RNA Silencing of Mycotoxin Production in Aspergillus and Fusarium Species

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Mycotoxins are natural fungal products that are defined by their harmful effects on humans and animals. Aflatoxin contamination of maize by Aspergillus species and trichothecene contamination of small grains by Fusarium species are two of the most severe mycotoxin problems in the United States. We are investigating RNA silencing in an effort to identify novel ways to control mycotoxin contamination of crops. Transformation of two Aspergilli (A. flavus and A. parasiticus) and a Fusarium (F. graminearum) with inverted repeat transgenes (IRT) containing sequences of mycotoxin-specific regulatory genes suppressed mycotoxin production in all three plant-pathogenic fungi. This atoxicogenic phenotype was stable during infection on corn and wheat, and importantly, F. graminearum IRT strains were less virulent on wheat than were wild type. The IRT did not alter physiological characteristics of the fungi, such as spore production and growth rate on solid media. These results indicate that RNA silencing exists in Aspergillus and Fusarium plant pathogens and suggest that RNA silencing technology may be a useful tool for eliminating mycotoxin contamination of agricultural products.

Additional keyword: deoxynivalenol.

Aspergillus and Fusarium plant pathogens produce toxic secondary metabolites during infection of grain and legume crops that lead to direct (e.g., yield loss) and indirect (e.g., health loss) costs in agriculture, animal husbandry, and human health. Commonly called mycotoxins, these metabolites are introduced into the diet through consumption of contaminated produce, most frequently seed or seed products, and are toxic to human beings and livestock by damaging specific organ systems. Detrimental properties exhibited by two toxins, aflatoxin produced by Aspergillus species and deoxynivalenol (DON) produced by Fusarium graminearum, include carcinogenesis, mutagenesis, teratogenesis, oestrogenesis, or immunosuppression, singly or in combination (Richard and Payne 2003). Because conventional approaches to controlling plant diseases have not worked consistently to eliminate or even substantially lower mycotoxin contamination, alternative means to reduce toxin formation in crops are being explored.

The posttranscriptional regulation of genes through interference and degradation of mRNA is known as RNA silencing (Denli and Hannon 2003; Pickford and Cogoni 2003; Waterhouse et al. 2001). This eukaryotic mechanism is activated by an RNase III enzyme (Dicer), which digests double-stranded RNA (dsRNA) molecules into 21- to 25-bp fragments (Bernstein et al. 2001). These fragments, called siRNAs, are incorporated into a complex of proteins known as the RNA induced silencing complex (RISC), which uses the incorporated siRNAs to target and degrade mRNA with complementary sequences (Elbashir et al. 2001; Hammond et al. 2001).

It has recently been demonstrated that inverted repeat transgenes (IRT) are efficient activators of RNA silencing in several fungal species, including Neurospora crassa, Magnaporthe oryzae, Cryptococcus neoformans, Histoplasma capsulatum, Aspergillus fumigatus, and A. nidulans (Catalanotto et al. 2004; Hammond and Keller 2005; Kadotani et al. 2003; Liu et al. 2002; Mouyna et al. 2004; Rappleye et al. 2004). Presumably, IRT produce hairpin RNA (hpRNA) that is digested into siRNAs by an RNase III enzyme such as Dicer, thus activating RNA silencing of endogenous genes that share identity with the sequences contained in the hpRNA. In a previous study focused on the genetic requirements of RNA silencing in A. nidulans, we showed that IRT containing sequences of a gene encoding a mycotoxin regulatory factor suppressed mycotoxin production through RNA silencing (Hammond and Keller 2005). These results led us to hypothesize that IRT could be used to inhibit mycotoxin production by plant pathogens.

Here, we report that IRT containing sequences corresponding to mycotoxin regulatory genes inhibit mycotoxin production in three plant pathogens: A. flavus, A. parasiticus, and F. graminearum. We also demonstrate that toxin inhibition is stable during pathogenesis and that the IRT used in this study appear to have no physiological effects on these fungi other than the intended inhibition of mycotoxin production.

RESULTS

RNA silencing of toxin production in Aspergillus plant pathogens.

AflR is a positive acting transcription factor required for the expression of aflatoxin biosynthetic genes in A. flavus and A. parasiticus (Woloshuk et al. 1994) and the expression of biosynthetic genes of a similar mycotoxin, sterigmatocystin (ST), in A. nidulans (Fernandes et al. 1998; Yu et al. 1996). Because loss of this transcription factor results in a loss of aflatoxin and ST production and an aflR-specific IRT was successfully used to suppress the ST pathway in A. nidulans (Hammond and Keller 2005), an IRT consisting of A. flavus aflR sequences was constructed to test if the RNA silencing pathway could be used to inhibit mycotoxin production in Aspergillus plant pathogens. AflR sequences are nearly identical in A. flavus and A. parasiticus (data not shown), therefore it was predicted that an IRT designed from A. flavus aflR sequences would also silence A.
parasiticus aflR. To facilitate the construction of the aflR IRT, we created a plasmid with an approximately 280-bp gfp spacer fragment placed between a high-expression fungal promoter and a fungal terminator (pTMH44.2) (Fig. 1A). Two nearly identical approximately 670-bp fragments of A. flavus aflR (Fig. 1B) were then placed in an inverted orientation on either side of the pTMH44.2 gfp spacer to create the aflR IRT (pTMH52.1) (Fig. 1A). For the A. flavus experiment the aflR IRT was transformed into an aflatoxin-producing strain, A. flavus 8610. For the A. parasiticus experiment, the aflR IRT was transformed into a versicolorin A–accumulating strain, A. parasiticus CS10. Versicolorin A is a biosynthetic intermediate in the aflatoxin biosynthetic pathway, and thus, its production is also dependent on AflR function. In both transformations, the aflR IRT was transformed along with pBZ5, a plasmid containing the A. parasiticus pyrG cassette (Skory et al. 1990), as a selectable marker.

Several transformants of both species integrated the aflR IRT into their genomes. Southern analysis indicated that a single copy of the aflR IRT had integrated into the genome of two out of 30 A. flavus transformants (IRT20 and IRT25) and two out of 50 A. parasiticus transformants (IRT15 and IRT33) without disrupting the native aflR locus (data not shown). An additional A. flavus transformant (IRT3) contained multiple inserts of the IRT construct (data not shown). As predicted, the A. flavus and A. parasiticus transformants carrying both the native aflR gene and the aflR IRT displayed an aflR-silencing phenotype, as inferred by the significant decrease of aflatoxin production by A. flavus IRT20 and IRT25 (no aflatoxin detected, except for a minimal amount of aflatoxin in one replicate) (Fig. 2A) and a significant decrease of versicolorin A production by A. parasiticus IRT15 (no versicolorin A detected) (Fig. 2B) and IRT33 (a minimal amount of versicolorin A detected) (Fig. 2B) relative to the parent strains and control transformants (Fig. 2A and B). Interestingly, A. flavus IRT3, a transformant with multiple copies of the aflR IRT, produced an intermediate level of aflatoxin compared with transformants with zero copies of the aflR IRT (normal aflatoxin production) and transformants with one copy of the aflR IRT (no aflatoxin detected except for a minimal amount of aflatoxin in one replicate) (Fig. 2A).

The AflR IRT correlates with loss of aflatoxin biosynthetic gene expression.

A. parasiticus transformants containing the aflR IRT were examined for expression of the aflatoxin biosynthetic gene verA. VerA is a biosynthetic gene required for the conversion of versicolorin A to the next intermediate in the aflatoxin biosynthetic pathway, and it requires AflR for its expression (Keller et al. 1994, 1995; Woloshuk et al. 1994). Northern analysis indicates that verA is expressed by the parental strain (CS10) and by the two pyrG-only transformant strains (C27 and C28) and that either verA is not expressed by the two A. parasiticus aflR IRT transforms (IRT15 and IRT33) or it is expressed at levels below the limits of detection by Northern blotting (Fig. 3).

Stability of the IRT silencing phenotype during Aspergillus pathogenesis.

To test if the aflR IRT-induced atoxigenic phenotype was stable during a host infection, viable corn seed was inoculated with either an A. parasiticus pyrG transformant control (C27) or A. parasiticus IRT15. After a 5-day incubation, Versicolorin A was extracted from the infected corn seed and was analyzed by thin-layer chromatography (TLC). In all four replicates, A. parasiticus C27 produced high levels of versicolorin A, whereas versicolorin A was only detected, at a low level, in one of the IRT15 replicates (Fig. 2C).

RNA silencing of mycotoxin production impairs ability of F. graminearum to cause wheat head blight.

Like AflR in Aspergillus species, F. graminearum Tri6 is a pathway-specific, positive-acting, DNA-binding protein required

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**Fig. 1.** Aspergillus and Fusarium inverted repeat transgene (IRT) constructs. A, Schematic representation of IRT plasmids pTMH44.2, pTMH52.1, and pTRM-tri6. Vertical bars, A. nidulans gpdA promoter; horizontal bars, A. nidulans trpC terminator; white bars, gfp spacer fragment. Restriction enzyme sites: E = EcoRI; Nc = NcoI; As = Ascl; Ba = BamHI; No = NotI; Hi = HindIII; and X = XhoI. B, A fragment of A. flavus aflR (from approximately 445 to 1,116 nt (sense and antisense) of the predicted aflR open reading frame [ORF]) was placed on either side of the spacer fragment of pTMH44.2 in an inverted orientation to give pTMH52.1. C, A fragment of F. graminearum tri6 (from nt 21 to 623 (sense) and 47 to 635 (antisense) of the predicted tri6 ORF) was placed on either side of the spacer fragment in pTMH44.2 to give pTRM-tri6. The black boxes in B and C represent the fragments of aflR and tri6 used to construct the IRT.
for the transcription of mycotoxin (trichothece) biosynthetic genes, and loss of this gene results in atoxigenic strains (Hohn et al. 1999). Therefore, to test if RNA silencing could be used to create atoxigenic strains in *Fusarium* species, an IRT consisting of *F. graminearum* trit6 sequences (Fig. 1A and C) was constructed. Transformation of *F. graminearum* R5317 with this IRT (pTRM-trit6-Hyg) (Fig.1A) resulted in four strains that were shown by Southern analysis to carry the trit6 IRT: NK8, NK9, NK10, and NK11 (data not shown). Transforms NK9, NK10, and NK11 integrated the trit6 IRT at random ectopic locations distinct from the native trit6 locus, whereas it appeared the trit6 IRT may have integrated near the native trit6 locus in NK8 (data not shown).

Previous studies have shown that DON is required for the spread of *F. graminearum* from the initially infected spikelet to adjacent spikelets (Bai et al. 2002; Bushnell et al. 2003). Therefore, to determine if DON production was suppressed in the trit6 IRT-carrying strains, they were tested for their ability to cause wheat head blight in comparison with a strain known to be virulent on wheat, *F. graminearum* R5317, and a control strain, *F. graminearum* NK3. Strain NK3 appears to have developed spontaneous resistance to hygromycin during the transformation process, as Southern analysis suggests it did not integrate either the trit6 IRT or the hygB portion of pTRM-trit6-Hyg (data not shown). *F. graminearum* R5317, NK3, and the four trit6 IRT strains (NK8 through NK11) were assayed for their ability to cause wheat head blight in either one or two greenhouse tests. Wheat heads were scored for the average percentage of blighted spikelets per head at 18 days after fungal inoculation. Strain R5317 and NK3 both produced 34% head blight in test 1 and 32 and 38% head blight, respectively, in test 2 (Table 1). Control wheat heads inoculated only with water did not show any head blight in either test (Table 1). All trit6 IRT-containing strains caused head blight within the infected floret and adjacent floret; however, head blight did not progress to flanking spikelets. In addition, DON was only detected in wheat seed infected with control strains R5317 and NK3 (Table 1). The failure of disease symptoms to spread to neighboring spikelets in all wheat infected with the four *F. graminearum* trit6 IRT strains is a characteristic shared by *F. graminearum* GZT40, a mutant strain that is unable to synthesize DON (Bai et al. 2002; Bushnell et al. 2003).

**Trit6 IRT and aflR IRT strains exhibit normal physiology.**

Macroscopically, the *Aspergillus* and *F. graminearum* IRT transformants were indistinguishable from their respective wild-type parents. To more accurately assess this observation, assays of conidia production (Fig. 4) and growth rate on solid media (data not shown) were conducted. No difference in either parameter was seen among the parents, the control transformants, and the IRT strains, with the exception of *A. flavus* IRT3, the transformant carrying multiple copies of the aflR IRT (Fig. 4). Overall, these results suggest that the effects of the IRT are limited to toxin production and do not adversely affect overall fitness.

**DISCUSSION**

In this paper, we demonstrate that IRT can suppress mycotoxin production in three plant pathogens, *A. parasiticus*, *A. flavus*, and *F. graminearum*. Targeting a mycotoxin biosynthetic pathway-activating transcription factor in each of these three fungi inhibited the downstream genes required for mycotoxin production, as determined by gene expression (verA) and toxin analysis (aflatoxin, versicolorin A, and DON). Additionally, we show that this IRT induced loss of toxin production is stable during infection of host plants (corn for *Aspergillus* and wheat for *Fusarium*) and, in the case of *F. graminearum*, a decrease in virulence results from DON suppression. Because growth diameter and spore production levels did not change in the IRT strains relative to control strains, it is likely that the aflR- and trit6-specific IRT did not have significant off-target effects.

![Fig. 2. An aflR inverted repeat transgene (IRT) inhibits mycotoxin production in *Aspergillus flavus* and *A. parasiticus*.](image1.png)

![Fig. 3. An Aspergillus aflR inverted repeat transgene (IRT) inhibits expression of verA in *A. parasiticus*.](image2.png)
effects in these fungi, and it is likely that the only genes affected in the strains were the intended targets.

The available evidence suggests that the IRT used in this study silence their respective mycotoxin regulatory genes through the well-characterized pathway of RNA silencing. First, our previous studies of RNA silencing in the genetic model fungus *A. nidulans* indicate that an *aflR* IRT correlates with the presence of *aflR*-specific approximately 25-nt siRNAs and the loss of *aflR* expression (Hammond and Keller 2005). Thus, the IRT-based mycotoxin suppression observed in the three plant pathogens studied here, two of which are also Aspergilli, is likely due to loss of endogenous *aflR* or *tri6* transcripts via degradation by RISC-associated siRNAs derived from the *aflR* or *tri6*-specific IRT. Second, IRT in other species of fungi have been firmly linked to RNA silencing (Catalanotto et al. 2004; Kadotani et al. 2003; Liu et al. 2002). Finally, a search of the available *F. graminearum* and Aspergillus sequence databases indicates the presence of Dicer and other RNA silencing enzymes in these species (data not shown). Therefore, the studies reported here strongly suggest that *Fusarium* and *Aspergillus* plant pathogens can be added to the growing list of RNA silencing–capable fungi.

Although the RNA silencing phenotype was stable during host infection in *A. flavus*, *A. parasiticus*, and *F. graminearum*, there was some loss of silencing in one replication of *A. parasiticus* IRT15 on corn (Fig. 2C). One possible explanation is that an IRT could be lost during normal growth. Support for this hypothesis comes from studies of *A. nidulans*, in which we have observed that sometimes up to 10% of progeny from a sexual cross lose an IRT carried by both parents (T. M. Hammond and N. P. Keller, unpublished data).

The loss of toxin production and cessation of biosynthetic gene expression in the IRT strains is similar to that observed by traditional gene knock outs of *Aspergillus* *aflR* (Butchko et al. 2003; Woloshuk et al. 1994; Yu et al. 1996) and *Fusarium* *tri6* (Proctor et al. 1995a). In addition, the *F. graminearum* *tri6* IRT strains were significantly reduced in their ability to cause disease, a phenotype identical to that of a *tri5* deletion strain that is unable to produce DON (Bai et al. 2002). *Tri5* encodes the first biosynthetic gene in the trichothecene pathway and gives the same trichothecene-negative phenotype as a *tri6* mutant (Hohn et al. 1999; Proctor et al. 1995a and b). Although pathogenicity tests for a *tri6* knockout have not been reported, the pattern of pathogenicity is expected to be the same as for a *tri5* mutant. The decrease in virulence of the *F. graminearum* IRT strains demonstrates this to be the case (Table 1).

RNA silencing shows promise as a molecular genetic tool in pathogenic fungi for several reasons. First, this technology opens genetic manipulation to formerly genetically intractable

![Fig. 4. Aspergillus and Fusarium mycotoxin-specific inverted repeat transgenes (IRT) do not alter spore production levels. Aspergillus and Fusarium strains were analyzed for the production of conidia (*Aspergillus*) or macroconidia (*Fusarium*) after a 5-day incubation. Five replicates were performed for each strain. *IRT3* carries multiple inserts of the IRT, perhaps increasing the likelihood that a gene directly or indirectly involved in spore production was altered during the transformation process.](image-url)

**Table 1. Disease assessment of F. graminearum infected wheat**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Average % blighted*</th>
<th>DON (ppm)*</th>
<th>Total seed mass*</th>
<th>Mass per seed*</th>
<th>Average % yield loss*</th>
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<td></td>
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<td>Water</td>
<td>0.0</td>
<td>Nd</td>
<td>10.6</td>
<td>33 (1.6)</td>
<td>0.0</td>
</tr>
<tr>
<td>R5317</td>
<td>33.2 (23.3)</td>
<td>3.7</td>
<td>8.1</td>
<td>25 (6.7)</td>
<td>24.2</td>
</tr>
<tr>
<td>NK3</td>
<td>33.7 (24.0)</td>
<td>4.4</td>
<td>7.7</td>
<td>27 (8.9)</td>
<td>18.2</td>
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<tr>
<td>NK8</td>
<td>6.3 (0.2)</td>
<td>Nd</td>
<td>10.8</td>
<td>34 (2.2)</td>
<td>+3.0</td>
</tr>
<tr>
<td>NK11</td>
<td>6.4 (0.4)</td>
<td>Nd</td>
<td>10.3</td>
<td>34 (2.0)</td>
<td>+3.0</td>
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<tr>
<td><strong>Experiment 2</strong></td>
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<td></td>
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<td>Nd</td>
<td>11.4</td>
<td>33 (3.2)</td>
<td>0.0</td>
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<tr>
<td>R5317</td>
<td>31.6 (22.1)</td>
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<td>8.0</td>
<td>27 (5.7)</td>
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<td>NK11</td>
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<td>Nd</td>
<td>12.4</td>
<td>34 (2.6)</td>
<td>+3.0</td>
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</tbody>
</table>

* R 5317 = wild type, NK3 = transformant control, NK8-11 = *tri6* IRT strains.
* Average percentage of blighted = average percentage of the wheat head that was blighted (10 heads).
* DON = deoxynivalenol, measured in parts per million, Nd = not detected (<2 ppm).
* Total seed mass = total mass of seeds harvested from 10 heads of one treatment (grams).
* Mass per seed = average seed mass (milligrams).
* Average percent yield loss = average percent yield loss per seed compared with the seed mass yield observed in the water control. + refers to an increase in yield relative to the water control treatment.
fungi. Species in which homologous recombination is rare can now be subjects for gene silencing as an IRT construct need not be targeted to any specific location within the genome to function. Second, gene silencing makes genetic manipulation easier for tractable fungi. The need to identify the clipping regions, promoter, or terminator of a gene is not necessary, only a large-enough portion of the gene to form a stable hair-pin dsRNA species. Although the smallest fragments of aflR and tri6 used in these experiments were approximately 600 bp, it is possible that smaller fragments would work. This could limit time-consuming species-specific optimization of transformation. Furthermore, it is possible to silence several genes with one construct. We have successfully concatenated portions of three genes within one IRT construct to silence a class of enzymes in the human pathogen A. fumigatus with one transformation event, eliminating numerous cloning steps (J. W. Bok and N. P. Keller, unpublished data). Also, we have shown that perfect homology is not required, as an A. flavus construct silenced genes in A. parasiticus. This portability of construct between closely related species will also speed genetic manipulation.

Finally, IRT-based silencing is likely to be broadly applicable and, thus, a preferred method of gene silencing for future studies of many different fungi based on the advantages discussed above. So far, this type of silencing has been demonstrated in only one plant pathogen, Magnaporthe oryzae. To this list, we add three plant-pathogenic fungi; including two Aspergilli and a Fusarium. The presence of a functional RNA silencing pathway in all four of these fungal plant pathogens leads us to speculate on the potential of RNA silencing as a not-just-molecular tool but as a broad-spectrum control strategy for phytopathogenic fungi. For such a control strategy to work, it will first be necessary to devise a method for delivering specific RNA silencing triggers into fungal hyphae in an agricultural setting.

MATERIALS AND METHODS

Fungal strains and growth conditions.

The strains used in this study included A. parasiticus CS10 (pyrG, verA, courtesy of J. Linz), A. flavus 8610 (argB, pyrG, courtesy of G. Payne), F. graminearum R5317 (NRRL 5908 or MRC 1781 group 2, courtesy of J.-H. Yu and D. Geiser), and F. graminearum PH-1 (NRRL 31084 or FGSC 9075, courtesy of F. Traili). A. flavus, A. parasiticus, and F. graminearum strains were grown in a 12-h light and dark cycle at 29°C and were maintained on V8 agar (200 ml of V8, 800 ml of H2O, 3 g of CaCO3, 20 g of agar) with appropriate supplements.

Nucleic acid manipulations.

Oligonucleotides used in this study are listed in Table 2. Southern analysis. Aspergillus strains were grown for 36 h in liquid glucose minimal media plus supplements (Shimizu and Keller 2001). Fusarium strains were grown for 36 h in YEP (2% yeast extract, 2% peptone). DNA was extracted by the alkali method described by Tag and associates (2001). Northern analysis. Aspergillus strains were grown in liquid PMS (6% peptone, 2 g of (NH4)2SO4, 2 g of MgSO4, 10 g of KH2PO4, trace elements), an aflatoxin-repressing medium, for 18 h, as described by Buchanan and Lewis (1984) and Woloshuk and associates (1994). RNA was extracted after 22 h, using Trizol as described by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.). Probes. A. flavus and A. parasiticus transformants were screened with an approximately 670-bp HindIII/BamHI aflR fragment from pTMH52.1. An EcoRI verA fragment from pBverA (Liang et al. 1996) was used to probe Aspergillus mRNA. F. graminearum transformants were screened with an approximately 600-bp NcoI/AscI fragment amplified from F. graminearum PH-1, using primers listed in Table 2.

Vector construction.

Primers used in vector construction are listed in Table 2. Construction of pTMH44.2. An approximately 280-bp fragment of gfp (green florescent protein) was amplified from pPRgT-T (Zolotukhin et al. 1996) with a forward primer containing an NcoI site and nested AscI site (Gfs5’/NcoI/AscI) and a reverse primer containing a HindIII site, nested NotI site, and nested BamHI site (Gfp3’/Hind/NotI/BamI). This fragment was cloned into the NcoI/HindIII sites of pAN52-3 (Punt et al. 1991), which contains the A. nidulans gpdA promoter and trpC terminator, to produce pTMH44.2 (Fig. 1A).

Construction of pTMH52.1. An approximately 670-bp fragment of A. flavus aflR was amplified from pRB3 (R. A. Butcho and N. P. Keller, unpublished data), using a forward primer containing a HindIII site (aflR-HindIII) and a reverse primer containing a BamHI site (aflR-BamHI) and was cloned into pBluescript II SK- (pBS, Stratagene, La Jolla, CA, U.S.A.) to create pTMH32.1. The HindIII/BamHI fragment from pTMH32.1 was then cloned into pTMH44.2 downstream of the gfp spacer to create pTMH46.1. A forward primer containing an NcoI site (aflR-NcoI) and a reverse primer containing an AscI site (aflR-AscI) were then used to amplify an approximately 670-bp fragment of aflR from pTMH46.1, which was subsequently cloned into pTMH46.1 upstream of the gfp spacer to create pTMH52.1 (Fig. 1A).

Construction of pTRM-tri6-Hyg. An approximately 610-bp fragment of tri6 from F. graminearum PH-1 was amplified from genomic DNA, using a forward primer containing an NcoI site and nested Smal site (tri6-NcoI/Smal) and a reverse primer containing an AscI site (tri6-AscI). This polymerase chain reaction (PCR) product was digested and ligated into the NcoI/AscI sites of pTMH44.2 to produce pTRM20. A similar approximately 610-bp fragment of tri6 was amplified by PCR, using a forward primer containing a HindIII and nested NotI site (tri6-HindI/NotI) and a reverse primer containing an NotI site (tri6-NotI). This PCR product was digested and ligated in reverse orientation into the HindIII/NotI sites in pTRM20 to produce pTRM-tri6 (Fig. 1A). Finally, the gpdA promoter, tri6 inverted repeat with gfp spacer, and trpC terminator were released from pTRM-tri6 with an EcoRI/Xbal digest, were filled in with Klenow polymerase (NEB, Beverly, MA, U.S.A.), and were ligated into Smal-digested pUCH2-8 (Alexander et al. 1998), to produce pTRM-tri6-Hyg. pUCH2-8 encodes hygroycin B phosphotransferase, which was the selectable marker used for transformation of Fusarium sp.

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<th>Name</th>
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<td>tri6-NotI</td>
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* Restriction sites are underlined.
Fungal transformation.

Aspergillus protoplasts were produced and transformed using the polyethylene glycol method (Miller et al. 1985). A. parasiticus and A. flavus protoplasts were cotransformed with pBZ5, a plasmid containing A. parasiticus pyrG (Skory et al. 1990) and pTMH52.1. Transformants were screened for uridine and uracil prototrophy and by Southern analysis for the presence of the aflR IRT. Fusarium protoplasts were produced and transformed as described by Butchko and associates (2003). Hygromycin resistant Fusarium transformants were then purified by dilution-plating, followed by hyphal tip isolation, and were then screened by Southern analysis to detect the trIT IRT.

Physiology.

Spore counts. A. flavus, A. parasiticus, and F. graminearum strains (5 ml of a 10^6 spores/ml suspensions) were spread-plated on five V8 media plates. The cultures were incubated at 29°C in a 12-h light and dark cycle for 5 days. For each plate, three randomly chosen 1.4-cm diameter cores were homogenized in 3 ml of water. Conidia or macroconidia were then counted.

Colony diameters. A. flavus, A. parasiticus, and F. graminearum strains (100 µl of a 10^5 spores/ml suspensions) were point-inoculated on V8 media. Plates were incubated at 29°C in a 12-h light and dark cycle for 5 days. Diameters of colonies from three replicates per strain were then averaged. All statistical analysis was performed with the Statistical Analysis System (SAS Institute, Cary, NC, U.S.A.).

Maize infections.

Maize seeds were surface-sterilized for 1 min in 10% Chlorox bleach solution, followed by a 1-min rinse with distilled water. Seeds were wounded in the embryo and were inoculated with 5 µl of a 10^5 spores/ml suspension of A. parasiticus. Ten seeds per replicate were transferred to a humid chamber consisting of moist filter paper on the bottom of a covered glass dish. A reservoir of distilled water was placed in the center of the dish. Seeds were moistened daily, and germinating seeds were transferred to a humid chamber containing 70:30 acetone and water, followed by an equal volume of acetone with a mortar and pestle. The resulting slurry was vortexed for 1 min. The mixture was incubated at 29°C. Versicolorin A was analyzed by dilution-plating, followed by hyphal tip isolation, and were then screened by Southern analysis to detect the trIT IRT.

Analysis of versicolorin A and aflatoxin production.

A. parasiticus and A. flavus cultures were point-inoculated on YES medium (2% yeast extract, 6% sucrose, pH 5.8) and were grown for 5 days at 29°C in a 12-h light and dark cycle. One 1.4-cm diameter core was harvested from the center of each plate and was homogenized in 1 ml of distilled water. Acetone and chloroform (1 ml each) were added, and the mixture was vortexed for 1 min. The mixture was incubated at room temperature for 1 h, was vortexed again, and was centrifuged for 10 min. The lower organic layer was removed and evaporated. Residue was resuspended in 500 µl of chloroform, and 10 µl of the suspension were spotted onto a TLC plate (Whatman Ltd., Kent, England). This experiment was done in triplicate. Versicolorin A was resolved with a toluene, acetic acid, and ethyl acetate solvent system (80:10:10), and aflatoxin was resolved in a chloroform and acetone (95:5) solvent system. Versicolorin A standards were previously prepared from extracts of A. parasiticus SRRC 164 (Keller et al. 1994), and aflatoxin standards were purchased from Sigma-Aldrich (St. Louis). Aflatoxin and versicolorin A were visualized using long-wave (366 nm) UV light, and digital photographs were taken and cropped using Adobe Photoshop 5.5 (Adobe Systems, Inc. San Jose, CA, U.S.A.). The auto balance function was used to facilitate viewing of the toxins in the digital pictures, thus slight differences might be perceived in the toxin colors between figures.

Wheat head blight assays and deoxynivalenol analysis.

Two greenhouse virulence tests were conducted in 2004 on cultivar Wheaton, essentially as described by Desjardins and associates (2004). F. graminearum inocula were generated by growth on mung bean liquid medium for 4 days at 29°C (Bai and Shaner 1976). The macroconidia were filtered through Miracloth (Calbiochem, EMD Biosciences, Inc., San Diego, CA, U.S.A.), were concentrated by centrifugation, were rinsed and suspended in water, and were counted using a hemocytometer (Improved Neubauer, Hauser Scientific, Horsham, PA, U.S.A.). Each fungal strain was inoculated into 10 heads by injecting approximately 10^7 macroconidia into one floret of a spikelet located in the lower third of each head. Control heads were injected with water. Disease severity was calculated as a percentage of blighted spikelets in each head 18 days after inoculation. Mature heads were threshed individually. Seeds collected from each of the 10 heads per treatment were combined and ground and were extracted with 4 ml of acetonitrile-water (86:14) per gram with shaking for 2 h. The solvent extract was filtered through Whatman filter paper and stored at 4°C until analyzed. The concentration of DON was determined by liquid chromatography-mass spectrometry as previously described (Plattner and Maragos 2003).

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Literature Cited


