RNA Silencing of Mycotoxin Production in *Aspergillus* and *Fusarium* Species

Tami McDonald,^{1,3} Daren Brown,² Nancy P. Keller,¹ and Thomas M. Hammond¹

¹ Department of Plant Pathology, University of Wisconsin, Madison, U.S.A.; ² USDA-ARS-NCAUR, Peoria, IL, U.S.A.

Submitted 20 October 2004. Accepted 22 January 2005.

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 atoxigenic 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 interception 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.*

Current address of T. McDonald: Program in Genetics, Duke University, Durham, North Carolina, U.S.A.

Corresponding author: N. P. Keller; Department of Plant Pathology, 1630 Linden Drive; Telephone: +1.608.262.9795; Fax: +1.608.263.2626; E-mail: npk@plantpath.wisc.edu

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 transformants (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 replications, *A. parasiticus* C27 produced high levels of versicolorin A, whereas versicolorin A was only detected, at a low level, in one of the IRT15 replications (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

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 = *AscI*; Ba = *BamHI*; No = *NotI*; Hi = *HindIII*; and X = *XbaI*. **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 (trichothecene) 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 tri6* sequences (Fig. 1A and C) was constructed. Transformation of *F. graminearum* R5317 with this IRT (pTRM-*tri6*-Hyg) (Fig. 1A) resulted in four strains that were shown by Southern analysis to carry the *tri6* IRT: NK8, NK9, NK10, and NK11 (data not shown). Transformants NK9, NK10, and NK11 integrated the *tri6* IRT at random ectopic locations distinct from the native *tri6* locus, whereas it appeared the *tri6* IRT may have integrated near the native *tri6* 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 *tri6* 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 *tri6* IRT or the *hygB* portion of pTRM-*tri6*-Hyg (data not shown). *F. graminearum* R5317, NK3, and the four *tri6* 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 *tri6* 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 tri6* 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).

Tri6 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 *tri6*-specific IRT did not have significant off-target

Fig. 3. An *Aspergillus aflR* inverted repeat transgene (IRT) inhibits expression of *vera* in *A. parasiticus*. Total RNA was isolated from *Aspergillus* strains grown for 18 h in aflatoxin-inducing medium, was separated by denaturing-gel electrophoresis, blotted to a nylon membrane, and hybridized to a probe specific for *vera*. The rRNA bands on the ethidium bromide-stained gel are shown to demonstrate the equal loading of RNA. P, parent strain CS10; C27 and C28, *pyrG*-only transformants; IRT15 and IRT33, *aflR* IRT transformants.

Fig. 2. An *aflR* inverted repeat transgene (IRT) inhibits mycotoxin production in *Aspergillus flavus* and *A. parasiticus*. **A**, *A. flavus* 8610 (parent strain), an *A. flavus pyrG*-only transformant (C1), and three *A. flavus aflR* IRT transformants (IRT3, IRT20, and IRT25) were analyzed for aflatoxin production in vitro by thin-layer chromatography (TLC). Qualitative amounts of aflatoxin B1 standard (Af) were loaded onto the TLC plate as a migration control. **B**, *A. parasiticus* CS10 (parent strain), two *A. parasiticus pyrG*-only transformants (C27 and C28), and two *A. parasiticus aflR* IRT transformants (IRT15 and IRT33) were analyzed for versicolorin A production in vitro by TLC. **C**, Maize was inoculated with *A. parasiticus* IRT15 or *A. parasiticus* C27. Versicolorin A production on the infected kernels was then analyzed by TLC. In B and C, qualitative amounts of versicolorin A standard were loaded as a migration control. Ve = versicolorin A standard. Ve appears light orange in B and dark orange in C due to contrast enhancements using photo-editing software. The blue compounds in B and C were not determined and are assumed to be unrelated to *A. parasiticus* aflatoxin production.

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.

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

Strain ^a	Average % blighted ^b	DON (ppm) ^c	Total seed mass ^d	Mass per seed ^e	Average % yield loss ^f
Experiment 1					
Water	0.0	Nd	10.6	33 (1.6)	0.0
R5317	33.2 (23.3)	3.7	8.1	25 (6.7)	24.2
NK3	33.7 (24.0)	4.4	7.7	27 (8.9)	18.2
NK8	6.3 (0.2)	Nd	10.8	34 (2.2)	+3.0
NK11	6.4 (0.4)	Nd	10.3	34 (2.0)	+3.0
Experiment 2					
Water	0.0	Nd	11.4	33 (3.2)	0.0
R5317	31.6 (22.1)	9.4	8.0	27 (5.7)	18.2
NK3	38.1 (26.1)	24.0	7.5	22 (7.2)	33.3
NK8	5.5 (0.2)	Nd	13.0	33 (3.2)	0.0
NK9	5.7 (0.5)	Nd	11.7	34 (1.6)	+3.0
NK10	5.7 (0.4)	Nd	13.3	33 (3.2)	0.0
NK11	5.8 (0.5)	Nd	12.4	34 (2.6)	+3.0

^a R 5317 = wild type, NK3 = transformant control, NK8-11 = *tri6* IRT strains.

^b Average percentage of blighted = average percentage of the wheat head that was blighted (10 heads).

^c DON = deoxynivalenol, measured in parts per million, Nd = not detected (<2 ppm).

^d Total seed mass = total mass of seeds harvested from 10 heads of one treatment (grams).

^e Mass per seed = average seed mass (milligrams).

^f 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 flanking 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 *afIR* 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. Trail). *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 72 h, then switched to an aflatoxin-inducing media, PMSG (PMS plus 6% glucose) 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 afIR* 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 fluorescent protein) was amplified from pPRgf-T4 (Zolotukhin et al. 1996) with a forward primer containing an *NcoI* site and nested *AscI* site (*Gfp5'NcoI/AscI*) and a reverse primer containing a *HindIII* site, nested *NotI* site, and nested *BamHI* site (*Gfp3'Hind/NotI/Bam*). 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 afIR* was amplified from pRB3 (R. A. Butchko and N. P. Keller, *unpublished data*), using a forward primer containing a *HindIII* site (*afIR-HindIII*) and a reverse primer containing a *BamHI* site (*afIR-BamHI*) and was cloned into pBluescript II SK- (pBS, Stratagene, La Jolla, CA, U.S.A.) to create pTHM32.1. The *HindIII/BamHI* fragment from pTHM32.1 was then cloned into pTMH44.2 downstream of the *gfp* spacer to create pTHM46.1. A forward primer containing an *NcoI* site (*afIR-NcoI*) and a reverse primer containing an *AscI* site (*afIR-AscI*) were then used to amplify an approximately 670-bp fragment of *afIR* from pTHM46.1, which was subsequently cloned into pTMH44.1 upstream of the *gfp* spacer to create pTHM52.1 (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 *SmaI* site (*tri6-NcoI/Sma*) 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 *SacI* site (*tri6-Hind/SacI*) 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/XbaI* digest, were filled in with Klenow polymerase (NEB, Beverly, MA, U.S.A.), and were ligated into *SmaI*-digested pUCH2-8 (Alexander et al. 1998), to produce pTRM-*tri6*-Hyg. pUCH2-8 encodes hygromycin B phosphotransferase, which was the selectable marker used for transformation of *Fusarium* sp.

Table 2. Primers used in this study

Name	Sequence ^a
Gfp5' <i>NcoI/AscI</i>	AACCATGGGGCGCGCGAGGGCTATGTG CAGGAG
Gfp3' <i>Hind/NotI/Bam</i>	TTAAGCTTGGCGGCCGACGGATCCATCCT CAATGTTG
<i>afIR-BamHI</i>	CGGATCCGCGGCTGGTCTTCC
<i>afIR-NcoI</i>	ACCATGGTCCCCGCCACTCG
<i>afIR-HindIII</i>	CAAGCTTCCCCGCCACTCGTG
<i>afIR-AscI</i>	AGGCGCGCCCTCGCGGCTGGTCTTC
<i>tri6-NcoI/Sma</i>	TTTCCATGGCGCCCGGGTCTCACT
<i>tri6-Hind/SacI</i>	TCACTACGAAGCTTGGAGCTCCTT
<i>tri6-AscI</i>	TCGGCGCGCCATGTTATCCACCCTG
<i>tri6-NotI</i>	TATGAGCGGCGCGCTAGTGATCTC

^a 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 *afIR* 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 *tri6* IRT.

Physiology.

Spore counts. *A. flavus*, *A. parasiticus*, and *F. graminearum* strains (5 ml of a 10^5 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% Clorox 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 petri dish. A reservoir of distilled water was placed in the center of the dish. Seeds were incubated in the dark at 29°C for 5 days. Seeds were moistened daily, and germinating seeds were removed daily. After 5 days, six seeds from each replicate were selected for versicolorin A analysis. Seeds were ground in acetone with a mortar and pestle. The resulting slurry was transferred to a 13-ml round-bottom tube, and then extracted once with 70:30 acetone and water, followed by an equal volume hexane wash to remove lipids. The resulting extract was removed to a clean vial and was dried and dissolved in 100 µl of chloroform, and chemical components were analyzed by TLC as described below.

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 28°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, Hausser Scientific, Horsham, PA, U.S.A.). Each fungal strain was inoculated into 10 heads by injecting approximately 10^3 macroconidium 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).

ACKNOWLEDGMENTS

We thank D. Shane, E. Mays, and R. Stessman for providing technical assistance. Funding for this project was provided by the University of Wisconsin Graduate School (Hatch Funds), the University of Wisconsin Alumni Research Foundation, and the U.S. Department of Agriculture under agreement number 59-0790-3-081 through a cooperative project with the U.S. Wheat and Barley Scab Initiative.

LITERATURE CITED

- Alexander, N. J., Hohn, T. M., and McCormick, S. P. 1998. The TRI11 gene of *Fusarium sporotrichioides* encodes a cytochrome P-450 monooxygenase required for C-15 hydroxylation in trichothecene biosynthesis. *Appl. Environ. Microbiol.* 64:221-225.
- Bai, G.-H., and Shaner, G. 1976. Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. *Plant Dis.* 80:975-979.
- Bai, G. H., Desjardins, A. E., and Plattner, R. D. 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia* 153:91-98.
- Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363-366.
- Buchanan, R. L., and Lewis, D. F. 1984. Regulation of aflatoxin biosynthesis: Effect of glucose on activities of various glycolytic enzymes. *Appl. Environ. Microbiol.* 48:306-310.
- Bushnell, W. R., Hazen, B. E., and Pritsch, C. 2003. Histology and physiology of *Fusarium* head blight. Pages 44-83 in: *Fusarium Head Blight of Wheat and Barley*. K. J. Leonard and W. R. Bushnell, eds. American Phytopathological Society, St. Paul, MN, U.S.A.
- Butchko, R. A., Plattner, R. D., and Proctor, R. H. 2003. *Fum13* encodes a short chain dehydrogenase/reductase required for C-3 carbonyl reduction during fumonisin biosynthesis in *Gibberella moniliformis*. *J. Agric. Food Chem.* 51:3000-3006.
- Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M. S., Vayssie, L., Macino, G., and Cogoni, C. 2004. Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Mol. Cell. Biol.* 24:2536-2545.

- Denli, A. M., and Hannon, G. J. 2003. RNAi: An ever-growing puzzle. *Trends Biochem. Sci.* 28:196-201.
- Desjardins, A. E., Brown, D. W., Yun, S. H., Proctor, R. H., Lee, T., Plattner, R. D., Lu, S. W., and Turgeon, B. G. 2004. Deletion and complementation of the mating type (MAT) locus of the wheat head blight pathogen *Gibberella zeae*. *Appl. Environ. Microbiol.* 70:2437-2444.
- Elbashir, S. M., Lendeckel, W., and Tuschl, T. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes. Dev.* 15:188-200.
- Fernandes, M., Keller, N. P., and Adams, T. 1998. Sequence-specific binding by *Aspergillus nidulans* AfIR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol. Microbiol.* 28:1355-1365.
- Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293:1146-1150.
- Hammond, T. M., and Keller, N. P. 2005. RNA silencing in *Aspergillus nidulans* is independent of RNA dependent RNA polymerases. *Genetics* 169:607-617.
- Hohn, T. M., Krishna, R., and Proctor, R. H. 1999. Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. *Fungal Genet. Biol.* 26:224-235.
- Kadotani, N., Nakayashiki, H., Tosa, Y., and Mayama, S. 2003. RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Mol. Plant-Microbe Interact.* 16:769-776.
- Keller, N. P., Kantz, N. J., and Adams, T. H. 1994. *Aspergillus nidulans* *verA* is required for production of the mycotoxin sterigmatocystin. *Appl. Environ. Microbiol.* 60:1444-1450.
- Keller, N. P., Segner, S., Bhatnagar, D., and Adams, T. H. 1995. *stcS*, a putative P-450 monooxygenase, is required for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans*. *Appl. Environ. Microbiol.* 61:3628-3632.
- Liang, S. H., Skory, C. D., and Linz, J. E. 1996. Characterization of the function of the *ver-1A* and *ver-1B* genes, involved in aflatoxin biosynthesis in *Aspergillus parasiticus*. *Appl. Environ. Microbiol.* 62:4568-4575.
- Liu, H., Cottrell, T. R., Pierini, L. M., Goldman, W. E., and Doering, T. L. 2002. RNA interference in the pathogenic fungus *Cryptococcus neoformans*. *Genetics* 160:463-470.
- Miller, B. L., Miller, K. Y., and Timberlake, W. E. 1985. Direct and indirect gene replacements in *Aspergillus nidulans*. *Mol. Cell Biol.* 5:1714-1721.
- Mouyna, I., Henry, C., Doering, T. L., and Latge, J. P. 2004. Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. *FEMS (Fed. Eur. Microbiol. Soc.) Lett.* 237:317-324.
- Pickford, A. S., and Cogoni, C. 2003. RNA-mediated gene silencing. *Cell Mol. Life Sci.* 60:871-882.
- Plattner, R. D., and Maragos, C. M. 2003. Determination of deoxynivalenol and nivalenol in corn and wheat by liquid chromatography with electrospray mass spectrometry. *J. Assoc. Off. Anal. Chemists Int.* 86:61-65.
- Proctor, R. H., Hohn, T. M., and McCormick, S. P. 1995a. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol. Plant Microbe Interact.* 8:593-601.
- Proctor, R. H., Hohn, T. M., McCormick, S. P., and Desjardins, A. E. 1995b. *Tri6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* 61:1923-1930.
- Punt, P. J., Zegers, N. D., Busscher, M., Pouwels, P. H., and van den Hondel, C. A. 1991. Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans* *gpdA* gene. *J. Biotechnol.* 17:19-33.
- Rapplee, C. A., Engle, J. T., and Goldman, W. E. 2004. RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. *Mol. Microbiol.* 53:153-65.
- Richard, J. L., and Payne, G. A. 2003. Mycotoxins: Risks in Plant, Animal, and Human Systems: Task force report No. 139. Council for Agricultural Science and Technology, Ames, IA, U.S.A.
- Shimizu, K., and Keller, N. P. 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* 157:591-600.
- Skory, C. D., Horng, J. S., Pestka, J. J., and Linz, J. E. 1990. Transformation of *Aspergillus parasiticus* with a homologous gene (*pyrG*) involved in pyrimidine biosynthesis. *Appl. Environ. Microbiol.* 56:3315-3320.
- Tag, A. G., Garifullina, G. F., Peplow, A. W., Ake, C., Jr., Phillips, T. D., Hohn, T. M., and Beremand, M. N. 2001. A novel regulatory gene, *tri10*, controls trichothecene toxin production and gene expression. *Appl. Environ. Microbiol.* 67:5294-5302.
- Waterhouse, P. M., Wang, M. B., and Lough, T. 2001. Gene silencing as an adaptive defence against viruses. *Nature* 411:834-842.
- Woloshuk, C. P., Foutz, K. R., Brewer, J. F., Bhatnagar, D., Cleveland, T. E., and Payne, G. A. 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 60:2408-2414.
- Yu, J. H., Butchko, R. A., Fernandes, M., Keller, N. P., Leonard, T. J., and Adams, T. H. 1996. Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. *Curr. Genet.* 29:549-555.
- Zolotukhin, S., Potter, M., Hauswirth, W. W., Guy, J., and Muzyczka, N. 1996. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J. Virol.* 70:4646-4654.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute website: www.broad.mit.edu
The Institute for Genomic Research (TIGR) databases: www.tigr.org