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Altered Regulation of 15-Acetyldeoxynivalenol Production in *Fusarium graminearum*

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Most *Fusarium graminearum* isolates produce low or undetectable levels of trichothecenes in liquid shake cultures, making it difficult to perform biochemical studies of trichothecene biosynthesis. To develop strains with higher levels of trichothecene production under liquid shake conditions we transformed *F. graminearum* with both a reporter gene containing a homologous trichothecene pathway gene promoter (*TRI5*) and a gene encoding a heterologous trichothecene pathway transcription factor (*TRI6*). The *TRI5* and *TRI6* genes are part of the trichothecene pathway gene clusters of both *Fusarium sporotrichioides* and *F. graminearum*. These genes encode trichodiene synthase (encoded by *TRI5*), the first enzyme in the trichothecene pathway, and a transcription factor (encoded by *TRI6*) required for pathway gene expression. Transformation of *F. graminearum* with plasmids containing either an *F. graminearum TRI5* promoter fragment (*FGTRI5^P*) or *FGTRI5^P* coupled with the β-D-glucuronidase (GUS) reporter gene resulted in the identification of several transformants capable of producing 45 to 200 mg of 15-acetyldeoxynivalenol (15-ADON)/liter in liquid shake culture after 7 days. Increased 15-ADON production was only observed in transformants where plasmid integration occurred through the *FGTRI5^P* sequence and was not accompanied by increased GUS expression. 15-ADON production was further increased in liquid culture up to 1,200 mg/liter following introduction of the *F. sporotrichioides TRI6* gene (*FSTRI16*) into *F. graminearum*. The effects of *FSTRI6* on 15-ADON production also depended on plasmid integration via homologous recombination of the *FGTRI5^P* fragment and resulted in a 100-fold increase in GUS expression. High-level production of 15-ADON in liquid shake cultures provides a convenient method for large-scale trichothecene preparation. The results suggest that targeting transformation vector integration to *FGTRI5^P* alters pathway gene expression and are consistent with the proposed conservation of TRI6 function between *Fusarium* species.

Fusarium graminearum Schwabe (*Gibberella zeae* [Schw.] Petch) is an important pathogen of crop plants such as maize (ear and stalk rot), wheat, and barley (head blight) (20). Trichothecene mycotoxins produced by *F. graminearum* reduce the quality of infected grain and are the major source of trichothecene contamination in these crops. Deoxynivalenol (DON) and its acetylated derivatives are the most commonly found trichothecenes in *F. graminearum*-infected plant materials. DON and other *Fusarium* trichothecenes are potent phytotoxins, and several lines of evidence indicate that they play a role in *F. graminearum* diseases of maize and wheat (5). Mutant strains lacking a functional trichothecene pathway due to disruption of *TRI5*, which encodes the first enzyme in the trichothecene pathway, produce significantly less disease on both maize ears (7) and wheat heads (16) than wild-type *F. graminearum*.

In *Fusarium sporotrichioides* NRRL 3299, nine trichothecene pathway genes are present in a gene cluster (10) while at least one other pathway gene, *TRI101* (*TRI^r*), appears to be unlinked (12, 13). Pathway gene expression requires the transcription factor TRI6, a C2H2-type zinc finger protein (18). Trichothecene production levels in *F. sporotrichioides* can be altered through the introduction of cosmids carrying different portions of the pathway gene cluster (11). Depending on the cosmid used for transformation, a 2- to 10-fold increase in trichothecene production was observed.

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Limited information concerning the nutritional and environmental factors that influence trichothecene biosynthesis is available. In *F. sporotrichioides* changes in the carbon/nitrogen ratios of the growth medium can greatly increase trichothecene accumulation in liquid shake cultures (22). Similar studies with *F. graminearum* reported low levels of trichothecene production for all media tested in liquid shake cultures although static liquid cultures and solid substrate fermentations resulted in much higher production levels (15). The lack of a liquid shake culture method for trichothecene production in *F. graminearum* has restricted biochemical studies of trichothecene biosynthesis in this fungus and has increased the difficulties associated with the large-scale preparation of trichothecenes such as DON (2).

To develop strains with increased levels of trichothecene production under liquid shake culture conditions, we introduced into *F. graminearum* a reporter gene consisting of the *F. graminearum TRI5* (*FGTRI5*) promoter (*TRI5^P*) coupled with the β-D-glucuronidase (GUS) coding region (*TRI5^P-GUS*) and/or the *TRI6* gene from *F. sporotrichioides* (*FSTRI6*). Both genes altered trichothecene production levels, and their effects appear to be dependent on whether plasmid integration occurred within the trichothecene pathway gene cluster.

MATERIALS AND METHODS

Strains, media, and culture conditions. *F. graminearum* GZ3639 isolated from scabby wheat in Kansas (3) was kindly provided by R. Bowden (Kansas State University) and maintained on V-8 juice–agar slants (21). Transformants of *F. graminearum* were grown on V-8 juice–150 μg of hygromycin B (Sigma, St. Louis, Mo.)/ml slants. Conidia were washed from V-8 juice–agar plates and used to inoculate YD medium (1% yeast extract, 2% peptone, 2% glucose) at a concentration of 10^5 conidia/ml for DNA isolation and GYEP medium (5% glucose,

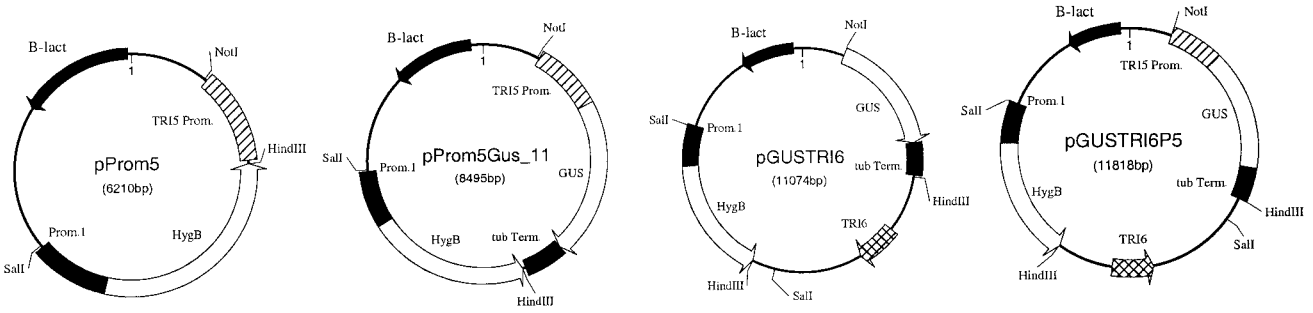

FIG. 1. Plasmids used in this study. Locations for *F. graminearum TRI5*^{P} (TRI5 Prom.), *E. coli* GUS coding region (GUS), hygromycin B coding region (HygB), Promoter 1 fragment from *Cochliobolus heterostrophus* (Prom. 1), the transcription termination sequence from the *benA* gene of *A. nidulans* (tub Term.), the β-lactamase coding region (B-lact), and the *FSTRI6* gene (TRI6) are shown. Selected restriction sites are also shown.

0.1% yeast extract, 0.1% peptone) (22) for trichothecene production and GUS activity.

GUS assays. GUS activity due to expression of the *Escherichia coli* GUS coding region in transformants was determined as described previously (9). Cultures were grown in GYEP medium for 24 h, after which mycelia were harvested by filtration, frozen in liquid N_2, and stored at −80°C. Mycelial mats were ground to a powder in liquid N_2 and resuspended in homogenization buffer (50 mM $NaHPO_4$ [pH 7.0], 2 mM EDTA, 1 mM 2-mercaptoethanol). Homogenates were centrifuged for 5 min at 12,000 × *g*, and the supernatants were collected for GUS analysis. Protein concentrations were determined with protein assay reagent (Bio-Rad, Hercules, Calif.) using bovine serum albumin as the standard (4). GUS assays were performed using 4-methylumbelliferyl-β-D-glucuronide (MUG) as the substrate in a total volume of 150 μl. Reaction mixtures consisting of the same components as the breakage buffer in addition to 2 mM MUG were incubated at 37°C for 10 min, and fluorescence was read in a DNA Quant 200 fluorometer (Hoefer, San Francisco, Calif.) precalibrated with 7-hydroxy-4-methylcoumarin. Transformant cultures were grown in duplicate, and the assays were performed in triplicate for each culture.

Plasmid constructions and fungal transformation. Plasmids were constructed using standard recombinant techniques (19), and PCRs were performed with *Pfu* (Stratagene, La Jolla, Calif.) DNA polymerase. To construct plasmids with a reporter gene suitable for monitoring trichothecene pathway gene expression, we amplified the 749-bp *TRI5*^{P} from *F. graminearum* in pADD6-1 (17) using primers 948 (5′-CAATGGAGATCTTGGCTCAG-3′) and 949 (5′-GGCGAGCTCGTAACAGTTATTCAATAAATTAAC-3′). Following digestion at the *Bgl*II and *Ecl*136III sites located within these primers (underlined) the *TRI5*^{P} fragment was cloned into the *Bam*HI and *Sma*I sites upstream of the GUS coding region in pGUS2-7 (9) or into the fungal transformation vector pUCH2-8 (1). pPROM5GUS-11 contained the *TRI5*^{P}-GUS fusion followed by the transcription termination sequence from *benA* of *Aspergillus nidulans* present in pGAP-4 (23), and pProm5 contained *TRI5*^{P} alone (Fig. 1). The 3′ end of the *TRI5*^{P} fragment in these plasmids begins 18 bp upstream from the *F. graminearum TRI5* (*FGTRI5*) start codon and contains three copies of the core binding sequence (TNAGGCCT) for the pathway transcription factor TRI6 (9). Plasmids carrying the *TRI6* gene of *F. sporotrichioides* were constructed by cloning the 3.32-kb *Hin*dIII fragment from COS9-1 (11) containing the entire *FSTRI6* gene into the *Hin*dIII sites of pGUS2-7 and pProm5GUS-11 to form plasmids pGUSTRI6 and pGUSTRI6P5, respectively. The *FSTRI6 Hin*dIII fragment includes the entire *TRI4*-to-*TRI6* intergenic region. The 5′ flanking sequences extend 910 bp upstream from the *TRI6* start codon, and the 3′ flanking sequences extend 1,763 bp downstream from the *TRI6* translational stop.

Fungal transformations were performed as described previously (17). DNA was isolated from single-spored cultures of transformants (8) and was characterized by PCR and Southern blotting to determine the location and number of plasmid copies and the presence of intact reporter genes. Characterization by PCR was performed with primers specific for either the *TRI5* gene or the GUS coding region. Southern blots were probed with PCR products amplified from either the *E. coli* β-lactamase gene (582 bp) or the *FGTRI5* coding region (575 bp). The β-lactamase gene was amplified using primers 689 (5′-CATCGAACTGGATCTCAACAGCG-3′) and 690 (5′-CTGCAATGATACCGCGAGACC-3′), and the *FGTRI5* coding region was amplified using primers 63 (5′-TGGAGAACTTTCCCACC-3′) and 176 (5′-CTGGGAATCCTCCAAAGTTG-3′). Southern blotting and labeling of the hybridization probes were performed as previously described (18).

RESULTS

***FGTRI5*^{P} transformants.** *F. graminearum* typically produces low or undetectable levels of the trichothecene 15-acetyldeoxynivalenol (15-ADON) under liquid shake culture conditions (15). We analyzed four pProm5GUS-11 transformants after 7 days of growth in shake cultures of GYEP medium for trichothecenes using a method described previously (14). Three of the transformants (D1-2, D6-2, and D8-3) produced between 25 and 225 mg of 15-ADON/liter, while transformant D4-1 and the parental strain, GZ3639, produced less than 5 mg of 15-ADON/liter.

The presence of the *TRI5*^{P} sequence in pProm5GUS-11 can lead to the generation of two different classes of transformants depending on whether or not plasmid integration occurs through homologous recombination with *TRI5*^{P}. Southern blotting analysis revealed that both classes of integration events were represented by the transformants (Fig. 2B). All

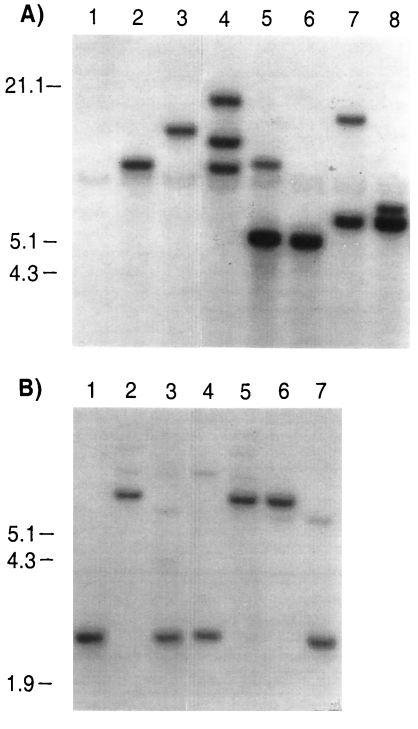

FIG. 2. Southern blots of transformants. (A) *Ecl*136II digest with a portion of the β-lactmase gene as the probe. Lanes: 1, GZ3639; 2, B4-1; 3, B9-1; 4, C11-1; 5, D4-1; 6, D6-2; 7, G11-1; 8, G15-1. (B) *Hin*dIII digest with the *FGTRI5* coding region as the probe. Lanes: 1, GZ3639; 2, B4-1; 3, B9-1; 4, D4-1; 5, D6-2; 6, G11-1; 7, G15-1. In panel B the major band for lanes 1, 3, 4, and 7 is 2.6 kb while the major band in lanes 2, 5, and 6 is 7.0 kb.

three transformants that produced high levels of trichothecenes (D1-2, D6-2, and D8-3) were also found to have *TRI5*^{P}-localized integration of pProm5GUS-11. In contrast, the transformant displaying the parental-strain level of trichothecene production (D6-2) carried an ectopic copy of pProm5GUS-11. Southern blots for two of the four pProm5GUS-11 transformants are shown in Fig. 2B. Plasmid integration at *TRI5*^{P} is demonstrated in a *Hin*dIII digest for D6-2 (Fig. 2B, lane 5) by the loss of a parental-strain *TRI5* band (Fig. 2B, lane 1) and the appearance of a higher-molecular-weight band of the predicted size for pProm5GUS-11 integration. The major band for transformant D4-1 (Fig. 2B, lane 4) comigrates with the band in the parental strain (Fig. 2B, lane 1). No significant differences in GUS expression between these transformants and untransformed GZ3639 were observed.

We also transformed *F. graminearum* with two plasmids that were identical to pProm5GUS-11 except that they lacked either *TRI5*^{P} (pGUS2-7) (9) or GUS gene (pProm5) sequences (Fig. 1). All three pGUS2-7 transformants analyzed had unaltered trichothecene production phenotypes. One of two pProm5 transformants (G11-1) produced between 50 and 75 mg of trichothecenes/liter in GYEP medium, while another (G15-1) produced <5 mg of 15-ADON/liter. Southern blotting data indicate that in G11-1 pProm5 integrated at both ectopic and *TRI5*^{P} sites. This conclusion is based on the presence of two bands in a blot probed with a DNA fragment unique to the transforming plasmid (Fig. 2A, lane 7) coupled with loss of the parental-strain *TRI5*^{P} band (Fig. 2B, lane 6). G15-1 appears to carry two ectopic copies of pProm5 (Fig. 2A, lane 8).

***FSTRI6* transformants.** *F. graminearum* was transformed with plasmids containing *FSTRI6* alone (pGUSTRI6) or *FSTRI6* combined with the *TRI5*^{P}-GUS reporter gene (pGUSTRI6P5; Fig. 1). Trichothecene production increased significantly in three of five pGUSTRI6 transformants and four of four pGUSTRI6P5 transformants. One pGUSTRI6 transformant (C11-1) and two pGUSTRI6P5 transformants (B4-1 and B9-1) were selected for further study. Based on Southern blotting, C11-1 carries three copies of pGUSTRI6 (Fig. 2A, lane 4) all of which are ectopic due to the absence of the *TRI5*^{P} sequence in this plasmid. Both B4-1 and B9-1 carry a single copy of pGUSTRI6P5 (Fig. 2A, lanes 2 and 3) but differ in the site of plasmid integration. In B4-1, integration of pGUSTRI6P5 occurs within *TRI5*^{P} (Fig. 2B, lane 2), but integration is ectopic for B9-1 (Fig. 2B, lane 3). We analyzed trichothecene production by these transformants and by C11-1, G11-1 (pGUS2-7 transformant; see above), and the parental strain at several time points (at 1, 2, and 7 days) over a time course of 7 days. The parental strain, GZ3639, produced undetectable levels of 15-ADON (<5 mg/liter) after 7 days. Transformant B9-1 had slightly higher levels of 15-ADON than C11-1 and G11-1 at day 1, but after 7 days the mean trichothecene levels for B9-1, C11-1, and G11-1 all fell within a range of 47 to 54 mg/liter. Transformant B4-1 had markedly higher trichothecene levels at each time point, resulting in a 20-fold increase (1,200 mg/liter) over the other transformants after 7 days.

GUS activity levels for transformant B9-1, which carries an ectopic copy of the *TRI5*^{P}-GUS reporter gene, were two- to fivefold higher at all time points than the activities measured for transformants lacking the *TRI5*^{P}-GUS reporter gene (C11-1 and G11-1) and the parental strain. However, GUS activity for B9-1 was only 1 to 2% of B4-1 levels at all time points. GUS activities for B9-1 and B4-1 did not change significantly over the 7-day time course.

DISCUSSION

While *F. graminearum* isolates typically produce moderate amounts of trichothecenes under various solid-substrate and static liquid culture conditions, it has been difficult to identify suitable liquid shake culture conditions for trichothecene production (15). This result contrasts sharply with those for other closely related *Fusarium* species, e.g., *F. sporotrichioides*, that typically produce high levels of trichothecenes under these conditions (22). Liquid shake cultures provide a convenient means for generating physiologically homogeneous cultures. Lack of suitable liquid shake culture methods for *F. graminearum* presents an obstacle to biochemical studies of trichothecene biosynthesis. We observed increased production of trichothecenes in liquid shake culture when plasmids carrying *TRI5*^{P} integrated at the genomic *TRI5* site. Transformants carrying ectopic copies of the *TRI5*^{P}-containing plasmids or transformed with plasmids lacking the *TRI5*^{P} sequences produced trichothecenes at wild-type levels. These results suggest that integration of the transforming plasmid at the *TRI5*^{P} site altered the regulation of trichothecene biosynthesis. Surprisingly, increases in trichothecene production were not accompanied by correspondingly higher levels of reporter gene activity in transformants carrying *TRI5*^{P}-GUS integrated within *TRI5*^{P}. One explanation for this result is that the mechanism responsible for increased trichothecene production in these transformants is independent of TRI6-mediated pathway gene activation (8). Alternatively, another uncharacterized regulatory mechanism, perhaps involving the up-regulation of isoprenoid biosynthesis and thereby the supply of farnesyl diphosphate for trichothecene biosynthesis, might have been altered by integration at *TRI5*^{P}.

Introduction of the *FSTRI6* gene into *F. graminearum* also might increase trichothecene production in a liquid shake culture. *FSTRI6* encodes a trichothecene pathway transcription factor required for pathway gene expression in *F. sporotrichioides*, and an apparent *TRI6* homologue has been identified in *F. graminearum* (12). The core sequence for *F. sporotrichioides* TRI6 binding in *FSTRI5*^{P} (TNAGGCCT) occurs three times in the *FGTRI5* promoter (9), so we anticipated that *FSTRI6* would function as a transcription activator of trichothecene pathway gene expression in *F. graminearum*. Integration of the *FSTRI6* gene at both ectopic (B9-1 and C11-1) and *TRI5*^{P} sites (B4-1) increased 15-ADON production in liquid cultures. *FSTRI6* integration at the *TRI5*^{P} site resulted in trichothecene production levels that were almost 20-fold higher than those observed with transformants carrying an ectopic copy of the same vector (pGUSTRI6P5) or a vector carrying *FSTRI6* without the *TRI5*^{P} sequence (pGUSTRI6). Thus, both introduction of the *FSTRI6* gene and *TRI5*^{P} positional effects are important for altering the regulation of trichothecene production.

Increases in trichothecene production in all *FSTRI6* transformants were accompanied by increased GUS activity. For the transformant with the *TRI5*^{P}-localized copy of *FSTRI6* (B4-1) the GUS levels were high at all time points, indicating that trichothecene pathway gene expression may be constitutive. These results suggest that the increased trichothecene production observed involves higher levels of pathway gene expression and that these changes could in turn be due to increases in TRI6 levels. Both *FSTRI6* and *FGTRI6* expression could be involved in the overproduction phenotype, particularly if *FGTRI6* is autoregulated due to its proximity to the *TRI4* promoter region (8). The increases in trichothecene production resulting from the introduction of *FSTRI6* support the proposed conservation of TRI6 function within *Fusarium* spe-

cies and indicate that the failure of *F. graminearum* strains to produce trichothecenes in liquid shake culture may be addressed by manipulating *TRI6* expression.

Trichothecene production is necessary for full virulence of *F. graminearum* on both maize and wheat (6), and changes in trichothecene regulation could affect virulence. The transformants described in this study with altered trichothecene regulation provide an opportunity to better characterize the role of trichothecene production in plant diseases caused by *F. graminearum*. In addition, transformants carrying the *FGTRI5*^{P}-GUS reporter gene could be useful in studies of trichothecene pathway gene expression during infection.

Like *F. graminearum*, a number of fungi produce low levels of trichothecenes under liquid shake culture conditions, making it difficult to characterize the distinctive toxin profiles and biosynthetic pathways of these fungi. High levels of trichothecene production increase the chances of detecting minor pathway intermediates and end products. The ability to significantly increase trichothecene production in *F. graminearum* through the introduction of a heterologous *TRI6* gene suggests a means for increasing trichothecene production in other fungi.

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