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RESEARCH PAPER

Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*

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Abstract

Genes encoding pathogenesis-related (PR-) proteins isolated from a cDNA library of *Fusarium graminearum*-infected wheat spikes of scab-resistant cultivar 'Sumai-3' were transformed into susceptible spring wheat, 'Bobwhite' using a biolistic transformation protocol, with the goal of enhancing levels of resistance against scab. Twenty-four putative transgenic lines expressing either a single PR-protein gene or combinations thereof were regenerated. Transgene expression in a majority of these lines (20) was completely silenced in the T_1 or T_2 generations. Four transgenic wheat lines showed stable inheritance and expression of either a single transgene or transgene combinations up to four generations. One line co-expressing a chitinase and β-1,3-glucanase gene combination, when bioassayed against scab showed a delay in the spread of the infection (type II resistance) under greenhouse conditions. This line and a second transgenic line expressing a rice thaumatin-like protein gene (*tlp*) which had moderate resistance to scab in previous greenhouse trials, along with susceptible and resistance checks were evaluated for resistance to scab under field conditions. None of the transgenic lines had resistance to scab in the field under conditions of strong pathogen, suggesting these plants lacked effective resistance to initial infection (type I resistance) under these conditions. AS far as is known, this is the first report of field evaluation of transgenic wheat expressing genes for PR-proteins against disease resistance.

Key words: Chitinase, field evaluation, genetic transformation, β-1,3-glucanase, transgene silencing, wheat scab.

Introduction

Fusarium head blight (FHB) or scab, caused by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*) is a major disease of wheat in many areas of wheat production in USA and throughout the world (Bai and Shaner, 1994; Parry *et al*., 1995; McMullen *et al*., 1997). Under favourable conditions this disease can cause losses of up to $1 billion per year (McMullen *et al*., 1997). Host resistance and cultural practices over a long time have been used to manage the disease with only moderate success. Effective control of FHB will require better knowledge of the epidemiology of the disease and the use of the modern tools of genetic engineering (Parry *et al*., 1995; Shah, 1997; Shaner and Buechley, 1999; Patnaik and Khurana, 2001). One strategy is to enhance the natural defences of wheat for disease control. The transcripts levels for many pathogenesis-related (PR-) protein genes were shown to be increased upon scab infection of wheat suggesting their importance in plant defence (Li *et al*., 2001; Pritsch *et al*., 2000, 2001). Different classes of PR-proteins including PR-1, PR-2 (β-1,3-glucanases), PR-3 (chitinases), PR-5 (thaumatin-like protein), and PR-9 (peroxidases) were induced within 6–12 h of inoculation and reached peak levels within 36–48 h after inoculation. Similarly, a 3-fold increase in the polyphenol oxidase (PPO) activity was detected in the resistant wheat cultivars upon inoculation with *F. graminearum* conidia when

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compared with the non-inoculated controls (Mohammadi and Kazemi, 2002).

The availability of genes encoding PR-proteins and the demonstration that PR-proteins exhibit strong *in vitro* antifungal activity (Mauch *et al*., 1988) has led to their deployment for enhancing disease resistance in crop plants. Several groups have reported that the introduction of a single transgene encoding different antimicrobial proteins including PR-proteins in wheat resulted in enhanced resistance (as measured in greenhouse trials) to a wide range of disease resistance including powdery mildew (Bliffeld *et al*., 1999; Schweizer *et al.,* 1999; Bieri *et al*., 2000; Oldach *et al*., 2001), *Tilletia tritici* (Clausen *et al*., 2000), barley stripe mosaic virus (Zhang *et al*., 2001), scab (Chen *et al*., 1999), and other fungal pathogens (Leckband and Lorz, 1998). The increase in resistance to the pathogen varied widely and in most cases resistance was only partial.

Following the initial reports of significant improvement in disease resistance in transgenic plants expressing PR-proteins (Broglie *et al*., 1991; Zhu *et al*., 1994; Jach *et al*., 1995; Jongedijk *et al*., 1995; Lin *et al*., 1995) there have been numerous studies that have exploited a similar strategy (reviewed in Datta *et al*., 1999). The results have been mixed with some notable failures (Neuhaus *et al*., 1991). The current consensus is that combinations of PR-proteins are required to achieve effective disease control.

This paper reports the production of four fertile transgenic wheat lines which stably and constitutively express one or both of two PR-protein genes (for a chitinase and a β-1,3-glucanase). As will be described, among the different combinations of genes for PR-proteins tested, only a line expressing a rice thaumatin-like protein and a specific combination of a wheat chitinase and a wheat glucanase had partial resistance to scab in greenhouse evaluations. However, field trials of the same lines failed to show any improved resistance under heavy inoculum loads. This is the first report of the field evaluation of transgenic wheat plants expressing PR-proteins.

Materials and methods

Plasmids

The plasmid constructs pAHCUbi194 and pAHCUbi383 (containing wheat chitinase genes) and pAHCUbi289 and pAHCUbi638 (containing wheat β-1,3-glucanase genes) were used to transform wheat calli. The clones encoding the chitinases and β-1,3-glucanases used in this study were isolated from a cDNA library constructed from scab-infected Sumai-3 wheat (Li *et al*., 2001). These cDNAs, a 2.0 kb fragment containing the maize ubiquitin promoter-intron and another 0.25 kb fragment containing the cauliflower mosaic virus transcription terminator (CaMV polyA) fragment were ligated to pBluescript vector. The *Hin*dIII fragments carrying the promoter–cDNA–CaMV polyA cassette were recovered and inserted into the unique *Hin*dIII site of the plasmid pAHC20 (Christensen *et al*., 1992). The plasmid pAHC20 contains the *bar* gene under the control of the maize ubiquitin promoter-intron. The *bar* gene confers resistance to the herbicide glucosinate (Liberty®, Aventis, Research Triangle Park, NC). The plasmids, pAHCUbi194 and pAHCUbi383, contain the opening reading frames (ORFs) from the two wheat cDNA clones 194 and 383 encoding a class VII and a class IV chitinase, respectively (Fig. 1A, B). Plasmids pAHCUbi289 and pAHCUbi638 (Fig. 1C, D) contain the ORFs from the two wheat glucanase genes 289 and 638, respectively (Li *et al*., 2001). These plasmids were used singly or in different combinations for wheat transformation.

Plant materials and transformation

Wheat plants (*Triticum aestivum* L. cv. 'Bobwhite') were grown in growth chambers with a 16 h light period, at a light intensity of 600 μE m^{-2} s^{-1} and a temperature regime of 20:18 °C (light:dark). Spikes were collected approximately 14 d post-anthesis and immature caryopses were sterilized in a solution containing 20% commercial bleach and 0.1% Tween 20 for 20 min and then rinsed three times with sterile, distilled water. Immature embryos were excised and placed on CM4 medium (Zhou *et al*., 1995), with the embryo-side in contact with the medium, for 5–7 d in the dark at 25 °C to initiate callus formation. Embryogenic calli were transferred to CM4 + osmoticum (0.2 M mannitol, 0.2 M sorbitol) 4–16 h prior to genetic transformation. Genes were introduced into embryogenic calli with the particle inflow gun (Finer *et al*., 1992).

The methods for selection and recovery of transgenic plants were similar to those of Altpeter *et al*. (1996) with slight modifications. Sixteen hours after particle bombardment, wheat calli were placed on CM4 medium containing 5 mg l^{-1} glufosinate and cultured in the dark at 25 °C for 8–10 d. Cultures were then transferred to shoot production medium (MSP), containing MS (Murashige and Skoog, 1962) basal salt mixture, B5 vitamins (Gamborg *et al*., 1968), 0.2 mg l^{-1} 2,4-D, 40 g l^{-1} maltose, 100 mg l^{-1} ascorbic acid, 10 mM MES, 5 mg l^{-1} glufosinate, and 2.0 g l^{-1} Gelrite, pH 5.7) and cultured in a 16 h light period at 25 °C for 2 weeks. Upon induction of green shoots, the cultures were transferred to elongation and rooting medium (MSE) containing 5 mg l^{-1} glufosinate and cultured for 2–3 weeks in the light (MSE medium is the same as MSP except for the omission of 2,4-D). Healthy looking plantlets were transferred to soil and grown in environmentally controlled growth rooms (16 h light periods, at a light intensity of 600 μE m^{-2} s^{-1}).

Leaf painting assay

To examine the expression of the *bar* gene in the transgenic plants, a freshly prepared aqueous solution of the herbicide, Liberty® (0.2% v/v) was applied on the mid-lamina portion (about 2.5 cm long) of the second/third youngest leaf using a cotton plug. The painted area was marked using a marker pen and visual observations were recorded after 3–4 d after painting.

Molecular characterization of the transgenic plants

Plant materials and methods of protein extraction, SDS-polyacrylamide gel electrophoresis and western and Southern blot analyses were carried out as described in Chen *et al*. (1998). For western blot hybridization, polyclonal antibodies raised in rabbits against a barley chitinase (Swegle *et al*., 1992) and a barley β-1,3-glucanase (a gift from Dr Murray Ballance, University of Manitoba, Winnipeg) respectively, were used. The antibodies were used at 1:1000 (v/v) dilutions. For RNA blot hybridization, total RNA was extracted from 150 mg leaves or spikes with TRIZOL® reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Aliquots of RNA (10 μg) were analysed by formaldehyde-agarose gel electrophoresis and blotted on to Hybond-N^+ nylon membranes (Amersham, Arlington Heights, IL). Membranes were probed with

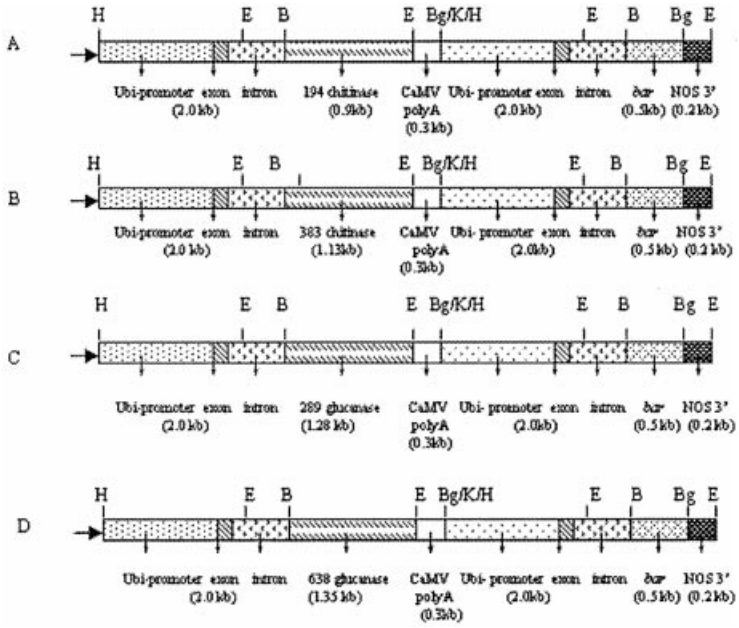

Fig. 1. Schematic presentation of the plant transformation plasmids; (A) pAHC20Ubi194 chitinase; (B) pAHC20Ubi383 chitinase; (C) pAHC20Ubi289 β-1,3-glucanase; and (D) pAHC20Ubi638 β-1,3-glucanase used for transforming wheat embryos. Both the gene of interest and the selectable marker gene *'bar'* are driven by the maize ubiquitin promoter-intron. The gene cassettes with the promoter, transgene(s), cauliflower mosaic virus (CaMV polyA) terminator region and nopaline synthetase (NOS) terminator are shown as boxes drawn approximately to scale. The plasmid vector backbone (pUC8) is not shown. Relevant restrictions sites are indicated: H, *Hin*dIII; E, *Eco*R1 B, *Bam*H1 K, *Kpn*1 Bg, *Bgl*II.

^{32}P-labelled coding region fragments of the wheat chitinase or glucanase genes. The other steps for RNA hybridization were similar to those for DNA hybridization (Chen *et al*., 1998). Reverse-transcriptase (RT-) PCR reactions were performed with 0.5 μg of total RNA using the Advantage One-Step, RT-PCR kit (Clonetech, Palo Alto, CA) as per the manufacturer's instructions. For distinguishing the transgene-specific transcripts, a forward primer in the first exon of the ubiquitin promoter, 5′-CGTGTTGTTCGCAGCG-CACAC-3′, and a reverse primer in the CaMV polyA fragment 5′-GCTCAACACATGAGCGAAACCC-3′ (Fig. 1), were used in the RT-PCR reaction. The reverse transcriptase reactions were performed in a thermal cycler at 50 °C for 1 h, followed by PCR amplification of 15–25 cycles at 94 °C for 0.5 min, 64.7 °C for 0.5 min and 1 min at 72 °C. A complete final extension for the PCR products was performed at 72 °C for 10 min. A semi-quantitative competitor RT-PCR assay (Igaz *et al*., 1998) using concentrations ranging from 10 pg to 80 pg of plasmid DNA used for transformation as the competitor were performed to quantify the transgene-specific mRNAs. After the reverse transcriptase reaction the competitor DNA were added into the reaction mixture and PCR amplified for 15 cycles. The transgene-specific transcripts ranged in sizes from 1.1 kb to 1.4 kb for the different cDNA clones, which were resolved on 1.6% w/v agarose gels.

The detection of the *bar* gene and genes of interest by PCR amplification was carried out using 200 ng of total genomic DNA as template and BIOLASE™ DNA polymerase (Bioline, Springfield, NJ) as per the manufacturer's instruction using the following primer combinations; Bar F, 5′-CCTGCCTTCATACGCTATTTATTT-GCC-3′ (forward primer); Bar R, 5′-CTTCAGCAGGTGGGTGTA-GAGCGTG-3′ (reverse primer) and Ubi A, 5′-GCCCTGCCT-TCATACGCTAT-3′ (forward primer in the intron of ubiquitin promoter) and PolyA-R, 5′-GGAATTCAAGCTTCATCGAGCT-CGGTA-3′ (reverse primer in the CaMV polyA fragment). DNA amplifications were performed in a thermal cycler using initial denaturation at 94 °C for 5 min, followed by 30 cycles of 1.0 min at 94 °C, 1.5 min at 62.1 °C (for *bar* gene)/62.4 °C (genes of interest) and 2.0 min at 72 °C. One additional complete extension cycle was performed for 10 min at 72 °C. The transgene products ranged in sizes from 0.6 kb, 1.1–1.4 kb for the *bar* gene and different cDNA clones, which were resolved on a 0.8% or 1.4% w/v agarose gel and visualized by ethidium bromide staining.

Scab bioassay

Resistance to scab in greenhouse-grown plants was evaluated by a 'single floret injection inoculation method' (Xu and Chen, 1993) with the indicated number of plants. Two plants of each transgenic line and of the controls were transplanted per one gallon (8-inch) pot. A 4′ high×4′ wide×10′ long, plastic-covered, mist chamber was constructed on a greenhouse bench. A single central floret of the spikelets of wheat entries were inoculated at anthesis with 10 μl of a conidial suspension of isolate Z-3639 of *Fusarium graminearum*. The pots were randomly picked for each entry at anthesis and inoculated with the conidial suspension. In the first experiment, the conidial suspension contained 1.6×10^4 conidia ml^{-1} (a minimal concentration was used in the initial experiment for standardizing the inoculum procedure and detection of the disease), while in the second and third experiments, the conidial suspension contained 5×10^5 conidia ml^{-1}. New sets of plants at similar physiological stage of anthesis were picked randomly on three different days and injected with the same inoculum. A minimum of six heads per entry was inoculated on each day. Plants were then placed overnight in the mist chamber with 15 s of mist every 12 min. The chamber was opened during the day and misted each night for a total of three nights. The plants were then placed in a greenhouse. The infected spikelets per inoculated head were counted 7, 10 and 14 d after inoculation. The percentage of infected spikelets per head was also calculated during the same period. Data collected from different dates were analysed separately by analysis of variance (ANOVA)

with mean separation by LSD (P=0.05) using Statistical Analysis System (SAS Institute, 1998).

Field testing of transgenic and control lines were carried out in spring 2002 at the Plant Pathology Experimental Farm located near Manhattan, KS, USA. A randomized complete block design was used with 20 replicates for each treatment. Each plot consisted of a 'hill' separated in all directions by 45 cm. One to three plants of each entry were grown in the greenhouse in 15 cm pots for about 3 weeks and then transplanted to the field on March 15 (one pot per 'hill'). Corn kernels (93 g m^{-2}) colonized by *F. graminearum* were applied to the soil on April 1. To provide head wetness, impact sprinklers irrigated the area during anthesis for 3 min h^{-1} from 21.00–06.00 h. When scab began to develop, the percentage of spikelets affected was rated for each hill at 3 d intervals for about 2 weeks. Data collected from different dates were analysed separately by analysis of variance (ANOVA) with mean separation by LSD (P=0.05) using Statistical Analysis System (SAS Institute, 1998).

Results

Transformation and characterization of T_0 plants

Twenty-four independent, fertile, primary transgenic lines with the selectable marker *bar* gene and single PR-protein gene or gene combinations were identified. PCR analysis using gene-specific primer combinations designed for detection of the *bar* gene (data not shown) and PR-protein genes was used for characterization of the 24 transgenic lines (results from a few T_1 plants transformed with single PR-protein gene or their combinations are shown in Fig. 2). Four transgenic lines contained only the 383 chitinase and three had only the 638 glucanase gene, while the remaining 17 lines came from the co-bombardment of three different PR-protein pairs (194:383, 383:638 and 289:383) of transgene constructs (Table 1). PCR analysis for the genes of interest in 17 lines from the multiple plasmid bombardment experiments detected the presence of all the expected transgenes in all lines except two that had one plasmid (194 chitinase or 638 glucanase). high co-transformation efficiency (15 out of 17) in the transgenic wheat plants could thus be demonstrated.

Western blot analyses were subsequently carried out with extracts from leaves of these T_0 transgenic plants for detecting the transgene-encoded proteins using appropriate controls including the escapes (*bar*-minus plants) and untransformed parental lines. All 24 lines (T_0 plants) were positive for the *bar* gene by PCR and had expression (to varying extents) of either one or two transgenes as expected from the PCR results (data not shown).

Progeny analyses of transgenic plants

Ten to 20 seeds from each T_0 generation plant were germinated for the 24 transgenic lines containing the *bar* gene. The leaf painting assay and PCR analyses of the genes of interest were performed to detect the segregation of Liberty resistant/sensitive and genes of interest (plus/minus) plants. T_1 progeny of 18 lines were completely susceptible to Liberty even though a majority (about 75%) had the *bar* gene and transgene as revealed by PCR. The presence of expected transgenes was confirmed (in most, but not in all cases) by carrying out PCR analysis of genomic DNA as templates using Ubi A and PolyA-R primers which yielded PCR-fragments of distinct sizes for each of the four transgenes (Fig. 2). Progenies from only six lines survived painting with Liberty. The segregation patterns for Liberty resistance/susceptibility of T_1 plants from these six lines were consistent with one or two transgene loci (segregation ratio of 3:1 or 15:1, Table 2). Results of PCR analyses for the *bar* gene and genes of interest, as well as the data from RT-PCR, Southern blotting and western blot analysis for transgene-encoded proteins were also consistent with the presence of one or two transgene loci (data not presented). The 18 Liberty-sensitive lines had no detectable transgene-encoded proteins as revealed by western blot analyses, presumably resulting from silencing of *bar* and transgenes. These

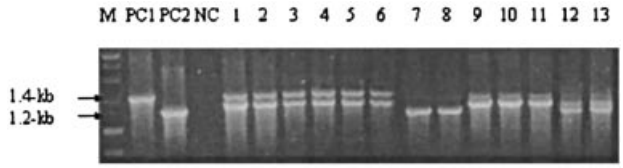

Fig. 2. Analysis of the transgenic T_1 plants, plasmid controls and an untransformed Bobwhite control (NC) for detecting the presence of PR-protein gene(s). The putative (T_0) transgenic plants and their progenies were analysed by a PCR-based assay of genomic DNA using transgene-specific primers, Ubi A and PolyA-R which yielded PCR-fragments of distinct sizes for each of the four transgenes. The PCR products were fractionated on 1.4% agarose gel and identified based on their migration pattern. Lanes: 1–6, detection of the 638 glucanase:383 chitinase transgenes; 7–8, detection of the 194 chitinase transgene; 9–11, detection of the 289 glucanase:383 chitinase transgenes; 12–13, detection of the 383 chitinase:194 chitinase transgenes; PC1, 2.5 ng of the plasmid DNA from pAHCUbi638; PC2, 2.5 ng of plasmid DNA from pAHCUbi194; and NC, DNA from non-transgenic 'Bobwhite' control. This PCR-based assay was further used for characterizing the T_0, T_1, and the subsequent generations of transgenic wheat plants.

Table 1. *Total number of transgenic lines and stability of transgene expression*

Event	Gene/gene combination[a]	Total no. of lines generated (T_0)	Lines stably expressing the transgene[b]
1	383	4	26 (T_2)[c]
2	638	3	82 (T_4)
3	194:383	8	62 (T_2)[c]
4	383:638	5	32A (T_4)
5	289:383	4	76, No. 78 (T_4)
Total		24	6

[a] 383 and 194 encode wheat chitinase; 289 and 638 encode wheat glucanase.
[b] Most advanced generations tested for transgene expression.
[c] Lines silenced for transgene expression in T_3 generation.

Table 2. *Segregation analyses of the transgenes in different transgenic lines based on the Liberty painting assay (0.2%) and by PCR for the detection of the expected PR-protein genes*

Line	Gene or combinations	T_1		T_2	
		Liberty (R/S)[a]	PCR detection of the transgenes (+/–)	Liberty (R/S)[a]	PCR detection of the transgenes (+/–)
26	383	10/2	10/2	12/3	12/3
32A	383:638	9/1	9/1	16/0	16/0
76	383:289	11/3	11/3	18/3	18/3
78	383:289	14/0	14/0	16/4	16/4
82	638	13/1	13/1	12/9	12/9
62	194:383	15/4	15/4	16/6	16/6

[a] R: resistant; S: susceptible. All Liberty-susceptible were also transgene-minus.

results indicated that a vast majority of the transgenic lines had undergone transgene inactivation in the T_1 generation.

Transgene silencing persists even in the advanced generations in some lines

All the six Liberty-resistant lines expressing the transgenes in the T_1 generation showed co-segregation for both the *bar* gene and the gene(s) of interest (Table 2). The T_1 plants expressing the highest levels of transgene-encoded protein(s) were selected by western blotting from each of the six lines and selfed to obtain T_2 progenies. Liberty painting, PCR for transgenes, RT-PCR, and western blotting were used for the analyses of progeny of subsequent generations to determine whether these lines stably expressed the transgene(s). When compared to their T_1 parents, T_2 progenies from two of these six lines (Nos 26 and 62) had very low levels of transgene-encoded protein and transcripts as revealed by RT-PCR and western blotting. Presumably, additional transgene silencing has occurred in the T_2 generation plants. In the T_3 generation, no evidence for a functional transgene was found even though the transgenes were detected by PCR in these two silenced transgenic lines.

Generation of lines homozygous for transgene loci

For the identification of homozygous lines, seeds were collected from all the T_2 plants of the four lines that showed high levels of transgene expression and from line 26 that had low activity of transgenes. Analyses of seedlings of the T_3 generation of these transgenic lines (Nos 26, 32A, 76, 78, and 82), revealed that the progeny from the plant 32A-2–4 were homozygous. T_3 progeny from T_2 parents of the remaining four lines showed segregation in leaf painting assays and western blot analyses for protein expression for the transgenes. Homozygous T_3 parents were identified by analyses of T_4 generation plants for the four lines (Nos 26, 76, 78, and 82). As discussed in the last section, only low levels of transgene-encoded protein were detected in the T_3 progenies of line 26, and this line was completely silenced for the transgenes in homozygous T_4 progeny (data not shown).

Detection of transgenes in homozygous lines

Genomic DNA from representative T_3 (or T_4) generation plants was digested with *Hin*dIII (which releases the fragment containing the intact ubiquitin-promoter linked to PR-protein transgene from the 5.7 kb vector-*bar* gene fragment; Fig. 1) and subjected to Southern blotting using a *bar* probe or PR-protein cDNA probes. The six lines had distinct patterns of transgene bands (except 32A and 32C which had identical banding patterns for four different enzyme digests; data not presented). The estimation of the transgene copy number using known amounts of transforming plasmids as standards ranged from 3 (line 32A) to over 10 (line 76) for the intact *bar* gene, in addition to rearranged copies (Fig. 3A, B). Even though it was difficult to estimate the copy number of the PR-protein genes accurately, because of the presence of endogenous homologous genes in wheat (Fig. 3C), comparison of the intensities of the 3.2 kb band (containing the intact ubiquitin promoter-PR protein gene-polyadenylation signal sequences) and upon digestion with different enzyme combinations indicated that copy numbers also ranged 3–10 (data not presented).

Transgene expression in the homozygous lines

Detection of transgene-encoded chitinase and glucanase was carried out using appropriate polyclonal antibodies. A low level of 26 kDa (expected size) of 383 chitinase protein was detected in the non-transgenic control and line No. 82. In the heterozygous T_2 plants of line No. 26, and in the homozygous plants of lines 32A, 76 and 78 the intensity of this 26 kDa chitinase band was higher. Line No. 32A had the highest levels of 383 protein followed by line No. 78 (Fig. 4A). With the β-1,3-glucanases antibody, multiple bands within the size range of approximately 30–32 kDa (expected size range for 638 and 289 glucanases) was detected in the transgenic lines 32A, 76, 78, and 82 (basal level expression was detected in line 26 and non-transgenic controls). The highest levels of 638 protein were seen in line 32A followed by line 82 (which had no chitinase transgene expression). Even though the predicted size difference between the two transgenic glucanase proteins is 2 kDa, the presence of multiple bands precluded unambiguous identification of 638 and 289 glucanases in these blots (Fig. 4B).

The highest levels of both transgene-encoded proteins were found in line 32A which was transformed with the 383 chitinase and 638 glucanase genes. Transgene-specific mRNA levels in this line were estimated using a semi-quantitative competitor RT-PCR assay (Igaz *et al*., 1998)

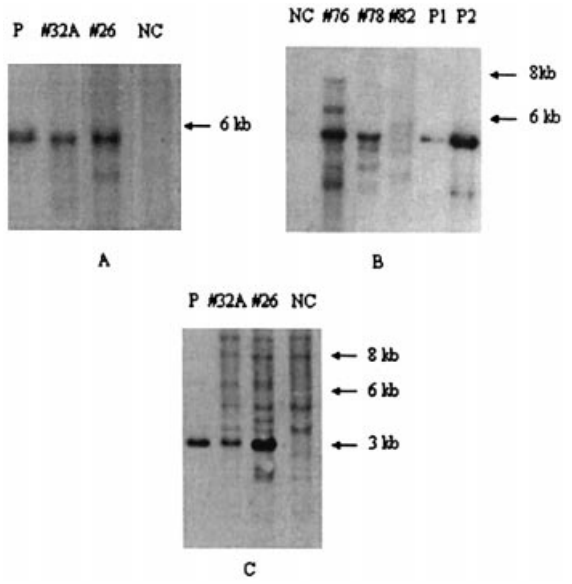

Fig. 3. Detection of the integration of *bar* and 383 chitinase transgene resulting from different transgenic events. Twelve micrograms of genomic DNA from the Liberty-resistant T_3 plants of lines 32A and 26 were digested with *Hin*dIII followed by hybridization with ^{32}P-labelled coding region fragment of *bar* gene and 383 chitinase cDNA. (A, B) Probed with *bar* gene, while (C) shows the hybridization pattern with 383 chitinase probe. The endogenous wheat homologues for the chitinase genes were also detected when probed with the 383 chitinase gene fragment (see lane NC). (A, C) P, 8 pg of linearized pAHC20 plasmid equivalent to two copies of the transgene. Lanes: 32A (383 chitinase and 638 glucanase); lane 26 (383 chitinase); NC, untransformed 'Bobwhite' plant. (B) P1 and P2, 4 and 20 pg of linearized pAHC20 plasmid equivalent to 1 and 5 copies of the transgene. Lanes: 78 (383 chitinase and 289 glucanase); 76 (383 chitinase and 289 glucanase); 82 (638 glucanase); NC: DNA from the untransformed 'Bobwhite' plant. The migration positions of some size standards are indicated by arrows.

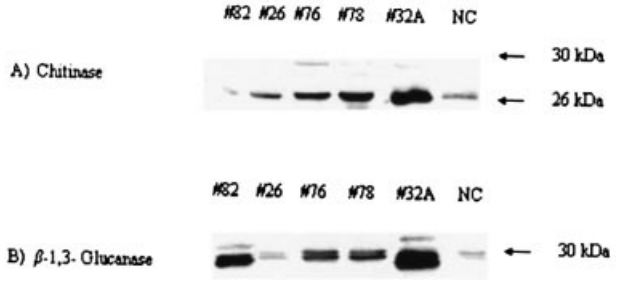

Fig. 4. Detection of the chitinase and β-1,3-glucanase gene expression in transgenic lines by western blot analysis. 250 µg of total leaf protein extract was loaded in each lane. Chitinase (A) and glucanase (B) expression in the Liberty-resistant plants of transgenic lines. Lanes: 82 (homozygous 638 glucanase); 26 (heterozygous 383 chitinase); 76 (homozygous 383 chitinase and 289 glucanase); 78 (homozygous 383 chitinase and 289 glucanase); 32A (homozygous 383 chitinase and 638 glucanase) and NC, non-transgenic 'Bobwhite'. The expected sizes of the transgene-encoded proteins are indicated by arrows.

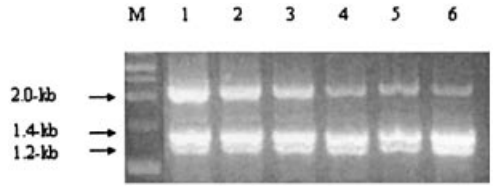

Fig. 5. Semi-quantitative competitive RT-PCR for quantifying the transgene-specific transcripts in line 32A (expressing the 383 chitinase/638glucanase transgenes). Known amounts of the plasmid pAHCUbi383 were added as competitor DNA to the RT-PCR reaction after the first step of reverse transcriptase (RT). Following the RT, 15 PCR cycles were performed and the amplified products were resolved on a 1.6% w/v agarose gel. The intensities of the individual PCR products were calculated using the BioRad gel documentation unit and were used to deduce the amount of the transgene-transcripts in total mRNA. Lanes: M, 1 kb DNA molecular marker; 1, 80 pg; 2, 40 pg; 3, 20 pg; 4, 10 pg; 5, 5 pg, 6, 2.5 pg of the competitor plasmid DNA. Arrows indicate the expected sizes of the amplified products, 1.4 kb (638 glucanase), 1.2 kb (383 chitinase) and 2.0 kb plasmid pAHCUbi383, respectively.

using concentrations ranging from 10–80 pg of the plasmid DNA used for the transformation as the competitor. The intensities of the PCR bands derived from the transgenes were compared to those derived from the plasmid (which includes the intron) using the BioRad gel documentation unit. Based upon these observations it is predicted that about 0.8% and 0.25% of the total mRNA (assuming mRNA is 1% of the total RNA and the amplified transcripts are close to the average sizes of mRNA) came from the contributions of 638 glucanase and 383 chitinase transgenes (Fig. 5). This line contained approximately three copies of each of the transgenes (Fig. 3C; and data not shown). RT-PCR using 0.5 µg of total RNA from line No. 32A yielded strong PCR bands for the transgene specific transcripts within 15 cycles. However, in all the other lines using similar amounts of total RNA, 20–25 cycles of PCR following the reverse transcription were required to amplify and detect the transgene transcripts. Northern blot analyses also confirmed the expression of both the chitinase and glucanase genes in all the four expressing lines (data not shown).

The T_3 and T_4 generation plants of this line (32A) that were homozygous for the transgene locus had a lesion-mimic phenotype. These symptoms developed at the booting stage and appeared to be dependent on the accumulation of high levels of the transgene expression, since heterozygous plants from this line did not have these lesions. The plants derived from the backcross with the parental line, 'Bobwhite', which are heterozygous for the transgene loci did not show this phenotype. The stable inheritance and expression of the transgenes was confirmed by the leaf painting assay, PCR and western blotting in heterozygous plants.

Scab bioassay and disease resistance

Three trials were conducted to evaluate the resistance of transgenic lines to scab under greenhouse conditions. In

Table 3. *Reaction to scab of selected transgenic T_2 lines: 1st greenhouse experiment*[a]

Entry	Total no. of plants inoculated	Mean number of infected spikelets/head[b]	
		Days after inoculation	
		10	14
32A	14	1.6 b	3.6 b
26	16	2.5 ab	11.5a
Bobwhite	19	3.8 a	9.8 a

[a] Macroconidia of *Fusarium graminearum* isolate Z-3639 were applied in a suspension containing spore density applied was 1.6×10^4 conidia ml^{-1}; heterozygous T_2 plants identified by their expression were tested in the greenhouse. Non-transformed 'Bobwhite' plants were used as controls. The experimental results were analysed by *t*-test with SAS software. Values within a day and column, when followed by a common letter are not significantly different according to ANOVA followed by LSD (P=0.05).

[b]The average number of spikelets/head varied between 14 and 16.

Table 4. *Reaction to scab of homozygous transgenic plants co-expressing wheat chitinase and β-1,3-glucanase genes: (A) 2nd greenhouse experiment and (B) 3rd greenhouse experiment*[a]

(A)			
Entry	Total no. of plants inoculated	Mean number of infected spikelets/head[b]	
		Days after inoculation	
		10	14
32A	25	3.7 b	7.4 b
32C	31	6.6 a	12.5 a
MN99112	24	2.0 c	4.2 c
Bobwhite	42	6.8 a	13.8 a
(B)			
76	44	6.5 bc	11.8 d
78	51	6.1 c	14.0 ba
82	70	7.1 a	14.6 a
MN99112	42	2.05 d	3.1 e
Bobwhite	64	6.8 ba	13.0 c

[a] Macroconidia of *Fusarium graminearum* isolate Z-3639 were applied in a suspension containing spore density applied was 1.6×10^4 conidia ml^{-1}; heterozygous T_2 plants identified by their expression were tested in the greenhouse. Non-transformed 'Bobwhite' plants were used as controls. The experimental results were analysed by *t*-test with SAS software. Values within a day and column, when followed by a common letter are not significantly different according to ANOVA followed by LSD (P=0.05).

[b]The average number of spikelets/head varied between 14 and 16.

the first set of experiments, two heterozygous T_2 lines, line 26 (low expression for 383 chitinase), line 32A (high expression for both 383 chitinase and 638 glucanase) and susceptible check 'Bobwhite' plants were tested. In the second set of experiments, homozygous T_3 plants of lines 32A, 32C (genetically identical to line 32A, but with no expression for 383 chitinase and 638 glucanase, used as an epigenetic control), a resistant check line, MN99112 (an experimental line received from Dr James Anderson, University of Minnesota, with resistance from Wuhan 3 wheat) and 'Bobwhite' were bioassayed in the autumn of 2001. In the third experiment, homozygous T_4 plants of lines 76, 78 (moderate expression for both 383 chitinase and 289 glucanase) and line 82 (moderate expression 638 glucanase) with the resistant check (MN99112) and 'Bobwhite' were bioassayed against scab in the spring of 2002. Symptoms characteristic of scab appeared on the spikes of transgenic and control plants in the greenhouse on the third day after inoculation with a conidial suspension. In the first greenhouse experiment, the segregating population of T_2 progenies of lines 32A and 26 were compared with the control non-transgenic parental line (Table 3). The conidial density used in this experiment (1.6×10^4 conidia ml^{-1}) resulted in moderate infection. Hyphal growth in the inoculated spikes could be detected within 3–4 d after inoculation (DAI). The mean number of infected spikelets in line 32A was one-third to half the number in line 26 and the susceptible 'Bobwhite' control on 10 DAI. The mean number of infected spikelets/head were also found to be still significantly lower (3.6) in line 32A by 14 DAI, when compared to the susceptible check (9.8) or line 26 (11.5). Since no significant differences in disease severity were observed between line 26 and the non-transgenic controls and because the plants homozygous for the transgene loci in this line were completely silenced, line 26 was excluded from further bioassay analyses.

In the second greenhouse experiment (Table 4A), the resistant check MN99112 had the lowest levels of disease as expected. The number of infected spikelets/head on 10 and 14 DAI in line 32A (3.7 and 7.2) and the resistant check (2.0 and 4.2) were significantly lower than the values for the silenced line 32C and the non-transgenic controls (Table 4A). A delay in both the onset and progression of the infection was seen in line 32A when compared with the susceptible control. The greater disease incidence and faster spread in line 32C, which is genetically identical to line 32A indicates that the observed increase in disease resistance is dependent on transgene expression and is not due to the tissue culture-related effects.

In the third greenhouse experiment, homozygous T_4 plants from three other lines; lines 76 and 78 which express both the chitinase and β-1,3-glucanase transgenes at moderately high levels, and line 82 which expresses only a β-1,3-glucanase at a moderately high level, did not show any improved resistance when compared to the susceptible control (Table 4B). The resistant check MN99112, showed minimal infection.

Table 5. *Mean percentage of the scab symptoms in the transgenic 'Bobwhite' wheat plants versus control spring wheat plants tested in the field near Manhattan, KS in spring 2002*[a]

Entry	Symptom rating 3rd day	Symptom rating 6th day	Symptom rating 10th day	Symptom rating 14th day
BT-14–18	17.5 a	25.6 a	59.7 a	65.0 ba
32A	9.4 c	23.0 ba	51.0 b	67.8 a
32C	13.2 b	16.5 c	54.7 ba	61.2 ba
Bobwhite	12.6 b	17.9 bc	53 b	60.8 b
Wheaton	6.7 c	12.2 c	49 b	52.7 c
MN99112	0.5 d	1.4 d	9.0 d	19.3 d
Scab-7	0.85 d	3.5 d	33.5 c	52.2 c

[a] Homozygous T_4 transgenic line expressing the transgene-encoded proteins was assayed. The percentage of affected spikelets was rated for each hill at 3 d intervals. BT-14-18: transgenic line expressing the thaumatin-like protein (*tlp*) transgene; 32A: transgenic line co-expressing the 383 chitinase/638 glucanase; 32C: silenced line co-transformed with 383 chitinase/638 glucanase; 'Bobwhite' untransformed control; Wheaton (susceptible check); MN99112 (resistant check); No. 5: Scab-7 (resistant check). The data were collected on 3, 6, 10, and 14 d after inoculation. Values within each column, when followed by a common letter were not significantly different according to ANOVA followed by LSD (P=0.05). Day 1 is when the symptoms for scab were visually observed in the entries.

Field evaluation of two transgenic lines

The following treatments were included in this experiment: homozygous T_4 plants of line BT-14-18 [(transgenic line expressing the rice *tlp* transgene) (Chen *et al*., 1999)]; line 32A; line 32C; non-transgenic 'Bobwhite' control plants; MN99112 (resistant check); Scab-7 (resistant check); and Wheaton (susceptible check). Field evaluation of lines 32A and BT-14-18 did not show any improved resistance. The disease ratings for these lines were similar to, or higher than, the non-transgenic 'Bobwhite' and susceptible controls (Table 5). A significant reduction in disease incidence was recorded on the third day rating in line 32A. However, by the sixth day of disease rating, the disease severity in the transgenic lines was either similar to or higher than the susceptible controls. Disease developed early and was severe in the transgenic lines and the 'Bobwhite' control (parental line) when compared with the susceptible check (Wheaton). The resistant check MN99112 had the lowest disease incidence. Another experimental line, Scab-7 also had low scores until day 14 when disease severity was similar to the susceptible check Wheaton (Table 5).

Discussion

Resistance to scab is usually inherited as a quantitative trait. Several chromosomes and QTLs are known to influence this resistance reaction (Bai and Shaner, 1994; Anderson *et al*., 2001), indicating that multiple genes affect the resistance. This finding is further supported by the induction of different PR-proteins including PR-2, PR-3, PR-4, PR-5, and other PR-genes within 12–24 h after initiation of infection (Li *et al*., 2001; Pritsch *et al*., 2001). However, there is no direct evidence to support the functional role of these genes in defence against scab. The aim of the present study was to introduce single PR-protein genes or gene combinations identified from a scab-infected Sumai-3 library (Li *et al*., 2001) into a susceptible wheat background and characterize their efficiency in defence against scab.

Previous studies involving genes-encoding PR-proteins for enhancing resistance to scab yielded transgenic lines over-expressing a rice *tlp* gene (PR-5) that showed a delayed resistance to spread in the head (type II resistance, Chen *et al*., 1999). This promising result encouraged the identification of candidate genes from a scab-resistant genotype (Sumai-3) and to transform wheat with these genes in order to enhance the levels of resistance to scab further in wheat. The major obstacle to this approach was encountered in generating enough lines with stable inheritance and expression of the transgenes. A majority of the lines underwent transgene-silencing at different generations. Of the 24 independent transgenic lines identified, only four lines (about 20%) stably expressed the transgenes in the T_3 generation. This report contradicts an earlier conclusion that ubiquitin promoter-driven genes are stably expressed over several generations, whereas CaMV promoter-driven genes were silenced in T_1 or subsequent generations (Chen *et al.,* 1998, 1999). The studies reported here indicate that the maize ubiquitin promoter-intron is also prone to random gene silencing in transgenic wheat plants, even though the frequency of gene silencing is somewhat less compared to genes under the control of the CaMV promoter.

Similarly high frequency of co-integration (90%) was detected in 15 of the 17 independent transgenic lines when co-bombarded with multiple plasmids. These results are in accordance with earlier reports on co-transformation of wheat with multiple genes (Barro *et al*., 1997; Bliffeld *et al*., 1999). There are previous reports on the co-expression of chitinase and β-1,3-glucanase genes in tobacco (Jach *et al*., 1995; Zhu *et al*., 1994) and tomato (Jongedijk *et al*., 1995) which are dicotyledenous plants that are more amenable to genetic transformation than is

wheat. This is the first report of stable co-expression of two different PR-protein genes (a chitinase and a β-1,3-glucanase gene) in transgenic wheat plants. Earlier attempts to co-express multiple PR-proteins for disease resistance in wheat resulted in the expression of only a single gene (Bliffeld *et al*., 1999; Chen *et al*., 1999; Schweizer *et al*., 1999). The accumulation of the two PR-protein genes in combination had no deleterious phenotypic effect on the plants, except for line No. 32A. This line, which had the highest levels of both chitinase and β-1,3-glucanase, was normal up to the T_2 generation when the transgene locus was still segregating. However, when this line was rendered homozygous for the transgene loci, expression of the transgene-encoded proteins reached the highest levels and the plants developed a lesion mimic phenotype. This line grew normally in the vegetative phase, but at the booting stage developed necrotic spots that appeared on the second and third leaves from the flag leaf and later spread throughout the plant. The homozygous plants from this line were backcrossed with the parental line 'Bobwhite' to determine whether the lesion mimic phenotype was dependent on 'gene-dosage'. The heterozygous plants derived from the backcross, did not develop the lesions and had a normal seed set.

Earlier attempts with expression of β-1,3-glucanase gene in wheat resulted in the failure to recover transgenic plants (Bliffeld *et al*., 1999). It was proposed that constitutive expression of β-1,3-glucanase interfered with plant regeneration and normal development of transgenic wheat plants. All the lines, including line 32A, set normal spikes (no lesions) with viable seeds. Lines 76 and 78 co-expressing the 383 chitinase and 289 glucanase at moderate levels, line 82 expressing the 638 glucanase only, and the heterozygous plants from line 26 expressing the 383 chitinase had normal phenotypes.

The availability of only four lines with stable expression of transgenes limited the number of combinations of PR-protein genes that could be assayed for their contributions to defence against scab. Among these four lines, only the line with the highest level of expression of 383 chitinase and 638 glucanase had a significant increase in scab resistance as measured in greenhouse trials. The other lines, with lower levels of expression of both transgenes and one line expressing only a β-1,3-glucanase showed no improvement in disease resistance. Thus, it appears that a combination of chitinase and β-1,3-glucanase genes is necessary to enhance host resistance to scab. Even then transgene expression must approach the levels seen in the heterozygous plants of line 32A because lines 76 and 78 which had lower levels of both chitinase and glucanase were quite susceptible to scab.

It was expected that plants homozygous for the transgene loci with higher levels of expression would have greater resistance to scab than heterozygous plants, however, disease severity was similar in both heterozygous and homozygous plants of line 32A (Tables 3, 4A) relative to the Bobwhite control. This may be the maximum level of protection that can be achieved, possibly because these enzymes only delay the spread of disease but do not eliminate it.

The greenhouse studies reported in this study are similar to the protective synergistic interactions of chitinase/glucanase as antifungal defences *in vitro* reported by other groups (Leah *et al*., 1991; Mauch *et al*., 1988) and *in vivo* studies with transgenic tobacco, tomato and carrots (Zhu *et al*., 1994; Jach *et al*., 1995; Jongedijk *et al*., 1995; Melchers and Stuvier, 2000). To improve the protective effects of over-expression of PR-protein genes, there are ongoing studies for combining different antifungal protein genes via crosses of homozygous expressing parents with different combinations of PR-protein and other antifungal genes. For example, crosses have been carried out between the moderately resistant *tlp* transgenic lines (Chen *et al*., 1999) with line No. 32A for pyramiding all three PR-protein genes into one line and to evaluate the interaction of these PR-protein genes as antifungal defences.

The two lines that were identified as moderately resistant to scab under greenhouse conditions (No. 32A and BT-14-18) did not show any improved resistance to scab when bioassayed against scab in a scab field nursery. No significant reduction in symptoms was observed except for line No. 32A on day 3. But on day 6 and beyond, the transgenic plants were overwhelmed by the disease. These differences between the results of greenhouse studies versus field evaluations are attributed to the differences in the inoculation techniques employed. In the greenhouse studies, a conidial suspension (10^4–10^5 conidia ml^{-1}) was injected into the young spikelets after anthesis as a single application. On the other hand, in the field inoculation, the corn kernels provide a continuous inoculum of ascospores, which land on the spikes, germinate and infect the spikes under conducive environmental conditions. The infection process is continuous and emerging spikes get infected at different stages and ages. Therefore, greenhouse assays screen for type II resistance (resistance to fungal spread from a single inoculation point), while field tests screen for both type II and type I (resistance to inoculation and spread). It is likely that the transgenic wheat line No. 32A displays type II resistance but not type I. Sharp *et al*. (2002) in an evaluation of transgenic wheat plants expressing viral coat proteins against wheat mosaic virus reported that while these plants were more resistant in greenhouse studies, they offered no protection in the field. As far as is known, this is the first report of field evaluation of a cereal plant expressing genes for PR-proteins against disease resistance.

The poor performance of line 32A in these field evaluations might have been due to the lesion-mimic phenotype. These plants which are presumably undergoing programmed cell death may have compromised ability to

withstand continuous pathogen pressure which occurs under field conditions. The lesion-mimic phenotype is only seen in the homozygous and not in heterozygous plants, which are equally resistant to scab in greenhouse bioassays. Next spring it is planned to conduct field evaluation of segregating populations of plants derived from line 32A to determine whether they will withstand the severe conditions of the disease in the scab nursery. In addition, there is a plan to evaluate plants with three different PR-protein genes namely chitinase, β-1,3-glucanase and *tlp*, because this combination of PR-protein genes would target not only the fungal cell wall, but also the fungal plasma membrane, the known target of TLP (Vigers *et al*., 1991; Yun *et al*., 1998). It is likely that PR-proteins may be effective only in combination with several other PR-proteins.

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References

Altpeter F, Vasil V, Srivastava V, Stöger E, Vasil IK. 1996. Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. *Plant Cell Reports* **16,** 12–17.

Anderson JA, Stack RW, Liu S, Waldron BL, Fjed AD, Coyne C, Moreno-Sevilla B, Mitchell JF, Song QJ, Froberg RC. 2001. DNA markers for *Fusarium* head blight resistance QTLs in two wheat populations. *Theoretical and Applied Genetics* **102,** 1164–1168.

Bai GH, Shaner G. 1994. Scab of wheat: prospects for control. *Plant Disease* **78,** 760–766.

Barro F, Rooke L, Bekes F, Gras P, Tatham AS, Fido F, Lazzeri PA, Shewry PR, Barcelo P. 1997. Transformation of wheat with high molecular weight subunit genes results in improved functional properties. *Nature Biotechnology* **15,** 1295–1299.

Bieri S, Potrykus I, Futterer J. 2000. Expression of active barley seed ribosome-inactivating protein in transgenic wheat. *Theoretical and Applied Genetics* **100,** 755–763.

Bliffeld M, Mundy J, Potrykus I, Futterer J. 1999. Genetic engineering of wheat for increased resistance to powdery mildew disease. *Theoretical and Applied Genetics* **98,** 1079–1086.

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais J, Broglie RM. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254,** 1194–1197.

Chen WP, Chen PD, Liu DJ, Kynast RJ, Friebe B, Velazhahan R, Muthukrishnan S, Gill BS. 1999. Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene. *Theoretical and Applied Genetics* **99,** 755–760.

Chen WP, Gu X, Liang GH, Muthukrishnan S, Chen PD, Liu DJ, Gill BS. 1998. Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the *bar* gene as a selectable marker. *Theoretical and Applied Genetics* **97,** 1296–1306.

Christensen AH, Schrrock RA, Quail PH. 1992. Maize ubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology* **18,** 675–689.

Clausen M, Krauter R, Schachermayr G, Potrykus I, Sautter C. 2000. Antifungal activity of a virally encoded gene in transgenic wheat. *Nature Biotechnology* **18,** 446–449.

Datta K, Muthukrishnan S, Datta SK. 1999. Expression and function of PR-protein genes in transgenic plants. In: Datta SK, Muthukrishnan S, eds. *Pathogenesis-related proteins in plants*. Boca Raton, FL: CRC Press, 261–277.

Finer JJ, Vain P, Jones MW, McMullen MD. 1992. Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports* **11,** 323–328.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50,** 151–158.

Igaz P, Fejer G, Szalai C, Toth S, Falus A. 1998. Development of competitive mRNA PCR for the quantification of interleukin-6-responsive *jun*B oncogene expression. *BioTechniques* **24,** 854–860.

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *The Plant Journal* **8,** 97–109.

Jongedijk E, Tigelaar H, van Roekel JSC, Bres-Vloemans SA, Dekker I, van den Elzen P JM, Cornelissen BJC, Melchers LS. 1995. Synergistic activity of β-1,3-glucanases and chitinases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85,** 173–180.

Leah R, Tommerup H, Svendsen I, Mundy J. 1991. Biochemical and molecular characterization of three barley seed proteins with anti-fungal properties. *Journal Biological Chemistry* **266,** 1464–1473.

Leckband G, Lorz H. 1998. Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance. *Theoretical and Applied Genetics* **96,** 1004–1012.

Li WL, Faris JD, Muthukrishnan S, Liu DJ, Chen PD, Gill BS. 2001. Isolation and characterization of novel cDNA clones of acidic chitinases and β-1,3- glucanases from wheat spikes infected with *Fusarium graminearium*. *Theoretical and Applied Genetics* **102,** 353–362.

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK. 1995. Genetic engineering of rice for resistance to sheath blight. *Bio/Technology* **13,** 686–691.

Mauch F, Mauch-Mani B, Boller T. 1988. Antifungal hydrolases in pea tissue: inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. *Plant Physiology* **88,** 936–942.

McMullen M, Jones R, Gallenberg D. 1997. Scab of wheat and barley: a re-emerging disease of devastating impact. *Plant Disease* **81,** 1340–1348.

Melchers LS, Stuiver MH. 2000. Novel genes for disease-resistance breeding. *Current Opinion in Plant Biology* **3,** 147–152.

Mohammadi M, Kazemi H. 2002. Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. *Plant Science* **162,** 491–498.

Murashige T, Skoog FA. 1962. A revised medium for rapid growth

and bioassays with tobacco tissue cultures. *Plant Physiology* **15,** 473–497.

Neuhaus JM, Ahl-Goy P, Hinz U, Flores S, Meins FJ. 1991. High-level expression of a tobacco chitinase gene in *Nicotiana sylvestris*: susceptibility of transgenic plants to *Cercospora nicotianae* infection. *Plant Molecular Biology* **16,** 141–151.

Oldach KH, Becker D, Lorz H. 2001. Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. *Molecular Plant Microbe Interactions* **14,** 832–838.

Parry DW, Jenkinson P, McLeod L. 1995. Fusarium ear blight (scab) in small grain cereals—a review. *Plant Pathology* **44,** 207–238.

Patnaik D, Khurana P. 2001. Wheat biotechnology: a minireview. *European Journal of Biotechology* **4,** 1–29.

Pritsch C, Muelbauer GJ, Bushnell WR, Somers DA, Vance CR. 2000. Fungal development and induction of defence response genes during early infection of wheat spikes by *Fusarium graminearum*. *Molecular Plant Microbe Interactions* **13,** 159–169.

Pritsch C, Vance CP, Bushnell RW, Somers AD, Hohn TM, Muehlbauer GJ. 2001. Systemic expression of defence response genes in wheat spikes as a response to *Fusarium graminearum* infection. *Physiological and Molecular Plant Pathology* **58,** 1–12.

SAS Institute. 1998. *System Version 7 for Windows*. Cary, NC: SAS Institution.

Schweizer P, Christoffel A, Dudler R. 1999. Transient expression of members of the *germin*-like gene in epidermal cells of wheat confers disease resistance. *The Plant Journal* **20,** 541–552.

Shah DM. 1997. Genetic engineering for fungal and bacterial diseases. *Current Opinion in Biotechology* **8,** 208–214.

Shaner G, Buechley G. 1999. Control of wheat diseases with foliar fungicides, 1998. *Fungicide and Nematicide Tests* **54,** 337–338.

Sharp GL, Martin JM, Lanning SP, Blake NK, Brey CW, Sivamani E, Qu R, Talbert LE. 2002. Field evaluation of transgenic and classical sources of wheat streak mosaic virus resistance. *Crop Science* **42,** 105–110.

Swegle M, Kramer J, Muthukrishnan S. 1992. Properties of barley seed chitinase and release of embryo-associated isoforms during early stages of inhibition. *Plant Physiology* **99,** 1009–1014.

Vigers AJ, Roberts WK, Selitrennikoff CP. 1991. A new family of plant antifungal proteins. *Molecular Plant Microbe Interactions* **4,** 315–323.

Xu Y, Chen L. 1993. *Wheat scab*; *theory and practice of control* (in Chinese). Jiangsu, China: Science and Technology Press of Jiangsu, 201–216.

Yun DJ, Ibeas JI, Lee H, Coca MA, Narasimhan ML, Uesono Y, Hasegawa PM, Pardo JM, Bressan RA. 1998. Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. *Molecular Cell* **1,** 807–812.

Zhang L, French R, Langenberg WG, Mitra A. 2001. Accumulation of barley stripe mosaic virus is significantly reduced in transgenic wheat plants expressing a bacterial ribonuclease. *Transgenic Research* **10,** 13–19.

Zhou H, Arrowsmith JW, Fromm ME, Hironaka CM, Taylor ML, Rodriguez D, Pajeau ME, Brown SM, Santino CG, Fry JE. 1995. Glyphosate-tolerant CP4 and GOX genes as a selectable marker in wheat transformation. *Plant Cell Reports* **15,** 159–163.

Zhu Q, Maher EA, Maced M, Dixon RA, Lamb C. 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Bio/Technology* **12,** 807–812.