number of functional transgene copies. Additionally, multiple genes can be expressed in an operon-like fashion from one promoter, thus allowing for "stacking" of genes in a single transformant. From an environmental standpoint, plastid transformation is preferable to nuclear transformation, as in most angiosperm plant species plastid genes are maternally inherited and therefore transgenes present in these plastid genomes are not susceptible to dispersal to other plant species via pollen dissemination (Maliga, 2004; Daniell, 2007). One of the major hurdles left with respect to plastid transformation technology is the development of efficient transformation systems for specific plant species. Though plastid transformation has been achieved in many plant species, there are still a great number that have not been transformed up to this time (Verma and Daniell, 2007). This is especially true for many crop plants that are susceptible to mycotoxin contamination such as maize, peanut, wheat, barley, grape, and tree nuts. In many cases difficulty in transforming these species arises because non-green tissues must be used as targets for biolistic transformation. Little is know about the regulation of the transcriptional and translational machinery in pro-plastids present in these tissues and pro-plastids are much smaller than that of chloroplasts found in green tissues, thus making successful integration of the transgene more difficult (Verma and Daniell, 2007). Given time, plastid transformation protocols will most probably be optimized for these plant species.

Genetic engineering of crop species for resistance to **Aspergillus flavus**

In most instances where a novel antifungal gene is being initially studied for its ability to control fungal growth and mycotoxin production, the investigator may utilize a readily transformable model plant such as *Arabidopsis* or tobacco to test transgene expression and perform in vitro bioassays using extracts from transgenic tissues. Genetic engineering of host plants for resistance to fungal diseases has been an ongoing area of investigation that has seen many small-scale laboratory/greenhouse successes, but success with

Table 2. Comparison of Chloroplast and Nuclear Genetic Engineering.

Transgenic Traits	Chloroplast Genome	Nuclear Genome
Transgene copy number	up to 10,000 copies/cell	usually less than 10 copies/cell
Level of gene expression	high abundance with high accumulation of foreign protein	gene expression often too low to be efficacious
Gene transcription	genes can be arranged in operon-form allowing multiple genes to be expressed from one promoter	difficult to stack multiple genes into one construct. Requires multiple promoters
Position effect	site-specific recombination eliminates positional effects on transgene expression	random insertion into genome results in variable transgene expression levels
Gene containment	maternal inheritance results in high level of containment-transgene not carried by pollen	possible outcrossing via pollen drift
Toxicity of foreign proteins	potential for minimization of adverse effects of transgenic proteins due to compartmentation	accumulation of toxic proteins in the cytosol may be deleterious to host

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respect to wide-scale commercialization is yet to be seen (Rajasekaran et al., 2002; Michelmore, 2003; Punja, 2004; Rajasekaran, Bhatnagar, et al., 2005; Punja, 2006). We have provided below some selected examples of transgenic expression of antifungal genes with promising effects on mycotoxigenic fungi.

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. The following section provides a brief account of different antifungal gene constructs that have been tested using crop or model test plants for resistance to the mycotoxigenic fungi.

One of the obvious transgenic approaches has been to overexpress potentially useful native antifungal peptides/proteins that are produced either constitutively or in response to fungal attack in plants as discussed in the previous section. Prime examples include chitinases and β1,3glucanases, protease inhibitors, thionins, RIPs, lectins, and polygalacturonase inhibitor proteins (PGIPs). For example, several laboratories have identified and characterized native antifungal genes from corn. Identifying resistance in corn makes it possible to correlate resistance with many endogenous small molecular weight compounds and biomacromolecules in kernel tissues already implicated as antifungal at various stages of kernel development in grain crops. However, compounds with activity against other fungal species are often ineffective against the saprophytic *A. flavus*, and thus it is important to select the best candidate inhibitory compounds and identify and characterize their respective genes before plant genetic engineering procedures are initiated. One such antifungal protein, corn trypsin inhibitor (TI) (Chen et al., 1998) has been shown to be correlated with kernel resistance to *A. flavus* infection of corn and when expressed in transgenic tobacco demonstrated enhanced resistance to the tobacco pathogen *Colletotrichum destructivum* (Rajasekaran et al., 2002). Evidence from several International Institute of Tropical Agriculture (IITA) corn genotypes (progeny of U.S. and African parental lines) resistant to preharvest aflatoxin contamination indicates that TI plays a key role in imparting this resistance. In addition, leaf extracts from transgenic tobacco expressing TI inhibited the growth of other phytopathogens, such as *Verticillium dahliae*. When cotton was transformed with the TI gene under the control of CaMV 35S promoter, the expression level was sufficient to control *Verticillium,* but not *A. flavus* (Rajasekaran et al., 2002; Rajasekaran, Cary, Chen, et al., 2008a). Perhaps higher seed-specific expression of this gene would be helpful. Recently, we identified the usefulness of corn PR10, a pathogenesis-related protein with antifungal and RNase activity (Chen et al., 2006) and glyoxalase I, a stress-related protein with demonstrated potential to directly inhibit aflatoxin accumulation (Chen et al., 2004). A synthetic version of a corn ribosome inhibiting protein (RIP) gene (called *mod1*) has been shown to control *A. flavus* in corn and peanuts (Boston et al., 1994, 1996; Weissinger et al., 2003). Lozovaya and colleagues (1998) reported that the resistance of a corn hybrid to *A. flavus* infection was correlated with an elevated level of β-1,3-glucanase in transgenic cells.

A full-length polyphenol oxidase cDNA from walnut embryos of resistant lines has been identified and is being tested for its antifungal or anti-insect activity. Recently, Mahoney and Molyneux (2004) demonstrated that precursor(s) of hydrolysable tannins (HT) such as gallic acid present within the pellicle tissues are responsible for inhibiting aflatoxin biosynthesis. Dandekar and McGranahan (2004) have initiated a program to identify and isolate genes responsible for HT/gallic acid biosynthesis with the objective of genetic engineering of susceptible walnut lines (and other crops) which lack or underexpress gallic acid in the seed coat.

The utility of genes coding for haloperoxidases (Wolffram et al., 1988) for plant disease resistance has been documented in transgenic tobacco plants (Rajasekaran et al., 2000). In bioassays using *A. flavus* as the test organism, addition of a myeloperoxidase greatly enhanced (90-fold) the lethality of H_2O_2 by catalyzing its conversion to sodium hypochlorite (Jacks et al., 1991). A bacterial chloroperoxidase also greatly reduced the viability of *A. flavus* conidia in vitro (Jacks et al., 1999; Rajasekaran et al., 2000). One of the bacterial chloroperoxidases, CPO-P, was found to confer antifungal traits against *Aspergillus* spp. and other phytopathogens in transgenic tobacco, cotton, and peanut (Rajasekaran et al., 2000; Jacks et al., 2004; Niu et al., 2004). H_2O_2 is produced by plants under pest attack and often serves as the substrate for these unique peroxidases in the specific host plant tissues (Jacks and Hinojosa, 1993; Rajasekaran et al., 2000). In fact, several investigators attempted to increase host plant resistance by increasing the level of H_2O_2

production. For example, Wu and colleagues (1995) introduced a glucose oxidase (GO) gene from *A. niger* to generate large amounts of hydrogen peroxide in transgenic potato plants to augment natural defense mechanisms. The transgenic potato plants showed an increased level of resistance to soft rot caused by *Erwinia carotovora* and to potato late blight caused by *Phytophthora infestans*. In contrast to these observations, a similar attempt with transgenic cottons expressing the *Talaromyces flavus* GO gene resulted in limited antifungal activity against the root pathogen, *V. dahliae* (Murray et al., 1999). However, these authors also discovered that the expression of GO in cottons resulted in phytotoxicity and reduced yield. Corn embryonic cultures also have been successfully co-transformed with the wheat oxalate oxidase (OXO) gene (Simmonds et al., 1998). OXO is a source of extracellular production of H_2O_2 in plants.

Recently Emani and colleagues (2003) transformed cotton plants with a cDNA clone encoding a 42 kDa endochitinase from the mycoparasitic fungus *Trichoderma virens*. Homozygous T2 plants of the high endochitinase-expressing cotton lines showed significant resistance against a soil-borne pathogen, *Rhizoctonia solani*, and a foliar pathogen, *Alternaria alternata*.

Peanut is also currently being transformed with antifungal genes; for example, with a gene encoding for Mod-1, a synthetic version of corn RIP (Weissinger et al., 2003), while walnut has been successfully transformed with barley lectin and nettle lectin antifungal genes (Mendum et al., 1995). Transgenic peanuts expressing the Mod-1 gene have demonstrated antifungal traits when peanut cotyledons were infected with *A. flavus*. Insect-resistant peanut lines expressing *CryIA*(c) have been evaluated by Ozias-Akins and colleagues (2002) as a possible means of inhibiting *A. flavus* inoculation into peanut pods by lesser cornstalk borer (LCB) (*Elasmopalpus lignosellus*), since it has been clearly documented that aflatoxin contamination is positively correlated with insect damage (Lynch and Wilson, 1991). Ozias-Akins and colleagues (1999) are also evaluating the potential of soybean *lox1* gene in suppressing the aflatoxin biosynthetic pathway. Soybean *lox1* gene encodes an enzyme that catalyzes the formation of a specific lipoxygenase metabolite of linoleic acid, 13(S)-β-hydroperoxyoctadecadienoic acid (HPODE), that has been shown to suppress the aflatoxin biosynthetic pathway in vitro (Burow et al., 1997; Keller et al., 1999).

Small MW peptides have been isolated from organisms other than plants that also show promise as antifungal agents (Rao, 1995; Broekart et al., 1997; Hancock and Chapple, 1999; Reddy et al., 2004). Some examples include the cecropins (Bowman and Hultmark, 1987) and magainins (Zasloff, 1987) of insect and amphibian origins, respectively, and their synthetic analogs (Cary et al., 2000; DeGray et al., 2001; Li et al., 2001; Rajasekaran et al., 2001; Chakrabarti et al., 2003).

Certain small lytic peptides have demonstrated convincing inhibitory activity against *A. flavus* and show promise for transformation of plants to reduce infection of seed. In our laboratory, we (Cary et al., 2000; Rajasekaran et al., 2001) reported that a synthetic lytic peptide (D4E1) gene, when transformed into tobacco, greatly enhances resistance *in planta* to *Colletotrichum destructivum*. The broad-spectrum antifungal activity of the synthetic peptide D4E1 is given in Table 3 (Rajasekaran et al., 2001). Treatment of germinating *A. flavus* spores with tobacco leaf extracts from plants, transformed with the D4E1 gene, significantly reduced spore viability (colony-forming units) relative to results obtained using extracts from nontransformed (control) plants. Similarly, in recent tests with cottonseed expressing the D4E1 gene, we demonstrated resistance to penetration of seed coats by a GFP reporter gene-containing *A. flavus* strain (Rajasekaran, Cary, et al., 2005; Rajasekaran, Cary, Cotty, and Cleveland, 2008). In addition to inhibiting the germination of *A. flavus* spores, D4E1 caused severe abnormal lytic effects on mycelial wall, cytoplasm, and nuclei. The expression of D4E1 gene in the progeny of transgenic cotton was sufficient to inhibit the growth in vitro of *Fusarium verticillioides* and *Verticillium dahliae* or *in planta* of *Thielaviopsis basicola* (Rajasekaran, Cary

Table 3. Broad spectrum antimicrobial activity of D4E1 in vitro.

Phytopathogen	IC_{50} (μM)	MIC (μM)
Alternaria alternata	12.39	> 25.0
Aspergillus flavus	7.75	25.0
Aspergillus flavus 70-GFP	11.01	25.0
Cercospora kikuchii	8.67	> 25.0
Colletotrichum destructivum	13.02	> 25.0
Claviceps purpurea	1.60	20.0
Fusarium graminearum	2.10	25.0
Fusarium moniliforme	0.88	12.5
Fusarium oxysporum	2.05	12.5
Penicillium italicum	5.92	> 25.0
Phytophthora cinnamomi	nd	4.67
Phytophthora parasitica	nd	4.67
Pseudomonas syringae pv. *tabaci*	0.52	2.25
Pythium ultimum	nd	13.33
Rhizoctonia solani	nd	26.7
Thielaviopsis basicola	0.52	6.0
Verticillium dahliae	0.60	5.25
Xanthomonas campestris pv. *malvacearum*	0.19	1.25

nd = not determined

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et al., 2005) and provide a good germination stand in a field infected with *Fusarium oxysporum* f.sp. *vasinfectum* (Rajasekaran et al., 2006). Transformation of peanut with another antifungal peptide, D5C, has been reported (Weissinger et al., 1999). Although the pure D5C showed strong activity in vitro against *A. flavus*, it was shown that the transgenic peanut callus showed poor recovery of plants because of possible phytotoxicity of the peptide.

In addition to nuclear transformation, we are currently exploring the possibility of expressing the antifungal genes in plastids with the objective of higher expression and preventing transgene escape through pollen. For example, the antimicrobial peptide MSI-99, an analog of magainin 2, was expressed via the chloroplast genome (DeGray et al., 2001) of tobacco. Leaf extracts from T2 generation plants showed 96% inhibition of growth against the bacterial pathogen *P. syringae* pv. *tabaci*. In addition, leaf extracts from T1 generation plants inhibited the growth of pregerminated spores of three fungal species, *A. flavus*, *Fusarium verticillioides*, and *Verticillium dahliae*, by more than 95%, compared with nontransformed control plant extracts. *In planta* assays with the bacterial pathogen *P. syringae* pv *tabaci* resulted in areas of necrosis around the point of inoculation in control leaves, whereas transformed leaves showed no signs of necrosis, demonstrating high-dose release of the peptide at the site of infection by chloroplast lysis. *In planta* assays with the fungal pathogen, *Colletotrichum destructivum*, showed necrotic anthracnose lesions in nontransformed control leaves, whereas transformed leaves showed no lesions. In addition to lytic peptide genes, a variety of other candidate antifungal genes from bacterial, plant, and mammalian sources have a good probability of being active against *A. flavus* upon transformation into plants.

Conclusion

With the advanced genomic, proteomic, and genetic transformation techniques available, the identification of resistance traits in aflatoxin-susceptible crops through marker-assisted breeding can facilitate a rapid development of resistant, commercially useful germplasm. Plant transformation facilitates the advantage of rapidly assessing the efficacy of a particular antifungal protein. In addition, identification of potent antifungal proteins and peptides from different sources will be valuable in transferring them through genetic engineering to susceptible crops to combat fungal infection resulting in preharvest aflatoxin contamination compromising food and feed safety. Genetic engineering by nuclear or organelle transformation thus provides a tool especially useful in testing gene functions, either using conventional gene constructs or RNAi constructs in a tissue, developmental, or environmental stimuli–specific manner. It also provides a means of enhancing the resistance of aflatoxin-susceptible crop species in a relatively short time frame compared with traditional breeding, as evidenced by the few examples presented in this review.

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