Expression of 3-OH trichothecene acetyltransferase in barley (Hordeum vulgare L.) and effects on deoxynivalenol

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Abstract

Fusarium head blight (FHB), caused primarily by Fusarium graminearum, has been the most destructive disease of barley (Hordeum vulgare L.) in the USA since the early 1990s, resulting in large economic losses for growers. The fungus produces the mycotoxin deoxynivalenol (DON), a protein synthesis inhibitor, which is harmful to humans and livestock. Chemically modifying DON could reduce DON accumulation in the grain. We introduced Tri101, which encodes a 3-OH trichothecene acetyltransferase that converts DON to a less toxic acetylated form, into the cultivar Conlon through particle bombardment of callus in an attempt to detoxify DON. Southern analyses confirmed six independent integrations of Tri101 into the barley genome. Northern, Western and trichothecene acetyltransferase activity analyses confirmed the inheritance and expression of Tri101 in the progenies of three independent transgenic lines. Greenhouse tests of T3 and T4 transgenic lines showed a reduction in DON concentration; however, field tests of T4 transgenic lines showed no reduction in DON accumulation. The field tests also showed the presence of somaclonal variation in the transgenic plants. The backcrossed transgenic lines were tested in the field and showed no reduction in DON accumulation. The backcrossed transgenic lines had reduced trichothecene acetyltransferase activity compared to the T4 lines and DON levels comparable to wild-type Conlon barley under field tests.

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Keywords: Fusarium head blight; Disease resistance; Transformation; Detoxification

1. Introduction

Fusarium head blight (FHB), primarily caused by the fungus Fusarium graminearum Schwabe [teleomorph Gibberella zeae (Schweinitz) Petch], is a devastating disease of barley and wheat (Triticum species) that inflicts heavy economic damage to the growers and industry [1]. Direct and indirect economic losses in the US due to FHB in barley and wheat from 1998 to 2000 are estimated at $2.7 billion, of which two northern states, North Dakota and Minnesota, account for about 55% of the total loss [2]. Combined losses for barley alone in North Dakota, South Dakota and Minnesota from 1998 to 2000 are estimated at $136 million.

F. graminearum can infect spike tissues from emergence of the spike from the flag leaf sheath through grain maturity [3] and causes necrosis of spikelet and seed cells, resulting in reduced yield and poor grain quality. In addition to reducing productivity, FHB infection results in the production of a number of trichothecene mycotoxins in the infected grains that
pose serious health hazards such as vomiting, dermatitis, and hemorrhagic septicemia in humans and livestock. High concentrations of these mycotoxins limit the utilization of the infected grains [4]. Trichothecenes are potent inhibitors of protein synthesis in eukaryotes and prevent polypeptide chain initiation or elongation by binding to 60 S ribosomal subunits [5,6]. Trichothecenes, produced by a number of Fusarium species, include deoxynivalenol, nivalenol, T-2 toxin, HT-2 toxin and fusarenon-X. Deoxynivalenol (DON) is the most common mycotoxin associated with FHB. The US Food and Drug Administration has set wheat advisory concentrations of 1 and 5 μg/g of DON in food for human and livestock consumption, respectively. DON-free or low-DON grain is required for malting barley as DON carries through malting and brewing into finished beer [7].

Many genes involved in the trichothecene pathway have been identified. The ‘Tri cluster’ contains a central core of genes such as Tri5, which is responsible for the first key step in the trichothecene pathway [8,9]. Tri101 is located outside of the Tri5 cluster and is under the transcriptional control of Tri6, a regulatory gene within the Tri5 cluster [10]. It has been well documented that Tri101 catalyzes the conversion of toxic Fusarium trichothecenes to less-toxic products and thus are involved in the protection of F. graminearum against its own toxin [9,10]. Tri101 is involved in the modification of a number of trichothecenes and, when expressed in Schizosaccharomyces pombe, confers resistance to trichothecenes [11]. Consequently, it was proposed that the transgenic plants expressing Tri101 might detoxify DON and reduce the use of agricultural chemicals [11].

The objective of the research described in this report was to produce Conlon barley plants expressing Tri101 from F. sporotrichioides and test whether the gene reduces DON accumulation via the conversion of DON to a less toxic acetylated form.

2. Materials and methods

2.1. Plant material

Immature embryos (2–4 mm in length) from barley cv. Conlon (two-rowed barley) were isolated from greenhouse [21–26 °C, 16/8 h (day/night) photoperiod supplemented by sodium halide lights] and growth chamber grown plants (16 h 20 °C day, 8 h 16 °C night photoperiod with light supplied by a mix of fluorescent and incandescent lights at approximately 150 W/m²). Caryopses were surface sterilized by soaking for 1 min in 70% ethanol, 5 min in 2.6% sodium hypochlorite, followed by three 5 min rinses in sterile distilled water. The embryo axis was removed by cutting the embryo into two halves and the scutellum cultured on callus-induction medium [12] for 10 days in the dark at 23 °C.

2.2. Plasmid construction

A cDNA clone of Tri101 [10] was excised from pT7Blue3 using PstI restriction enzyme and inserted into the PstI cloning site of pUB-LIT1 to generate the expression construct pUBR1 [13]. Tri101 was driven by the maize ubiquitin (ubi1) promoter and its intron (ubi1l), and terminated by nos. The monocot transformation vector pAHС25 [14] was used for co-bombardment to select the transgenic plants. pAHС25 contains the uidA (gus) gene and the bar gene for herbicide resistance under the control of the maize ubiquitin (ubi1) promoter and its intron (ubi1l), followed by the nos terminator.

2.3. Regeneration of transgenic plants

The protocol for particle bombardment and selection of transgenic plants was described in Manoharan and Dahleen [15]. The primary regenerated plants (T0) and their progeny (T1) were sprayed with the herbicide FINALE (Hoehst Roussel Agri-Vet Co., Somerville, NJ), diluted to contain 0.05% ammonium glufosinate (active ingredient), plus 0.1% Tween 20, using a spray bottle on 15-day old plants. The plants were sprayed to runoff three times at 4-day intervals and subsequently scored as resistant or susceptible 4 days after the final spraying.

2.4. Molecular and expression assays

DNA from putative transgenic plants was isolated from leaves according to Dellaporta et al. [16]. A part of the Tri101 gene was amplified with the following primers: UBI (5'-CCTGCTTCTACGGTATTTATTTGC-3') and TRI (5'-GGAGACTGAATTGGGTAGATCG-3') that produced a 240 bp fragment. PCR was carried out in a 25 μl reaction volume using the following temperature regime: one cycle at 96 °C for 30 s; 35 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; and finally one cycle at 72 °C for 15 min. Amplified products were electrophoresed on 1.0% agarose-thidium bromide gels and observed under UV light.

Southern analyses were conducted on genomic DNA (10 μg) digested with AfII, or EcoRV, separated on a 1% agarose gel and transferred onto Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ). A 1.4 kb PstI fragment of the Tri101 gene was used as a probe. Hybridization was performed at 60 °C. The probe sequence was labeled with α-32P dCTP using the Redi-prime labeling kit (Amerham Biosciences), following the manufacturer’s instructions. Membranes were washed in a buffer solution containing 2 × SSC and 0.1% SDS at 50 °C three times for 30 min each (medium stringency), or in a buffer solution containing 0.5 × SSC and 0.1% SDS at 60 °C three times for 30 min each (high stringency), and subjected to autoradiography with X-ray film.

Northern analyses were conducted on total RNA isolated from young leaves using TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH). Ten micrograms of RNA was fractionated on a 1.5% (w/v) agarose-formaldehyde gel according to Sambrook et al. [17]. The RNA was blotted onto a Hybond-N+ nylon membrane by overnight capillary transfer in 10 × SSC. Solutions used for RNA work were treated with 0.1% (v/v) diethyl-pyrocarbonate (DEPC) overnight and autoclaved.
Probe labeling and hybridization were the same as for Southern analysis.

Antibodies to the Tri101 protein were produced as follows. The Tri101 gene was amplified from the plasmid using the primer set: MP101S1 (5'-CGCGGATCCATGGCCGCA-CAAGCAGC-3') and MP101A2 (5'-TCACCGCTCGAGC-TACCCAATTACTTGGC-3'). The PCR product was digested with BamHI and XhoI, and cloned into the BamHI/ XhoI cloning site of pGEX-4T3 (Amersham Bioscience, Piscataway, NJ) to generate the protein expression vector pGEX-Tri101. The GST-Tri101 fusion protein was purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (Amersham Bioscience) and the Tri101 protein was obtained by thrombin digestion according to the manufacturer’s protocol. The rabbit polyclonal Tri101 antibody was made against the purified Tri101 protein plus Freund’s adjuvant at the University of California at Davis Center for Laboratory Animal Science, essentially as described by Harlow and Lane [18]. Subcutaneous injections of 200 μg protein per boost were carried out every 2 weeks in two rabbits. Serum was collected at 2-week intervals and titers determined.

Immunological detection of Tri101 production in transgenic plants was conducted on seedling tissues. Leaves (0.4 mg) were ground in liquid nitrogen with a mortar and pestle, mixed with 0.4 ml protein extraction buffer (50 mM Tris, 500 mM NaCl, pH 7.5) containing protease inhibitor cocktail (Roche, Indianapolis, IN), and incubated on ice for 30 min. The supernatant was removed after centrifugation (10,000 × g for 10 min, 4 °C) and used for immunoblot analysis. Twenty micrograms of protein from independent plants and 1 ng of purified Tri101 protein as a positive control were separated on SDS-PAGE using 10–20% Tris–glycine gel (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membrane (Molecular Probe, Eugene, OR). After transfer, the membrane was blocked in TBS-T (10 mM-Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) + 5% dried nonfat milk for 1 h. After washing (2 × 15 min) in TBS-T, the rabbit Tri101 antibody (1:2000 dilution in blocking buffer) was added and incubated for 2.5–3 h. After washing in TBS-T, the membrane was incubated in goat anti-rabbit HRP conjugate IgG (Amersham Bioscience) at 1:2000 dilution for 1 h at room temperature and washed as indicated above. Labeling was monitored by chemiluminescence (Pierce, Rockford, IL) according to the manufacturer’s instructions.

Spike tissues were collected from the 2005 field plots as mature, ground in liquid N2, and proteins according to the manufacturer’s instructions monitored by chemiluminescence (Pierce, Rockford, IL). The T2 progeny from T1 plants that survived herbicide spraying were planted and screened by PCR to identify homozygous plants. These were advanced by self-pollination to generate seed for greenhouse and field testing. T3 plants were crossed with wild-type Conlon, then the F1 backcrossed again to Conlon. The resulting progeny were self-pollinated and the BCF2 plants were sprayed with herbicide to identify those with the transgenes. Surviving plants were self-pollinated and BCF3 homozygous lines were identified by PCR for the Tri101 gene.

2.6. Greenhouse tests

The spikes of homozygous T3 and T4 transgenic plants and non-transformed Conlon (control) along with the resistant checks (CIho4196, Zhedar 2) were inoculated in the greenhouse with macroconidia of F. graminearum strain R010 (provided by Dr. R. Stack, NDSU) produced on PDA (Difco, Michigan) in Petri plates. R010 was selected for its consistent and high spore production in vitro. Two plants per pot were grown for a total of 20 pots for each independent event. Three primary spikes per pot were spray inoculated with 1 ml of inoculum of 7000–8000 spores per ml in deionized water plus 1 drop of Tween 20 per liter (Fischer Biotech, New Jersey). Inoculated plants were placed in a closed chamber under 100% humidity at 25 °C in the dark. After 2 days, the plants were transferred to the greenhouse at 21–28 °C, 14/10 h (day/night) photoperiod supplemented by sodium halide lights with a minimum photon flux density of 250 μmol m⁻² s⁻¹. Two weeks after inoculation the disease response was determined by counting the total and infected number of seed on each inoculated spike. Two independent tests were carried out at different times (January 2002 and 2003).

2.7. Field tests

Field trials of the T4 transgenic plants were conducted at Langdon ND (N48°45′, W98°19′) for disease and toxin testing. Wild-type Conlon, CIho4196, and Zhedar 2 were included for testing. Four inoculated misted replicates and four un-inoculated un-misted replicates were tested. Field trials were also conducted for T5 lines, and null and transgenic backcross lines (T5/Conlon/Conlon) for events 5 and 6 that had been selected for the wild-type Conlon phenotype to remove somaclonal variation. Three replicates each were planted in the misted, inoculated nursery and in the adjacent un-inoculated, un-misted nursery, with Conlon and CIho4196 included as checks. An augmented block design was used with the checks planted every 10 rows. The misted nursery was inoculated with a mixture of five F. graminearum isolates three times at two weekly intervals beginning 2
weeks prior to heading. From inoculation until grain ripening, the misted nursery was irrigated for 20 min at late afternoon and early morning each day. Nozzles used were Rain Bird XS-360 Xerispray (Rain Bird Corp., Glendora, CA). Application rate and droplet size depended on water pressure at the site, but a typical application was 15 l/m²/h. Inoculum was 500 kg/ha of a mix of equal proportions of Fusarium colonized wheat, barley and maize (Zea mays L.) spread between the plots [20]. In each year, surrounding fields had moderate to high FHB as a result of naturally produced spores, so that field trials were an uncontrollable combination of natural and inoculated isolates. FHB severity was evaluated approximately 3 weeks after anthesis, by counting the total and infected number of seed on ten randomly selected spikes per row. Height was measured at maturity. Seeds from each row were threshed from 100 randomly selected spikes and 20 g used for DON analysis. Analysis of variance (ANOVA) and mean separation [21] were used to detect differences between genotypes. There were no significant genotype by nursery interactions, so data from inoculated and non-inoculated nurseries were combined, resulting in eight replicates for the T4 lines and six replicates for the T5 and BC lines.

DON concentrations in the barley samples were determined by gas chromatography with electron capture detection using the method of Tacke and Casper [22]. 3-ADON concentrations were measured on seed by the same methods. Results were analyzed by ANOVA [21] to detect differences between control and transgenic lines.

3. Results

3.1. Transformation of barley with Tri101

Forty-seven plants were regenerated after bombardment and selection. A 0.24 kb fragment of the introduced Tri101 gene was amplified by PCR in all of the transgenic plants, which were subsequently analyzed by Southern hybridization. Genomic DNA digested with AffIII releases a 3.6 kb fragment consisting of the ubiquitin promoter, the coding region of Tri101 and the nos terminator. The expected 3.6 kb fragment was found in all of the tested transgenic plants (six are shown in Fig. 1A). Apart from the 3.6 kb fragment, different sized fragments were hybridized that may be rearranged or truncated copies of the plasmid. A total of seven independent transgenic events were identified, resulting in a transformation frequency of 3.25% of the calli bombarded regenerating transgenic plants. Plants from four of the six independent transgenic events (# 1–3) exhibited morphological abnormalities such as thick roots, broad leaves, and reduced fertility. Cytological analysis of the root tips and microspore mother cells of the abnormal T0 plants indicated chromosome doubling from diploid to tetraploid [15]. Southern analysis of genomic DNA digested with EcoRV, which cuts once in the plasmid, indicated that three to five copies of Tri101 were integrated into the genome of three independent transgenic diploid lines (# 4–6; Fig. 1B).

![Image](image_url)

Fig. 1. Southern analysis of the putative transgenic plants (T0). Genomic DNA digested with AffIII, which releases a 3.6 kb fragment spanning the promoter and coding region of Tri101 (A), and EcoRV, which cuts once in the plasmid (B), was hybridized to a radioactively labeled 1.4 kb Tri101 sequence. Lines 1–3 are tetraploid and lines 4–6 are diploid (P: plasmid DNA; C: genomic DNA from non-transformed barley; 1–6: transgenic barley lines).

3.2. Progeny and expression analyses

Twenty T1 plants from each T0 were sprayed with the herbicide FINALE to identify the null segregants, and analyzed by PCR to confirm the presence of Tri101 in the resistant plants (Fig. 2). A 0.24 kb fragment of the introduced Tri101 gene was amplified by PCR in all of the FINALE resistant transgenic plants. Both the herbicide application and PCR data indicated that the progenies of the T0 plants followed Mendelian inheritance for a single dominant transgene (data not shown). The 3:1 segregation in tetraploid plants indicated gene insertion after chromosome doubling [23]. Only plants from the three diploid events (# 4–6) were used for further analysis.

![Image](image_url)

Fig. 2. PCR analysis of herbicide-resistant T1 transgenic plants. A 0.24 kb fragment of the introduced Tri101 gene was amplified by PCR in all of the FINALE resistant transgenic plants tested, and separated on 1.0% agarose-ethidium bromide gels (M: molecular size marker (lambda DNA-HindIII digest), P: plasmid DNA, C: genomic DNA from non-transformed barley, 1–4: transgenic lines from event # 4, 5–8: transgenic lines from event #5, and 9–11: transgenic lines from event # 6).
Expression of *Tri101* was confirmed by Northern, Western and trichothecene acetyltransferase assays. A 1.4 kb *Tri101* sequence hybridized to a specific band from total RNA isolated from leaves only in the T1 transgenic plants (#4–6) and not in the non-transformed control plant (Fig. 3). Western analysis detected the *Tri101* protein only in the transgenic lines (T4) from three independent diploid events (#4–6) and not in the non-transformed control line (Fig. 4A). Antibodies also cross-hybridized to the Rubisco large subunit protein in the transformed and non-transformed plants. This was confirmed by staining the Western blot with Ponceau S Red which detected a slightly larger protein than the transgene protein. The *Tri101* protein was detected in spike tissues of all three T5 lines and two backcross lines tested but not in wild-type Conlon (Fig. 4B). Higher background staining and non-specific binding to proteins in control and transgenic lines was evident in this figure, likely from insufficient blocking and extended color development. As the protein used for antibody development was produced in bacteria and the extra bands in Fig. 4B are present in wild-type and transgenic lines, it is unlikely that they are related to *Tri101*. Expression of *Tri101* also was detected in T2–T5 seeds by assaying trichothecene acetyltransferase enzyme activity (data not shown). Enzyme activity was similar from year to year. Low levels of enzyme activity were detected in wild-type Conlon (0.16–0.19 mg isotrichodermin/mg protein). Transgenic lines showed a two- to four-fold increase in conversion of isotrichodermol to isotrichodermin (0.25–0.88 mg isotrichodermin/mg protein), indicating that the *Tri101* enzyme was active. However, the backcrossed lines showed enzyme activity similar to wild-type Conlon (Table 2).

### 3.3. Greenhouse and field tests

T3 and T4 transgenic barley lines expressing *Tri101* showed reduced FHB infection incidence compared to the untransformed susceptible parent Conlon (Table 1). The disease reduction was equivalent to that of the best resistant lines (CIho4196 and Zhedar 2). In T3 lines, where samples were bulked and not statistically analyzed, the trend was for reduced DON accumulation in transgenic lines compared to both the susceptible parent and resistant checks. In the T4, all the three transgenic lines showed reduced FHB infection and DON accumulation. Both resistant checks had increased DON accumulation compared to Conlon. FHB severity scores in the field were more variable between replicates than in the greenhouse as similar symptoms can be caused by other pathogenic fungi. The field tests conducted in 2003 did not show a reduction in FHB or DON (Table 1). Two of the *Tri101* lines had significantly higher FHB and all had significantly higher DON and 3-ADON. Somaclonal variation was evident in reduced height (2–14 cm shorter) and reduced seed weight (18–45 g lower/100 spikes) in the transgenic plants compared to wild-type Conlon. Clearly the environmental, epidemiological, expression or other factors in the field are influencing the reaction of the transgenic lines to both FHB infection and DON accumulation.
Because of concerns that somaclonal variation may mask transgene effects, two T₃ lines (5 and 6) were backcrossed to wild-type Conlon. The backcross-derived lines were more similar to Conlon for the traits observed than the T₃ lines (data not shown). This was especially true for height; all the T₃ lines were significantly shorter than Conlon while none of the backcross-derived lines were shorter (Table 2). Field testing of T₅ and newly developed backcross-derived lines, plus checks indicated that none of the lines expressing Tri101 showed differences from wild-type Conlon for FHB infection, and DON and 3-ADON accumulation (Table 2).

4. Discussion

In F. graminearum, trichothecene 3-O acetyltransferase (Tri101) converts 3-hydroxy trichothecenes like DON into less toxic 3-O-acetyl forms, 3-ADON. The acetylation of the C-3 hydroxyl group by Tri101 through the transfer of its acetyl moiety and reduced toxicity of DON [24]. 3-ADON has approximately a 10-fold lower effect on human leukocytes than does DON [25]. 3-ADON also was less phytotoxic than DON based on cell culture experiments in wheat [26]. Trichothecenes such as deoxynivalenol also play an important role as virulence factors in the pathogenicity of F. graminearum in wheat [27,28]. In barley, recent reports indicate that DON is not essential for fungal spread [29,30]. Langevin et al. [29] reported that three of the four cultivars tested showed no difference in spread between the trichothecene non-producing F. graminearum (Tri5−) and the Tri5+ strain, suggesting DON is not important in the pathogenicity of the fungus in barley. Jansen et al. [30] had similar results, with the Tri5+ and Tri5− strains showing no difference in five genotypes, supporting the hypothesis that DON is not a pathogenicity factor in barley, which could explain the high FHB levels even when significant Tri101 activity was detected (Table 2).

In the present study, we attempted to detoxify DON in barley by introducing Tri101 from F. sporotrichioides. Results from the greenhouse tests indicated that Tri101 could reduce DON concentration. In the T₃ and T₄, the transgenic lines consistently showed lower DON than the wild-type Conlon and other resistant lines. Unfortunately, the data on 3-ADON were not available that could have provided useful information on the mechanism of DON detoxification. Nevertheless, reduced DON concentrations in the transgenic lines may indicate the role for Tri101 in the detoxification of DON. In contrast, the field tests showed no effect on DON concentration. Although low levels of DON detoxification did occur in transgenic lines (Table 1) as evidenced by increased 3-ADON level in transgenic lines, the results are confounded by the presence of higher DON concentrations than the wild-type Conlon and resistant checks. Some of the differences between greenhouse and field results are likely caused by the differences in F. graminearum strains. Tri101 could have been more effective under the relatively uniform temperature, humidity and inoculum in the greenhouse tests. In the field tests, variation in temperature, humidity, inoculum, and disease pressure could have overwhelmed any effects of Tri101 against DON. Similar results were obtained when the transgenic wheat plants were tested in the greenhouse and field conditions [31,32]. A transgenic wheat line (32A) containing chitinase and glucanase genes showed moderate level of FHB resistance in the greenhouse. However, under field conditions, the same transgenic line did not show resistance to FHB, despite the high level of the transgenes’ expression [31]. The differences between the results of greenhouse studies versus field evaluations were attributed to the differences in the inoculation techniques employed. In the greenhouse studies, a conidial suspension (10⁴ to 10⁵ conidia ml⁻¹) was injected into the young spikelets as a single application, while in the field inoculation, the corn kernels provide a continuous inoculum of ascospores that land on the spikes, germinate and infect the spikes at different stages and ages under conducive environmental conditions overwhelming any resistance provided by the transgenes [31]. Similar differences were observed in

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Greenhouse</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FHB</td>
<td>DON</td>
</tr>
<tr>
<td>Conlon</td>
<td>19 b</td>
<td>12.5</td>
</tr>
<tr>
<td>CIho4196</td>
<td>5 a</td>
<td>6.0</td>
</tr>
<tr>
<td>Zhdar 2</td>
<td>5 a</td>
<td>7.0</td>
</tr>
<tr>
<td>Event 4</td>
<td>6.5 a</td>
<td>1.7</td>
</tr>
<tr>
<td>Event 5</td>
<td>6 a</td>
<td>2.2</td>
</tr>
<tr>
<td>Event 6</td>
<td>6 a</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Means within a column followed by the same letter are not significantly different (P < 0.05). Field tests included two lines per event, with eight replications per experiment.

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of lines</th>
<th>Height</th>
<th>FHB</th>
<th>DON</th>
<th>% activity</th>
<th>3-ADON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conlon</td>
<td>1</td>
<td>109</td>
<td>14.0</td>
<td>8.0</td>
<td>12.3</td>
<td>0.20</td>
</tr>
<tr>
<td>Event 5 T₃</td>
<td>2</td>
<td>97 ***</td>
<td>13.5</td>
<td>11.8</td>
<td>18.1</td>
<td>0.23 ns</td>
</tr>
<tr>
<td>Event 5 BCtrans</td>
<td>5</td>
<td>107 ns</td>
<td>11.6</td>
<td>7.2</td>
<td>14.7</td>
<td>0.18 ns</td>
</tr>
<tr>
<td>Event 5 BCnull</td>
<td>3</td>
<td>109 ns</td>
<td>12.8</td>
<td>8.9</td>
<td>11.2</td>
<td>0.24 ns</td>
</tr>
<tr>
<td>Event 6 T₄</td>
<td>2</td>
<td>98 ***</td>
<td>14.1</td>
<td>11.3</td>
<td>21.8</td>
<td>0.23 ns</td>
</tr>
<tr>
<td>Event 6 BCtrans</td>
<td>5</td>
<td>109 ns</td>
<td>13.6</td>
<td>7.8</td>
<td>12.3</td>
<td>0.19 ns</td>
</tr>
<tr>
<td>Event 6 BCnull</td>
<td>3</td>
<td>110 ns</td>
<td>14.4</td>
<td>9.4</td>
<td>12.6</td>
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<tr>
<td>CIho4196</td>
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<td>135 ***</td>
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<td></td>
<td>8.5</td>
<td>10.3</td>
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</table>

*, **, *** significantly different from wild-type Conlon at p < 0.05, 0.01 and 0.001 level; ns non-significant, respectively.
transgenic wheat plants expressing viral coat proteins against wheat mosaic virus, which showed more resistance in greenhouse studies but offered no protection in the field. The differences between greenhouse and field-testing were again attributed to the use of different inoculum techniques [32]. Further, the maize ubiquitin promoter used to drive Tri101 can be regulated by heat shock, increasing expression under high temperatures followed by a sharp decrease in gene expression within an hour or two [33,34] that may reduce effectiveness of Tri101 under field conditions. Moreover, somaclonal variation was evident in field-tested transgenic lines, but not in greenhouse tested lines. Under field conditions, transgenic plants exposed to the stresses and pathogens of the natural environment were visibly weaker than wild-type Conlon, with reduced height and seed weight. Variation can be caused by activated transposons, altered methylation patterns, point mutations, or other genetic changes, which also can influence transgene expression [35].

To reduce the chance that somaclonal variation was influencing Tri101 expression, transgenic lines from events 5 and 6 were backcrossed to Conlon and homozygous lines selected. Somaclonal variation was removed but, while the Tri101 protein was produced, the activity of acetyltransferase was significantly reduced in the backcrossed lines and they did not show reduced DON when tested in the field. Several studies have shown that the Ubil promoter drives gene expression in spike tissues and immature embryos [36–38], and the Tri101 protein was clearly produced (Fig. 4B) so it is unknown why the activity levels were so low. Perhaps the plant cell environment was sufficiently different from the fungal cells to prevent optimal Tri101 activity. Genes from Fusarium differ from monocots such as wheat in the usage of specific codons, a factor with potential impact on cellular mRNA stability and translatability [13]. Perhaps use of a modified Tri101 gene with monocot codons may significantly improve expression levels for efficient detoxification of DON in barley. It is also possible that the Tri101 protein from F. sporotrichioides has greater specificity to the group A trichothecene toxins it produces rather than the group B trichothecenes from F. graminearum. The fungal regulation mechanisms for Tri101 are still unknown and could involve cis- or trans-acting factors or other fungal components lacking in the plant. The ongoing Fusarium genomics projects should help elucidate these mechanisms.

In conclusion, we have transformed the commercial malting barley cultivar Conlon with Tri101 through particle bombardment. The integration and expression of Tri101 was observed through the T3 and backcross generations. Transgenic lines showed reduced FHB infection and DON accumulation under greenhouse testing; however, the transgenic lines showed no effect on FHB and DON levels when tested in the field with high stress and disease levels. Somaclonal variation was observed in the field-tested transgenic lines. Backcrossed transgenic lines had reduced trichothecene acetyltransferase activity compared to T5 lines and did not detoxify DON or offer protection against FHB in barley under field tests.

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