JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Fusarium graminearum TRI14 Is Required for High Virulence and DON Production on Wheat but Not for DON Synthesis in Vitro

REX B. DYER, RONALD D. PLATTNER, DAVID F. KENDRA, AND DAREN W. BROWN*

Mycotoxin Research Unit, USDA/ARS, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, Illinois 61604

Fusarium head blight (FHB) of wheat (*Triticum aestivum* L.), caused by the fungus *Fusarium graminearum*, is a major concern worldwide. FHB grain is reduced in yield, may fail to germinate, and is often contaminated with deoxynivalenol, a trichothecene mycotoxin linked to a variety of animal diseases and feed refusals. Annual losses in the tens of millions of dollars due to FHB underscore the need to develop improved methods of disease control and prevention. Previous research has identified deoxynivalenol biosynthesis as a virulence factor on wheat. Recently, we found that the *TRI14* gene of *F. sporotrichioides*, closely related to *F. graminearum*, was not required for synthesis of a related trichothecene, T-2 toxin. *TRI14* does not share similarity with any previously described genes in the databases. In this study, we examined the role that *F. graminearum TRI14* may play in both deoxynivalenol synthesis and in virulence on wheat. *TRI14* deletion mutants synthesize deoxynivalenol on cracked maize kernel medium and exhibit wild-type colony morphology and growth rate on complex and minimal agar media. However, FHB assays on greenhouse-grown wheat indicate that FgΔTri14 mutants cause 50−80% less disease than wild type and do not produce a detectable quantity of deoxynivalenol on plants. We discuss a number of possible roles that *TRI14* may play in the disease process.

KEYWORDS: Gene cluster; gene complementation; pathogenicity; secondary metabolite; transport

INTRODUCTION

Fusarium graminearum (teleomorph *Gibberella zeae*), the causal agent of Fusarium head blight (FHB) or scab, is an international problem. Severe outbreaks in the U.S. between 1991 and 1997 led to direct losses of $1.3 billion, and epidemics in China have resulted in grain yield losses of more than 1 million tons (*1*). Economic losses are exacerbated by contamination of infected grain with toxins, especially the trichothecene, deoxynivalenol. Deoxynivalenol is a protein synthesis inhibitor and is associated with a variety of animal diseases. Subacute to acute oral toxicity is characterized by vomiting, feed refusal, weight loss, and diarrhea (*2*). In addition to emesis, effects on the central nervous system lead to anorexia, and high doses can cause necrosis in multiple tissues including the gastrointestinal tract, bone marrow, and lymphoid tissue (*2*). Contaminated grain often demands a lower price and, depending on the level of contamination, may be rejected at grain elevators entirely (*3*).

Deoxynivalenol, produced by *F. graminearum*, and T-2 toxin, produced by *F. sporotrichioides*, are structurally related trichothecenes that share an orthologous biosynthetic gene cluster containing 10 and 12 genes, respectively (*4*−*6*). Cluster gene transcription in both species is governed by *TRI6*, which is located in the cluster and encodes a Cys_2His_2 transcription factor (*4*, *7*, *8*). Gene deletion studies have determined roles for all of the coregulated cluster genes except *TRI9* and *TRI14* (*6*). Roles for *TRI9* and *TRI14* could not be predicted because neither shares sequence similarity with any previously described sequences in the databases and because *F. sporotrichioides* FgΔTri14 mutants produce T-2 toxin in culture (*5*). It is possible that *TRI14* is not required or involved in T-2 toxin synthesis in culture or that its function is redundant.

The negative impact of FHB necessitate comprehensive methods for combating the problem. Applications of fungicides and biocontrol agents, breeding and engineering resistant cereal lines, and molecular genetic studies aimed at elucidating new targets to limit *F. graminearum* growth or toxin synthesis are all being pursued (*9*−*12*). To date, five genetic loci have been described in the literature as required for pathogenicity by *F. graminearum* on wheat (*13*−*17*). Over 10 years ago, the *TRI5* gene was found to be involved in deoxynivalenol biosynthesis and gene deletion mutants (e.g. GZT40) unable to synthesize deoxynivalenol are 90% reduced in ability to cause disease. FHB symptoms occur in infected spikelets but fail to spread to

* Corresponding author. Tel: (309) 681-6230. Fax: (309) 681-6689. E-mail: browndw@ncaur.usda.gov.

10.1021/jf051441a
Published on Web 10/15/2005

Table 1. *Fusarium Graminearum* Strains Used in This Study

strain	genotype	deoxynivalenol (ppm) SD (no.)
GZ3639	wild-type	204 ± 31 (4)
GZT40 (FgΔTri5)	TRI5 deletion mutant	NA
FgΔTri1427 and 50	TRI14 deletion	115 ± 87 (7)
FgT14E36 and E57	wild-type *TRI14*	270 ± 36 (4)
FgT14AB3, 6, 8, 18, 30 and 33	complemented *TRI14* mutant	173 ± 109 (6)

neighboring spikelets (*18*). Two loci encode different MAP kinases (*MGV1* and *gpmk1*) and gene disruption mutants display a complicated phenotype which includes significant impairments to normal growth as well as reduced ability to cause FHB (*14*, *15*). The *CPS1* locus encodes a putative adenylate-forming enzyme, and gene disruption mutants appear to grow normally but are significantly reduced in their ability to cause FHB (*16*). How *CPS1* contributes to disease is unknown. It is possible that the failure of the MAP kinase and *CPS1* mutants to cause FHB is linked to a failure to synthesis deoxynivalenol. The *MGV1* mutant was unable to synthesis deoxynivalenol in planta while the ability of the *CPS1* or *gpmk1* mutants to synthesis deoxynivalenol were not reported. Recently, it has been shown that a secreted lipase, encoded by the gene *FGL1*, is required for virulence on wheat (*17*). Most infection with Δ*fgl1* strains were restricted to the inoculated spikelet suggesting that this gene is involved in fungal spread within the wheat head (*17*). On the basis of the function of the FGL1, it is unlikely that the reduced virulence generated by Δ*fgl1* strains is connected to deoxynivalenol synthesis. In this study, we examined the possibility that *TRI14* plays a role in virulence in *F. graminearum*.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions. *Fusarium* strains used in this study are described in **Table 1**. *F. graminearum* strain GZ3639 (FGSC 8630) was isolated from scabby wheat in Kansas (*19*). This strain is highly virulent on wheat and synthesizes a significant amount of deoxynivalenol when grown on wheat and in cracked corn cultures (*20*). GZ3639 is the progenitor strain for all transformant strains generated in this study. GZT40 was derived from GZ3639 and contains a nonfunctional *TRI5* gene (*13*). Cultures for DNA isolation were grown in YPG medium (0.3% yeast extract, 1% peptone, and 2% glucose) at 28 °C with shaking at 200 rpm for 3−5 days. Small scale cultures of *F. graminearum* strains to screen for deoxynivalenol production were grown on 10 g of cracked corn at 22 °C in the dark for 3 weeks. Macroconidia for inoculation and viability assays were collected from strains grown in mung bean medium at 28 °C with shaking at 200 rpm for 4 days (*21*). Conidia concentration was determined by counting on a haemacytometer. Mung bean medium was prepared by boiling 40 g of mung beans in 1 L of deoinized water for 10 min, filtering the liquid through Miracloth (Calbiochem), and autoclaving the filtrate. Spore viability assays were performed on potato dextrose agar (PDA) prepared according to the manufacturer's instruction (Sigma, St. Louis, MO). General fungal growth rates were examined on two complete media (PDA and V-8 agar) and on the minimal medium Czapek agar (*22*).

Nucleic Acid Manipulation and Primers. DNA analysis used standard techniques (*23*). DNA probes were labeled with α-^{32}P-dCTP (DuPont NEN, Boston MA) using the Prime-A-Gene labeling system (Promega, Madison, WI). Primers for PCR amplification were synthesized on a PerSeptive Biosystems Expedite Nucleic Acid Synthesis System (PE Biosystems, Foster City, CA) and processed according to the manufacturers recommendations. DNA probes were prepared by PCR using appropriate primers and template DNA. Fungal mycelium was collected on Miracloth by filtration and dried, and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Inc. Valencia, CA) according to the manufacturer's instructions. Sequence similarity searches of the nonredundant (NR) database maintained by the National Center for Biotechnology Information (NCBI) were performed using the BLAST program (*24*, *25*).

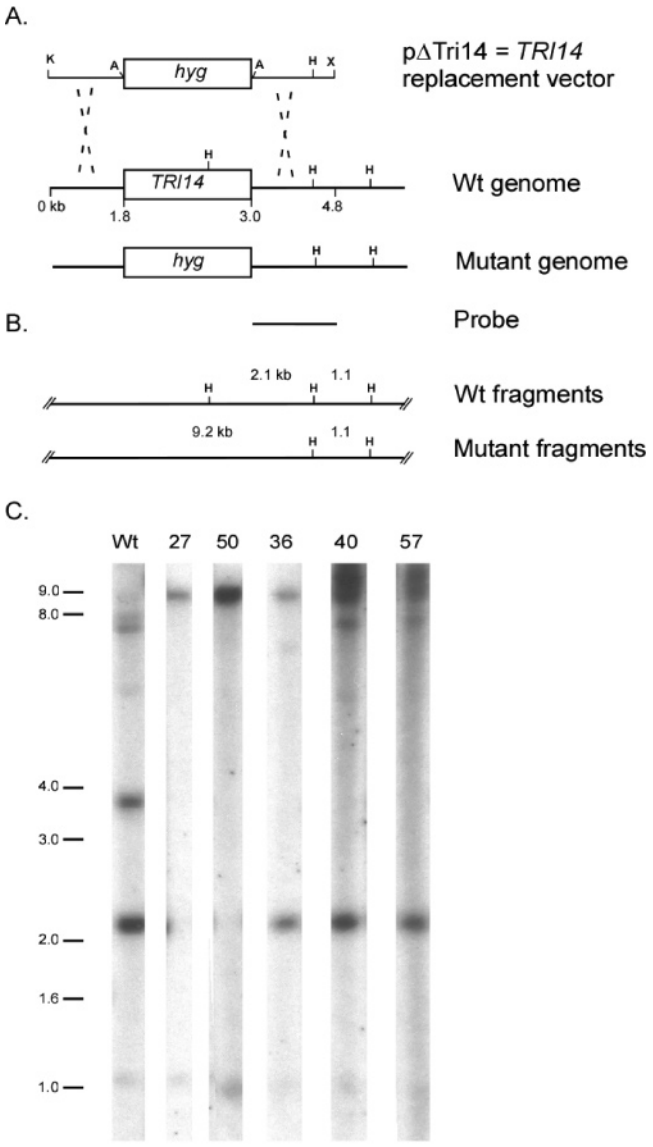

Figure 1. *TRI14* gene replacement. (A) Schematic diagram depicting replacement of *TRI14* with *hyg* by a double homologous recombination event represented by the crossed, dashed lines. Restriction site K = *Kpn* I, A = *Asc* I, X = *Xba* I, and H = *Hind* III. (B) Representation of probe used for southern analysis and predicted size of fragments expected for the wild-type (Wt) and the mutant genome digested with *Hind* III. (C) Southern analysis of genomic DNA digested with *Hind* III from hygromycin resistant transformants and the wild-type (Wt) parent strain. The numbers 27 and 50 refer to the FgΔTri14 mutants FgΔTri1427 and -50, respectively. The numbers 36, 40, and 57 refer to the nondeletion transformants FgΔTri1436, -40, and -57, respectively, where the vector integrated via a single homologous or a nonhomologous event. Evidence of gene replacement was the loss of the 2.1 kb band and the gain of a 9.2 kb band. A 1.1 kb band was observed in all lanes. The bands at 3.5 kb and 7.5 kb were observed in ∼20% of the genomic DNA examined and are likely artifacts due to nonspecific DNA hybridization or incomplete digestion of genomic DNA.

Plasmid Construction and Transformation. Plasmid pΔTri14 was constructed to delete the *F. graminearum TRI14* coding region by a double homologous recombination event (**Figure 1**). The general strategy used to construct the deletion vector has been described (*5*). In brief, a 1827 bp DNA fragment upstream of the predicted start codon of the target gene and a 1,805 bp fragment downstream of the predicted

stop codon of the target gene were amplified by PCR and cloned separately into cloning vector pT7Blue-3 with the Perfectly Blunt Cloning Kit (Novagen, WI). The upstream fragment was amplified with primers Fg14LKpn (5′-CAggtaccAAGTGCTGGAGTGAGGATG -3′) and Fg14LAsc (5′-CAggcgcgccGTGTATACTATTCAATAGC-3′). The downstream fragment was amplified with primers Fg14RAsc (5′-CAggcgcgccGCTCAACTGTAAGATGATGG-31) and FgRXho (5′-CActcgagGGTGATCGGATGTAAGGG-3′). *Asc* I sites (denoted by lower case letters) were engineered into both PCR products at the end proximal to the start or stop codon, respectively. *Xho* I and *Kpn* I sites were engineered into the opposite end of each PCR product to enable one of the products to be cloned into the plasmid containing the second product such that the original orientation of the fungal-derived DNA fragments was maintained. The final disruption vector was generated by cloning the *Asc* I Hyg/P1 fragment from pHygAsc into the *Asc* I site located in the plasmid containing the two PCR products. The Hyg/P1 fragment consists of a promoter from *Cochliobolus heterostrophus* (P1) and a gene encoding for resistance to hygromycin (Hyg) (*26*). Sequence analysis of the *F. graminearum* portion of the disruption vector verified the orientation of the PCR fragments and did not identify any nucleotide differences from the corresponding genomic sequence.

Plasmid pTri14AB was constructed to complement the FgΔTri1450 mutant strain. pTri14AB carried a 2027 bp DNA fragment that included the entire *TRI14* coding region plus 612 bp upstream of the predicted translational start site and 242 bp downstream of the predicted stop codon. The *TRI14* containing PCR fragment was amplified with primers FgT14CFa (5′-GAggcgcgccGACAATCAGTCGAGTAAC-3′) and FgT14CR (5′-GTAACTGTGAAGTTAGGAGTCGC-3′) and cloned into pT7Blue-3 with the Perfectly Blunt Cloning Kit (Novagen). A fragment of DNA encoding for resistance to Geneticin (*27*) was then cloned in the *Asc* I site introduced by primer FgT14CFa. Sequence analysis of the *F. graminearum* portion of the disruption vector did not identify any nucleotide differences from the corresponding genomic sequence.

Transformation of *F. graminearum* GZ3639 and FgΔTri1450 and selection and isolation of transformants were performed essentially as described (*13*). Protoplasts were generated by digestion of germinating macroconidia with Driselase (Sigma, St. Louis, MO), Chitinase (Sigma), and Lysing Enzyme (Sigma). Transformants were selected for by resistance to either hygromycin B (Sigma) or Geneticin (Gibco, Invitrogen Corp. Carlsbad, CA). Confirmation of *TRI14* gene deletion of transformants was performed by southern analysis. Confirmation of transformants receiving pTri14AB was performed by PCR using primers rp650 (5′-TGCTGAAACCTCCGTATGCCTG-3′) and rp654 (5′-CCA-GATCATCCTGATCGACAAG-3′) and template DNA with Geneticin was predicted to generate a 873 bp DNA fragment. GZ3639 was maintained on V-8 juice agar slants, hygromycin-resistant strains were maintained on slants containing 300 μg/mL hygromycin B, and geneticin-resistant strains were maintained on slants containing 300 μg/mL of Geneticin.

Wheat Head Blight Assays. Five greenhouse virulence tests or assays were conducted on wheat cultivar Wheaton and one test was condeucted on cultivar Norm essentially as follows. Four assays were conducted with two pΔFgTri14 mutant strains and the wild-type GZ3639 parent strain while two assays were conducted with GZT40. A total of 10 seeds were sown in plastic pots (18.7 cm diameter) filled with pasteurized soil (Scotts Redi-Earth Plug and Seedling Mix, Scotts Co., Marysville, OH), placed into a growth chamber (EconoAir, model GC-16, Ecological Chambers, Inc., Winnipeg, Mb, Canada), and incubated at 15 °C with a 12 h light/dark cycle. After seedlings emerged, plants were fertilized weekly with 200 mL of an aqueous solution containing 0.125% Peter's 20−20−20 (Spectrum Brands, St. Louis, MO) and 0.008% iron sulfate. After 5 weeks, the pots were transferred to a greenhouse where temperatures ranged from 15 to 20 °C at night and 23−28 °C during the day. Supplemental high-pressure sodium lights were used to maintain a 14 h day. After 1 week, the number of plants/pot were reduced to 4. Anthesis began roughly 2 weeks later after which the infection process was initiated.

Macroconidia prepared from mung bean medium were harvested by low-speed centrifugation and washed once with water. Heads were inoculated by injecting 10−20 μL of spore suspension (1×10^5 macroconidia/mL) into one floret in the third or fourth spikelet up from the base of the wheat head. Control heads were inoculated with an equal amount of sterile water. Following injection of 10 replica heads/treatment, 3−4 heads from one treatment were enclosed in plastic bags for 3 days. Disease ratings were scored at 2−3 day intervals, and disease severity (e.g. symptoms) was determined as percentage of diseased spikelets at 18 or 21 days post inoculation (dpi). Heads were allowed to mature prior to harvest and then were individually threshed. The number of seeds and seed weight per head were determined. Yield loss refers to the average percent yield loss/seed relative to the water control.

Extraction and Analysis of Deoxynivalenol. The 3 week old cracked corn cultures of *F. graminearum* were extracted with 5 mL of acetonitrile/water (86:14)/g of cracked corn. Wheat seeds from each treatment protocol were pooled, chopped into small pieces in a model M-2 Stein Laboratory Mill (Steinlite Corp., Atchison, KS), ground to a fine powder with a mortar and pestle, and extracted with 4 mL of acetonitrile/water (86:14)/g of material. Extracts were analyzed for deoxynivalenol by gas chromatography mass spectroscopy (GC-MS) as described (*18*).

Statistical Analysis. The experimental design for four greenhouses tests comparing percentages of head blight for wild-type parent and five transformants is a random incomplete block with unequal strain occurrence. Levene's homogeneity of variance tests were performed to check for transformation necessity before running analyses. All subsequent analyses were performed on transformed data where necessary, but raw data means are presented for ease of interpretation. Single-factor, mixed model analyses of variance (ANOVAs) for each greenhouse test were used to analyze strain differences in average percent disease symptoms and average percent yield loss (SAS version 9.1.3, 2002−2003, SAS Institute, Inc., Cary, NC). *F*-test statistical results were considered significant at $p \leq 0.05$. Comparisons of significant strain means were performed using differences of least squares means at the $p \leq 0.05$ level if a significant *F*-test statistic was obtained from an ANOVA.

RESULTS

***TRI14* Gene Deletion and Complementation.** Wild-type *F. graminearum* strain GZ3639 was transformed with the deletion vector pΔFgTri14 and 43 hygromycin-resistant isolates were recovered. Genomic DNA from 33 transformants was analyzed by Southern blot to determine if gene deletion had occurred. The replacement of *TRI14* with Hyg/P1 by double homologous integration would result in the loss of a *Hin*d III restriction site. Southern analysis of *Hin*d III digested wild-type genomic DNA probed with a 1.8 kb fragment of DNA downstream of the *TRI14* stop codon hybridized to the expected 1.1 kb and 2.1 kb bands. Deletion of *Hin*d III restriction site within the *TRI14* coding sequence would result in an altered hybridization pattern where the 2.1 kb fragment was lost and a 9.2 kb fragment was gained. Two deletion mutants, designated FgΔTri1427 and FgΔTri1450, had the hybridization pattern predicted for the double homologous integration event indicating that the *TRI14* coding sequence was deleted (**Figure 1**). Three strains, designated FgT14E36, FgT14E40, and FgT14E57, had the hybridization pattern predicted for the wild-type plus at least one additional band indicating that the *TRI14* coding sequence was intact and that the deletion vector had integrated either by single homologous integration or ectopically (or both).

FgΔTri1450 was transformed with the *TRI14* gene complementation vector pTri14AB, and 34 geneticin-resistant strains were recovered. Genomic DNA from 22 transformants was analyzed by PCR to determine if plasmid integration had occurred. A total of 19 of the 22 stains examined tested positive for the Geneticin gene with primers rp650 and rp654, and six strains, designated FgT14AB3, FgT14AB6, FgT14AB8, FgT14AB18, FgT14AB30, and FgT14AB33, were arbitrarily chosen for further analysis.

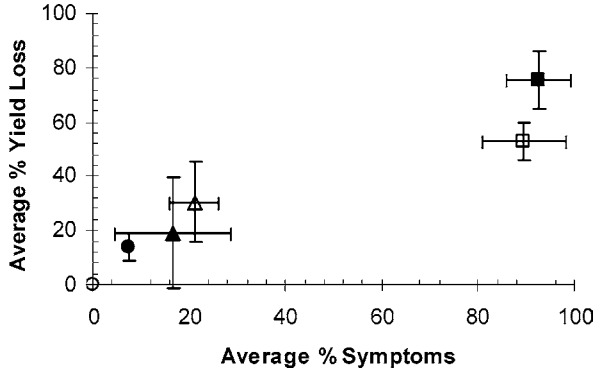

Figure 2. *TRI14* deletion results in significant reduction in Fusarium head blight symptoms and yield loss. GZ3639 (■) is the parent strain to the FgΔTri14 (△ = FgΔTri1427, and ▲ = FgΔTri1450) and FgΔTri5 (● = GZT40) mutant strains and the three nondeletion transformant control strains (□ = ectopic). ○ = water mock infection. Not all strains were assayed in all four assays. Standard deviations along both axis are indicated.

Growth Characteristics and Deoxynivalenol Analysis on Cracked Corn. Both FgΔTri14 strains appeared identical to the wild-type parental strain GZ3639 with regard to growth in liquid and on solid media. No significant differences in mycelial morphology or growth rate were noted on complex media such as V-8 agar, PDA, liquid YPG, and cracked corn or the less complex Czapek media. Sexual reproduction was unaffected as both FgΔTri14 mutants produced perithecia and ascospores similar to wild-type on autoclaved corn stalks. Conidial production in mung bean medium and germination on PDA plates was not affected by *TRI14* deletion. Conidial germination for the FgΔTri14 mutants was between 67 and 87%, and that of wild type was 71%. Chemical analysis of solvent extracts from growth on cracked corn indicated that FgΔTri14 mutants FgTri1427 and FgTri1450 produced deoxynivalenol similar to wild-type strain GZ3639, three nondeletion transformants, and six FgTri1450 *TRI14* add-back strains (**Table 1**).

Virulence Tests. We conducted five independent virulence tests of FgΔTri14 mutants, nondeletion transformants, and complemented FgΔTri14 mutants for virulence in the greenhouse on wheat cultivars Wheaton (**Tables 2** and **3**; **Figure 2**). The FHB disease symptoms measured for each treatment were the average percent symptomatic spikelets, average seed yield loss/head, and level of deoxynivalenol contamination in seed extracts and are referred collectively as FHB. In the first and second tests, virulence of GZ3639 and FgΔTri14 mutants FgTri1427 and FgTri1450 was examined on Wheaton. In both tests, heads infected with GZ3639 suffered over 90% FHB and seeds were contaminated with significant levels of deoxynivalenol while heads inoculated with the two FgΔTri14 mutants were significantly less diseased and no deoxynivalenol was detected in any of the harvested seeds (**Table 2** and **Figure 3**). In a second test, macrocondia from the nondeletion transformants FgTri14E36 and FgTri14E57 was examined and was found to generate FHB similar to GZ3639 (**Table 2** and **Figure 2**). In the third and fourth tests, we compared FHB symptoms generated by GZT40, a strain that is unable to synthesis deoxynivalenol and is reduced in virulence (*13*), to FHB symptoms generated by FgΔTri14 mutants on Wheaton. In these tests, heads infected with GZ3639 and both FgΔTri14 mutants had levels of FHB comparable to similarly infected heads in tests 1 and 2. It is important to note that although the level of FHB in heads inoculated with GZT40 were not statistically different than the FgΔTri14 mutants (**Table 2** and **Figure 2**),

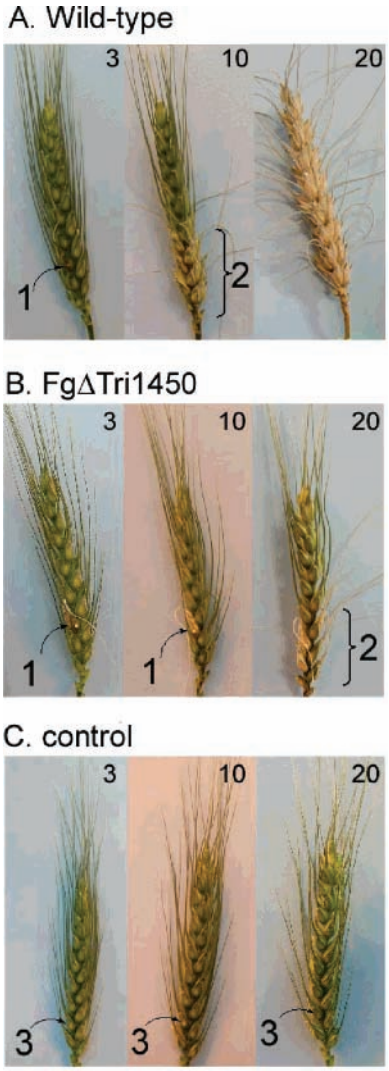

Figure 3. FHB wheat virulence assay. The wheat heads in each panel were inoculated with *F. graminearum* wild-type GZ3639 (A), FgΔTri1450 (B), or water (C), and heads were photographed at 3 days post inoculation (dpi), 10 dpi, and 20 dpi as indicated. (1) Fungal mycelia and plant necrosis was often observed in injected florets at 3 dpi. In a majority of heads infected with FgΔTri1450, FHB symptoms did not progress to neighboring spikelets. (2) FHB progresses rapidly in wild-type infected heads with most spikelets becoming bleached at 20 dpi. On the occasions that FHB symptoms generated by FgΔTri1450 did progress, it was minimal. (3) Water or mock infected spikelets show minimal damage, and seed development was not affected.

Table 2. FHB Results for *Tri14* Deletion Strains

strain	treatments	symptoms (%)	yield loss (%)	DON (ppm)
GZ3639	4	92.5 aa	75.2 aa	110.2 ± 70.7
FgΔTri1427	4	15.5 bc	11.3 b	nd[b]
FgΔTri1450	4	18.3 b	17.4 b	nd
FgTri14E36	1	79.6 a	55.5 a	32.3
FgTri14E57	1	94.9 a	61.1 a	69.6
GZT40	2	7.9 c	14.1 b	nd
water	4	0.1 d		nd

[a] Average percent disease or yield loss for strains followed by the same letter is not statistically different on the basis of differences of least-squares means at the $p \leq 0.05$ level. [b] nd indicates none detected.

disease progression differed significantly. For FgΔTri14 mutants, disease progressed to neighboring spikelets in 36% of the infected heads (29 of 80) while disease progressed in only 10%

Figure 4. Representative seeds harvested from one wheat head with 40% FHB at 20 dpi. (A) Seeds from the injected spikelet were significant reduced in size. (B) Seeds from spikelets showing disease symptoms ranged in disease severity. Note the shriveled appearance often referred to as "tombstones". (C) Seeds from healthy looking spikelets appear normal.

Table 3. Virulence Test of *Tri14* Complemented Strains

treatment	symptoms (%)	yield loss (%)	DON (ppm)
GZ3639	100	83.5	251.3
FgΔTri1450	42.7	22.9	nd[a]
FgT14AB3	95.8	74.3	189.5
FgT14AB6	95.6	73.1	348.1
FgT14AB8	100	76.7	374.0
FgT14AB18	96.0	75.5	248.5
FgT14AB30	88.1	77.0	149.4
FgT14AB33	77.1	55.5	130.7
Water	0	0	nd

[a] nd indicates none detected.

(2 of 20) of heads infected with GZT40. Concomitant with the fourth test on Wheaton, we assayed FHB on cultivar Norm by wild-type, FgΔTri14 mutants and GZT40. Similar reduction in virulence was observed (data not shown), indicating the reduction of virulence in the FgΔTri14 mutants is not host dependent. The affect of disease on seed development was observed upon harvest (**Figure 4**). Seeds collected from spikelets displaying significant symptoms up to 20 dpi were significantly reduced in size or yield as compared to seeds from spikelets with little disease symptoms.

We combined the results from the first four tests and found that the FgΔTri14 mutants caused significantly less disease than the wild-type or nondeletion transformants (**Table 2**). **Figure 2** depicts the average percent symptomatic spikelets and percent yield loss for the nondeletion transformants, FgΔTri14 mutants, wild-type, and deoxynivalenol deficient strain from the first four tests. The FgΔTri14 mutants caused significantly less disease than the wild-type or nondeletion transformants. In the fifth test, virulence of GZ3639, FgTri1450, and six *TRI14* complemented strains were examined on Wheaton. In this test, heads inoculated with GZ3639 and all six *TRI14* complemented strains exhibited substantial FHB while heads inoculated with FgTri1450, the parent strain of the complemented strains, exhibited significantly less FHB (**Table 3**).

DISCUSSION

TRI14 is located adjacent to the trichothecene biosynthetic gene cluster in *F. graminearum* and *F. sporotrichioides* and was considered part of the cluster because its transcription was coregulated with the cluster genes (*5*). Despite these observations, we found that the *F. sporotrichioides TRI14* (*FsTRI14*) is not required for T-2 toxin synthesis (*5*). In this report, we further explore *TRI14* function and have shown that *F. graminearum TRI14* (*FgTRI14*) is required for wild-type levels of virulence on wheat (**Figure 1**) and that it is not required for deoxynivalenol synthesis in cracked corn culture (**Table 1**). The *TRI14* mutant strains were different from the *TRI5* mutant strain (FgΔTri5) GZT40 with regard to virulence and ability to produce deoxynivalenol. *TRI5* encodes a trichodiene synthase which catalyzes the first step in trichothecene biosynthesis, and thus *TRI5* mutants cannot synthesize deoxynivalenol on cracked corn or wheat (*13*). FHB symptoms generated by *TRI5* mutants on wheat are significantly reduced as compared to wild-type and were only rarely observed in spikelets adjacent to the inoculated spikelet. In contrast, although FHB symptoms generated by FgΔTri14 mutants on wheat are significantly reduced compared to wild-type, symptoms were three times more likely to be observed in neighboring spikelets. Despite apparently significant levels of disease on occasion, we never observed any deoxynivalenol in *TRI14* mutant-inoculated wheat. Two possible explanations for these results are that *TRI14* is required for deoxynivalenol production in planta but not in culture or that there is not sufficient fungus present in TRI14 mutant-inoculated heads to produce detectable levels of deoxynivalenol.

What role does *FgTRI14* play in the wheat disease process? One possibility is that *TRI14* acts as a positive regulator of deoxynivalenol synthesis. For example, *TRI14* could affect transcription or translation of deoxynivalenol biosynthetic genes or interact with the DNA binding transcriptional regulator TRI6. The *A. parasiticus* AflJ is involved in the expression of aflatoxin biosynthetic genes in conjunction with a DNA binding protein encoded by *AflR* (*28*, *29*). Detection of *TRI4* expression (a trichothecene gene encoding an enzyme involved early in the pathway) in planta by *TRI5* mutants but not by FgΔTri14 mutants would support the involvement of *TRI14* in transcription. Alternatively, *TRI14* may increase overall fungal metabolism leading indirectly to more deoxynivalenol synthesis. It does not appear that *TRI14* is a transcription factor as no DNA binding domains were found in the *TRI14* predicted protein (Tri14p) using the Simple Modular Architecture Research Tool (SMART) (*30*, *31*). *TRI14* could influence deoxynivalenol synthesis via protein−protein interactions mediated though one or multiple phosphorylation sites. Analysis of potential Tri14p phosphorylation sites with the program NetPhos 2.0 identified 5 out of 22 serine residues, 4 of 26 threonine residues, and 3 of 19 tyrosine residues with scores well above (≥ 0.7) the cutoff at 0.5 (*32*). Experiments are underway to determine if Tri14p is a phosphoprotein and to identify proteins that may interact with it.

Another possible role that *TRI14* may play is in the export of deoxynivalenol outside of the mycelia. The detection of deoxynivalenol on cracked corn after growth of the FgΔTri14 strains may reflect an inefficient transport mechanism or even simple diffusion out of the mycelia. The minimal and delayed disease symptoms generated by FgΔTri14 strains on wheat could be due to a limited quantity of deoxynivalenol that is sufficient to partially overcome plant defenses and cause disease and is below our limit to detect in seed extracts. It should be noted

that if *TRI14* is involved in trichothecene transport, it could be either independent or function in consort with *TRI12*, a major facilitator superfamily (MFS) type chemical transporter that is located in the cluster (*33*).

The quantity of deoxynivalenol found in extracts after growth on cracked corn could reflect the large quantity of fungal mycelia produced in culture. Cracked corn cultures typically include significant fungal growth while there was little physical evidence of fungal growth on infected wheat. But fungal growth may not be tightly coordinated with production of secondary metabolites. *Neotyphodium lolii*, an endophyte of ryegrass, is able to maintain a high metabolic rate without new hyphal growth *in planta* (*34*). Experiments measuring fungal levels in planta via ergosterol or by a real-time quantitative PCR method could provide insights to help understand the connection between fungal growth or fitness and pathogenicity in planta. It is possible that the failure of protein kinase mutants (*MGV1* and *gpmk1*) to cause disease could be linked to their reduced ability to grow (*14*, *15*). In the case of the FgΔTri14 strains, since we did not observe any growth defects in culture, it is unlikely that the reduced pathogenicity is due to a defect in growth. Experiments with the wild-type, FgΔTri14 strains and various mutants in the deoxynivalenol pathway measuring deoxynivalenol levels in relation to fungal DNA load in plant material may lead to a better idea of how such levels influence pathogenesis in wheat. Another possibility that should be mentioned is the possible involvement of *TRI14* in the synthesis of another pathogenicity factor. Perhaps *TRI14* serves to bridge or coordinate deoxynivalenol synthesis with other factors that contributes to virulence. Experiments are underway to characterized the function of *TRI14* in the wheat disease process.

ABBREVIATIONS USED

PCR, polymerase chain reaction; EST, expressed sequence tag; Hyg, hygromycin B phosphotransferase gene; NR, nonredundant database; NCBI, National Center for Biotechnology Information; BLAST, basic local alignment sequence tool; YPG, yeast extract−peptone−glucose medium; PDA, potato dextrose agar.

ACKNOWLEDGMENT

We thank R. Proctor and R. Butchko for critically reading the manuscript, E. Mays and D. Shane for technical assistance, A. Morgan for synthesizing oligonucleotides, R. Stessman for assistance with virulence assays, and D. Palmquist for statistical analysis.

LITERATURE CITED

(1) Bai, G.; Shaner, G. Management and resistance in wheat and barley to Fusarium head blight. *Annu. Rev. Phytopathol.* **2004**, *42*, 135−161.

(2) Schlatter, J. Toxicity data relevant for hazard characterization. *Toxicol. Lett.* **2004**, *153*, 83−89.

(3) Bai, G.-H.; Shaner, G. Scab of wheat: prospects for control. *Plant Dis.* **1994**, *78*, 760−766.

(4) Brown, D. W.; McCormick, S. P.; Alexander, N. J.; Proctor, R. H.; Desjardins, A. E. A genetic and biochemical approach to study trichothecene diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*. *Fungal Genet. Biol.* **2001**, *32*, 121−144.

(5) Brown, D. W.; McCormick, S. P.; Alexander, N. J.; Proctor, R. H.; Desjardins, A. E. Inactivation of a cytochrome P-450 is a determinant of trichothecene diversity in *Fusarium* species. *Fungal Genet. Biol.* **2002**, *36*, 224−233.

(6) Brown, D. W.; Dyer, R. B.; McCormick, S. P.; Kendra, D. F.; Plattner, R. D. Functional demarcation of the *Fusarium* core trichothecene gene cluster. *Fungal Genet. Biol.* **2004**, *41*, 454−462.

(7) Proctor, R. H.; Hohn, T. M.; McCormick, S. P.; Desjardins, A. E. *TRI6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* **1995**, *61*, 1923−1930.

(8) Hohn, T. M.; Krishna, R.; Proctor, R. H. Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. *Fungal Genet. Biol.* **1999**, *26*, 224−235.

(9) Kruger, W. M.; Pritsch, C.; Chao, S.; Muehlbauer, G. J. Functional and comparative bioinformatic analysis of expressed genes from wheat spikes infected with *Fusarium graminearum*. *Mol. Plant Microbe Interact.* **2002**, *15*, 445−455.

(10) Mesterhazy, A.; Bartok, T.; Lamper, C. Influence of wheat cultivar, species of *Fusarium*, and isolate aggressiveness on efficacy of fungicides for control of Fusarium head blight. *Plant Dis.* **2003**, *87*, 1107−1115.

(11) Snijders, C. H. A. Resistance in wheat to *Fusarium* infection and trichothecene formation. *Toxicol. Lett.* **2004**, *153*, 37−46.

(12) Trail, F.; Xu, J. R.; San Miguel, P.; Halgren, R. G.; Kistler, H. C. Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*). *Fungal Genet. Biol.* **2003**, *38*, 187−197.

(13) Proctor, R. H.; Hohn, T. M.; McCormick, S. P. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol. Plant Microbe Interact.* **1995**, *8*, 593−601.

(14) Hou, Z.; Xue, C.; Peng, Y.; Katan, T.; Kistler, H. C.; Xu, J. R. A mitogen-activated protein kinase gene (MGV1) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Mol. Plant Microbe Interact.* **2002**, *15*, 1119−1127.

(15) Jenczmionka, N. J.; Maier, F. J.; Losch, A. P.; Schafer, W. Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase *gpmk1*. *Curr. Genet.* **2003**, *43*, 87−95.

(16) Lu, S. W.; Kroken, S.; Lee, B. N.; Robbertse, B.; Churchill, A. C.; Yoder, O. C.; Turgeon, B. G. A novel class of gene controlling virulence in plant pathogenic ascomycete fungi. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5980−5985.

(17) Voigt, C. A.; Schafer, W.; Salomon, S. A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. *Plant J.* **2005**, *42*, 364−375.

(18) Bai, G.; Desjardins, A. E.; Plattner, R. D. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia* **2001**, *153*, 91−98.

(19) Bowden, R. L.; Leslie, J. F. Nitrate nonutilizing mutants of *Gibberella zeae* and their use in determining vegetative compatibility. *Exp. Mycol.* **1992**, *16*, 308−315.

(20) Desjardins, A. E.; Brown, D. W.; Yun, S. H.; Proctor, R. H.; Lee, T.; Plattner, R. D.; Lu, S. W.; Turgeon, B. G. Deletion and complementation of the mating type (*MAT*) locus of the wheat head blight pathogen *Gibberella zeae*. *Appl. Environ. Microbiol.* **2004**, *70*, 2437−2444.

(21) Bai, G.-H.; Shaner, G. Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. *Plant Dis.* **1996**, *80*, 975−979.

(22) Thom, C. *The Penicillia*; The Williams and Wilkins Co.: Baltimore, MD, 1930; p 644.

(23) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: A laboratory manual*, 3rd ed. ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 2001.

(24) Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI−BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389−3402.

(25) Zhang, Z.; Schaffer, A. A.; Miller, W.; Madden, T. L.; Lipman, D. J.; Koonin, E. V.; Altschul, S. F. Protein sequence similarity searches using patterns as seeds. *Nucleic Acids Res.* **1998**, *26*, 3986−3990.

(26) Turgeon, B. G.; Garber, R. C.; Yoder, O. C. Development of a fungal transformation system based on selection of sequences with promoter activity. *Mol. Cell. Biol.* **1987**, *7*, 3297−3305.

(27) Marek, E. T.; Schardl, C. L.; Smith, D. A. Molecular transformation of *Fusarium solani* with an antibiotic resistance marker having no fungal DNA homology. *Curr. Genet.* **1989**, *15*, 421−428.

(28) Meyers, D. M.; Obrian, G.; Du, W. L.; Bhatnagar, D.; Payne, G. A. Characterization of *aflJ*, a gene required for conversion of pathway intermediates to aflatoxin. *Appl. Environ. Microbiol.* **1998**, *64*, 3713−3717.

(29) Chang, P. K. The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. *Mol. Genet. Genomics* **2003**, *268*, 711−719.

(30) Schultz, J.; Copley, R. R.; Doerks, T.; Ponting, C. P.; Bork, P. SMART: A web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* **2000**, *28*, 231−234.

(31) Schultz, J.; Milpetz, F.; Bork, P.; Ponting, C. P. SMART, a simple modular architecture research tool: Identification of signaling domains. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5857−5864.

(32) Blom, N.; Gammeltoft, S.; Brunak, S. Sequence- and structure-based prediction of eukaryotic protein phoshorylation sites. *J. Mol. Biol.* **1999**, *294*, 1351−1362.

(33) Alexander, N. J.; McCormick, S. P.; Hohn, T. M. TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: Gene isolation and expression in yeast. *Mol. Gen. Genet.* **1999**, *261*, 977−984.

(34) Tan, Y. Y.; Spiering, M. J.; Scott, V.; Lane, G. A.; Christensen, M. J.; Schmid, J. In planta regulation of extension of an endophytic fungus and maintenance of high metabolic rates in its mycelium in the absence of apical extension. *Appl. Environ. Microbiol.* **2001**, *67*, 5377−5383.

Received for review June 17, 2005. Revised manuscript received September 2, 2005. Accepted September 6, 2005.

JF051441A