

Review

Evidence for an association in corn between stress tolerance and resistance to *Aspergillus flavus* infection and aflatoxin contamination

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Aflatoxins are carcinogenic secondary metabolites produced mainly by *Aspergillus flavus* during infection of susceptible crops, such as corn. *A. flavus* infection and subsequent aflatoxin contamination is a serious issue in the southern US, especially during a drought. Field studies demonstrate that reduction of drought stress by irrigation reduces aflatoxin contamination in corn and peanut. Drought tolerant corn varieties were also found to produce significantly less aflatoxins in the field under drought conditions compared to aflatoxin-resistant controls. Genetic studies to identify QTLs for low levels of aflatoxin accumulation observed significant environmental effects on the location and number of QTLs between studies conducted at different locations and during different years. Proteomic comparisons of kernel proteins between corn genotypes resistant or susceptible to *A. flavus* infection have identified stress-related proteins along with antifungal proteins as associated with kernel resistance. Gene expression studies of plants in response to biotic or abiotic stress also found that disease resistance-related genes could be upregulated by abiotic stresses and *vice versa*. Further examination of host plant and pathogen interactions revealed that plant responses to abiotic stresses and pathogen infections were mediated through several common regulatory genes or factors. The presence of “cross-talk” between responses to abiotic stress and biotic stress provides new approaches for enhancing host resistance to biotic stresses through the upregulation of key signal transduction factors.

Key words: Plant hormone, fungal infection, gene regulation, transcription factor.

INTRODUCTION

Aflatoxins are toxic, highly carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*, during infection of susceptible crops both in the field and after harvest (Brown et al., 1999). Aflatoxins are found to contaminate a wide variety of

important agricultural products such as corn, peanuts, tree nuts and cottonseed especially under extreme heat and drought conditions (Payne, 1998) and cause hundreds of millions of dollars in losses in the US. Foodstuffs contaminated with aflatoxins have also been associated with increased incidence of liver cancer in humans (Hsieh, 1989).

The discovery of natural resistance in corn in the past two decades (King and Scott, 1982; Scott and Zummo, 1988; Widstrom et al., 1987; Campbell & White, 1995) has led to a renewed effort to eliminate aflatoxin contamination from food and feed through the enhancement of host resistance. Due to the lack of understanding of host resistance mechanisms, and the

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Abbreviations: QTL, quantitative trait loci; ABA, abscisic acid; HSPs, heat shock proteins; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase.

lack of markers known to be consistently associated with resistance, the transfer of resistance traits into corn lines with good agronomic traits has been difficult. Recent efforts to identify molecular and genetic markers for corn kernel resistance as well as studies in host plant-pathogen interactions have suggested a correlation between stress tolerance and plant disease resistance. This article reviews evidence for an association of stress tolerance and aflatoxin resistance in corn based on field, genetics, proteomics, microarray, and host-pathogen interaction studies, and summarizes the latest approaches to enhance plant resistance to a broad spectrum of biotic and abiotic stresses.

THE EFFECT OF ABIOTIC STRESSES (DROUGHT AND HEAT) ON AFLATOXIN PRODUCTION IN CORN AND PEANUT

High temperature and drought, which often occur together during the growing season and likely contribute to poor kernel development, have been reported to increase growth of the fungus and toxin production (Payne, 1998). Jones et al. (1981) found that irrigating corn fields to reduce drought stress also reduced fungal infection and aflatoxin contamination. Irrigation not only relieved drought stress, but also reduced soil temperature. Lower soil temperature was found to reduce aflatoxin contamination in peanut (Hill et al., 1983). Increased aflatoxin contamination was observed in drought-treated peanuts with increased soil temperatures (Cole et al., 1985). Dorner et al. (1989) also concluded that a higher soil temperature favors *A. flavus* growth and aflatoxin production.

A study on the effect of drought on peanut resistance to *A. flavus* by Wotton and Strange (1987) found that fungal colonization was inversely related to water supply, as was aflatoxin production. In a four-year study on the influence of irrigation and subsoiling on infection and aflatoxin production in corn, Payne et al. (1986) also concluded that water stress appears to be a major factor affecting aflatoxin contamination. Studies of aflatoxin and fumonisin contamination of corn grown under high or moderate heat stress (Abbas et al., 2002) demonstrate that heat stress also plays an important role in the susceptibility of corn to both aflatoxin and fumonisin contamination. In an effort to determine if a relationship exists between drought-tolerance and aflatoxin resistance, Tubajika and Damann (2001) compared ear rot and aflatoxin production between nine drought-tolerant and two aflatoxin-resistant corn lines under field and laboratory conditions. They found that drought-tolerant lines all had significantly lower levels of ear rot and aflatoxin contamination compared to the aflatoxin-resistant controls, when grown under drought conditions (Tubajika and Damann, 2001). This suggests a possible association between drought tolerance and aflatoxin

resistance in corn.

Recent genetic studies also highlight a relationship between environmental factors and kernel resistance. In an effort to identify genetic markers or chromosome regions associated with kernel resistance, a quantitative trait loci (QTL) mapping approach has been used in the past several years. Paul et al. (2003) located QTLs for lower aflatoxins in five separated bins in their 1997 mapping population (Tex6 x B73) x B73 BC1S1. However, it was found that the environment strongly influenced the detection of these QTLs, since there were very few chromosome regions consistently associated with QTLs from year to year (Paul et al., 2003). A similar environmental effect on QTL was observed in another study (Davis et al., 1999), where 227 F2:F3 lines of Mp313E x Va35 were evaluated in 1997 and 1998. Here, six QTLs for reduced aflatoxin level were identified in 1997 samples, four of which were contributed by Mp313E, the resistant parent, and two by Va35, the susceptible parent. However, only two of the QTLs identified in 1997 samples reappeared in 1998 samples.

MOLECULAR EVIDENCE FOR AN ASSOCIATION BETWEEN STRESS TOLERANCE AND DISEASE RESISTANCE

Proteomics studies reveal that aflatoxin-resistant corn genotypes contain high levels of stress-related proteins

A proteomics approach was recently employed to identify proteins whose level of expression associated with kernel resistance against *A. flavus* infection and aflatoxin production (Chen et al., 2002). Endosperm and embryo proteins from several resistant and susceptible genotypes have been compared using large format 2-D gel electrophoresis, and over a dozen protein spots, either unique or 5-fold upregulated in resistant lines, have been identified and sequenced (Chen et al., 2002). These proteins can be grouped into three categories based on their peptide sequence homology: (1) storage proteins, such as globulin 1, globulin 2, and late embryogenesis abundant proteins (LEA3, LEA14); (2) stress-related proteins, such as an aldose reductase (ALD), a peroxiredoxin antioxidant (PER1), a cold regulated protein, a water-stress inducible protein, an anionic peroxidase, a glyoxalase I protein (GLX I), and several small heat shock proteins (HSP), and (3) antifungal proteins, which include a trypsin inhibitor and a pathogenesis-related protein 10.

Although the objective of the proteomics investigation was to identify antifungal proteins associated with host resistance, the majority of those identified were stress-related proteins and highly hydrophilic storage proteins. These data suggest that kernel resistance may require

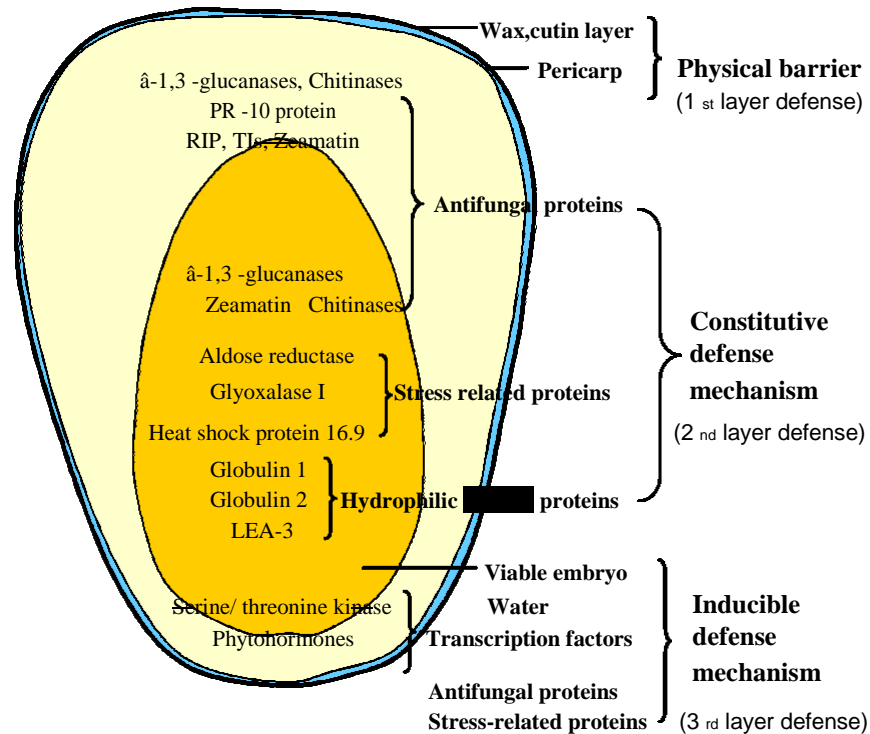


FIGURE 1. A model of host resistance mechanisms in corn against *Aspergillus flavus* infection and aflatoxin production. The level of constitutive and inducible antifungal proteins, some hydrophilic storage proteins and stress related proteins, viable embryos, regulatory factors such as protein kinases and phytohormones involved in the signal transduction pathways, and physical barriers are believed to be important for host resistance. Different locations of these resistance factors indicate the existence of three possible layers of defense. LEA-3, group 3 late embryogenesis abundant protein; PR, pathogenesis-related protein; RIP, ribosomal-inactivating protein; TI, trypsin inhibitor.

not only the presence of high levels of antifungal proteins, but also that of high levels of stress-related proteins and highly hydrophilic storage proteins. Storage proteins have been reported to play an important role in stress tolerance. Members of the LEA genes family have been associated with plant responses to many different stresses including drought, salt, cold, heat, and wounding (Thomann et al., 1992). Transgenic expression of an LEA protein from barley demonstrated increased tolerance to water and salt stress in rice (Xu et al., 1996).

Some stress-related proteins have been reported to not only confer stress-tolerance, but also enhance disease resistance. The expression of heat shock proteins, especially the small HSPs under stress, has been widely studied (Vierling, 1991) and shown to possess molecular chaperone activity (Jacob et al., 1993). Aside from heat stress, HSPs are also induced by other stresses such as cold, drought, or salinity (Sabehat et al., 1998). The transgenic expression of an aldose reductase, which is involved in the synthesis of an osmolyte, sorbitol, and temporally correlated with its acquisition of desiccation tolerance in barley embryo (Roncarati et al., 1995), was found to protect transgenic

tobacco plants against lipid peroxidation under chemical (paraquat and heavy metal) and drought stress (Oberschall et al., 2000). The role of glyoxalase in stress-tolerance is also highlighted in a recent study using transgenic tobacco plants over-expressing a *Brassica juncea* glyoxalase I (Veena et al., 1999). Further investigation suggests a direct role for glyoxalase I in corn resistance against aflatoxin accumulation through the removal of its aflatoxin inducing substrate, methylglyoxal (Chen et al., 2004). Sugarbeets carrying a superoxide dismutase transgene from tomato exhibited increased tolerance to oxidative stress, as well as to leaf infection with the fungus *Cercospora beticola* (Tertivanidis et al., 2004). Studies by Park et al. (2001) and Shin et al. (2002) also found that transgenic expression of the tobacco stress-inducible gene 1 (Tsi1) induced expression of several pathogenesis-related genes under normal conditions, resulting in improved tolerance to salt and pathogens. Possession of unique or higher levels of these constitutively expressed, stress-related proteins may put resistant lines in an advantageous position over susceptible ones in the ability to synthesize proteins and defend against pathogens

while under stress. The contribution of various factors from three different layers to resistance in corn kernels is summarized in Figure 1.

Gene expression studies reveal that abiotic stress and infection induce similar plant responses

Biotic or abiotic stress alone was able to induce the expression of genes involved in both biotic and abiotic stress responses (Cheong et al., 2002; Mengiste et al., 2003). In response to a combination of heat and drought stress, *Arabidopsis* induces several resistance-related genes, such as two putative disease resistance proteins and a thaumatin-like protein. It also induces proteins involved in signal transduction and stress (Rizhsky et al., 2004). Also in *Arabidopsis*, differential expression was observed for genes coding for a late embryogenesis abundant protein LEA-5, a putative protein kinase, and a DNA-binding protein in response to the application of a fungal protein known to cause necrosis (Keates et al., 2003). Another study by Rao et al. (2002) identified 35 genes significantly up- or down- regulated in rice after inoculation with *Magnaporthe grisea*. Sequence homology analysis showed that these genes had known functions in defense reactions, signal transduction, stress response, photosynthesis and sugar metabolism. Differential expression of genes with diverse functions, including genes known to be involved in resistance or stress responses and genes known to be involved in primary or secondary metabolism has also been observed in two potato lines varying in resistance to *Phytophthora infestans* (Evers et al., 2003). All of these studies clearly indicate similarities and distinctions in host responses to biotic and abiotic stresses, as well as cross-talk (both overlap and interference) between pathogenesis-related responses and plant responses to other types of stresses (Wan et al., 2002). In another word, fungal infection represents a unique kind of stress to host plants. In response to such stress, plants not only induce specific antifungal genes, but also upregulate general stress-related genes.

SIGNAL TRANSDUCTION AND CROSS-TALK OF HOST PLANTS IN RESPONSE TO BIOTIC AND ABIOTIC STRESS

The mechanisms plants use to adapt to abiotic and biotic stress have been widely studied in a number of plants. Current research effort has focused on the isolation of stress-responsive genes and their regulation as a means to understand the molecular events underlying the adaptation process. An increasing body of evidence suggests that a subset of plant responses to biotic and abiotic stress is shared, such as the generation of reactive oxygen species (ROS), the activation of mitogen-

Activated protein kinases (MAPKs), and hormone modulations.

Reactive oxygen species (ROS)

ROS production is recognized as a common event in plant response to biotic and abiotic stresses (Kovtun et al., 2000; Lamb and Dixon, 1997; Mithöfer et al., 2004). The hypersensitive response (localized plant cell death at the infection site) to halt pathogen invasion during an incompatible host-pathogen interaction has also been reported to involve the production of ROS (Wojtaszek, 1997). The mechanism of how ROS leads to downstream responses is still not clear, however, the requirement of specific MAPKs has been implicated (Kovtun et al., 2000), possibly mediated through a serine/threonine kinase (OXI1) in *Arabidopsis* (Rentel et al., 2004). The activity of this kinase was induced in vivo by H₂O₂ and its expression was upregulated by a wide range of H₂O₂-generating stimuli (Rentel et al., 2004), suggesting this kinase (OXI1) is an essential part of the signal transduction pathway linking oxidative burst signals to diverse downstream responses.

Mitogen-activated protein kinases

Plant-pathogen recognition causes the rapid activation of appropriate defenses. Some of the components in the signal transduction pathways have been identified and characterized. One good example is the mitogen-activated protein kinase (MAPK) cascades that are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses. All plant MAPKs have a Thr-Glu-Tyr activation motif, except members of subfamily V, where Glu is replaced by Asp (Zhang and Klessig, 2001). Recently, a MAPK kinase 2 (MKK2) from *Arabidopsis*, specifically activated by cold and salt stress and by the stress-induced MAPK kinase kinase MEKK1, was found to increased freezing and salt tolerance in transgenic plants (Teige et al., 2004), suggesting the importance of MAPK cascades in plant responses to multiple stresses.

One of the mechanisms by which different stimuli converge onto one MAPK is believed to involve several unrelated kinases that function as MAPKKKs to initiate the MAPK cascade (Widmann et al., 1999). Based on the homology of the kinase domain, several plant kinases have been identified as MAPKKKs, including EDR1 and NPK1/ANPs (Zhang and Klessig, 2001). Although the downstream MAPKK is unknown, tobacco NPK1 and its *Arabidopsis* homologs (ANPs) have recently been shown to activate MPK3 and MPK6, two *Arabidopsis* MAPKs (Kovtun et al., 2000). A recent review by Hammond-Kosack and Parkerz (2003) provides a comprehensive list of MAPKs identified from different plant-pathogen systems.

Cis-acting elements and other transcription factors

Studies of transcriptional activation of some stress responsive genes have also led to the identification of cis-acting elements ABRE (ABA responsive element) and DRE (dehydration responsive element)/CRT (C-repeat) that function in ABA-dependent and ABA-independent gene expression in response to stress, respectively (Seki et al., 2003). Transcription factors belonging to the ethylene-responsive element binding factor family that bind to DRE/CRT were also isolated (Stockinger et al., 1997; Liu et al., 1998). The genes encoding these transcription factors are induced early and transiently in response to cold, and these transcription factors, in turn, activate the expression of target genes. Similar transcription factors (DREB2A and DREB2B) are also induced by dehydration and promote the expression of various genes involved in drought stress tolerance (Liu et al., 1998). The expression of a new DNA-binding protein DBF1 that specifically interact with the DRE2 cis-element of a corn rab17 gene promoter, is induced by ABA, dehydration and high salinity (Kizis and Pages, 2002).

Another example of a transcription factor is calcium-dependent protein kinases (CDPKs). CDPKs are implicated as important sensors of Ca²⁺ flux in plants in response to stress (Ludwig et al., 2004). CDPKs are encoded by multigene families, and expression levels of these genes are spatially and temporally controlled throughout development. In addition, subsets of CDPKs are involved in signal transduction during stress including cold, salt, and drought or pathogen infection. For example, the NtCDPK2, which is essential for Cf-9-specified resistance to the *Cladosporium fulvum* Avr9 peptide, was found to play a role in the perception of abiotic stresses in tobacco plants as well (Romeis et al., 2001). Recently, a new transcription factor, BOS1 (*Botrytis susceptible 1*) was found to be required for both biotic and abiotic stress responses in *Arabidopsis* (Mengiste et al., 2003).

Phytohormones

A variety of plant hormones, including salicylic acid (SA), jasmonate (JA), ethylene, and abscisic acid, have been implicated in mediating responses to a wide range of biotic and abiotic stresses. (Diaz et al., 2002; Thomma et al., 1998; Audenaert et al., 2002). The roles of these hormones are dependent upon the particular host-pathogen interaction (Knoester et al., 1998). On the basis of the interactions that have been studied, a general rule for hormonal action has been proposed in which resistant responses to biotrophs require SA, whereas responses to necrotrophs require JA and ethylene (Feys and Parker, 2000). In some instances, these hormones are involved in determining the level of host basal resistance (Delaney et al., 1994). In other cases, their actions are only involved in production of disease symptoms and do not

affect the growth of the pathogen. In tomato, ethylene, JA, and SA all independently contribute to its resistance to *Botrytis cinerea* (Diaz et al., 2002). Also in tomato, the host plant actively regulates the *Xanthomonas campestris* pv *vesicatoria* -induced disease response via the sequential action of at least three hormones (JA, ethylene, and SA), which promote expansive cell death of its own tissue (O'Donnell et al., 2003).

Further, the effect of phytohormones is also regulated by other factors. For example, the MAPK kinase kinase, EDR1, negatively regulates SA-inducible defenses (Frye et al., 2001), whereas MAPK 4 appears to differentially regulate SA and JA signals (Petersen et al., 2000). These findings also suggest that MAPK modulates cross-talk between different plant defense pathways (Hammond-Kosack and Parkerz, 2003).

NEW APPROACHES OF ENHANCING HOST RESISTANCE TO FUNGAL DISEASES THROUGH ENHANCEMENT OF PLANT STRESS TOLERANCE

In the past few years, studies trying to understand signal transduction of plants under biotic or abiotic stress demonstrate that signaling elements isolated from one species might work in others, and that a broad-spectrum disease resistance may be obtained by manipulating the signal cascade to strengthen defense capability and the durability of multiple host defense systems. This enhanced understanding of signal transduction has led to new approaches to advance host resistance.

Recently transgenic expression of a tobacco MAPKKK (NPK1) was shown to confer enhanced drought tolerance in transgenic corn (Shou et al., 2004). Under drought conditions, transgenic corn plants maintained significantly higher photosynthesis rates than did the non-transgenic control, suggesting that NPK1 induced a mechanism that protected photosynthesis machinery from dehydration damage. In addition, drought-stressed transgenic plants produced kernels with weights similar to those under well-watered conditions, while kernel weights of drought-stressed non-transgenic control plants were significantly reduced when compared with their non-stressed counterparts.

DREB1A, which specifically interacts with a cis-acting element DRE and induces expression of stress tolerance genes (Liu et al., 1998), has been shown in *Arabidopsis thaliana* to play a crucial role in promoting the expression of drought-tolerance genes. Overexpression of the cDNA encoding DREB1A from the stress inducible rd29A promoter caused minimal effects to plant growth while providing a high level of tolerance to drought, salt, and freezing in transgenic plants (Kasuga et al., 1999; Pellegrineschi et al., 2004). Another study by Shin et al. (2002) reported that transgenic hot pepper plants expressing the tobacco stress-induced gene 1 (Tsi1) exhibited resistance to Pepper mild mottle virus (PMMV) and Cucumber mosaic virus (CMV). Furthermore, the

plants also demonstrated increased resistance to other pathogens.

CONCLUSIONS

Evidence from field studies, from proteomic comparisons of differences between resistant and susceptible corn genotypes, from gene expression analysis of plants in response to biotic and abiotic stresses, and from examination of signal transduction components involved in biotic and abiotic stress responses indicates the existence of an association between stress tolerance and disease resistance against *A. flavus* infection and aflatoxin production in corn. This understanding not only highlights the importance of expression of stress-related proteins in resistance, but also leads to new approaches to enhance plant resistance to a broad-spectrum of pathogens, such as overexpression of transcriptional regulatory genes..

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