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Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene

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Abstract Fusarium head blight occurs in cereals throughout the world and is especially important in humid growing regions. Fusarium head blight (FHB) has re-emerged as a major disease of wheat and barley in the U.S. and Canada since 1993. The primary causal agents of FHB, *Fusarium graminearum* and *Fusarium culmorum*, can produce deoxynivalenol (DON), a trichothecene mycotoxin that enhances disease severity and poses a health hazard to humans and monogastric animals. To reduce the effects of DON on wheat, we have introduced *FsTRI101*, a *Fusarium sporotrichioides* gene formerly known as TriR, into the regenerable cultivar Bobwhite. *TRI101* encodes an enzyme that transfers an acetyl moiety to the C3 hydroxyl group of trichothecenes. Four different transgenic plants carrying the *FsTRI101* gene were identified. Although expression levels varied among the four lines, all of them accumulated *FsTRI101* transcripts in endosperm and glume. *TRI101*-encoded acetyltransferase activity was detected in endosperm extracts of a single plant that accumulated *FsTRI101* mRNA. Greenhouse resistance tests indicated that the accumulation of *FsTRI101*-encoded acetyltransferase in this plant confers partial protection against the spread of *F. graminearum* in inoculated wheat heads (spikes).

Keywords Fusarium head blight · Mycotoxin · Scab · Transformation

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Introduction

In recent years, Fusarium head blight (FHB or scab) of wheat and barley has re-emerged as a serious disease in North America (Windels 2000), particularly in regions of the United States and Canada that are subjected to summer rains. Occurrences of FHB epidemics since 1993 have been attributed to a combination of factors that favor fungal propagation, including increased inoculum from greater levels of corn and wheat debris resulting from the adoption of reduced tillage systems (Dill-Macky and Jones 2000), increased rainfall between anthesis and flowering (Sung and Cook 1981) and cyclic plantings of wheat and maize. FHB is a complex disease that often involves more than one host species and has been associated with 17 different species of *Fusarium* (Parry et al. 1995). *Fusarium graminearum* is the primary causal agent of FHB in the midwestern United States, and worldwide (Sitton and Cook 1981). Infection by *F. graminearum* causes necrosis of florets that gives the head a "scabby" appearance, and results in moderate to severe reductions in grain yield (reviewed in Sutton 1982; Bai and Shaner 1994).

Some strains of the pathogenic fungus produce a trichothecene cytotoxin, deoxynivalenol (DON), that poses a health threat to humans and monogastric (non-ruminant) animals. Trichothecenes, such as T-2 toxin, scirpentriol and trichodermol, interact with the peptidyl transferase site of eukaryotic ribosomes and are potent inhibitors of translational initiation, elongation or termi-

nation (McLaughlin et al. 1977). Mutants of *Saccharomyces cereviseae* showing tolerance to DON were found to have an altered ribosomal protein L3 (Grant et al. 1976; Fried and Warner 1981; Fernandez-Lobato et al. 1990), indicating that DON exhibits the same mode of action shown for other trichothecenes. In recent reports, DON has been shown to inhibit protein synthesis in vitro (Yuan et al. 1999) and to act at the level of the ribosome *in planta* (Harris and Gleddie 2001). The effects of DON exposure on animals include cell necrosis and smooth muscle paralysis, hence its common name, vomitoxin. As a result, the U.S. Food and Drug Administration has set advisory levels of 1 ppm and 5 ppm of DON in food for human and livestock consumption, respectively.

Studies by Proctor et al. (1995) provide strong evidence that DON is a virulence factor in host-*Fusarium* interactions. Disruption of the gene encoding trichodiene synthase (*Tri5*), the enzyme catalyzing the first step in the DON biosynthetic pathway, reduced disease severity and progression by *Gibberella zeae* (teleomorph) on both wheat heads (Desjardins et al. 1996) and maize ears (Harris et al. 1999). Restoration of *Tri5* gene function to *Tri5*-minus mutants resulted in increased bleaching of flowering heads, higher trichothecene levels in the seeds, and reduced seed weight in field-inoculated wheat (Desjardins et al. 1996). Toxin production and disease severity on wheat seedlings (Proctor et al. 1997) and maize (Harris et al. 1999) were also restored to wild-type levels in a *Tri5* revertant and in a strain transformed with the wild-type *Tri5*.

Levels of DON in severely infected grain can exceed 60 ppm in the field, amounts that are unacceptable to the milling and brewing industries. Since reduced tillage offers clear benefits for soil and water conservation, and since the pathogen is so abundant in wheat-growing regions, cultivars able to limit *Fusarium* infection and/or detoxify DON are needed. Multigenic (QTL) sources of scab resistance have been identified in wheat and barley (Faris et al. 1999; Waldron et al. 1999, Bai et al. 2000; Anderson et al. 2001; Kolb et al. 2001); however, these genes show complex patterns of inheritance, and the levels of protection that they confer (30–40%) are not adequate for the control of FHB under high inoculum pressure. Partial resistance to FHB in one wheat cultivar, Frontana, was characterized by Miller and Arnison (1986). They found that cultured Frontana cells showed three-fold higher levels of DON degradation compared to susceptible cell suspensions, suggesting that reduced DON levels could be at least part of the resistance mechanism for this cultivar.

Although partial resistance has been identified in wheat varieties, no sources of immunity to *F. graminearum* have yet been found. In these circumstances, new effective genes as well as genes with complementary modes of action will be beneficial. Such genes can be introduced by genetic transformation and are potentially valuable sources of single-locus, co-dominant antifungal activities. Harris and Gleddie (2001) modified a rice 60S ribosomal protein L3 gene to mimic a *rpl3* mutation in a *S. cerevisiae* strain that was found to be tolerant to the trichothecene isotrichodermin. Calli and protoplasts derived from transgenic tobacco carrying the modified rice gene showed significantly greater regeneration and viability in the presence of DON as compared to those from tobacco expressing the unmodified gene.

We are investigating an alternative strategy, the expression of a trichothecene acetyltransferase gene, *FsTRI101*, from *Fusarium sporotrichioides*. *F. sporotrichioides* is a close relative of the pathogenic *F. graminearum*, and both species produce enzymes that transfer an acetyl group to the C3-OH of trichothecenes (Kimura et al. 1998), including DON (McCormick et al. 1999). *S. cerevisiae* expressing *TRI101* from *F. graminearum* can grow in the presence of 3- to 5-fold higher levels of DON than yeast without the gene (Adams and Lemmens 1996). We hypothesize that FsTRI101 activity will curtail the accumulation of DON and limit hyphal spread in wheat heads infected with *F. graminearum*. Enhanced trichothecene resistance in tobacco seedlings expressing *FsTRI101* has been demonstrated (Muhitch et al. 2000). Hence this gene might provide a similar benefit to wheat. In this paper, we report the fusion of the *FsTRI101* coding sequence to the maize constitutive *Ubi-1* promoter/exon 1/intron 1 (UBI) (Christensen and Quail 1996), its stable integration into the wheat genome, and the expression of *FsTRI101* in wheat-floret organs.

Materials and methods

Vector and plasmid constructs

To assemble the monocot expression vector pUB-LIT, a 2.6-kb *Pst*I fragment containing maize UBI (Christensen and Quail 1996) was excised from pUBK and ligated to *Nsi*I-treated pLITMUS-29 (New England BioLabs, Beverly, Mass.). The orientation of UBI was determined by restriction nuclease analysis using *Eco*RI. The 3′ NOS terminator, originally from pBI121 (Clontech, Palo Alto, Calif.), was excised from the plant transformation vector pPLANT5 by cleavage with *Stu*I and *Bsi*WI, and inserted into pUB-LIT at the *Sna*BI and *Asp*718 restriction sites. The resulting 5,069-bp plasmid was designated pUB-LIT1. The monocot expression vector pUBK was derived from pACH20 (Christensen and Quail 1996), in which the *bar* gene conferring resistance to the herbicide bialaphos (de Block et al. 1987) is regulated by the maize UBI and NOS 3′ terminator. The origin of replication and the ampicillin resistance gene (*bla*) of pAHC20 was replaced with the corresponding portion of pBGS9 (Spratt et al. 1986), which encodes kanamycin resistance (*npt*II).

A cDNA clone of *FsTRI101*, TriR1/pYES2, was described in earlier studies (McCormick et al. 1999). The *FsTRI101* coding region was amplified from TriR1/pYES2 by PCR, using primers 955 and 956 (Table 1) and *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The resulting fragment was cloned into pT7Blue3 using the Perfectly Blunt Cloning Kit (Novagen, Madison, Wis.). The coding region was excised using *Pst*I restriction endonuclease and subsequently inserted into the *Pst*I cloning site of pUB-LIT1, to generate the wheat expression construct pUBR1 (Fig. 1). Following PCR and cloning, the *FsTRI101* coding region, the promoter-coding region junction and coding region-terminator fusion, were confirmed in the complete nucleotide sequence of pUBR1 (data not shown).

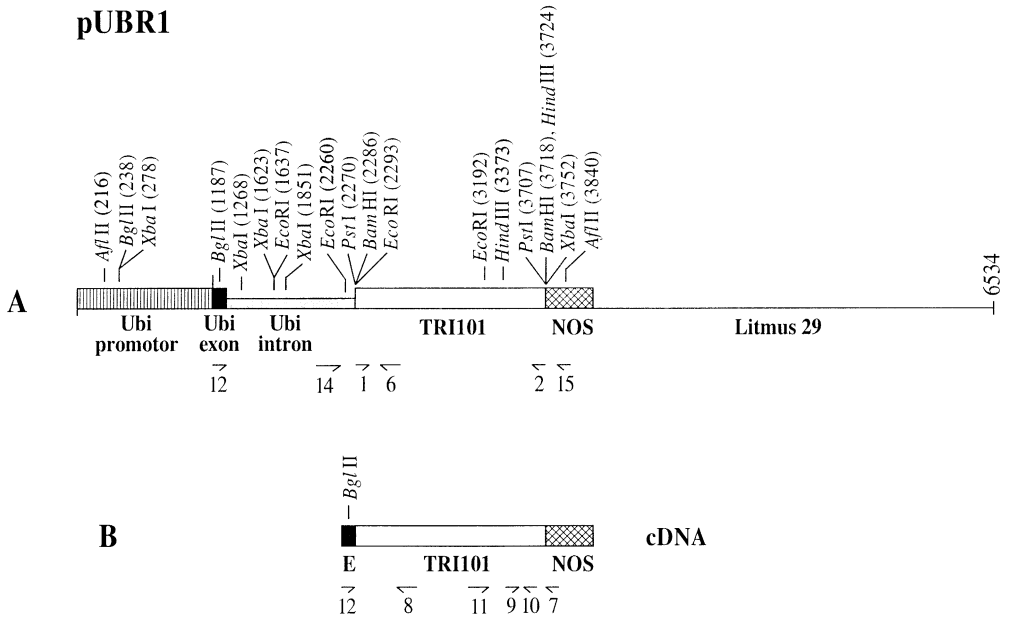


Fig. 1 The pUBR1 construct and deduced *FsTRI101* transcript. **Panel A**, Linear diagram of the plasmid, showing the 2.6-kb maize UBI (*vertical hatching*), exon (*black*) and intron (*gray*), the 1.4-kb *FsTRI101* coding region (*white*), the NOS 3′ untranslated region (*cross-hatched*) and the Litmus 29 backbone. Primers used for the PCR amplification of the *FsTRI101* gene are indicated by *numbered arrows* corresponding to the list in Table 1. Primer 13 (not shown) is specific to *F. graminearum TRI101* and occurs in approximately the same location as Primer 6 in *FsTRI101* Restriction sites are indicated above the plasmid diagram. **Panel B**, The approximately 1,700 nt *FsTRI101* cDNA (mRNA) deduced from pUBR1. Primers used for detection of *FsTRI101* transcripts are indicated by *numbered arrows* and listed in Table 1

Table 1 PCR primers used for site-directed mutagenesis, sequencing, transgene detection, and RT-PCR

Primer		Sequence (5′ to 3′)	Application
1	955	GCGCTGCAGATCAAAATGGCCGCAACAAGC	Cloning of *TRI101* coding region
2	956	CGCTCTGCAGTAGTCTAGTAAACTATCTACC	Cloning of *TRI101* coding region
3	ActA	CACTGGAATGGTCAAGGCTG	Actin RT-PCR (forward)
4	ActB	CTCCATGTCATCCCAGTTG	Actin RT-PCR (reverse)
5	BAR-A	CTTCAGCAGGTGGGTGTAGAGCGTG	Detection of *bar* coding region
6	FsTRI	GGACGGGGTAAACGAGACTG	Detection of *FsTRI101* coding region
7	RTNOS	GCCAAATGTTTGAACGATCTGC	RT-PCR (3′ end)
8	RTTRI	GAAACCCGCCTTTCTCAACC	*FsTRI101* RT-PCR (5′ end)
9	RTTRI2	CCTGAGAGTGTGAGAAGACC	*FsTRI101* RT-PCR (3′ end)
10	RTTRI3	CTTTGTCCACTCCTCATCC	*FsTRI101* RT-PCR (3′ end)
11	RTTRI4	GATGCTTCCACACCTACTG	*FsTRI101* RT-PCR (3′ end)
12	RTUBI	CAACCTCGTGTTGTTCGGAG	RT-PCR (5′ end)
13	TRI-B2	GGAGACTGATTTGGGTGTAGATCG	Detection of *FgTRI101* coding region
14	UBI-A2	CCTGCCTTCATACGCTATTTATTTGC	Detection of UBI::*TRI101* DNA
15	NOS-A	CCCATCTCATAAATAACGTC	Detection of *TRI101::Nos* 3′ DNA

Nucleotide sequencing and sequence analysis

Cycle sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, Calif.) and a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, Mass.). Sequence data were collected and analyzed using the ABI Prism 310 Genetic Analyzer, Data Collection software (version 1.0.2) and the AutoAssembler 1.4.0 (Perkin Elmer Applied Biosystems, Foster City, Calif.).

Generation and selection of transformants

Constructs were introduced into excised immature embryos of cv Bobwhite using a Model PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, Calif.) and a derivative of protocols described in Weeks et al. (1993) and Blechl and Anderson (1996). Media contained 40 g/l of maltose instead of sucrose. For the transformation experiment that yielded lines 74, 156 and 176, plasmid pAHC20 (Christensen and Quail 1996), carrying the *bar* gene, was co-bombarded with pUBR1 in a 1:1 molar ratio. Calli were transferred to MMS media containing 2 mg/l of 2,4 D for 3.5 weeks, followed by MMS media containing 0.2 mg/l of 2,4 D to induce shoot regeneration plus 3 mg/l of bialaphos (Meiji Seika Kaisha, Ltd., Tokyo, Japan) for selection of resistant plantlets. Subsequent steps were carried out as in Weeks et al. (1993). In a second experiment, plasmid pUBK was co-bombarded as a selectable marker DNA instead of pAHC20. On the day following bombardment, calli were transferred to MMS containing 2 mg/l of 2,4 D without selection for 2 weeks. Calli were transferred to MMS con-

Table 2 Restriction endonuclease cleavage products of the *UBI::TRI101* RT-PCR species. Amplified by primers "12" and "8" (Fig. 1). *indicate the predicted species that were observed on 6% acrylamide gels

Treatment	Predicted cleavage products (bp)	
	upper band[a]	lower band[b]
None	1,500*	495*
*Bam*HI	1,140*, 360*	135, 360*
*Bgl*II	45, 1,050*	45, 450*
*Hin*dIII	1,500*	495*
*Eco*RI	625*, 495*, 355*	110*, 35, 355*
*Xba*I	125, 355*, 230*, 880*	495*

[a] Transcript with 1,008-bp *Ubi* intron
[b] Spliced transcript

taining 1 mg/l of bialaphos and 2 mg/l of 2,4 D for 2 weeks. This selection step was repeated before shoots were regenerated on MMS containing 0.2 mg/l of 2,4 D and 1 mg/l of bialaphos. This experiment resulted in Line 49.

To analyze bialaphos-resistant plants and their progeny for integration of the above constructs, total DNA was obtained from 1 cm^2 leaf sections of mature plants (Dellaporta et al. 1983). Control DNAs included untransformed cv Bobwhite (negative) and pUBR1 (positive). DNA was amplified using polymerase chain reaction (PCR) and primers (Table 1) specific to the *Ubi-1* intron (Primer 14, UBI-A2), and to either *FsTRI101* (Primer 6, FsTRI) or to *TRI101* from *F. graminearum* (Primer 13, TRI-B2; Kimura et al. 1998). Primer 13 is specific to a DNA segment of *F. graminearum TRI101* that occurs in approximately the same location as Primer 6 in *FsTRI101*. Amplification was carried out as recommended for *Taq* polymerase (Perkin-Elmer, Foster City, Calif.), at an annealing temperature of 62 °C. The PCR products were authenticated by treatment with restriction endonucleases, including *Pst*I, *Eco*RI, *Bam*HI, *Bgl*II, and *Xba*I (Table 2), that generated the cleavage products predicted from the nucleotide sequence of *FsTRI101*.

To identify homozygotes, a minimum of eight progeny were sampled from each of two successive generations and tested for growth on 3 mg/l of bialaphos (Weeks et al. 1993) and for amplification of the UBI::*FsTRI101* DNA. Homozygotes were further characterized for transgene copy number, transcript accumulation, trichothecene acetyltransferase activity and Type II FHB resistance in greenhouse assays.

Genomic DNA analyses

Genomic DNA was extracted (D'Ovidio et al. 1992) from mature leaves of transgenic plants. The DNA was exhaustively cleaved with either *Bam*HI or *Afl*II restriction enzymes, partitioned on agarose, transferred to a Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, Calif.), and hybridized to α-^{32}P-labeled coding sequence fragments as recommended by Bio-Rad. The hybridization probe consisted of a 1.4-kb *Bam*HI fragment of pUBR1 containing the *FsTRI101* coding sequence. Probe DNA was labeled to a specific activity of $1–2 \times 10^6$ cpm/ng ($1.8–3.7 \times 10^4$ Bq/µg) using the Multiprime DNA Labeling System (Amersham Pharmacia Biotech, Inc., San Francisco, Calif.). Hybridization was detected on pre-flashed XAR-5 film (Kodak, Rochester, N.Y.) with an intensifying screen at –80 °C.

RT-PCR

Endosperm total RNA (Altenbach 1998) was obtained from 15 to 30 seeds harvested 15 to 25 days post-anthesis (dpa), and from glumes of three heads harvested at approximately 15 dpa. Total RNA was treated with RQ1 RNAse-free DNAse (Promega, Madison, Wis.), prior to purification by passage through a RNeasy column (Qiagen, Inc., Valencia, Calif.). RT-PCR was carried out using 600 ng of RNA and either a GeneAmp RNA PCR Kit (Perkin Elmer, Foster City, Calif.) or a OneStep RT-PCR Kit (Qiagen, Inc.) at an annealing temperature of 58 °–60 °C and 35 cycles. Transcript-derived cDNA was amplified using Primer 12 plus Primer 8 or Primer 6 (for the 5′ end of the transcript), and Primer 7 plus Primer 9 or Primer 10 plus Primer 11 (for the 3′ end) (Fig. 1, Table 1). RT-PCR products were cleaved with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Xba*I for authentication (Fig. 1, Table 2).

For semi-quantitative RT-PCR of *FsTRI101* transcripts, cDNA made from 50 to 600 ng of total RNA was amplified for 25 cycles. Actin cDNA was generated from 5 to 60 ng of total RNA, and amplified using ActA and ActB primers (Table 1) that were designed from conserved regions of three actin cDNA clones from *Triticum* spp. (O. Anderson, GenBank accession nos. BE398871, BE425627, BE432906).

FsTRI101 enzyme-activity assay

FsTRI101 activity was determined essentially as described in McCormick et al. (1999). Endosperm tissue extruded from seed coats of developing seeds (15–25 dpa) was homogenized in 10 mM of potassium phosphate buffer, pH 7.0, and 1 mM of β-mercaptoethanol. After centrifugation at 18,000 g for 5 min at 4 °C, the supernatant was removed and frozen in powdered dry ice. Assays were incubated at 30 °C and were initiated by the addition of 100 µl of the thawed supernatant to a reaction mixture containing 250 µl of 500 mM potassium phosphate buffer (pH 7.0), 10 µl of isotrichodermol in acetone (1 mg/50 µl), 100 µl of 20 mM $MgSO_4$ and 50 µl of acetyl-CoA (Sigma) in water (25 µg/200 µl). Controls contained 100 µl of potassium phosphate buffer instead of the seed extract. Reaction mixtures were sampled at 1, 2 and 4 h after addition of the endosperm extract. Reaction products were analyzed by gas chromatography using flame ionization detection on a Hewlett-Packard 5890 Gas Chromatograph fitted with a 30-m fused silica capillary column (DB1; 0.25 µm; J& W Scientific Co., Palo Alto, Calif.). The column was held at 120 °C at injection; heated to 210 °C at 15 °C/min and held for 1 min; and finally heated to 260 °C at 5 °C/min and held for 8 min. Under these conditions, isotrichodermol is eluted at 10.3 min and isotrichodermin is eluted at 11.6 min. Three assays were run for each extract. Total protein was determined using the Bio-Rad Protein Assay reagent (Hercules, Calif.). The identity of the product was confirmed by GC-MS.

Greenhouse testing

Evaluation of transformed lines expressing *FsTRI101* for resistance to the spread of *Fusarium* within heads was conducted in greenhouse tests. The first experiment was planted in December 1999, and repeated twice with plantings in January and October 2000. Null segregants of the transgenic lines were not included. Resistant check lines were Sumai3 (seed source Shaanxi, P.R. China) and Fujian 5114; susceptible checks were Norm, Roblin and Wheaton. Transgenic lines, check lines and non-transformed Bobwhite were planted in 13-cm square plastic pots (13-cm, height) containing Metro-Mix 200 growth medium (Scotts-Sierra Horticultural Products, Maryland, Ohio). Five pots of each line were planted with two plants each, except in the third test in which three plants were planted in each pot. Each pot was fertilized at planting with 3 g of slow-release fertilizer (14-14-14, N-P-K). Temperatures in the greenhouse throughout the tests ranged from 20 to 25 °C.

Macroconidia were produced by culturing *F. graminearum*, isolate Butte86ADA-11 (Evans et al. 2000), on mung-bean agar [40 g of mung bean (var. Berken Improved)/liter, placed in boiling distilled water until the pericarp just starts to crack, filtered through two layers of cheesecloth, adjusted to 1 liter and 15 g of

agar] in Petri dishes (100 × 15 mm) using the method described by Evans et al. (2000). Isolates were grown for 14 days, and macroconidia were harvested by washing the culture surface with 10–20 ml of distilled water per Petri dish using a pressurized hand sprayer. The suspension of spores and mycelial fragments was filtered through one layer of cheesecloth, and spore concentrations adjusted to 1×10^5 spores/ml.

Wheat plants were inoculated when they were shedding anthers (Feekes growth stage 10.5, Large 1954) by carefully placing 10 µl of inoculum within the flower of a centrally located spikelet, using a Hamilton syringe fitted with a repeating dispenser [models 750RN (500 µl) and BB600-10; Hamilton Company, Reno, Nev.]. Only the primary spikes of each plant were inoculated. Inoculated plants were placed in a dew chamber providing approximately 98% relative humidity for 72 h. Lighting (12 h:12 h, light:dark) was provided using four 40-W fluorescent lights (12 µmol/m^{-2}/s^{-1}) during the dew period. Plants were returned to greenhouse benches. For each line or cultivar, approximately ten heads were inoculated in each of the three experiments.

Disease response was determined by visually examining heads 21 days after inoculation. Symptomatic spikelets (not including the inoculated spikelet) and total spikelets were recorded for each head. Symptomatic spikelet data were subjected to analysis of variance using Statistix (Statistix 7.0, Analytical Software, Tallahasse, Fla.). For cases in which the *F* Ratio was significant ($P < 0.05$), differences among treatments were determined using least significant differences. Data for individual tests were analysed separately and in a combined analysis.

Results

Transformation and transgene copy number

Co-transformation of a total of 1,400 *Triticum aestivum* cv Bobwhite embryos in two separate experiments with pUBR1 and the *bar* selectable marker gene yielded eight independent transgenic plants resistant to bialaphos. We used PCR analysis to show that four of these lines also carried the unselected *FsTRI101* transgene; these are designated 49, 74, 156 and 176. For this analysis, we used primers specific to the *Ubi-1* intron and *TRI101* (Primer 6 or the overlapping Primer 13 from *F. graminearum*) to amplify a 240-bp UBI::*FsTRI101* segment from pUBR1 (Table 1, Fig. 2). This PCR product was generated from genomic DNA of all four transformed lines but not of untransformed Bobwhite, and was identical to a product amplified directly from the pUBR1 plasmid. An example of such a result is shown in Fig. 2. Treatment of the 240-bp PCR product with *Pst*I generated two fragments of approximately 140 bp and 110 bp, which were predicted from the sequence of pUBR1 (Fig. 1). PCR using these primers was also carried out to follow the inheritance of *FsTRI101* and to identify progeny that were homozygous for the transgene.

Genomic DNA blots of Line 49 (heterozygous when tested) and three homozygous lines (74, 156, 176) indicated that more than one copy of pUBR1 had integrated into the wheat genome (Fig. 3). Cleavage with *Bam*HI was expected to produce a 1.4-kb fragment containing just the coding region. A fragment of this size hybridized most intensely to the *FsTRI101* coding sequence probe in all four lines. Additional hybridizing *Bam*HI species, in lines 156 and 176, indicated that some copies of

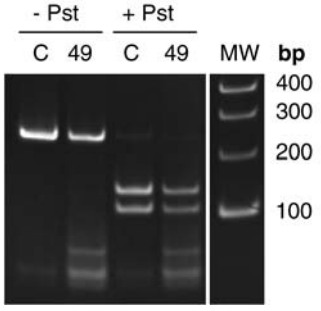


Fig. 2 PCR detection of stably integrated *FsTRI101* DNA in Line 49, and authentication of the PCR product by restriction endonuclease digestion. The UBI::*FsTRI101* PCR products were generated from pUBR1 plasmid (*C*) or genomic DNA from transformed Line 49 using primers 14 and 13 (Table 1), and were partitioned on 6% acrylamide in TBE, with (+) or without (–) treatment with *Pst*I restriction endonuclease. MW = 100-bp DNA ladder (GibcoBRL)

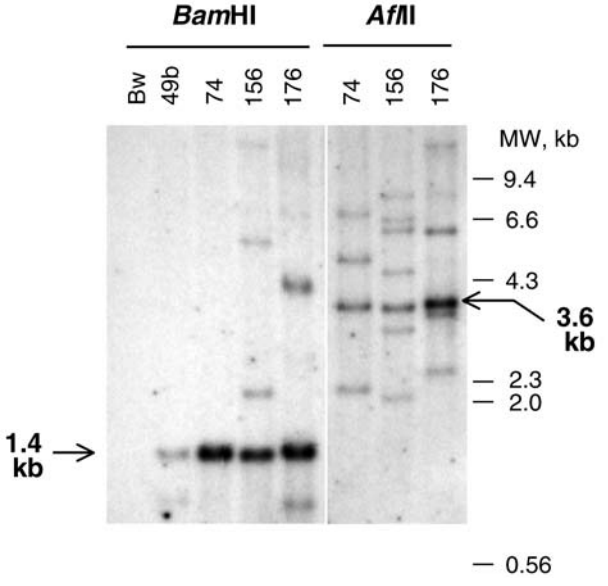


Fig. 3 Blot of genomic DNA from *FsTRI101* lines (49, 74, 156, 176) and untransformed Bobwhite (*Bw*) hybridized to radiolabeled 1.4-kb *Bam*HI DNA segments containing the *FsTRI101* coding sequence. Approximately 10 µg of DNA was exhaustively cleaved with either *Bam*HI or *Afl*II restriction endonuclease. Autoradiography was carried out at –80 °C for 70 h. The sizes and locations of fragments expected from intact pUBR1 transgenes are indicated by *arrows*. Sizes of molecular-weight markers are indicated on the right

FsTRI101 had undergone rearrangements in these lines. *Afl*II cleaves twice within pUBR1 to give a 3.6-kb fragment that spans the *Ubi1* promoter/exon/intron, *FsTRI101* coding sequence, and the *Nos* 3′ transcriptional terminator (Fig. 1). *Afl*II treatment of genomic DNA from the three homozygous lines verified that at least one copy of the intact 3.6-kb fragment was present, indicating that these lines had the potential for expressing *FsTRI101*. Each of these lines also contained fragments of different sizes, most likely rearranged or truncated

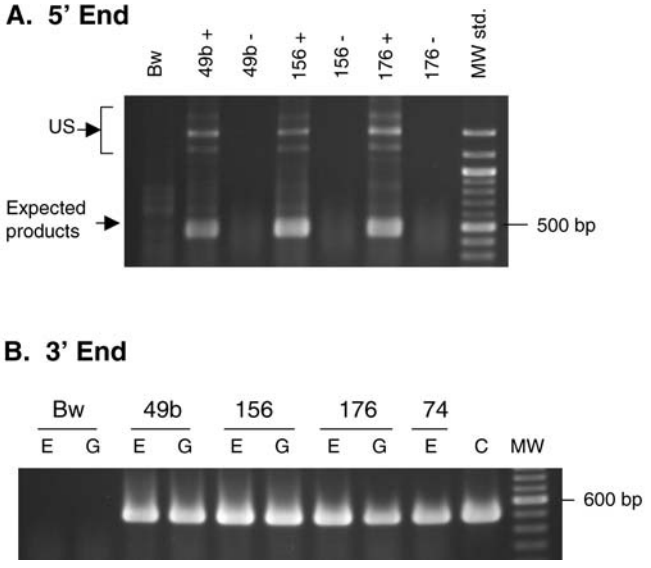


Fig. 4A, B RT-PCR analyses for 5′ and 3′ portions of *FsTRI101* transcripts expressed in transformed wheat lines. **Panel A**, a major 500-bp product representing the 5′ end of the transcript was detected with primers :: "12" and "8" (Fig. 1, Table 1) in cDNA (+) derived from 600 ng of total RNA from endosperm harvested at 25–30 dpa, but not in RNA samples without reverse transcription (–). "*Bw*" = cDNA from untransformed Bobwhite; US = RT-PCR products of unspliced transcripts containing the 1,008-bp *Ubi-1* intron. From sequence data, the RT-PCR products were predicted to be 500 bp for the mature *FsTRI101* message, and about 1,500 bp for products with the *Ubi* intron. *MW* = 100-bp ladder from New England BioLabs, of which the intense bands are 500 bp and 1,000 bp. **Panel B**, 500-bp product representing the 3′ portion of the coding region, amplified from cDNA synthesized from 600 ng of endosperm (*E*) and glume (*G*) total RNA from untransformed (*Bw*) and transformed lines. Primers "10" and "11" were used (Fig. 1, Table 1). C = pUBR1 control template; *MW* = 100-bp ladder from GibcoBRL, of which the intense band is 600 bp

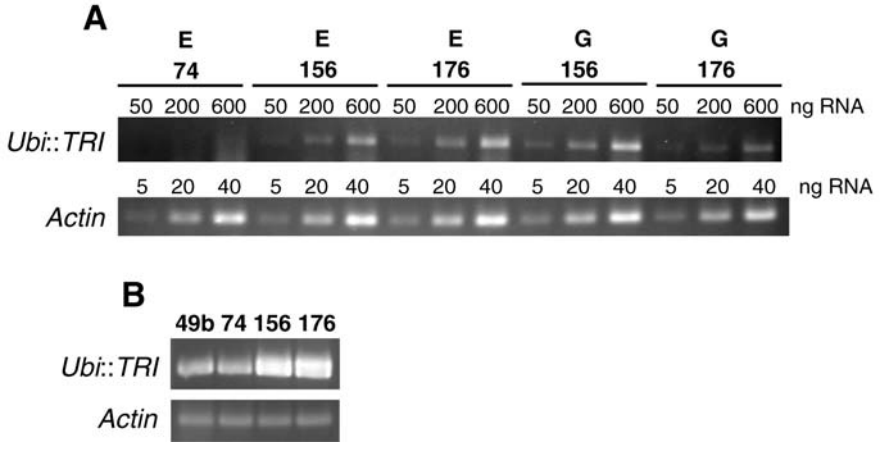
copies of the plasmid. DNA from the untransformed cv Bobwhite did not hybridize to the *FsTRI101* probe.

Accumulation of *FsTRI101* transcripts

We used RT-PCR to evaluate the expression of *FsTRI101* in floret organs of transgenic wheat plants. We initially focused on endosperm, an organ in which the *Ubi-1* promoter was known to be active (Stoger et al. 1999; Rooke et al. 2000), and from which RNA could readily be extracted. Primers 12 and 8, designed to amplify the 5′ end of the *TRI101* cDNA, generated a major PCR product of approximately 500 bp in Lines 49, 156 and 176 (Fig. 4A), and in Line 74 (data not shown). This product was obtained only if cDNA synthesis was first carried out, indicating that it was derived from *FsTRI101* transcripts rather than from contaminating DNA. It was not detected in RNA from untransformed Bobwhite. Two or three additional PCR products of about 1.5 kb were amplified from the endosperm cDNA of all four lines. Treatment of these products with restriction enzymes that cleaved within the *Ubi-1* intron or within the *FsTRI101* coding region indicated that they represented transcripts that retained the *Ubi-1* intron (Table 2). In contrast to the endosperm, the glume showed no accumulation of unprocessed *FsTRI101* mRNA. The 3′ terminal portion of the coding sequence, detected with primers 10 and 11, was also represented in endosperm and glume transcripts from all four transformed lines, but not from untransformed Bobwhite (Fig. 4B). However, RT-PCR that was carried out with primers 9 and 7 (NOS), designed to amplify the 3′ untranslated region of the transcript, failed to produce amplification products (data not shown). The degree of amplification varied slightly with different RT-PCR protocols.

FsTRI101 was differentially expressed in each of the four lines, as observed in semi-quantitative RT-PCR experiments (Fig. 5). The amplification of *FsTRI101* cDNA was proportional to the amount of RNA template in the range of 50–600 ng, whereas linear amplification of actin cDNA required a 10-fold dilution of the same

Fig. 5A, B Semi-quantitative RT-PCR of *FsTRI101* transcripts in transgenic wheat lines. **Panel A**, amplification of UBI::*FsTRI101* cDNA (upper) made from different amounts of endosperm (*E*) and glume (*G*) total RNA from three transgenic lines, and actin cDNA (lower) from the same RNA preparations. Twenty five amplification cycles were performed. **Panel B**, a separate experiment was done using 35 amplification cycles to detect transcripts from lines 49 and 74 in endosperm RNA. PCR reactions showing comparable actin amplification levels were assembled to compare *TRI101* transcript levels



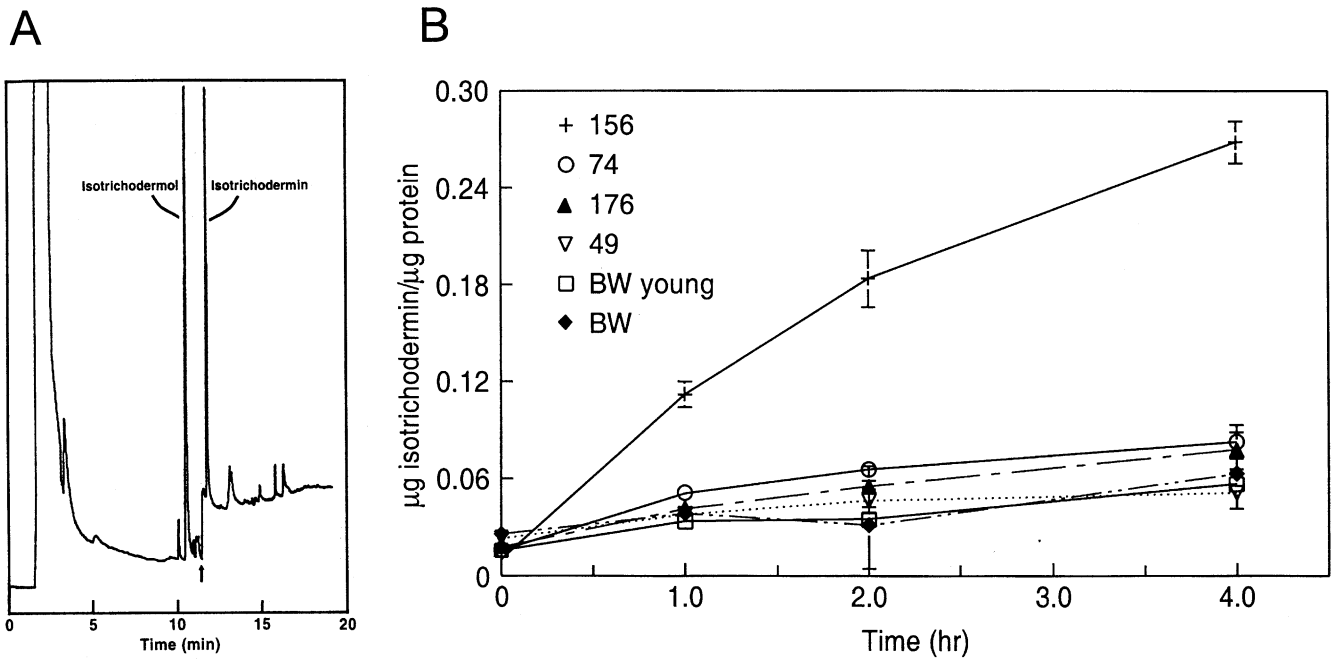


Fig. 6A, B FsTRI101 enzyme assay. **Panel A**, GC chromatogram of the substrate (isotrichodermol) and product (isotrichodermin) from a Line 156 endosperm extract after a 4-h incubation. Attenuation was increased at 11 min to better visualize isotrichodermin. **Panel B**, FsTRI101 activity in endosperm of non-transformed Bobwhite (BW) and transgenic wheat lines. "BW young" was harvested at 14–20 dpa. All other samples were harvested at 21–28 dpa. Conversion of isotrichodermol to isotrichodermin was monitored over a 4-h period. Each measurement represents the average of three assays with standard deviations shown by *vertical bars*

samples, or 5–40 ng of total RNA (Fig. 5A). Steady state levels of *FsTRI101* mRNA were higher in Line 156 and Line 176 than in lines 49 or 74 (Fig. 5A, B). Under conditions that resulted in linear amplification of cDNA from lines 49 and 74 (35 cycles), amplification was non-linear for comparable amounts of Line 156 and Line 176. Conversely, no amplification was obtained with cDNA from Line 74 under conditions (25 cycles) that resulted in linear amplification of cDNA from Lines 156 and 176. From these results, we estimate that the relative abundance of *FsTRI101* transcripts is at least 10-fold higher in Lines 156 and 176 than in Lines 49 and 74. In general, transcript levels in the glume were similar to those in the endosperm (Fig. 5A), as determined by comparison with the actin internal standard.

FsTRI101 acetyltransferase activity

FsTRI101 activity was measured in endosperm extracts of all four *FsTRI101* lines, using isotrichodermol as a substrate (Fig. 6A). Line 156, one of two that exhibited the highest levels of *FsTRI101* mRNA accumulation, showed trichothecene acetyltransferase activity that was significantly above background (Fig. 6B). No significant activity was observed in endosperm from lines 49, 74, 176, untransformed Bobwhite or two transgenic lines not carrying *FsTRI101* (data not shown).

Greenhouse resistance tests

Three independent greenhouse tests for resistance to the spread of *Fusarium* were performed on lines 156 and 176, and on resistant and susceptible check cultivars (Table 3). The combined results of the three tests are also presented (Table 3, Fig. 7). Disease symptoms were generally seen within 3 days in the inoculated spikelet, and where disease spread was observed; non-inoculated spikelets became symptomatic in 6 to 12 days after inoculation. The three tests ranked the lines in a similar order although the mean severity of Fusarium head blight was significantly lower ($P = 0.05$) in Test 2 (mean FHB severity of 40%) in comparison to Tests 1 and 3 (mean FHB severities of 56% and 50%, respectively). The variability among tests, as observed for Bobwhite, is not uncommon for lines with intermediate levels of resistance (Dill-Macky, unpublished data) and is attributed in part to the large contribution of the environment to the disease (e.g. Anderson et al. 2001).

The spread of symptoms from the inoculated spikelet was usually not observed in the resistant checks, Sumai3 and Fujian 5114 (Fig. 7). In contrast, the spread of symptoms in the susceptible checks, Norm, Roblin and Wheaton, frequently exceeded 75% (Fig. 7, Table 3), with means for the percent symptomatic spikelets of 61%, 84% and 70%, respectively. Non-transformed Bobwhite appeared to be moderately susceptible; most heads showed 50–100% symptomatic spikelets (Fig. 7), with mean percent symptomatic spikelets of 74%, 21% and 74% for the three tests.

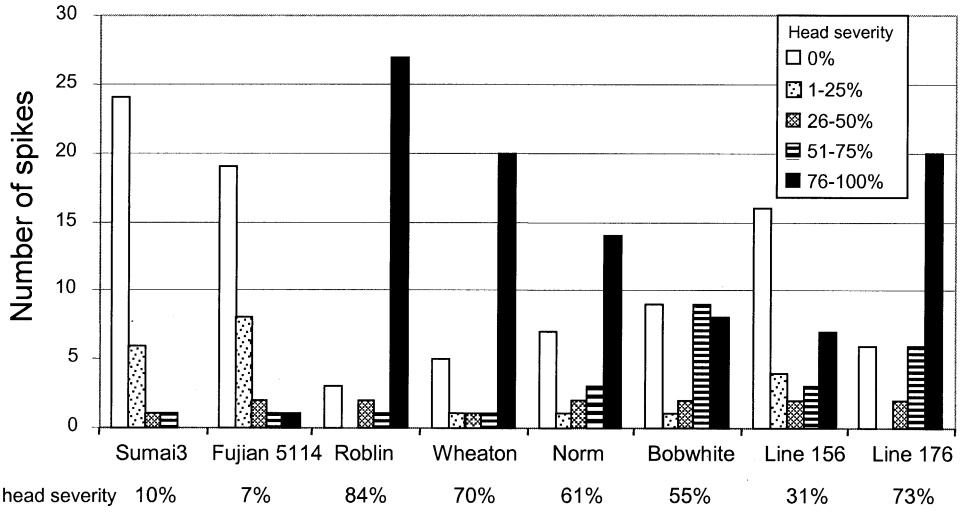


Fig. 7 Frequency distribution of spikes in five disease severity classes [0%, 1–25%, 26–50%, 51–75%, 76–100% symptoma tic spikelets (not including the inoculated spikelet)] for transgenic lines 156 and 176, non-transformed Bobwhite from the three trials in which lines 156 and 176 were tested (Bw3), and resistant (Sumai3 and Fujian 5114) and susceptible (Norm, Roblin, Wheaton) checks. A total of 30 numbers spikes were evaluated for each of the cultivars and test lines

Table 3 Fusarium head blight severity of *FsTRI101* lines and check cultivars point inoculated in greenhouse with *F. graminearum*

Line	Fusarium head blight severity (%)[a]							
	Test 1		Test 2		Test 3		Combined data tests 1–3	
Line 156	27[b]	a[c]	36[b]	bc	31[b]	b	31[d]	b
Line 176	74	b	69	de	77	c	73	de
Bobwhite	74	b	21	ab	74	c	55	c
Norm	68	b	49	cd	65	c	61	cd
Roblin	91	b	80	e	81	c	84	e
Wheaton	89	b	61	cde	65	c	70	de
Fujian 5114	8	a	5	a	9	ab	7	a
Sumai 3	24	a	3	a	2	a	10	a

[a] Fusarium head blight severity, (symptomatic spikelets-1/total spikelets) × 100
[b] Values represent the means of ten replicates
[c] Means followed by the same letter are not significantly different at $P \leq 0.05$
[d] Values represent the means of the combined data of three tests

Line 156, having a significant level of FsTRI101 enzyme activity, exhibited partial resistance to *F. graminearum*. Its level of resistance was significantly greater ($P = 0.05$) than that of line 176, which did not exhibit FsTRI101 activity (Table 3). The majority of heads of line 156 had less than 26% symptomatic spikelets (Fig. 7), and the mean percent of symptomatic spikelets for the three tests were 27%, 36% and 31%. Line 176 showed 79%, 73% and 61%. In tests 1 and 3 (Table 3), the level of resistance in line 156 was significantly greater than that of non-transformed Bobwhite (74%) and the susceptible checks. However, in Test 2, Bobwhite severity was only 21% and Line 156 was not more resistant. Combined data for the three tests showed significant differences in resistances among Line 156, Line 176 and non-transformed Bobwhite (Table 3).

Discussion

A *Fusarium* gene, *FsTRI101*, was successfully expressed in wheat under control of the maize UBI promoter. In Line 156, TRI101 (acetyltransferase) activity was detected. Line 156 also showed moderate but significant levels of protection against *Fusarium* spread in inoculated heads in greenhouse resistance assays. In contrast, Line 176 showed no evidence of FsTRI101 activity or enhanced resistance, even though it accumulated as much *FsTRI101* mRNA as Line 156. The basis for the lower trichothecene acetyltransferase activity in Line 176 compared to Line 156 is unknown, but could reflect additional levels of regulation, such as protein processing, stability and activity. Alternatively, transcript estimates generated from semi-quantitative RT-PCR are subject to a high variance that could account for the apparent high transcript levels in Line 176. As expected, protein activity rather than mRNA accumulation is the more accurate indicator of the tolerant lines.

The *FsTRI101* gene has been transferred to tobacco by Muhitch et al. (2000), who used the CaMV 35*S* promoter to obtain high levels of expression. Their transgenic tobacco plants showed enhanced tolerance to trichothecenes in germination tests, whereas our *TRI101*-expressing wheat lines did not (data not shown). No differences were observed between transgenic and untransformed wheat plants germinated in the presence of up to 5 mg/l (14 μM) of DAS (unpublished); both types of wheat germinated well at concentrations that were toxic

to untransformed tobacco. The refractory response of wheat in these assays points to basic differences in the uptake or metabolism of trichothecenes among plants species.

Steady state levels of endosperm *FsTRI101* mRNA, determined using semi-quantitative RT-PCR, varied among the four homozygous lines, and were about 10-fold lower than endogenous actin mRNA. *TRI101* mRNA expression levels do not appear to correlate with gene copy numbers determined by genomic Southern blots. In other transgenic lines, containing a wheat thaumatin-like protein (*tlp*) gene under regulation of maize UBI, *tlp* expression levels were comparable to those of actin in endosperm (unpublished), indicating that UBI is a strong promoter for this tissue. The differences in steady state mRNA levels for wheat vs *Fusarium* transgenes suggest that molecular attributes of the chimeric mRNA and/or the transgene coding region have roles in gene expression. For instance, genes originating from *Fusarium* differ subtly from genes from wheat in the usage of specific codons, a factor with potential impact on cellular mRNA stability and translatability.

Transcript accumulation was reduced in the endosperm of younger seeds harvested 15–20 dpa (data not shown), indicating that either UBI is less active or the *FsTRI101* mRNA is more labile in younger endosperm. No marked differences in glume and endosperm levels of *TRI101* mRNA were apparent for any of the four lines. In endosperm, but not in the glume, we observed incomplete intron processing as well as apparent mRNA truncation in our RT-PCR experiments. No plant consensus polyadenylation sites or intron processing consensus elements were present in the deduced *FsTRI101* mRNA. Incomplete transcript processing is associated with the expression of some genes in cells and tissues undergoing endoreduplication, including endosperm and symbiotic root nodules (discussed in Okubara et al. 1999). Therefore, unspliced *FsTRI101* transcripts in wheat endosperm are not wholly unexpected. Incomplete splicing was observed in transcripts from endosperm sampled at both 15–20 dpa and 20–25 dpa.

Amplification anomalies were noted for the 3′ end of *FsTRI101* transcripts from both endosperm and glume, specifically, involving the absence of amplification products when a NOS-specific primer was used. Either the NOS priming site was not present in these transcribed mRNAs or the amplification was sub-optimal, perhaps due to DNA or protein interactions in the 3′ untranslated portions of the messages.

FsTRI101 activity was most likely to be detected in the greenhouse assays that measure Type II resistance (spread in the head). The detection of partial FHB resistance in only one of four transgenic lines raises the formal possibility that tissue culture-induced somaclonal variation might be the basis for the resistance phenotype. However, since this line was the only one with detectable TRI101 activity, and five other transgenic lines containing constructs other than *FsTRI101* did not show improved resistance compared to non-transformed Bobwhite (data not shown), we favor the interpretation that FsTRI101 activity can confer partial resistance to FHB spread in wheat. In the single field trial conducted so far, neither lines 156 or 176 exhibited more overall resistance to *Fusarium* than non-transformed Bobwhite (data not shown). These apparently contradictory results are consistent with the role of DON as a virulence factor post-infection rather than in the initial infection process. However, the durability and magnitude of resistance will need to be tested in further field trials.

The moderate *in planta* tolerance associated with FsTRI101 activity is the first reported use of a DON modifying enzyme to combat FHB in cereals. Given the promise shown by *FsTRI101* in both wheat and tobacco, it would be beneficial to modify the gene for enhanced expression in wheat and other cereals. A modest reduction in DON levels will improve grain quality by lowering the accumulation of a potent trichothecene toxin, and might afford additional protection for existing varieties with moderate levels of resistance. Finally, FsTRI101 in combination with anti-*Fusarium* proteins having different modes of action, could produce lines of wheat with more effective resistance to FHB.

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