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Global gene regulation by *Fusarium* transcription factors *Tri6* and *Tri10* reveals adaptations for toxin biosynthesis

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Summary

Trichothecenes are isoprenoid mycotoxins produced in wheat infected with the filamentous fungus *Fusarium graminearum*. Some fungal genes for trichothecene biosynthesis (*Tri* genes) are known to be under control of transcription factors encoded by *Tri6* and *Tri10*. *Tri6* and *Tri10* deletion mutants were constructed in order to discover additional genes regulated by these factors *in planta*. Both mutants were greatly reduced in pathogenicity and toxin production and these phenotypes were largely restored by genetic complementation with the wild-type gene. Transcript levels for over 200 genes were altered ≥ twofold for *Δtri6* or *Δtri10* mutants including nearly all known *Tri* genes. Also reduced were transcript levels for enzymes in the isoprenoid biosynthetic pathway leading to farnesyl pyrophosphate, the immediate molecular precursor of trichothecenes. DNA sequences 5′ to isoprenoid biosynthetic genes were enriched for the Tri6p DNA binding motif, YNAGGCC, in *F. graminearum* but not in related species that do not produce trichothecenes. To determine the effect of trichothecene metabolites on gene expression, cultures were treated with trichodiene, the first metabolic intermediate specific to the trichothecene biosynthetic pathway. A total of 153 genes were upregulated by added trichodiene and were significantly enriched for genes likely involved in cellular transport. Differentially regulated genes will be targeted for functional analysis to discover additional factors involved in toxin biosynthesis, toxin resistance and pathogenesis.

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Introduction

Trichothecenes are a large group of toxic sesquiterpene epoxides produced as secondary metabolites by certain species of fungal genera *Fusarium*, *Myrothecium*, *Stachybotrys* and others (Bennett and Klich, 2003). They all share a 12, 13-epoxy-trichothec-9-ene (EPT) structure and their structural diversity arises from the position, number and types of functional groups attached to the EPT skeleton. Trichothecenes pose a health threat to human and animals as most induce toxic effects such as emesis, oral lesions, dermatitis and haemorrhaging (Pestka and Smolinski, 2005). They also have been implicated in incidents of systemic immuno-suppression and cancer (Bimczok *et al.*, 2007; Sakai *et al.*, 2007). Besides their effects on animals, trichothecenes also are virulence factors for fungi pathogenic to plants (Proctor *et al.*, 1997; Jansen *et al.*, 2005).

In addition to the complete genomic sequence of *Fusarium graminearum* (Cuomo *et al.*, 2007) and *F. verticillioides* as well as several *Fusarium* gene expression and sequence databases (Brown *et al.*, 2005 and others), many of the mycotoxin biosynthetic and regulatory genes have been identified. In *Fusarium sporotrichioides* and *F. graminearum*, genes involved in trichothecene biosynthesis have been shown to reside at more than one locus (Kimura *et al.*, 1998a; 2003a; Brown *et al.*, 2001; Jurgenson *et al.*, 2002; Meek *et al.*, 2003; Alexander *et al.*, 2004). The biosynthetic pathway for trichothecenes has been studied in detail and nearly all genes involved in trichothecene biosynthesis (*Tri* genes) have been identified (Desjardins *et al.*, 1993; Kimura *et al.*, 2001; 2007). Most *Tri* genes have been found within the 25 kb *Tri5* cluster (Kimura *et al.*, 2003b). Two trichothecene genes also have been found in a two-gene mini-cluster (*Tri1*-cluster) (Brown *et al.*, 2003; Peplow *et al.*, 2003; McCormick *et al.*, 2004) while another gene, *Tri101*, has been

identified at a separate locus (Kimura *et al*., 1998a). Nevertheless, not all of the genes necessary for trichothecene biosynthesis have been identified and the exact function of a few *Tri5* cluster genes have yet to be clarified (Kimura *et al*., 2007).

Among the characterized *Tri5* cluster genes involved in trichothecene biosynthesis, *Tri6* and *Tri10* were first shown to be regulators capable of influencing gene expression of other trichothecene biosynthetic genes in *F. sporotrichioides*. *Tri* genes, both within and outside the *Tri5* cluster, genes for three enzymatic steps in the isoprenoid biosynthetic pathway as well as many unidentified genes, were found to be controlled by these global regulators (Proctor *et al*., 1995a; Tag *et al*., 2001; Peplow *et al*., 2003). These results suggest that examining genes whose expression is controlled by *Tri6* and *Tri10* may lead to discovery of the currently unidentified genes important for trichothecene biosynthesis.

Fusarium graminearum is one of several fungi for which a whole genome sequence assembly is available (Galagan *et al*., 2005) and an Affymetrix oligonucleotide microarray has been designed to explore the transcriptome of this organism (Güldener *et al*., 2006a). To understand genome-wide regulatory control of toxin synthesis, we generated gene deletion mutants of *Tri6* and *Tri10* in *F. graminearum* and monitored gene expression in each mutant during plant infection when trichothecenes normally are produced. We found that the relationship between *Tri6* and *Tri10* in *F. graminearum* during plant infection was different from that previously described for *F. sporotrichioides* in culture. In addition to playing a more important role than *Tri10* in regulating trichothecene and isoprenoid biosynthesis, *Tri6* also controls the expression of many other genes that are related to housekeeping functions, secondary metabolism and pathogenesis. Our data also indicated that *Tri6* negatively regulated the expression of *Tri10* but deletion of *Tri10* had no obvious effect on *Tri6* expression. Furthermore, we found that putative Tri6p-binding sequences were enriched in the promoters of the genes of the isoprenoid biosynthetic pathway in *F. graminearum* but not in other non-trichothecene-producing *Fusarium* species. The co-regulation of isoprenoid and trichothecene biosynthesis by *Tri6* may represent an evolutionary adaptation in *F. graminearum*, which deploys trichothecene mycotoxins as important virulence factors.

Results

Generation of Tri6 *and* Tri10 *deletion mutants*

Tri6 and *Tri10* deletion mutant strains (PH1*Δtri6* and PH1*Δtri10*) were generated in the wild-type strain PH-1 by ligation-PCR mutagenesis (Figs S1 and S2). Strains containing integration of the deletion construction at an ectopic site in PH-1 were also generated. The mutations in PH1*Δtri6* and PH1*Δtri10* were complemented by re-introducing the wild-type allele at an ectopic locus by genetic transformation resulting in complement PH1*Δtri6* (PH1*Δtri6/Tri6*) and complement PH1*Δtri10* (PH1*Δtri10/Tri10*) strains. The pathogenicity of all strains was tested by point-inoculation of wheat spikelets. The mean number of symptomatic spikelets was significantly reduced for plants inoculated with either mutant compared with wild type (Table 1; Fig. 1). Each mutant could infect the tissue and cause localized necrosis at the point of inoculation but was greatly reduced in its ability to cause symptoms beyond the inoculated spikelet. Ectopic transformants that retain wild-type *Tri6* and *Tri10* loci were not significantly reduced in pathogenicity compared with PH-1 (data not shown). Complement strains PH1*Δtri6/Tri6* and PH1*Δtri10/Tri10* recovered the disease phenotype and were not significantly reduced in symptom expression compared with PH-1 under tested conditions. Reduction of pathogenicity in PH1*Δtri6* and PH1*Δtri10* was mirrored by reduction of trichothecene levels accumulating *in planta*. Plant tissue inoculated with PH1*Δtri10* accumulated < 2% of the deoxynivalenol (DON) concentration of

Table 1. Pathogenicity and trichothecene accumulation on wheat inoculated with wild-type and mutant strains.

		Trichothecene concentration[b]		
Strain	Pathogenicity[a]	DON	3ADON	15ADON
PH-1	8.10 ± 1.09	282 ± 83.3	4.09 ± 2.01	57.9 ± 24.7
PH1*Δtri6*	1.35 ± 0.51[c]	nd	nd	nd
PH1*Δtri6/Tri6*	7.63 ± 1.52	141 ± 41.0[c]	2.15 ± 0.81[c]	24.0 ± 10.0[c]
PH1*Δtri10*	1.40 ± 0.67[c]	4.89 ± 3.02[d]	nd	0.40 ± 0.28[d]
PH1*Δtri10/Tri10*	5.88 ± 1.45	169 ± 59.9[c]	2.22 ± 0.83[c]	46.0 ± 25.2[c]

a. Mean number of wheat spikelets per inflorescence exhibiting necrotic symptoms 14 days after inoculating a single central spikelet.
b. Mean and standard deviation of the concentration (μg g^{-1} dried infected tissue) of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON) or 15-acetyldeoxynivalenol (15ADON) 14 days after inoculation. nd, not detected (< 0.1 μg $^{-1}$).
c. Mean significantly less than value for PH-1 ($P < 0.05$).
d. Mean significantly less than value for PH-1 ($P < 0.001$).

tissue inoculated with wild type, 14 days after inoculation. Tissue inoculated with PH1*Δtri6* resulted in no detectable trichothecene accumulation under the same conditions. Strains PH1*Δtri6/Tri6* and PH1*Δtri10/Tri10* were largely able to recover the ability to produce toxins although accumulated less than that for the wild type, perhaps due to the introduction of the functional alleles at ectopic sites. Strains derived from ectopic integration of the *Tri6* or *Tri10* deletion vectors that retained the wild-type *Tri6* and *Tri10* genes were not significantly reduced in trichothecene accumulation ($P = 0.088$ and $P = 0.202$ respectively, data not shown), indicating that the process of genetic transformation itself does not alter toxin accumulation.

Tri *gene expression in* Δtri6 *and* Δtri10 *mutants*

Deoxynivalenol is synthesized in multiple steps beginning with the cyclization of farnesyl pyrophosphate mediated by trichodiene synthase (encoded by *Tri5*), which is the first step unique to DON biosynthesis. The pathway to DON is catalysed by at least six additional enzymes encoded by the genes *Tri4, Tri101, Tri11, Tri3, Tri1* and *Tri8* (Fig. 2). In addition to *Tri6* and *Tri10*, one transmembrane transporter (*Tri12*) and genes of unknown function (*Tri9* and *Tri14*) also may be involved in DON biosynthesis and/or virulence. During plant infection, the expression levels of nearly all known trichothecene genes (*Tri1, Tri3, Tri4, Tri5, Tri8, Tri9, Tri11, Tri12, Tri14* and *Tri101*) were reduced at least twofold with PH1*Δtri6* compared with the wild type (Fig. 2, Table S1). Indeed, the expression of the *Tri3, Tri5, Tri11, Tri12* and *Tri14* genes were not detected at significant levels in PH1*Δtri6* at all, suggesting a stringent regulation of those genes by *Tri6*. Conversely, the expression level of *Tri10* was significantly increased in PH1*Δtri6* compared with expression in the wild type suggesting a negative regulation of *Tri10* by *Tri6*.

The expression levels of most trichothecene biosynthetic genes also were reduced in PH1*Δtri10* in comparison with the wild type but to a lesser degree than in PH1*Δtri6* (Fig. 2; Table S2). Transcripts of most *Tri* genes were still abundant enough to be detected. Only the expression of *Tri9* was more reduced in PH1*Δtri10* than in PH1*Δtri6*; *Tri9* was not detected at significant levels in PH1*Δtri10*. Unlike the effect of *Δtri6* on *Tri10,* the expression of *Tri6* was not significantly altered in PH1*Δtri10*. Taken together, the results of *Tri* gene regulation are consistent with reduced trichothecene accumulation and pathogenicity in wheat infected with these mutants. Both *Tri6* and *Tri10* positively regulated the expression of other *Tri* genes during plant infection, but *Tri6* appeared to have a larger effect than *Tri10*. Results of the effect *Tri6* and *Tri10* on expression of *Tri5* was confirmed by quantitative PCR (Fig. S3).

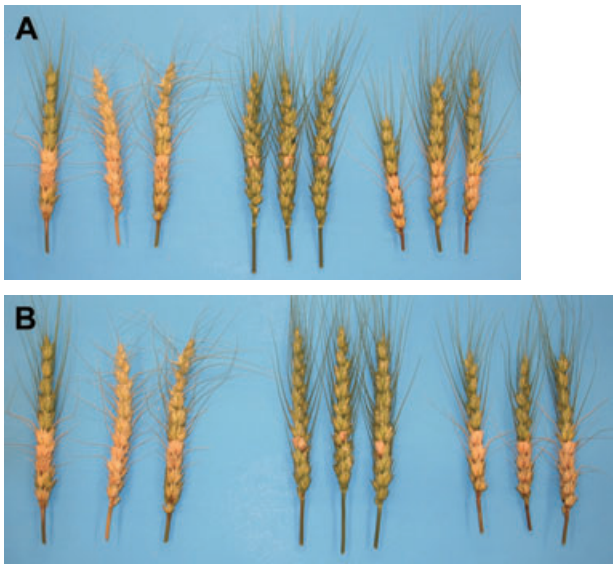

Fig. 1. Pathogenicity phenotypes of wild-type PH-1 and mutant strains. Wheat cultivar Norm was point-inoculated and incubated 14 days. Wheat heads inoculated with PH-1 (left three spikes – A and B) show tissue bleaching, deformed awns, and brown necrotic stem. Symptoms in plants inoculated with (A) PH1*Δtri6* (centre three) or (B) PH1*Δtri10* mutants are restricted to small necrotic lesions and deformed awns that are largely restricted to the inoculated spikelet. Plants inoculated with complement strains (A) PH1*Δtri6*/*Tri6* (right three) or (B) PH1*Δtri10*/*Tri10* are largely restored in pathogenicity and are able to spread beyond the point of inoculation.

Control of the expression of isoprenoid biosynthetic genes by Tri6 *and* Tri10

The immediate precursors to trichothecene biosynthesis are derived from the isoprenoid biosynthetic pathway (Fig. 3), which also leads to the production of primary metabolites such as sterols and haem by way of farnesyl pyrophosphate. Previous work in *F. sporotrichioides* has suggested that some genes involved in the isoprenoid biosynthetic pathway also may be regulated by *Tri6* and *Tri10* (Peplow *et al.*, 2003). We have found evidence from analysis of PH1*Δtri6* and PH1*Δtri10* during infection of wheat that *Tri6* significantly regulates the expression of genes encoding all eight enzymes catalysing the reaction steps from acetyl coenzyme A (CoA) to farnesyl pyrophosphate (Fig. 3), including acetyl CoA acetyltransferase, hydroxymethyl-glutaryl CoA synthase, hydroxymethyl-glutaryl CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase, isopentenyl pyrophosphate isomerase, and farnesylpyrophosphate synthetase. Similar to what was observed with *Tri* genes, *Tri6* appeared to have a larger effect than *Tri10* in the regulation of the isoprenoid biosynthesis genes (Fig. 3). All eight genes also had a reduced expression level in PH1*Δtri10* compared with the wild type but to a lesser degree than for PH1*Δtri6*. Only

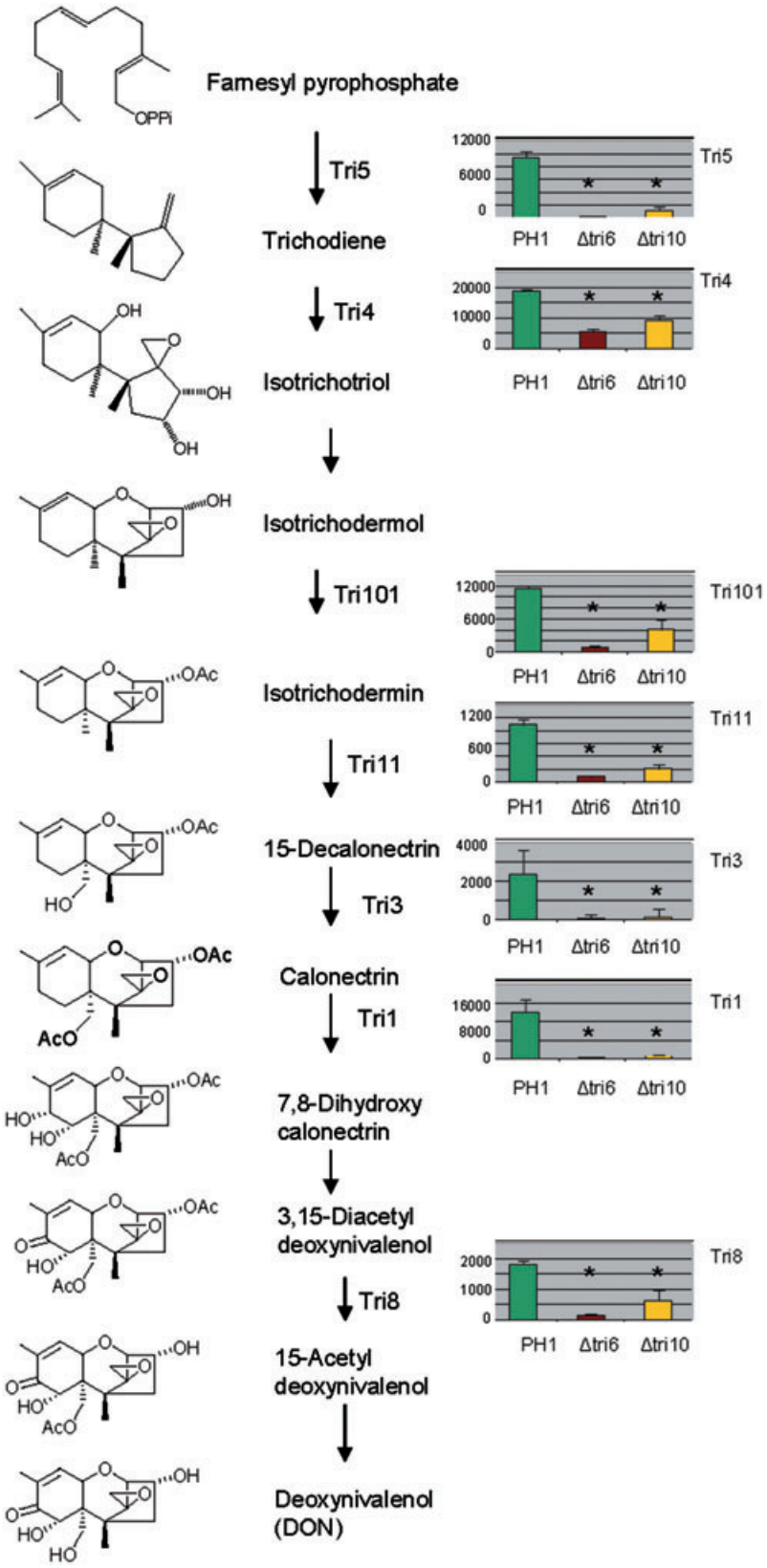

Fig. 2. Trichothecene biosynthetic pathway. A proposed pathway for trichothecene biosynthesis from farnesyl pyrophosphate to DON showing the genes known to encode enzymatically mediated steps. Histograms show relative expression levels of these genes in arbitrary units in wild type (green), and in PH1*Δtri6* (red) or PH1*Δtri10* (yellow) mutants. Asterisks indicate levels in the mutant are significantly reduced ($P < 0.05$) compared with wild type.

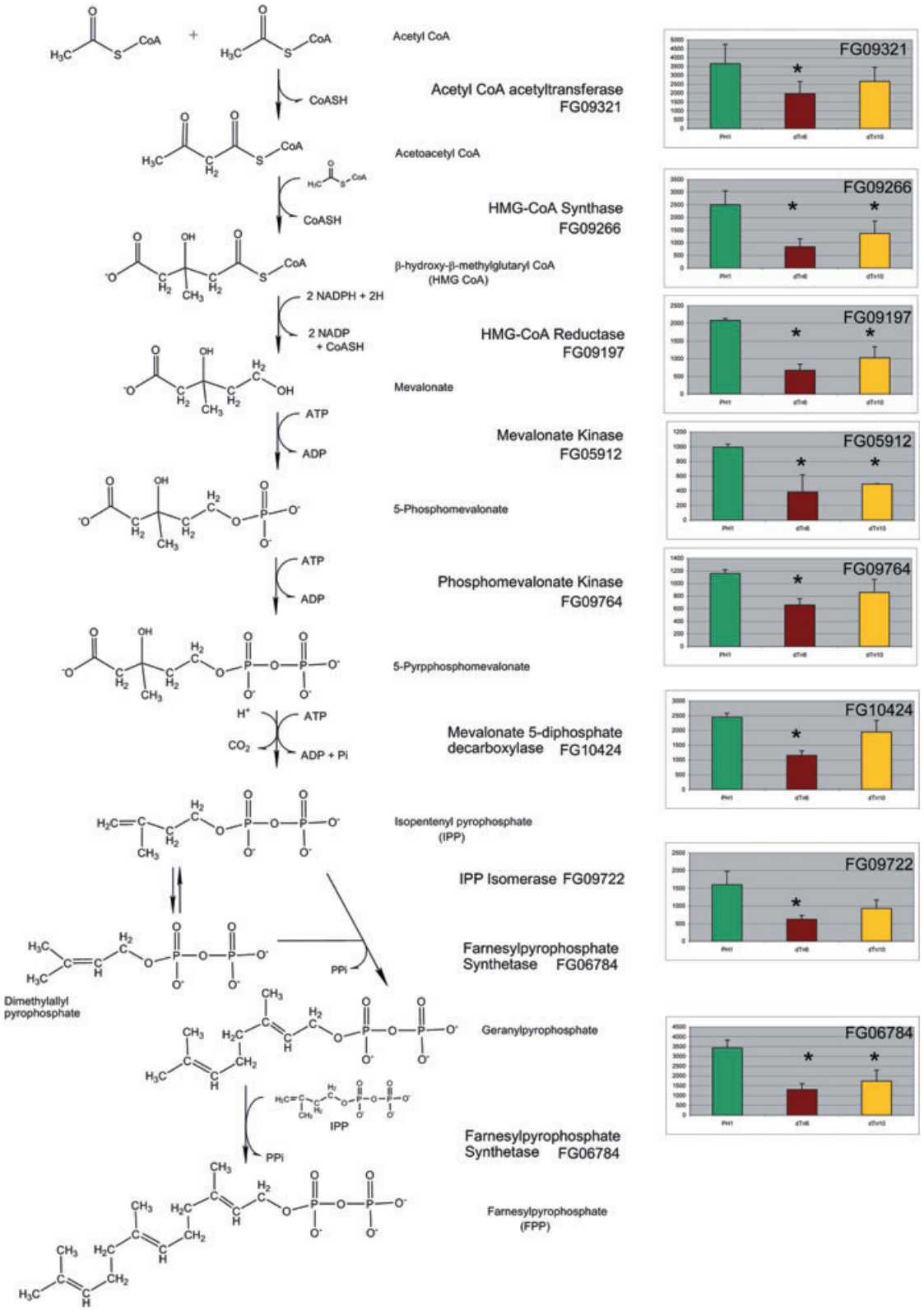

Fig. 3. Isoprenoid biosynthetic pathway. The pathway for isoprenoid biosynthesis from acetyl CoA to farnesyl pyrophosphate showing the genes known to encode enzymatically mediated steps. Histograms show relative expression levels of these genes in arbitrary units in wild type (green), and in PH1*Δtri6* (red) or PH1*Δtri10* (yellow) mutants. Asterisks indicate levels in the mutant are significantly reduced ($P < 0.05$) compared with wild type.

four of them, FG9266, FG09197, FG05912 and FG06784, had more than twofold reduction in PH1*Δtri10* in comparison with the wild type.

Genes other than Tri *genes regulated by* Tri6 *and* Tri10

In all, 208 genes were differentially expressed ≥ twofold in PH1*Δtri6* ($P < 0.04$) compared with wild type (Table S1). Among these, five genes had increased expression levels in PH1*Δtri6* whereas the remaining 203 genes were reduced in expression. Other than *Tri* genes, genes involved in isoprenoid biosynthesis (MIPS category 01.06.06; $P = 9.71e-06$) are the most significantly enriched category of genes differentially regulated by *Tri6*, followed by genes involved in disease virulence and defence (MIPS category 32.05; $P = 1.26e-04$), ABC transporters (MIPS category 20.03.25; $P = 5.39e-04$), and secondary metabolism (MIPS category 01.20; $P = 9.78e-04$).

Thirty-seven genes were differentially expressed ≥ twofold ($P > 0.04$) between wild-type strain and PH1*Δtri10* (Table S2). Among them, only one gene (Fg05754) was more highly expressed in PH1*Δtri10*. This gene has the highest sequence similarity to the *Magnaporthe grisea* pathogenicity gene *PTH11*. Of the 36 genes reduced ≥ twofold in the *Δtri10* mutant, seven were *Tri* genes and four were genes involved in the isoprenoid biosynthesis previously described. The remaining 25 are unclassified proteins of unknown function. In total, 27 out of 37 (73%) genes with altered expression levels in PH1*Δtri10* also had significant changes in expression levels in PH1*Δtri6* (Fig. 4). These observations further indicate that *Tri6* and *Tri10* must have overlapping yet distinct functions in *F. graminearum*. Besides playing a more important role than *Tri10* in regulating trichothecene and isoprenoid biosynthesis, *Tri6* also controls the expression of many other genes related to secondary metabolism and pathogenesis.

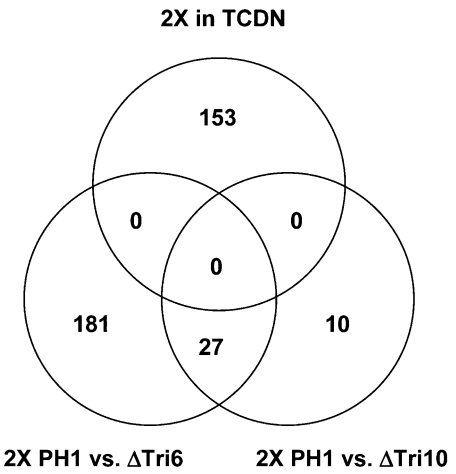

Fig. 4. Venn diagram comparing genes differentially regulated among three conditions. Genes with expression levels ≥ twofold higher in cultures treated with 250 μM trichodiene (TCDN) than untreated cultures (top), genes differentially expressed ≥ twofold in the *Δtri6* mutant versus wild type (below, left) or differentially expressed ≥ twofold in the *Δtri10* mutant versus wild type (below, right).

Promoter analysis

Using the binomial distribution to determine probabilities of word pattern frequency in the 1000 bp upstream sequences of genes controlled by *Tri6*, the motif AGGCC was among the most highly enriched ($P = 9.6e-07$). This sequence is at the core of the minimum DNA binding motif YNAGGCC previously established for the Cys_2His_2 zinc finger transcription factor *Tri6*p in *F. sporotrichioides* (Hohn *et al.*, 1999). The sequence YNAGGCC also was significantly overrepresented ($P = 0.0097$) 5′ to *F. graminearum* genes regulated by *Tri6* compared its occurrence upstream of all genes, indicating that the *Tri6*p binding domain is conserved between these trichothecene producing species. YNAGGCC was found 5′ to all *F. graminearum Tri* genes (Fig. S4) except *Tri10*, the only *Tri* gene not positively regulated by *Tri6*. This sequence also was found 5′ to all genes of the *F. graminearum* isoprenoid biosynthetic pathway (Fig. S5) where it was significantly enriched ($P = 0.001$). The sequence was not enriched upstream of the orthologous genes in *F. oxysporum* ($P = 0.594$) or *F. verticillioides* ($P = 0.434$), species which do not produce trichothecenes and lack a *Tri6* orthologue. Enrichment for YNAGGCC in the promoters of the genes of the isoprenoid pathway in *F. graminearum* thus is not likely to have occurred by chance ($P = 0.001$). We interpret this enrichment as a selectively favourable adaptation allowing for modulation of this pathway by *Tri6*.

Of the 203 genes positively regulated by *Tri6*, 116 genes have at least one occurrence of YNAGGCC in their upstream sequence (Table S3). All five genes negatively regulated by *Tri6* lack the word in their upstream sequence. The 87 genes positively regulated by *Tri6* but lacking the YNAGGCC motif, likely are influenced by indirect effects of the mutant on cellular metabolism and physiology.

Gene expression in response to trichodiene feeding

While genes may be altered in expression by *Tri6* and *Tri10* via direct transcriptional activation, regulation might also be mediated by metabolites that are end-products or intermediates in the trichothecene biosynthetic pathway. To address the possibility of metabolite feedback regulation, the first intermediate specific to trichothecene biosynthesis, trichodiene (Fig. 2), was added to cultures of a *tri5* knockout mutant (Proctor *et al.*, 1995b) that is inca-

pable of synthesizing trichothecenes *de novo* from farnesyl pyrophosphate. All other genes involved in trichothecene biosynthesis are intact in the *tri5* mutant and DON accumulated in these cultures indicating that exogenously added trichodiene was taken up and metabolized to DON (data not shown). We thus infer that genes with different expression levels in the *Δtri5* mutant in the presence or absence of exogenous trichodiene are likely to specifically respond to metabolites unique to the trichothecene biosynthetic pathway.

Surprisingly, expression levels for most *Tri* genes were not significantly affected by addition of 250 μM trichodiene to the *tri5* mutant. However, *Tri9*, *Tri10* and *Tri14*, were significantly upregulated in the presence of trichodiene (although < twofold).

Overall, 153 genes were increased in expression ≥ twofold upon addition of trichodiene compared with expression in the absence of trichodiene. Among the genes upregulated were eight that potentially encode transmembrane transporters (Table S4) including Fg03033 (related to a fluconazole resistance protein), Fg03882 (a probable ABC transport), and Fg01997 (manually annotated by MIPS as Fg13356 – related to tetracycline efflux protein, *otrb*). Interestingly, the gene for the presumptive trichothecene efflux transporter, *Tri12*, was not significantly upregulated by trichodiene. In fact, none of the 218 genes downregulated ≥ twofold in PH1*Δtri6* and/or *Δtri10* mutants *in planta* were among those upregulated in the presence of added trichodiene in culture (Fig. 4).

Discussion

Genes encoding the enzymes required for the biosynthesis of many secondary metabolites are physically clustered in fungal genomes. Besides the well-known gene clusters for trichothecene (Brown *et al*., 2001), zearalenone (Kim *et al*., 2005; Gaffoor and Trail, 2006; Lysøe *et al*., 2006) and aurofusarin (Malz *et al*., 2005) in *F. graminearum*, fungal genes for synthesis of penicillin (Hoffmeister and Keller, 2007), melanin (Kimura and Tsuge, 1993), HC-toxin (Ahn and Walton, 1996), stergmatocystin and aflatoxin (Yu *et al*., 1995; Brown *et al*., 1996), gibberellins (Tudzynski *et al*., 1998), ergot alkaloids (Tudzynski *et al*., 1999), AK-toxin (Tanaka *et al*., 1999), lovastatin (Kennedy *et al*., 1999), fumonisin (Seo *et al*., 2001), sirodesmin (Gardiner *et al*., 2004) and epipolythiodioxopiperazines (Patron *et al*., 2007) are clustered. While it is still unclear why fungal genes for secondary metabolites are clustered, models involving lateral gene transfer (Rosewich and Kistler, 2000) or the necessity of clustering for co-regulation of genes specific to the biosynthetic pathway (Zhang *et al*., 2004) have been proposed.

In several *Fusarium* species, genes involved in the trichothecene biosynthesis pathway have been found outside the main *Tri5* biosynthetic cluster (Kimura *et al*., 1998b; McCormick *et al*., 1999; Meek *et al*., 2003). Nevertheless, those genes outside the main cluster may be regulated by the transcription factors *Tri6* and *Tri10*, the two genes found flanking *Tri5* within the cluster (Tag *et al*., 2001; Peplow *et al*., 2003). The fact that some genes essential for trichothecene biosynthesis are not clustered, yet are co-regulated with those within the cluster, indicates that gene position may not be critical for appropriate trichothecene gene regulation.

Co-regulation of trichothecene genes outside the main biosynthetic cluster implies that the currently unknown trichothecene biosynthetic genes may be discovered on the basis of their co-regulation. Undiscovered biosynthetic genes may include those mediating the steps from 7, 8-dihydroxy calonectrin to 3, 15-diacetyl deoxynivalenol and from 15-acetyl deoxynivalenol to DON (Fig. 2). The strategy using co-regulation to identify new *Tri* genes was taken previously, leading to the isolation of several genes that were positively regulated by *Tri6* and/or *Tri10* (Tag *et al*., 2001; Peplow *et al*., 2003). Of the eight genes whose expression was previously reported to be influenced by *Tri10* (*Ibt1* – *Ibt8*) (Peplow *et al*., 2003), only *Ibt2* (Fg02218 – a predicted alpha/beta hydrolase fold protein) was confirmed by us to be regulated by both *Tri6* and *Tri10* in *F. graminearum*. Due to the technical limitations of differential cDNA library screening, only 22 transcripts and 26 transcripts, respectively, were previously reported to be regulated by *Tri6* or *Tri10*. By comparison, using hybridization to the *F. graminearum* Affymetrix GeneChip microarray (Güldener *et al*., 2006b), a larger number of differentially regulated genes in *F. graminearum* were detected: 208 genes were expressed ≥ twofold more in the wild-type strain compared with PH1*Δtri6* and 38 genes were expressed ≥ twofold more in the wild-type strain compared with PH1*Δtri10* ($P > 0.04$). Together 27 genes were expressed ≥ twofold in the wild-type strain compared with both *Δtri6* and *Δtri10* mutant strains.

The category of genes most significantly enriched among those positively regulated by *Tri6* were genes involved in isoprenoid metabolism. Previous reports proposed that genes for the isoprenoid biosynthetic enzymes acetyl CoA acetyltransferase (*ACAT*), hydroxymethylglutaryl CoA synthase, mevalonate kinase and farnesyl pyrophosphate synthetase were under transcriptional control of both *Tri6* and *Tri10* in *F. sporotrichioides* based on northern hybridization data (Tag *et al*., 2001; Peplow *et al*., 2003). Our data are consistent with these results except for *ACAT* regulation by *Tri10*; expression of the *ACAT* gene in *F. graminearum* (Fg09321) was reduced in PH1*Δtri10* but only at a significance *P*-value of 0.13. Additionally, we have also demonstrated that all eight genes

encoding the enzymes mediating isoprenoid biosynthesis are significantly regulated by *Tri6*. These additional genes include 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMR1 – Fg09197) which we have previously identified among random mutants screened for loss of pathogenicity (Seong *et al*., 2005; 2006). The HMR1 mutants studied resulted in a truncated gene transcript and were shown to be greatly reduced in trichothecene biosynthesis and pathogenicity. These results, together with the reduced pathogenicity of both PH1*Δtri6* and PH1*Δtri10*, indicate that proper regulation and metabolic flow through the isoprenoid pathway are essential for efficient accumulation of trichothecenes *in planta* and pathogenicity to wheat.

Promoters of the genes for both the isoprenoid and trichothecene biosynthetic pathways show adaptation for regulation by *Tri6*. The minimal Tri6p binding sequence YNAGGCC is found, usually in multiple copies, between −100 and −800 bp of the translational start site for all *Tri* genes except *Tri10*, the one *Tri* gene not significantly regulated by *Tri6*. Taken together the −1000 bp 5′ to the *Tri* genes are significantly enriched ($P = 0.000015$) for the *Tri6* binding sequence when compared with the −1000 bp 5′ to all genes in the genome. YNAGGCC is also found in all −1000 bp sequences of the genes in the isoprenoid pathway and is found in much greater abundance than would be expected by chance ($P = 0.0012$). The sequence was not enriched 5′ to the orthologous genes in the related fungi, *F. oxysporum* and *F. verticillioides*, fungi which lack *Tri6* and that do not produce trichothecenes. Using the criteria of regulatory sequence distribution (Hulzink *et al*., 2003) on the list of genes positively regulated by *Tri6*, it is possible to reduce the number genes that are candidates for direct regulation by this transcription factor.

Other categories of genes significantly enriched among those positively regulated by *Tri6* were those involved in disease, virulence and defence, C-compound and carbohydrate metabolism, ABC transporters and secondary metabolism. Among the regulated disease and virulence genes were *Tox1* and *Tox2* (Fg00060, Fg00061) that are related to the *Ustilago maydis*, mycovirus encoded killer toxin Kp4 (Gu *et al*., 1995). Members of this fungal family of toxins specifically inhibit voltage-gated calcium channels and may be toxic to other fungi (Gage *et al*., 2001) or to plants (Allen *et al*., 2008). Such gene products might be expected to contribute to the virulence of the pathogen, although not directly mediated by trichothecenes. Among regulated genes for C-compound and carbohydrate metabolism is a gene encoding acetyl CoA synthetase (Fg01743) which catalyses the synthesis of acetyl CoA, the precursor to the isoprenoid biosynthetic pathway feeding into trichothecene synthesis. On the other hand, genes for the pyruvate dehydrogenase complex, which produces acetyl CoA for the Krebs-TCA cycle, were not regulated by *Tri6*.

Among genes for secondary metabolism regulated by *Tri6* is a cluster of four genes centred on *NPS1* (Fg11026), the non-ribosomal peptide synthetase involved in intracellular siderophore accumulation (Tobiasen *et al*., 2007). A flanking gene, Fg11025, also known as *Tri15*, encodes a predicted zinc finger transcription factor that was originally described based on its induction by T-2 toxin and co-regulation with genes of the *Tri5* biosynthetic cluster. However, disruption of *Tri15* does not alter accumulation of trichothecenes in *F. sporotrichioides* (Alexander *et al*., 2004). Because of this and its proximity to and co-regulation with *NPS1*, we predict that *Tri15* actually may be a transcription factor associated with the siderophore biosynthesis gene cluster.

Also noticeable among the genes regulated by *Tri6* were many potentially encoding drug- or toxin-resistance transporters, ABC transporters and/or other genes involved in detoxification (Table S1). Four predicted ABC transport proteins (Fg07383, Fg08309, Fg08830 and Fg11028) and a major facilitator superfamily (MFS) transporter (Fg07802) were upregulated by *Tri6*. (Fg11028 is part of the co-regulated NPS1 gene cluster.) The trichothecene biosynthetic cluster also contains a gene for a known trichothecene efflux protein (another MFS transporter, *Tri12*, Fg03541) strongly regulated by *Tri6* and *Tri10*. However, deletion of *Tri12* in *F. sporotrichioides* resulted in only a partial decrease in trichothecene resistance suggesting that additional genes involved in self-protection likely exist (Alexander *et al*., 1999; Tag *et al*., 2001). Further functional characterization of MFS or ABC transport proteins regulated by *Tri6* will determine whether they are the additional self-protection mechanisms that operate during plant infection.

In addition to regulation by *Tri6* and *Tri10 in planta*, 153 genes were differentially expressed after addition of trichodiene, a key intermediate of the trichothecene biosynthesis pathway, to cultures of the fungus having a *Δtri5* mutation and thus incapable of making endogenous trichothecenes. Surprisingly there was no overlap between the group of genes differentially expressed ≥ twofold by *Tri6* or *Tri10* during wheat infection and those regulated by trichodiene in culture. Exogenous trichodiene was taken up by cells because it was metabolized to 15-acetyl DON (data not shown). Therefore, it appears that *F. graminearum* cells cope with DON very differently when it is encountered in the environment in culture versus when it is produced within the cell *in planta*.

Transcriptional activation by *Tri6* is the central element of our model for regulation of trichothecene biosynthesis in *F. graminearum* during plant infection (Fig. 5). *Tri6* is essential for proper transcript levels for all genes of the trichothecene and isoprenoid biosynthetic pathway as

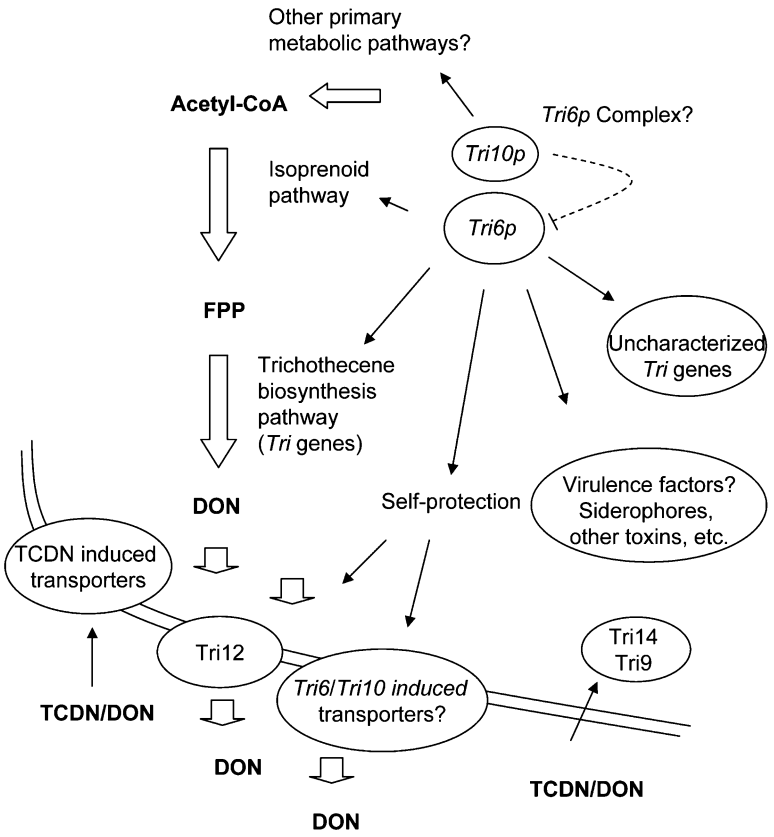

Fig. 5. Model of global regulation by *Tri6* and *Tri10*. Tri6p and Tri10p, perhaps as a transcriptional protein complex, regulate an overlapping set of genes involved in trichothecene and isoprenoid biosynthesis. Additionally regulated are known and predicted transporters involved in trichothecene efflux and tolerance, predicted siderophore and toxin gene clusters, and presumptive, uncharacterized Tri genes. Extracellular trichothecene compounds such as trichodiene (TCDN) appear to upregulate a non-overlapping set of transporters and other genes that may be responsive to the fungal environment.

well as genes upstream feeding acetyl CoA biosynthesis. It is also required for proper expression of the gene for the trichothecene efflux transporter, *Tri12,* and likely other genes for trichothecene export that may be necessary for avoiding the inhibitory effects of the toxin. Furthermore, *Tri6* is important for expression of *Tri14* which has been postulated to be involved in toxin export or to function as another component of the *Tri6p* transcriptional complex (Dyer *et al*., 2005). Scores of other genes including likely uncharacterized *Tri* genes are under positive control by *Tri6* as well. *Tri10* has a similar, yet less stringent influence on transcript levels for a largely overlapping set of genes with *Tri6*. We postulate that *Tri10p* serves as a component of the *Tri6p* transcriptional complex that intensifies but may only slightly modify its transcriptional effect.

Our model for regulation of trichothecene biosynthesis by *Tri6* and *Tri10* during plant infection differs from a previous model (Tag *et al*., 2001; Peplow *et al*., 2003) explaining trichothecene regulation in culture for *F. sporotrichioides*. The previous model proposed that *Tri10* is a transcription factor and the master regulator of trichothecene biosynthesis. By this model, *Tri10p* accepts signals for toxin production necessary for *Tri6* expression and subsequent activation of all *Tri* genes, the gene encoding farnesylpyrophosphate transferase, and hypothetical genes conferring toxin self-protection. *Tri6* also was proposed to negatively regulate *Tri10* in a regulatory feedback loop by binding *Tri6* recognition sites within the *Tri10* coding sequence (Tag *et al*., 2001; Peplow *et al*., 2003).

Our observation has been that during plant infection by *F. graminearum*, *Tri10* did not regulate *Tri6*; *Tri6* expression levels were not significantly reduced compared with wild type in PH1*Δtri10* ($P = 0.164$). However, *Tri6* did seem to influence *Tri10* gene expression as it does in *F. sporotrichioides*, although the increase in *Tri10* expression in a *Δtri6* background was relatively small (< twofold) but significant ($P = 0.015$). Also unlike in *F. sporotrichioides*, *F. graminearum Tri10* contains no *Tri6p* binding motifs either in its promoter or in its coding region. Therefore, in *F. graminearum*, *Tri10* does not appear to be a master regulatory factor but rather seems to function ancillary to *Tri6*, perhaps as a portion of a *Tri6p* transcription complex. Importantly our model proposes that *Tri6* may exert direct transcriptional control over a wide range of previously undescribed genes by directly binding DNA motifs found 5′ to *Tri* genes (except for *Tri10*), all genes for isoprenoid biosynthesis and other *Tri6* responsive genes currently of unknown function.

We have demonstrated that proper regulation of trichothecene biosynthetic and related genes by *Tri6* and *Tri10* is essential for full pathogenicity and the wild-type

accumulation of trichothecenes in wheat. The disease phenotypes of *Δtri6* and *Δtri10* mutants show localized necrosis at the point of infection with little or no spread of symptoms (Fig. 1), resembling that of *Δtri5* mutants incapable of producing trichothecenes altogether (Jansen *et al*., 2005). Efficient metabolic flow through the trichothecene biosynthetic pathway and the preceding isoprenoid pathway is essential for trichothecene accumulation *in planta*. Factors that alter this flow, whether mutations in genes for enzymes (Seong *et al*., 2006) or regulators of the pathways, result in near complete loss of pathogenic aggressiveness. Regulatory control of these pathways thus reflect evolutionary adaptation for pathogenicity and toxigenesis and present potential for disease control by perturbation any of a number of steps limiting to trichothecene accumulation.

Experimental procedures

Strains and culture condition

Fusarium graminearum strain NRRL 31084 (PH-1) and the deletion mutants generated in this study were grown at 25°C in liquid complete medium (Harris *et al*., 1994) or on V8 juice agar [200 ml V-8 juice (Campbell Soup Company, Camden NJ), 2 g $CaCO_3$, 15 g agar, water to 1 l]. Spores (macroconidia) were prepared as described previously (Seong *et al*., 2005). Spores grown in liquid medium for 5 days were harvested by centrifugation at 3500 r.p.m. for 5 min and washed twice with sterile distilled water prior to inoculation.

Fungal transformation, directed gene mutation and complementation

Protoplast preparation and fungal transformation were performed as described previously (Hou *et al*., 2002). V8 juice agar supplemented with 250 mg ml^{-1} hygromycin B (Calbiochem, La Jolla, CA) was used for isolation of transformants. The *Tri6* and *Tri10* genes were replaced with a gene conferring resistance to hygromycin B using the ligation-PCR approach (Zhao *et al*., 2004; Fig. S1). Using genomic DNA from strain PH-1 as a template, four primers (Table S1) were used for amplification of each flanking region for gene replacement of *Tri6*: CK_111, CK_112, CK_59 and CK_60. Another four primers (Table S1) CK_85, CK_86, CK_87 and CK_88 were used for amplification of each flanking region for gene replacement of *Tri10*. Hygromycin-resistant transformants were isolated and gene replacement mutations were confirmed by Southern hybridization (Fig. S2). Southern blots were probed with the gene for hygromycin B phosphotransferase amplified from pCX62 (Zhao *et al*., 2004) using primers HK_159 and HK_160 (Table S1). *Tri*6 deletion mutants were probed with the *Tri*6 coding region amplified from PH-1 genomic DNA using primers CK_130 and CK_131 (Table S1). *Tri*10 deletion mutants were probed with the *Tri10* coding region amplified from PH-1 genomic DNA using primers CK_132 and CK_133 (Table S1). DNA probes were directly labelled with alkaline phosphatase by denaturing amplified products into single strands and covalently attaching the enzyme to DNA with a cross-linker (GE HealthCare/Amersham Biosciences AlkPhos Direct Labeling System) and then detecting with CDP-Star (Amersham) as the chemiluminescent reagent.

Complementation of the tri6 and tri10 mutants

Using genomic DNA from strain PH-1 as a template, a 3 kb fragment containing the *Tri6* gene was amplified with primers Tri6-CF and Tri6-CR (Table S1). The BamHI and SalI sites introduced in the primers were underlined. The resulting PCR product was cloned between the BamHI and SalI sites of pHZ100, which was generated by cloning the neomycin resistant cassette into the XmnI site of pHZ100. For the *TRI10* gene, a 3.7 kb fragment was amplified with primers Tri10-CF and Tri10-CR (Table S1) and cloned into pHZ100 between the BamHI and NotI sites. The resulting complementation vectors pCTri6 and pCTri10 were transformed into protoplasts of the PH1*Δtri6* and PH1*Δtri10* deletion mutants respectively. Complemented strains were confirmed by Southern blot analyses (Fig. S2). Probes for blots were obtained using amplification of PH-1 genomic DNA with Tri6 NF and NR for *Tri6* and with Tri10 NF and NR for *Tri10* (Table S1).

Plant growth, inoculation, and mycotoxin analysis

Wheat plants (*Triticum aestivum*), cultivar Bobwhite, were grown as previously described (Goswami and Kistler, 2005). For RNA extraction, wheat spikelets, at mid-anthesis stage, were individually inoculated with 10 µl of conidial suspension of each strain in a 0.01% Triton ×–100 solution (10^6 spores ml^{-1}). After inoculation, wheat heads were placed in plastic bags for 48 h, after which inoculated wheat heads were incubated for an additional 48 h in a lighted growth chamber at 25°C. Infected wheat heads then were collected and immediately frozen at –80°C prior to RNA extraction. Four days after inoculation was chosen to study gene expression because at this time point DON is consistently accumulating in inoculated wheat yet has not reached its peak levels seen at later time points. For pathogenicity determination and mycotoxin measurement, single-point inoculated spikelets were scored for disease at 13–15 days after inoculation and then collected, weighed and stored at –20°C prior to mycotoxin analysis conducted as previously described (Goswami and Kistler, 2005).

Trichodiene feeding experiment

A trichodiene feeding experiment was conducted with three biological replications as follows. A *Tri5* knockout mutant of *F. graminearum* strain Z3639 (NRRL 29169) (Proctor *et al*., 1995b) was grown on V8 juice agar medium for 4 days. A 4 cm^2 agar block from the solid culture was macerated and inoculated in 50 ml of first-stage medium (3 g of NH_4Cl, 2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $FeSO_4 \cdot 7H_2O$, 2 g of KH_2PO_4, 2 g of peptone, 2 g of yeast extract, 2 g of malt extract, and 20 g of glucose in one litre of distilled water) in a 250 ml flask and grown for 3 days at 25°C in the dark with shaking at

150 r.p.m. Biomass was harvested by filtration with Miracloth and washed twice with sterile distilled water. The biomass was macerated and divided into two portions, each of which was used to individually inoculate 20 ml of second-stage production medium (1 g of $(NH_4)_2HPO_4$, 3 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 5 g of NaCl, 40 g of sucrose and 10 g of glycerol in 1 l of distilled water) in 250 ml flasks and grown under the same conditions as first-stage medium for 5 days. Trichodiene (final concentration 250 μM) was added to one flask upon transfer to the second stage medium. By 5 days, added trichodiene had consistently been converted to 15-acetyl deoxynivalenol.

Nucleic acid extraction, RNA labelling, and hybridization

Total RNA was isolated from infected wheat heads using TRIzol™ reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Total RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Ten micrograms of total RNA were treated according to the conventional Affymetrix eukaryotic RNA labelling protocols (Affymetrix, Santa Clara, CA). Prior to labelling, an Agilent 2100 bioanalyzer™ (Agilent, Palo Alto, CA) was used to examine the RNA quality. One GeneChip each was used for the three biological replications in each experiment. Hybridizations, washes, and chip reading followed standard Affymetrix procedures (http://www.bipl.ahc.umn.edu/) in use at the Biomedical Image Processing Facility at the University of Minnesota.

Analysis of microarray hybridization data

The hybridization signals were scanned with a GeneChip GCS 3000 scanner (Affymetrix, Santa Clara, CA) and the cell intensity (CEL) files were obtained from software GCOS 1.2 (Affymetrix, Santa Clara, CA). CEL files were loaded into the Expressionist Pro software version 1.0 (Genedata, San Francisco, CA). When present, defective areas on the chip and outliers were masked while the files were processed for further analysis. For experiments involving either fungal RNA or RNA from infected plants as a starting material, the Robust Multichip Analysis (RMA) algorithm (Irizarry *et al*., 2003) was used for condensing the data as implemented in the Refiner segment of Expressionist. Data were normalized using the RMA global normalization algorithm with the 100 control probe sets (having an 'AFFX' prefix) and 13 plant probe sets (with a 'Contig' prefix) as reference points. The above mentioned 113 probe sets and an additional 147 control probe sets which are not of fungal origin were subtracted from the *F. graminearum* gene list. Detection of RMA-normalized probe set signals was further validated using the Affymetrix MAS5.0 statistical algorithm (Hubbell *et al*., 2002) at a detection *P*-value < 0.04. A probe was called present when it was detected in at least two of the three replications at a detection *P*-value < 0.04 and was considered absent when it was not detected at $P < 0.04$ in any of the three replicates. Probe sets that yielded a present call at any time point of *Fusarium*-treated samples were selected and from them, ones that did not also satisfy an absent call in water inoculated control plants were subtracted. Probe set signal levels varying ≥ twofold between treatments and significance levels of differences as determined by *t*-tests were implemented in Expressionist. Data from microarray experiments are stored at PLEXdb (http://www.plexdb.org – Wise *et al*., 2007) under accession numbers as follows: gene regulation by *Fusarium* transcription factors *Tri6* and *Tri10* (accession number FG11); response to trichodiene treatment in *Fusarium graminearum* (accession number FG10).

qRT-PCR

RNA was purified as for microarray experiments and an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used for cDNA synthesis. *Tri5* was amplified using primers Tri5QF and Tri5QR (Table S1). The beta-tubulin gene of *F. graminearum* was amplified using primers TubQF and TubQR (Table S1) as the control. PCR reactions consisting of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C were performed with the MX3000 System (Stratagene, Cedar Creek, TX). Data were collected with the sequence Detector Software MXPro (Stratagene). Relative levels of *Tri5* transcripts were calculated by the comparative Ct method using the beta-tubulin gene as the endogenous reference and assigning the level of *Tri5* expression in the wild-type strain the value of 1 (Fig. S3).

Gene annotation and categorization

Gene prediction, annotation and assignment to functional category were conducted using the FGDB database as described previously (Güldener *et al*., 2006a). Genes identified as differentially regulated between wild type and mutants were categorized for function and a hypergeometric distribution was used to calculate the cumulative probability of each single category, drawing from the population of total genes in that category found in the genome as a whole.

Promoter analysis

To discover patterns in the promoter regions of selected gene sets, 1000 nucleotides upstream sequence of each gene were obtained from Broad Institute database (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/MultiDownloads.html) using a PERL script. Missing gene annotations were manually verified at MIPS FGDB (Güldener *et al*., 2006a). A word-based pattern discovery method was employed using the RSAT program (van Helden, 2003a,b), available online at http://rsat.ulb.ac.be/rsat/ whereby the patterns of tested oligonucleotide sequences are treated as 'words'. Background distribution of six, seven or eight character words was calculated based on the 1000 nucleotides upstream sequences for all predicted genes in the genome. Enrichment of motifs was calculated using a binomial distribution corrected by the RSAT program (*E*-value). Words with $E < 0.01$ (P-value < 0.0001) were considered. Sequence overlapping analysis and sequence common distribution on known *Tri* genes were the criteria for selecting the most probable sequence candidate for a regulatory role. Probability of over-representation in *Fusarium graminearum* and comparison with *F. oxysporum* and *F. verticillioides* genomes was calculated using two-tailed exact Fisher test available at

http://www.langsrud.com/fisher.htm. Background frequency was calculated on genome-wide 1000 bp upstream sequences of each species obtained from Broad Institute *Fusarium* database.

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