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RESEARCH PAPER

Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*

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Abstract

Fusarium head blight (FHB; scab), primarily caused by *Fusarium graminearum,* is a devastating disease of wheat worldwide. FHB causes yield reductions and contamination of grains with trichothecene mycotoxins such as deoxynivalenol (DON). The genetic variation in existing wheat germplasm pools for FHB resistance is low and may not provide sufficient resistance to develop cultivars through traditional breeding approaches. Thus, genetic engineering provides an additional approach to enhance FHB resistance. The objectives of this study were to develop transgenic wheat expressing a barley class II chitinase and to test the transgenic lines against *F. graminearum* infection under greenhouse and field conditions. A barley class II chitinase gene was introduced into the spring wheat cultivar, Bobwhite, by biolistic bombardment. Seven transgenic lines were identified that expressed the chitinase transgene and exhibited enhanced Type II resistance in the greenhouse evaluations. These seven transgenic lines were tested under field conditions for percentage FHB severity, percentage visually scabby kernels (VSK), and DON accumulation. Two lines (C8 and C17) that exhibited high chitinase protein levels also showed reduced FHB severity and VSK compared to Bobwhite. One of the lines (C8) also exhibited reduced DON concentration compared with Bobwhite. These results showed that transgenic wheat expressing a barley class II chitinase exhibited enhanced resistance against *F. graminearum* in greenhouse and field conditions.

Key words: Chitinase, *Fusarium graminearum*, Fusarium head blight, transformation, wheat.

Introduction

Fusarium head blight (FHB; scab), primarily caused by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch: synonym=*G. saubinetti*), is a serious disease of wheat and other small grains in hot and humid regions around the world. Between 1998 and 2000, FHB caused an estimated 2.7 billion dollar economic loss in the Midwestern United States (Nganje *et al*., 2004). FHB reduces yield through discoloured and shrivelled 'tombstone' kernels. Grain quality is also reduced due to accumulation of trichothecene mycotoxins such as deoxynivalenol (DON), and the estrogenic zearalenone (McMullen *et al*., 1997).

Host resistance in wheat has been considered the most practical and effective means of FHB disease control; however, wheat breeding programmes have been limited by a lack of effective resistance genes (Bai and Shaner, 1996). Two major types of FHB resistance have been classified. Type I resistance is a reduction in initial infection, and Type II resistance is reduced spread of

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Abbreviations: FHB, Fusarium head blight; GUS, β-glucuronidase; VSK, visually scabby kernels; DON, deoxynivalenol.

disease symptoms in the spike (Schroeder and Christensen, 1963). Quantitative trait loci (QTL) that confer Type I and Type II resistance have been identified (Waldron *et al*., 1999; Buerstmayr *et al*., 2003). To increase FHB resistance, wheat-breeding programmes select for both Type I and Type II resistance (Rudd *et al*., 2001). However, wheat germplasm sources identified to date exhibit partial resistance. Thus, genetic engineering provides an additional approach to increase the level of FHB resistance in wheat.

Several classes of genes have been used in a genetic engineering approach to develop resistance in wheat to fungal pathogens. One group of genes, referred to as defence response genes encode proteins such as: β-1,3-glucanases, chitinases, thaumatin-like proteins (tlps), ribosome-inactivating protein (RIPs), and thionins. The defence response genes function in a variety of ways to inhibit fungal infection and expression of these genes in transgenic plants has been shown to enhance fungal resistance (Muehlbauer and Bushnell, 2003). Expressing defence response genes in transgenic wheat resulted in enhanced resistance to the powdery mildew (Bliffeld *et al*., 1999; Oldach *et al*., 2001; Bieri *et al*., 2003) and leaf rust pathogens (Oldach *et al*., 2001). With respect to FHB, wheat lines expressing β-1,3-glucanase, thaumatin-like protein1 (tlp-1), ribosome-inactivating protein (RIP), α-1-purothionin, and *Arabidopsis NPR1* (At*NPR1*) transgenes exhibited enhanced resistance against *F. graminearum* in greenhouse and/or field trials (Chen *et al*., 1999; Makandar *et al*., 2006; Balconi *et al*., 2007; Mackintosh *et al*., 2007).

Chitinases (EC 3.2.1.14) break bonds between the C1 and C4 of two consecutive N-acetylglucosamines of chitin, which is a main component of the cell wall in fungi. Plant chitinases are characterized as pathogenesis-related proteins and are classified into seven classes (I–VII) based on their primary structures (Flach *et al*., 1992; Collinge *et al*., 1993). Chitinase genes are up-regulated during early infection of wheat and barley spikes by *F. graminearum* (Pritsch *et al*., 2000, 2001; Li *et al*., 2001; Kang and Buchenauer, 2002; Kong *et al*., 2005; Boddu *et al*., 2006, 2007; Bernardo *et al*., 2007; Golkari *et al*., 2007). Expression of a rice chitinase transgene in rice, Italian ryegrass, and grapevine resulted in enhanced resistance to the rice blast, crown rust, and powdery mildew pathogens, respectively (Nishizawa *et al*., 1999; Yamamoto *et al*., 2000; Takahashi *et al*., 2005). Transgenic wheat lines carrying an overexpressed wheat chitinase and β-1,3-glucanase combination showed partial resistance to FHB in greenhouse evaluations; however, the lines did not exhibit improved resistance under field conditions (Anand *et al*., 2003). Wheat plants constitutively expressing a barley class II chitinase transgene also showed resistance against the powdery mildew and leaf rust pathogens (Bliffeld *et al*., 1999; Oldach *et al*., 2001). The efficacy of transgenic wheat expressing a barley class II chitinase against the powdery mildew and leaf rust fungal pathogens made it an obvious choice to test against *F. graminearum*.

The objectives of this study were to develop transgenic wheat carrying a barley class II chitinase transgene and evaluate these lines during *F. graminearum* infection for resistance in the greenhouse and in the field. Seven transgenic wheat lines that exhibited enhanced Type II FHB resistance in the greenhouse have been identified. Two of these lines exhibited high levels of chitinase protein and enhanced FHB resistance in the field.

Materials and methods

Plant materials

The spring wheat cultivars Alsen, 2375, Roblin, Sumai 3, Wheaton, and Bobwhite were used for the experiments. Wheaton and Roblin are hard red spring wheat cultivars that are highly susceptible to FHB; Bobwhite was used for the transformations and is slightly less susceptible than Wheaton; 2375 is moderately susceptible to FHB; Alsen exhibits Type II resistance and is moderately resistant to FHB; Sumai 3 is a Chinese cultivar exhibiting Type I and Type II resistance (Bai and Shaner, 1996).

Plant transformation plasmids

pAHC25: The pAHC25 plasmid (Christensen and Quail, 1996; gift from Dr Peter Quail, USDA-ARS, Albany, CA) contains the *uidA* and *bar* genes driven by the maize ubiquitin promoter. The *uidA* gene encodes the β-glucuronidase (GUS) enzyme and the *bar* gene encodes the phosphinothricin acetyltransferase (PAT) enzyme. PAT activity confers resistance to phosphinothricin-containing herbicides.

pAHCBarChit: The 998 bp barley seed class II chitinase cDNA (GenBank accession number M62904; Leah *et al*., 1991; a gift from Dr John Mundy, Carlsberg Research Laboratory, Copenhagen, Denmark) was cloned into the *Bam*HI site of pAHC17. The barley class II chitinase cDNA sequence contains an 801 bp open reading frame, beginning with the first ATG initiation codon at nucleotide position 61 and ending with a TAA termination codon at position 862. The pAHC17 plasmid (Christenson and Quail, 1996; a gift from Dr Peter Quail, USDA-ARS, Albany, CA) contains the maize ubiquitin promoter/exon/intron (UBI-1) sequence and the *Agrobacterium tumefaciens* nopaline synthase 3,- end sequence. The orientation within the plasmid and open reading frame integrity of the barley chitinase gene were confirmed by sequencing.

Wheat transformation

The spring wheat cultivar Bobwhite was used as the host for transformation. All aspects of the transformation protocols including particle gun bombardment of embryos, tissue culture selection and plant regeneration was conducted according to Mackintosh *et al.* (2006). A 1:1 ratio of pAHC25 and pAHC-BarChit, 5 μg each, were cotransformed into Bobwhite. During the selection and regeneration process, the identity of the callus and shoots derived from each embryo was maintained. To ensure that each line was independent, only a single plant expressing the transgene from each embryo was advanced for testing.

RNA isolation and transcript analysis

Total RNA was isolated from 100 mg leaf tissue with TRIZOL® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription polymerase chain reactions (RT-PCR) were performed with 1 μg of total RNA using the Superscript™ III One-step RT-PCR kit (Invitrogen, Carlsbad, CA). For the chitinase transgene and actin control, the reverse transcriptase reactions were performed in a thermal cycler at 55 °C for 30 min, followed by 35 cycles of amplification (denature at 94 °C for 15 s, annealing at 60 °C for 1 min and extension at 68 °C for 1 min) and final extension of 68 °C for 5 min. The primer pair (5′-GATGCATATACATGATGGCATATGCAG-3′, and 5′-GTCCA-TAGTTGTAGTTGTGGGAGAG-3′) was used for amplification of the chitinase mRNA. The expected size for the amplified products from the chitinase mRNA was 742 bp. The primer pair (5′-GCCACACTGTTCCAATCTATGA-3′ and 5′-TGATGGAATTG-TATGTCGCTTC-3′) was used for amplification of the wheat actin control gene. The expected band size for the wheat actin gene was 369 bp. Sequence analysis of the chitinase and actin RT-PCR products confirmed that the correct transcripts had been amplified.

Southern blot analysis

Southern blot analysis was performed according to de la Peña *et al*. (1996). A radiolabelled portion of the ubiquitin promoter and chitinase transgene was used as a probe for the hybridizations. The probe was derived from the PCR primers used in the RT-PCR reactions, resulting in a 742 bp probe (Fig. 1).

Greenhouse screening of transgenic lines against F. graminearum *infection*

Seed from each wheat genotype were planted into Metro-Mix 200 growth medium (The Scotts Company, Marysville, OH) in 6′ square plastic pots in a greenhouse. Twenty seeds were planted for each line with each pot containing five seeds. Plants were fertilized with one teaspoon of Osmocote (14-14-14 N-P-K, Scotts Company, Marysville, OH) fertilizer per pot at the 3-leaf stage. At anthesis, a single central floret of the spikelet of the main stem was inoculated with 10 μl of a macro-conidial suspension (100 000 conidia ml^{-1}) of isolate Butte86Ada-11 (Evans *et al*., 2000) of *F. graminearum*. Inoculated spikes were bagged in plastic and the plants were placed in a dew chamber for 72 h and subsequently moved back to the greenhouse. The number of visually symptomatic spikelets, including the inoculated spikelet on each plant, were counted 20 d after inoculation (dai). The disease severity was determined as the percentage of infected spikelets on the inoculated spikes with visually detectable disease symptoms. In each greenhouse screen, non-transformed Bobwhite, Wheaton, and Sumai 3 were used. For statistical analysis, Student's *t* tests were used to compare each transgenic line to the parental Bobwhite controls. All analysis was performed with Microsoft Excel Version 2003 (Microsoft Corporation, Redmond, WA).

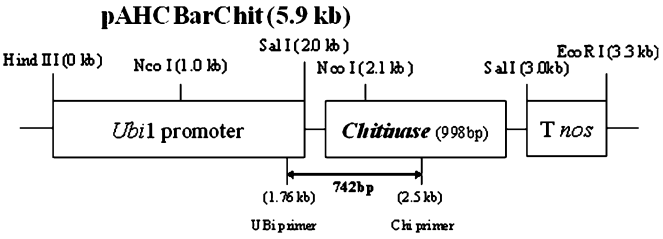

Fig. 1. The pAHCBarChit plasmid containing the barley class II chitinase transgene was used for wheat transformation. The arrow indicates the region amplified in the RT-PCR assays and the region used for the Southern blot probe. The ubiquitin 1 promoter and intron is from the maize ubiquitin gene and the T *nos* termination sequence is from the nopaline synthase gene from *Agrobacterium tumefaciens*.

Field screening of transgenic lines against F. graminearum *infection*

Transgenic wheat lines were evaluated in the field against *F. graminearum*. Seed for each transgenic line was derived from selfing plants that exhibited expression of the chitinase transgene. It is possible that the transgene was still segregating in the lines tested in the field. Bobwhite, Alsen, 2375, Norm, Roblin, and Wheaton were included in the experiment as disease checks. An additional non-inoculated treatment of Wheaton was also used to establish the background level of inoculum. Two experiments were conducted: one during the summer of 2005 at the University of Minnesota Agricultural Experiment Station in Crookston, MN and another in the summer of 2007 at the University of Minnesota Agricultural Experiment Station (UMore Park) in Rosemount, MN. T_6 and T_8 lines were used for the 2005 and 2007 field tests, respectively. The experimental design was a randomized complete block with four replications. Each genotype was planted in two-row plots; rows were 2.4 m long and were spaced 0.3 m apart. Within each row, 3.3 g m^{-1} of seed was sown.

The inoculum consisted of a mixture of 50 isolates of *F. graminearum* in 2005 and 41 isolates in 2007. The isolates were obtained from naturally FHB-infected commercial wheat and barley fields in Minnesota from 2004 and 2006. The plots were inoculated at anthesis and then 3 d later. Each row was inoculated with 33 ml m^{-1} of inoculum mixture (1×10^5 macroconidia ml^{-1}). Inoculum was applied using a CO_2-powered backpack sprayer fitted with a TeeJet® (Spraying Systems Co., Wheaton, IL) SS80015 flat-fan nozzle that was operated at a pressure of 276 kPa.

FHB disease severity was evaluated visually 21 dai. Twenty spikes from primary tillers were arbitrarily selected per plot and rated for disease severity. Disease severity was measured as the percentage of symptomatic spikelets per spike. After harvest, the grain was assessed for the percentage of visually scabby kernels (VSK) on a hand-cleaned 50 g sample. VSK values were assigned based on standards with a known percentage of scabby kernels (Jones and Mirocha, 1999). After VSK analysis, the samples were ground for 2 min with a Stein Laboratory Mill and deoxynivalenol (DON) concentration was determined using gas chromatography and mass spectrometry with the procedure described in Mirocha *et al*. (1998) with slight modifications. For statistical analysis, Student's *t* tests were used to compare each transgenic line to the parental Bobwhite controls. All analysis was performed with Microsoft Excel Version 2003 (Microsoft Corporation, Redmond, WA).

Western blot analysis

Protein was extracted by grinding spikes at anthesis in extraction buffer (50 mM NaH_2PO_4, pH 6.8, 100 mM PMSF) and cell debris was removed by micro-centrifugation. Total protein concentration was determined using Bio-Rad reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Protein extracts (10 μg) were separated by SDS-polyacrylamide electrophoresis (12% acrylamide) and transferred to PVDF transfer membrane (Amersham Biosciences, Piscataway, NJ). Affinity-purified polyclonal chitinase antibodies were developed from two peptides (SRAQFDRMLLHR-NDGAC and CGKRYYGRGPIQLSHNYNY) from the barley chitinase protein. The polyclonal chitinase antibody was developed by Quality Controlled Biochemicals, Inc., Hopkinton, MA. The blots were incubated with a 1:1000 dilution of the chitinase polyclonal antibody.

Chitinase protein was visualized using an ECL Western Blotting Reagent Pack (rabbit) (Amersham Biosciences, Piscataway, NJ).

Results

Generation of transgenic wheat plants

The pAHCBarChit plasmid (Fig. 1), which included the barley chitinase gene driven by the maize ubiquitin promoter, and pAHC25 which carries the *bar* gene as a selectable marker and the *uidA* (GUS) as a reporter gene, were co-bombarded into the wheat cultivar Bobwhite. Plants were selected on bialaphos and only plants that exhibited GUS expression were regenerated. To identify plants that exhibited expression of the barley chitinase transgene, RT-PCR analysis was conducted on the T_0 plants. Sixteen transgenic wheat plants expressing the chitinase transgene were identified. The 16 wheat plants were advanced to T_2 lines by selfing T_1 plants that expressed the transgene based on the RT-PCR assay.

Greenhouse screening of transgenic plants for FHB resistance

FHB resistance was evaluated in the greenhouse of these 16 transgenic wheat lines that exhibited barley chitinase transgene expression. Each line was examined in at least three generations spanning the T_2 to T_4 or T_2 to T_5 and each line was examined in at least three separate disease screens. Based on an RT-PCR assay for the chitinase transgene, advanced generations were obtained through selfing plants that expressed the barley chitinase transgene. Thus, in each disease screen it is still possible that the lines were segregating null, homozygous or hemizygous for the transgene. For each screen, 20 plants of each line were grown and the spikes were inoculated with *F. graminearum*. The central spiklelet in primary spikes were point inoculated and examined visually for the spread of disease 20 dai. RT-PCR was used to assay each plant in the transgenic lines for chitinase transgene expression. Based on the RT-PCR assays, only those plants expressing the chitinase transgene were used to evaluate the efficacy of chitinase against *F. graminearum*. FHB severity in the transgenic lines expressing the chitinase transgene was compared to the severity observed in the non-transgenic Bobwhite parent.

In the greenhouse experiments, seven transgenic lines (C3, C4, C6, C8, C12, C15, and C17) had significantly reduced FHB severity in at least two FHB disease screens when compared to the Bobwhite control ($P < 0.05$; Table 1). No significant difference was observed in the remaining nine transgenic lines in the FHB disease screens (data not shown). The transgenic C8 and C12 lines had significantly reduced FHB severity compared to the untransformed Bobwhite controls in three greenhouse screens ($P < 0.05$). The average reduction of disease severity across all the trials in C8 and C12 was 46% and 58%, respectively. The remaining lines C3, C4, C6, C15, and C17 lines exhibited reduced severity in two disease screens. C3, C4, C6, C15, and C17 exhibited an average reduction in severity of 40%, 30%, 36%, 52%, and 31%, respectively. Overall, all seven lines showed a similar level of resistance to FHB.

Molecular analysis of transgenic plants

To verify that the seven lines that exhibited enhanced resistance in the greenhouse screens were independently derived and transgenic, DNA gel blot analysis was conducted. Genomic DNA from the seven lines was digested with *Nco*I. Based on the plasmid map for

Table 1. *Percentage of Fusarium head blight severity in transgenic wheat carrying a barley chitinase in greenhouse evaluations*

Numbers in parenthesis represent the number of plants expressing the chitinase transgene, based on the RT-PCR assay, in the screen.

Genotype[a]	Greenhouse testing[b]						
	Autumn 2002	Winter 2003	Spring 2003	Autumn 2003	Autumn 2003	Spring 2004	Spring 2004
C3	34 (12)	–	72 (15)	–	35** (11)	–	12*** (12)
C4	48 (15)	–	53* (19)	–	23*** (16)	–	55 (10)
C6	50 (11)	–	56 (14)	–	32*** (15)	–	26*** (5)
C8	6** (8)	–	52* (13)	–	48* (16)	–	–
C12	–	35** (14)	–	38** (14)	–	8*** (10)	–
C15	–	48 (17)	–	21*** (17)	–	24*** (18)	–
C17	–	–	38*** (17)	–	47* (13)	–	57 (16)
Bobwhite	54 (36)	64 (18)	73 (33)	68 (46)	68 (46)	60 (22)	60 (22)
Wheaton	99*** (60)	91** (21)	94*** (57)	98*** (58)	98*** (58)	90*** (56)	90*** (56)
Sumai 3	7*** (61)	21*** (16)	16*** (46)	8*** (46)	8*** (46)	13*** (139)	13*** (139)

[a] C3, C4, C6, C8, C12, C15, C17 are transgenic wheat lines carrying the barley chitinase. Bobwhite was the untransformed control, Wheaton was the FHB susceptible check, and Sumai 3 was the FHB resistant check.

[b] Greenhouse testing in autumn 2002, winter 2003, spring 2003, autumn 2003, autumn 2003, spring 2004, and spring 2004 corresponded to T_2, T_2, T_3, T_3, T_4, T_4, and T_5 for the transgenic lines, respectively. FHB severity was measured as the percentage of infected spiklets per head 20 d after inoculation. – Indicates that this line was not examined in this screen. *, **, *** indicates significance at the 0.05, 0.01, and 0.001 level, respectively, compared to Bobwhite (Student's *t* test).

pAHBarChit this will result in 1.1 kb fragment. A portion of the ubiquitin promoter and chitinase transgene was used as a probe (Fig. 1). The probe hybridized weakly to the Bobwhite control; however, each line exhibited an approximately 1.1 kb fragment and each of the lines exhibited a different banding pattern, indicating that the seven lines were transgenic and resulted from independent transformation events (Fig. 2).

To examine transgene expression in seven lines (C3, C4, C6, C8, C12, C15, and C17) that exhibited resistance against *F. graminearum* in the greenhouse screens, RT-PCR and western blot analysis were conducted. Figure 3 is an example of the RT-PCR analysis of these seven lines. Western blot analysis of protein expression was also conducted. Protein from spikes was isolated at anthesis from transgenic lines C3, C4, C6, C8, C12, C15, and C17 and the blots were incubated with antibody for the barley chitinase protein. In the western blot shown in Fig. 4, the cross-reaction of the chitinase antibody with protein from Bobwhite and all the transgenic lines was observed. Three transgenic lines (C8, C15, and C17) had a clearly higher level of chitinase protein (Fig. 4). Similar results were observed in other western blots prepared with leaf and spike protein. These results indicate that C8, C15, and C17 were the only lines that exhibited a high level of chitinase protein.

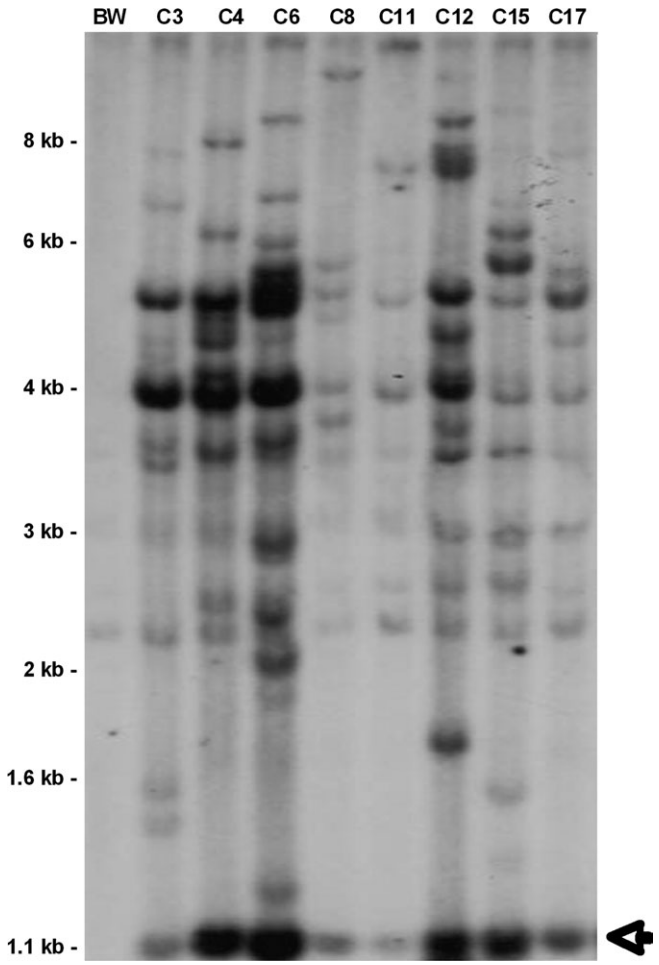

Fig. 2. Southern blot analysis of seven transgenic wheat plants carrying a barley chitinase. Genomic DNA from Bobwhite parent and transgenic lines were digested with *Nco*I, and hybridized with a probe that bridges the ubiquitin promoter and the chitinase transgene junction. The arrow indicates the expected size of the 1.1 kb hybridizing fragment from a *Nco*I digestion of the plasmid.

Field disease screening of transgenic plants for FHB

To examine the efficacy of the wheat transgenic lines expressing the chitinase transgene in providing improved levels of resistance to *F. graminearum*, field evaluations of seven lines (C3, C4, C6, C8, C12, C15, and C17) were conducted in the summers of 2005 and 2007. FHB severity, percentage of visually scabby kernels (VSK), and DON concentrations on these seven lines and the non-transgenic Bobwhite control were assessed (Table 2). In the 2005 and 2007 field tests, the C17 line exhibited significant reduction in percent FHB severity and VSK compared to Bobwhite. The C8 line showed significant reduction in percentage FHB severity, percentage VSK, and in DON concentration. The disease severity in the C3, C4, C6, C12, and C15 transgenic lines were either similar to or higher than the non-transgenic Bobwhite control.

Discussion

FHB is a serious disease of wheat and has resulted in significant economic losses around the world. Available FHB resistance in wheat is inherited in a quantitative manner and is partial. Several QTL in wheat have been identified that confer Type I and II resistance, with the largest QTL explaining variation for Type II resistance located on chromosome 3BS (Waldron *et al*., 1999; Anderson *et al*., 2001; Buerstmayr *et al*., 2003). Multiple studies have shown the induction of a large set of defence

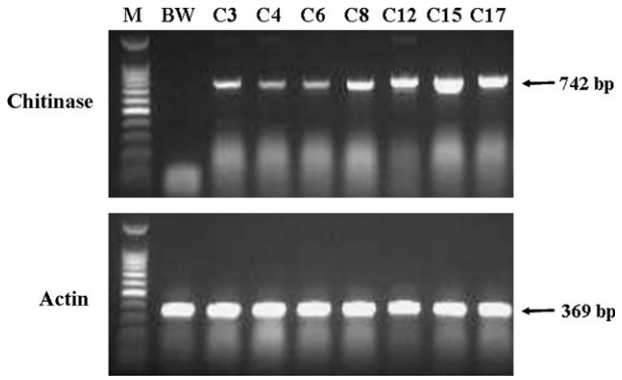

Fig. 3. RT-PCR analysis of transgenic wheat plants carrying a barley chitinase gene. The expected size of the chitinase transgene fragment was 742 bp. The wheat actin gene was used as a positive control and it exhibited the expected size of 369 bp.

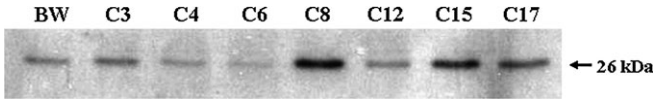

Fig. 4. Western blot analysis of transgenic wheat plants carrying a barley chitinase gene. Total protein (10 μg) extracted from spikes of transgenic lines was subjected to SDS-PAGE analyses. Molecular markers indicated the protein to be the expected 26 kDa size.

Table 2. *Percentage of Fusarium head blight (FHB) severity, visual scabby kernels (VSK), and deoxynivalenol (DON) concentration in transgenic wheat carrying a barley chitinase evaluated in the field in 2005 and 2007*

Genotype[a]	FHB severity (%)	VSK (%)	DON concentration (ppm)[b]
Wheaton	62.2[c]**	23.6***	15.8*
Roblin	64.1*	15.3	10.0
Alsen	14.2***	3.6***	3.5***
Sumai 3	1.9***	1.1***	0.8***
2375	33.0	6.8*	6.8*
Wheaton (non)	51.3	18.1	13.3
Bobwhite	42.0	10.1	10.1
C3	42.3	11.6	12.6
C4	43.2	9.1	9.1
C6	33.9	8.1	9.7
C8	25.8**	4.6***	6.6**
C12	39.1	10.5	11.5
C15	40.0	8.8	10.5
C17	25.6***	7.8*	8.4

[a] C3, C4, C6, C8, C12, C15, C17 are transgenic wheat lines carrying the barley chitinase transgene. T_6 and T_8 plants were evaluated in 2005 and 2007, respectively. Bobwhite was the untransformed control, Wheaton and Roblin are FHB susceptible checks, and Alsen and Sumai 3 are a FHB resistant check, and 2375 is a moderately resistant check. The non-inoculated treatment of Wheaton (non) was used to establish the background level of inoculum.

[b] ppm, parts per million.

[c] Values presented are the means of eight replicates (four replicates tested in each 2005 and 2007). *, **, *** indicates significance at the 0.05, 0.01, and 0.001 level, respectively, compared to Bobwhite (Student's *t* test).

response genes in wheat following *F. graminearum* infection (Pritsch *et al*., 2000, 2001; Li *et al*., 2001; Kang and Buchenauer, 2002; Kong *et al*., 2005; Hill-Ambroz *et al*., 2006; Bernardo *et al*., 2007; Golkari *et al*., 2007). Chitinase, one of the defence response genes identified in these studies, limits fungal growth by degrading the major structural polysaccharide of fungal cell walls (Leah *et al*., 1991). It has been proposed that overexpression of a chitinase transgene protein may function to provide fungal pathogen resistance on both direct and indirect levels. On the direct level it degrades chitin of growing hyphae, whereas on the indirect level it results in the release of chitin oligomers which can act as elicitors of plant defence mechanisms (Collinge *et al*., 1993). In this report, it is shown that transgenic wheat expressing a barley class II chitinase gene enhances resistance against *F. graminearum* under greenhouse and field conditions.

To date, complete resistance against fungal pathogens has not been achieved by the expression of single genes encoding defence response genes. Expression of chitinase transgenes of different origins resulted in enhanced resistance in rice (Nishizawa *et al*., 1999), Italian ryegrass (Takahashi *et al*., 2005), and grapevine (Yamamoto *et al*., 2000) to *Magnaporthe grisea*, *Puccinia coronata*, and *Uncinula necator*, respectively. Moreover, expression of a barley chitinase gene in transgenic wheat resulted in enhanced resistance to infection by *Erysiphe graminis*, *Blumeria graminis*, and *Pucinia recondita* (Bliffeld *et al*., 1999; Oldach *et al*., 2001; Bieri *et al*., 2003). Chen *et al*. (1999) showed that expression of a rice thaumatin like protein-1 (tlp-1) transgene in wheat resulted in enhanced FHB disease during the early stages of disease progression in the greenhouse, indicating a delay in FHB development. A transgenic wheat line carrying a chitinase transgene did not enhance FHB resistance compared to the non-transgenic controls in greenhouse experiments (Anand *et al*., 2003). However, the chitinase transgene exhibited a low level of expression and was probably silenced in the tested generation. Another wheat line carrying a chitinase and β-1,3-glucanase exhibited delayed susceptibility compared with non-transgenic controls in greenhouse screens, but this line did not exhibit delayed susceptibility in field screens (Anand *et al*., 2003). Balconi *et al*. (2007) showed that transgenic wheat plants expressing a maize RIP gene reduced the FHB disease symptoms 14 dai. These authors only detected enhanced FHB resistance during the early stages of disease progression in the greenhouse. In contrast to these reports, FHB resistance was detected during the late stages of disease progression (i.e. 20 and 21 dai for the greenhouse and field, respectively). Seven lines were identified expressing a barley chitinase transgene that exhibited enhanced FHB resistance compared to the non-transgenic Bobwhite control in greenhouse screens. Seven transgenic lines in field trials were evaluated, and two lines were identified that exhibited improved resistance against *F. graminearum* in the field trials. Thus, our results show that expressing defence response genes in transgenic wheat can result in enhanced resistance against *F. graminearum*. Consistent with these results, Mackintosh *et al*. (2007) also showed that expression of the defence response genes α-1-purothionin, tlp-1, and β-1,3-glucanase in transgenic wheat exhibited resistance against *F. graminearum* in the greenhouse and field trials.

Improved resistance was not detected in each of the seven transgenic lines in every greenhouse screen. Lines providing enhanced resistance were designated as those that exhibited resistance in at least two of the greenhouse screens. The lack of consistency in the disease screens is probably due to the variability inherent in FHB disease screens, which was also observed previously (Mackintosh *et al*., 2007). Therefore, to detect transgenic wheat lines

carrying enhanced levels of FHB resistance, our results demonstrate the importance of conducting multiple tests.

The transgenic lines that exhibited resistance in the greenhouse did not all exhibit resistance in the field. In the greenhouse, the spikes were point-inoculated and Type II resistance was evaluated, whereas in the field the spikes were spray-inoculated and disease severity, VSK, and DON concentration were evaluated. The transgenic wheat lines (C3, C4, C6, C12, and C15), which showed enhanced Type II resistance in the greenhouse evaluations, did not display detectable resistance in the field. However, the transgenic wheat lines C8 and C17 did show resistance in the field, and reduced the average disease severity compared to Bobwhite by 39%. Yield and grain quality reductions from FHB are due to fungal damage to kernels, and contamination of grain by DON. In our study, the C8 transgenic line also showed reduced VSK and DON concentration in harvested grain, whereas the C17 line exhibited reduced VSK. These results are probably due to the chitinase transgene delaying the onset of the FHB disease and thus reducing the colonization of the developing wheat kernels and the production of mycotoxins.

Chitinase protein levels in spikes of C8 and C17 were correlated with the field results. In our study, the C8 and C17 lines exhibited a high level of chitinase protein and they were the only lines that exhibited FHB resistance in the field. In contrast, the C15 line exhibited an increase in chitinase protein compared to Bobwhite. However, for unknown reasons this line did not result in enhanced resistance against *F. graminearum* in the field. It is possible that the inherent variation in FHB screens resulted in the C15 line not showing a difference when compared to Bobwhite. The chitinase protein levels in the C3, C4, C6, and C12 transgenic wheat lines were observable but indistinguishable from Bobwhite and exhibited enhanced FHB resistance only in the greenhouse. Interestingly, the C12 line exhibited the highest level of resistance in the greenhouse and a low level of chitinase protein, but did not show enhanced resistance in the field. Balconi *et al*. (2007) showed that reduced FHB symptoms in transgenic wheat lines carrying maize RIP gene did not depend on the level of RIP protein. However, Takahashi *et al*. (2005) showed that transgenic ryegrass plants with a higher level of chitinase mRNA accumulation and activity tended to have higher resistance to crown rust disease (*Puccinia coronata*). Except for the anomaly observed in C15, our results indicate that increased chitinase protein is sufficient to enhance host resistance to FHB in field-grown plants.

Wheat germplasm pools lack sufficient resistance to develop FHB-resistant varieties. Sumai 3 is widely used as a source of Type I and Type II resistance that limits initial infection and disease spread, respectively. QTL mapping in Sumai 3 and Sumai 3 derivatives have identified the location of Type I and Type II resistance and shown that Type II resistance is the major form of FHB resistance (Anderson *et al*., 2001; Buerstmayr *et al*., 2003). However, the level of Sumai 3-derived resistance is insufficient for cultivars in severe FHB epidemics. Thus, the transgenic lines presented here may provide a potential wheat germplasm source for enhanced FHB resistance.

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