Apoplastic Extracts from a Transgenic Wheat Line Exhibiting Lesion-Mimic Phenotype Have Multiple Pathogenesis-Related Proteins That Are Antifungal

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A transgenic wheat line constitutively expressing genes encoding a class IV acidic chitinase and an acidic β -1,3-glucanase, showed significant delay in spread of *Fusarium* head blight (scab) disease under greenhouse conditions. In an earlier work, we observed a lesion-mimic phenotype in this transgenic line when homozygous for transgene loci. Apoplastic fluid (AF) extracted from the lesion-mimic plants had pathogenesis-related (PR) proteins belonging to families of β -1,3-glucanases, chitinases, and thaumatin-like proteins (TLPs). AF had growth inhibitory activity against certain fungal pathogens, including *Fusarium graminearum* and *Gaeumannomyces graminis* var. *tritici*. Through a two-step ion-exchange chromatography protocol, we recovered many PR proteins and a few uncharacterized proteins. Three individual protein bands corresponding to a TLP (molecular mass, 16 kDa) and two β -1,3-glucanases (molecular mass, 32 kDa each) were purified and identified by tandem mass spectrometry. We measured the *in vitro* antifungal activity of the three purified enzymes and a barley class II chitinase (purified earlier in our laboratory) in microtiter plate assays with macroconidia or conidiophores of *F. graminearum* and *Pyrenophora tritici-repentis*. Mixtures of proteins revealed synergistic or additive inhibitory activity against *F. graminearum* and *P. tritici-repentis* hyphae. The concentrations of PR proteins at which these effects were observed are likely to be those reached in AF of cells exhibiting a hypersensitive response. Our results suggest that apoplastic PR proteins are antifungal and their antimicrobial potency is dependent on concentrations and combinations that are effectively reached in plants following microbial attack.

Additional keywords: antifungal assay, antifungal proteins, programmed cell death.

Incompatible plant-pathogen interactions result in a hypersensitive response (HR) and induce resistance both locally (around the site of infection) and systemically (in noninfected parts of the plant). These responses lead to the formation of necrotic spots, often referred to as “lesions,” containing dead cells (Dangl et al. 1996) that restrict further growth and spread of the pathogen to neighboring healthy cells (Dixon and Harrison 1992). Cells in and around the infection site accumulate patho-

genesis-related (PR) proteins such as chitinases and β -1,3-glucanases with antimicrobial activity (Ward et al. 1991; Yang et al. 1997). Many transgenic plants exhibiting “lesion-plus” phenotype show physiological and chemical changes similar to those associated with HR (Mittler and Rizhsky 2000). Constitutively elevated levels of defense-related proteins, including PR proteins, were detected in the lesion-plus transgenic plants in the absence of pathogen recognition and induction of plant defenses (Mittler and Rizhsky 2000). Such a phenotype often is referred to as transgene-induced lesion mimic (Mittler and Rizhsky 2000; Nishizawa et al. 2003).

PR proteins are the most intensively studied proteins induced in plants following microbial attack. These proteins were first detected in tobacco plants exhibiting HR upon *Tobacco mosaic virus* infection (Gianinazzi et al. 1970; van Loon and van Kammen 1970). PR proteins now are classified into 14 groups (van Loon and van Strien 1999), and 2 additional groups have been proposed (L. C. van Loon, *personal communication*). Some proteins in groups PR-1, PR-2, PR-3, PR-4, and PR-5 have antifungal activity (Jayaraj et al. *in press*). The mechanism or mechanisms of action and catalytic activity of some of these proteins, especially those of PR-1, are not known (Niedermann et al. 1995). Chitin and β -1,3-glucans that constitute the major cell wall components of most fungi (except possibly oomycetes) are degraded by chitinases and β -1,3-glucanases, respectively (Wessels and Sietsma 1981). These proteins inhibit the growth of certain fungi *in vitro* when applied either individually or in combinations (Leah et al. 1991; Mauch et al. 1988; Sela-Buurlage et al. 1993), which led to the hypothesis that these enzymes have specific roles in pathogen-induced defenses in plants. Synergistic interactions also are known in transgenic plants that express combinations of genes for PR proteins (Anand et al. 2003b; Jongedijk et al. 1995; Zhu et al. 1994).

The PR-5 proteins share significant sequence similarity to thaumatin (an intensely sweet protein from the arils of ripe fruits of *Thaumatococcus daniellii*) and also are known as the thaumatin-like proteins (TLPs) (Cornelissen et al. 1986). The proposed mechanism of their action involves plasma membrane permeabilization of the target fungal cell (Roberts and Selitrennikoff 1990; Vigers et al. 1992) and signal transduction (Yun et al. 1998). Some PR-5 proteins have strong antifungal activity (Abad et al. 1996; Velazhahan et al. 2002; Vigers et al. 1992), and disease onset was delayed in transgenic plants expressing heterologous TLPs (Chen et al. 1999; Liu et al. 1994; Zhu et al. 1996).

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Earlier, we coexpressed genes for a class IV chitinase and an acidic β -1,3-glucanase in wheat plants and recovered a transgenic wheat line that developed necrotic lesions in the T₂ and subsequent generations (Anand et al. 2003a and b). This transgenic line delayed the spread of infection (type II resistance) to *Fusarium* head blight or scab caused by *Fusarium graminearum* under greenhouse conditions (Anand et al. 2003b). The appearance of lesions in this transgenic line was associated with increased accumulation of salicylic acid, induction of other PR-protein genes, and activation of reactive oxygen intermediates (Anand et al. 2003a). Preliminary screening of protein extracts from leaf tissues of these plants detected not only the accumulation of the transgene-encoded proteins but also other host PR proteins, including TLP, PR-1, and additional isoforms of the chitinases and β -1,3-glucanases. The concentrations of the PR proteins that were recovered from the apoplastic fluid were in the range reported by Vigers and associates (1992) for other crops. In vitro antifungal assays against important fungal wheat pathogens demonstrated the antifungal activity of the purified PR proteins at relatively higher concentrations and were dependent on the combinations applied as suggested by many earlier workers (Mauch et al. 1988; Sela-Buurlage et al. 1993). A similar study on isolation and characterization of antifungal proteins from apoplastic fraction of

infected barley leaves details the analysis of several chitinases, glucanases, and TLPs and also the combinatorial effects of these PR-proteins (Zareie et al. 2002). The novelty of this report is in the simplified technique used for purification and characterization of some of the induced PR proteins from wheat and the test of the combinatorial antifungal activity in vitro with a number of fungi.

RESULTS

Host and transgene-encoded pathogenesis-related proteins accumulate in the apoplastic fluid.

With the rationale of enhancing disease resistance in wheat to fungal pathogens, an attempt was made to constitutively express single and combinations of genes encoding PR proteins in wheat using transgenic approaches (Anand et al. 2003b). In the process, a stable homozygous transgenic line 32A-2-3 coexpressing an acidic chitinase (383Chi) and an acidic β -1,3-glucanase (638Glu) (both transgenes are driven by the constitutive maize ubiquitin promoter-intron) was identified that had high levels of transgene-encoded proteins and several other host-encoded PR proteins. This line had a developmentally associated lesion phenotype. Interestingly, the development of the lesion-mimic phenotype was associated with the

Fig. 1. A, Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and **B**, **C**, and **D**, Western blot analysis of the apoplastic fluid from transgenic line 32A-2-3 coexpressing an acidic chitinase (383 Chi) and acidic β -1,3-glucanase (638 Glu). Crude apoplastic fraction (200 μ g) of and proteins from the flowthrough from the DEAE-Sepharose column (50 μ g) were fractionated on a 10 to 20% SDS-polyacrylamide gradient gel. The DEAE-Sepharose column was immunoblotted and detected using polyclonal antisera for **B**, chitinase, **C**, β -1,3-glucanase, and **D**, thaumatin-like protein. Molecular masses are indicated in kilodaltons (kDa).

presence of the functional transgene or transgenes and not due to disruption of host genes at the site of integration (Anand et al. 2003a). The overexpression of the transgenes also resulted in turning on other host PR genes, production of reactive oxygen species, and accumulation of high levels of salicylic acid (SA) just prior to the appearance of lesions (Anand et al. 2003a). Because we detected the induced expression of many PR proteins in the leaf tissues of this lesion-mimic line, it was hypothesized that the apoplastic leaf fractions (AF) of this line may serve as an excellent starting material for purification, characterization, and evaluation of antifungal potencies of many defense-related proteins.

We detected the expression of transgene-encoded chitinases, β -1,3-glucanases, and their endogenous isoforms in the AF of the transgenic wheat line 32A-2-3 (Anand et al. 2003a). The majority (>90%, based upon the band intensities) of the chitinases and β -1,3-glucanases was in the AF, with the remainder detected in the cell extracts (data not shown). Western blot

analysis of AF fraction from the line 32A-2-3 with polyclonal antibodies led to the detection of a few chitinases ranging in size from 26 to 35 kDa (Fig. 1B) and β -1,3-glucanases of $\approx$ 32 kDa (Fig. 1C). The AF fraction contained three TLPs with sizes of 14, 16, and 24 kDa (Fig. 1D). Based on the above finding, we predict that many PR proteins are expressed in the leaves of line 32A-2-3 and are predominately localized in the apoplastic compartment.

Purification and characterization of AF proteins.

We fractionated the AF proteins using ion-exchange column chromatography and few major protein bands (8 to 10) were eluted after DEAE-Sepharose column chromatography in the size range of 40 to 60 kDa. None of the eluted protein bands reacted with the polyclonal antibodies raised against chitinase, β -1,3-glucanase, or TLP (data not shown) and these bands were not studied further. We could detect 6 to 7 major protein bands (Fig. 1A) in the flowthrough fraction as visualized on

Fig. 2. A, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and **B**, Western blot analysis of purified protein fractions. Different fractions (250 ng) collected from CM-Sepharose column on 0 to 0.5 M NaCl salt gradient was fractionated on 12% SDS-PAGE and immunoblotted and detected with polyclonal antibodies for chitinase, β -1,3-glucanases, and thaumatin-like protein (TLP). Coomassie blue-stained gel and corresponding proteins detected in each lane by Western blots analyses are shown in parallel. Fractions 8 to 15 correspond to the purified wheat TLP and fractions 41 to 51 contain the β -1,3-glucanases. Lane 1, RPN 756 is the rainbow molecular weight protein marker, arrows indicate the molecular weights in kilodaltons (kDa), and legends marked 1 and 2 represent the two purified glucanases. The different pathogenesis-related proteins are identified as: Glu, apoplastic β -1,3-glucanases; BarChi, barley class II chitinase; TLP, apoplastic thaumatin-like protein; and Chi, apoplastic chitinase.

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The flowthrough fraction from a DEAE-Sepharose column was dialyzed and fractionated further using a CM-cellulose column. Three proteins were eluted from the carboxymethyl (CM)-cellulose column, including a 16-kDa TLP protein (fractions 8 to 15) and two β -1,3-glucanases (≈ 32 kDa, fractions 41 to 51) that were purified to homogeneity (Fig. 2). These proteins reacted strongly with the polyclonal antibodies raised against a mung bean TLP and a barley β -1,3-glucanase. Fractions 18 to 23 had three major protein bands, one of which (a 26-kDa protein) cross-reacted with the chitinase antibody (Fig. 2). Similarly, three major chitinases (26, 28, and 35 kDa) and two TLPs (16 and 24 kDa) were detected in the fractions 26 to 37 (Fig. 2) by Western blot analyses. Additional fractions that cross-reacted with antibodies raised against β -1,3-glucanase also were detected (data not shown). Purified fractions containing the two different glucanases, a single TLP protein, and fractions with mixtures of different chitinases and TLPs are depicted in Figure 2. The amount of total proteins in the different fractions based upon our recovery data is presented in Table 1.

The two pools of proteins (fractions 18 to 23 and 26 to 37) containing chitinases had specific activities of 142 and 188 U mg^{-1} proteins, respectively, whereas the specific activity of a pure barley chitinase prepared in our laboratory (Swegle et al. 1992) was 480 U mg^{-1} . Based on the intensities of the chitinase protein bands in stained gels (Fig. 2A), the single chitinase protein detected in fractions 18 to 23 accounted for approximately one-third of the total protein in the fraction. The specific activity of the pooled protein fractions (41 to 51) containing the β -1,3-glucanases was found to be 1,320 U mg^{-1} . This finding suggests that the fractions containing proteins recognized immunologically by the chitinases and β -1,3-glucanases antibodies also possessed the corresponding enzymatic activities.

Protein analysis and characterization of the purified PR proteins.

For protein analysis, the protein fractions containing a single TLP and the two β -1,3-glucanase bands ($>95\%$ purity based on staining) were excised, cut into sections, and digested with trypsin, and peptides were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) as detailed below. LC-MS/MS analyses of the protein digests of TLP and

the two β -1,3-glucanases bands were performed by nano-high-performance liquid chromatography (nHPLC) coupled to an ABI QSTAR Pulsar hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer, and representative LC-MS/MS data were recorded. Resultant mass spectral data were queried against the NCBI nonredundant protein database using MASCOT, a probability-based protein identification tool (Perkins et al. 1999). Two bands (Fig. 2, lane marked purified wheat glucanase; fractions 41 to 51) that cross-reacted with a polyclonal antibody raised against β -1,3-glucanase were identified as basic β -1,3-endoglucanase from wheat (GenBank accession number: CAA77085.1) with high molecular weight search (MOWSE) scores (>750), but with slightly different sequence coverages (53% for band 2 and 46% for band 1). A closer inspection of the matched peptides from these two bands further revealed that band two contains one peptide (YIAAGNEVQGGTTQSI VPAMR) that is not present in band one. This suggests that these two proteins do not have the same sequence and most likely are different isoforms of β -1,3-endoglucanase. Both proteins were found to migrate as separate bands in a gradient SDS-PAGE (10 to 20%), but were determined to have similar

Table 1. Fractionation of apoplastic fluid proteins

Fractions	Volume (ml)	Protein concentration ($\mu\text{g } \mu\text{l}^{-1}$)	Total protein (mg)
Apoplast	35	0.47	16.5
DEAE-Sepharose flowthrough	60	0.12	7.2
CM-Sepharose bound fractions			
Fractions 8–14	4.5	0.17	0.76
Fractions 18–23	4.2	0.2	0.84
Fractions 26–37	6.3	0.22	1.39
Fractions 41–51	6.0	0.21	1.26

Table 2. Characteristic features of the three apoplastic proteins that were purified and characterized from a lesion-mimic transgenic wheat line^a

Purified protein	Molecular weight	N-terminal amino acid sequence
AFTLP	16,234	ATFNKNNCGSTK
AFGLU1	32,619	IGVCYGVNGDGLPP
AFGLU2	32,524	IGVCYGVIGNNLP

^a Residues in bold represent those that are different i) among other well-characterized wheat thaumatin-like proteins (TLPs) (for purified TLP protein from the apoplastic fluid) and ii) between the two purified β -1,3-glucanases.

Fig. 3. Mycelial growth inhibition assay with apoplastic fluid (AF) preparations. Agar disks (5 mm) of the different fungal isolates were placed in the center of the petri dishes and allowed to grow in the dark at room temperature for 48 h. Apoplastic fraction and the crude protein extract from the transgenic line 32A-2-3 and nontransgenic plants were applied on filter paper discs at concentrations ranging between 100 and 250 μg and placed along the growing tips of the mycelium. Mycelial growth inhibition assay with AF preparations (left petri dish) and total protein extracts (right petri dish) from leaves of transgenic plants and nontransgenic control plants were monitored. Mycelial growth inhibition zones were observed 48 to 64 h after incubation with the protein fractions. **A**, Inhibition of *Fusarium graminearum* mycelial growth at protein concentrations of (a) 250 μg from non-transgenic wild-type control plants, 200 μg (b) and 250 μg (c) from the transgenic line 32A-2-3. **B**, Inhibition of *Gaeumannomyces graminis* var. *tritici* mycelium at protein concentrations of 250 μg from nontransgenic wild-type control plants (a), and 200 μg (b) and 250 μg (c) from the transgenic line 32A-2-3. Arrows indicate the inhibition zones.

A

B

i) 3h

ii) 10h

Fig. 4. Spore germination inhibition assay with different protein preparations. Known concentrations of the macroconidial suspension culture of *Fusarium graminearum* (10^6 spores ml⁻¹) were incubated with different amounts of the apoplastic fraction from transgenic-plants and wild-type plants, pure class II chitinases, thaumatin-like protein (TLP), β -1,3-glucanases, sodium phosphate buffer supplemented with bovine serum albumin (BSA), and water. Inhibition of spore germination of *F. graminearum* was scored as percentage of spore inhibition at hourly intervals. **A**, Purified protein fractions that were tested for their transient antifungal activity at different concentration ranges (10 to 100 μ g ml⁻¹) at hourly intervals. Data after 1 and 3 h of incubation on spore inhibition is illustrated in the Y axis, while the protein concentrations are represented along the X axis. The different pathogenesis-related proteins are identified as: AFGlu, apoplastic β -1,3-glucanases; BarChi, barley class II chitinase; AFTLP, apoplastic thaumatin-like protein; and BSA. **B**, Spore inhibition assay in microtiter plates. **B i** **1**, Pure class II barley chitinase (50 μ g ml⁻¹); **2**, the AF (250 μ g ml⁻¹) from line 32A-2-3; and **3**, AF from nontransgenic wild-type wheat plants (250 μ g ml⁻¹); observations were made after 3 h of incubation with the proteins. Arrows indicate the germinating macroconidia. **B ii** **1**, Sodium phosphate buffer supplemented with BSA (250 μ g ml⁻¹); **2**, purified β -1,3-glucanases (150 μ g ml⁻¹); **3**, purified β -1,3-glucanases plus barley chitinase (each protein fraction at 37.5 μ g ml⁻¹); and **4**, purified β -1,3-glucanases plus barley chitinase (each protein fraction at 75 μ g ml⁻¹).

molecular masses (32,619 for band 1 and 32,524 for band 2) by matrix-assisted laser desorption-ionization time of flight (MALDI-TOF)/MS. Further, 2-DE gel electrophoresis identified the two glucanases as basic proteins (pI 8.7 and 9; data not presented) that were significantly different from that of the transgene-encoded acidic 638 glucanase (pI 4.3) (Li et al. 2001). The N-terminal sequences of the purified glucanases (AFGlu) showed significant similarity to other known wheat and barley glucanases (Table 2); however, to our knowledge, this is the first report on identification of β -1,3-glucanases from wheat leaf tissue.

LC-MS/MS analysis of a third band from SDS-PAGE (Fig. 2, lane marked as purified wheat TLP; fractions 8 to 15) identified this protein as an 18-kDa wheat thaumatin-like protein (Mingeot and Jacquemin 1998) with sequence coverage of 11%. The MALDI-TOF/MS analysis showed that this TLP protein had a molecular mass of 16,234, indicating that this protein could be the processed form of the 18-kDa wheat thaumatin-like protein (minus the signal peptide region). The N-terminal sequence of the purified TLP protein from the AF (AFTLP) was similar to that of other known TLPs of similar molecular masses previously reported in wheat, and is almost identical to that of a wheat TLP described earlier (GenBank accession AF384146; L. Yu, J.-S. Niu, Z. Q. Ma, P. D. Chen, and D. J. Chen *unpublished data*), except for the 13th residue (Table 2).

Effects of AF proteins on fungal growth.

In vitro antifungal assays were carried out to test the effectiveness of AF proteins from transgenic line 32A-2-3 against fungal pathogens of wheat. No mycelial growth inhibition was detected in the protein-disk assays at concentrations ranging from 100 to 250 μ g of total AF proteins against wheat fungal pathogens: *Pyrenophora tritici-repentis*, *Rhizoctonia solani*,

and *Stagonospora nodorum*; whereas, at concentrations of 200 μ g and above, AF from the transgenic line inhibited mycelial growth of *Fusarium graminearum* and *Gaeumannomyces graminis* var. *tritici* substantially (Fig. 3A and B). The AF from nontransgenic wild-type plants failed to inhibit growth at similar concentrations against both fungi.

We also performed germination assays to measure the effect of the unfractionated AF, individual purified PR proteins, and various combinations of the purified PR proteins against *F. graminearum* and *P. tritici-repentis*. The rationale behind selecting these pathogens are i) these fungi are important from an agronomic point of view because they cause significant yield losses in wheat, and ii) the limited number of previous studies involving PR proteins and these pathogens. It is extremely difficult to raise spore cultures of another important wheat pathogen, *G. graminis* var. *tritici*, under in vitro conditions; therefore, we had to exclude this pathogen from evaluation of effects of AF and PR proteins on growth inhibition. We could not obtain a wheat chitinase in highly purified form and, therefore, utilized a closely related barley chitinase that had been purified to homogeneity through chitin affinity chromatography and isoelectric focusing that was available in our laboratory (Swegle et al. 1992). This barley class II chitinase (75 μ g ml⁻¹) and AF from the transgenic line exhibited strong antifungal activity against *F. graminearum* (Fig. 4A). Spore germination was completely inhibited with AF proteins at 250 μ g ml⁻¹ for up to 3 h (Fig. 4B); whereas, at concentration of 100 μ g ml⁻¹, the same AF fraction inhibited germination of 40 to 50% of the spores (data not shown). Bovine serum albumin (BSA), distilled water, and fractions containing the AF from wild-type nontransgenic wheat plants (Fig. 4A and B), did not inhibit spore germination. A longer incubation of the spores with AF (≥ 250 μ g ml⁻¹) for ≥ 6 h resulted in 100% germination;

Fig. 5. Effect of different pathogenesis-related (PR) proteins on spore germination of *Fusarium graminearum*. The macroconidial germination was observed under the microscope at hourly intervals after incubation of the spore suspension (10^6 spores ml⁻¹) with individual purified proteins or in combinations. The data represent the mean percent inhibition from three independent experiments and the standard error values (indicated by bar) after 10 h of incubation. The values on the X axis represent protein concentrations (μ g ml⁻¹) applied. The different PR proteins are identified as: AFGlu, apoplastic β -1,3-glucanases; BarChi, barley class II chitinase; and AFTLP, apoplastic thaumatin-like protein.

however, in this extended incubation, no more than 50% of the spores germinated in the presence of barley chitinase at $75 \mu\text{g l}^{-1}$ (data not shown). We could observe the antifungal potency of the AF fraction at lower concentrations (as low as $75 \mu\text{g ml}^{-1}$); however, quantifying this data was impossible because the spores germinated rapidly after a short period of apparent inhibition, and replicating these observations became difficult. The AF fractions from the wild-type and the transgenic line had no inhibitory effect on the growth of *P. tritici-repentis* conidio-

phores, whereas pure barley chitinase at concentrations as low as $20 \mu\text{g ml}^{-1}$ showed growth inhibition of the conidiophores for shorter incubation periods.

Combinatorial effects of PR proteins on fungal spore germination.

In germination assays against *F. graminearum*, the individual AF protein fractions and barley class II chitinase (BarChi) were effective at concentrations of $\geq 75 \mu\text{g ml}^{-1}$, resulting in any-

Fig. 6. Effect of different pathogenesis-related (PR) proteins on the growth of conidiophores of *Pyrenophora tritici-repentis*. A suspension containing few conidia and majority of conidiophores (10^6 conidiophores ml^{-1}) was incubated with individual purified proteins or in combinations and observed under a microscope ($\times 20$) for hyphal growth. The data represent the mean percent hyphal growth inhibition from three independent experiments and the corresponding standard error values (indicated by bar) after 3, 6, and 9 h of incubation. The values on the X axis represent protein concentrations ($\mu\text{g ml}^{-1}$) applied. The different PR proteins are identified as: I, apoplastic β -1,3-glucanases (AFGlu); II, barley class II chitinase (BarChi); III, apoplastic thaumatin-like protein (AFTLP); IV, AFGlu:BarChi; V, AFGlu:AFTLP; VI, AFTLP:BarChi; VII, AFGlu:AFTLP:BarChi. Lanes I to VII represent data for 3 h of incubation, while lanes VIII and IX represent the combined effect of the three purified proteins (AFGlu:AFTLP:BarChi) on the growth inhibition at 6 and 9 h, respectively.

Fig. 7. Morphological changes occurring in the growing hyphae of *Pyrenophora tritici-repentis* when incubated with antifungal proteins. Known concentrations of the conidia or conidiophore suspension cultures (10^6 spores ml^{-1}) were incubated with thaumatin-like protein (TLP), chitinase, β -1,3-glucanases, or a combination of these proteins, and their ability to inhibit germination of *P. tritici-repentis* was recorded 3 h after the addition of pathogenesis-related proteins. The percentage of hyphae with obvious growth was recorded at hourly intervals in each treatment. **A**, Aggregation of the growing hyphae after germination in TLP fraction at 3 h; **B**, hyphal lysis and bulging of the hyphal tip in assays with protein fraction with a combination of wheat β -1,3-glucanases, wheat TLP, and barley chitinase (each at $20 \mu\text{g ml}^{-1}$) after 3 h of incubation. Arrows indicate the bulged hyphal tips and lysis of germinating conidiophores.

where between 50 and 85% inhibition of spore germination at 3 h of incubation (Fig. 4A). Purified wheat TLP (AFTLP), BarChi, and wheat β -1,3-glucanases (AFGLu) preparations completely inhibited spore germination of *F. graminearum* at concentrations of 150 and 200 $\mu\text{g ml}^{-1}$ for over a 6-h period (data not shown). Concentrations below these levels resulted in over 40 to 50% germination with different purified proteins. Therefore, we took incubation periods over 6 h as effective time points to score the differences in levels of inhibition among different enzymes against *F. graminearum*. AFGLu ($\geq 150 \mu\text{g ml}^{-1}$) inhibited spore germination to a greater extent (80%) after 10 h of incubation (Figs. 4B and 5). At similar concentrations, pure BarChi and AFTLP were less effective and resulted in only 51 and 56% inhibition of spore germination, respectively (Fig. 5). Combinations of BarChi (100 $\mu\text{g ml}^{-1}$)/AFTLP (150 $\mu\text{g ml}^{-1}$) and AFGLu (100 $\mu\text{g ml}^{-1}$)/AFTLP (150 $\mu\text{g ml}^{-1}$) decreased spore germination relative to single PR proteins (Fig. 5). Strikingly, the combination of BarChi and AFGLu inhibited spore germination by up to 62% at concentrations as low as 37.5 $\mu\text{g ml}^{-1}$ of each of these proteins. This combination of BarChi/AFGLu (each protein at 37.5 $\mu\text{g ml}^{-1}$) was as effective at inhibiting spore germination as the levels achieved by the combinations BarChi/AFTLP or AFGLu/AFTLP at concentrations as high as each protein at 100 $\mu\text{g ml}^{-1}$ (Fig. 5). Doubling the concentration of the BarChi/AFGLu (each at 75 $\mu\text{g ml}^{-1}$) resulted in almost complete inhibition of spore growth (Figs. 4B and 5). A combination of all three purified proteins (each at 37.5 $\mu\text{g ml}^{-1}$) did not result in any further spore germination inhibition over and above the levels observed in the combinations of BarChi/AFGLu at similar concentrations (data not shown). Therefore, the chitinases and glucanases appear to act synergistically against *F. graminearum* and this is consistent with their coordinate transcriptional induction of these genes in the scab-infected plants (Li et al. 2001).

Even though our initial results indicated that AF proteins did not inhibit the growth of *P. tritici-repentis* conidiophores, we still decided to investigate the fungistatic potential of the purified protein fractions based upon the observed effects of pure barley chitinase against the fungus. Interestingly in assays with *P. tritici-repentis*, the individual protein fractions delayed conidiophore growth for 3 to 6 h (Fig. 6). Nearly 80 to 100% of conidiophores germinated within 6 h in assays with the individual PR proteins or the combinations tested (data not shown). The BarChi/AFGLu/AFTLP combination inhibited conidiophore germination for up to 3 h at a concentration of individual proteins as low as 20 $\mu\text{g ml}^{-1}$ (Fig. 6). In most of the cases, aggregation of the germinating hyphae began within 6 h of incubation (Fig. 7A). The growth inhibition also was accompanied by lysis or bulging at the tips of the germ tubes at protein concentrations of 20 $\mu\text{g ml}^{-1}$ (Fig. 7B). After 6 to 9 h of incubation, the conidiophores recovered and hyphal growth was resumed.

DISCUSSION

Two different β -1,3-glucanases and a TLP protein were identified and purified from the AF of the lesion-mimic transgenic wheat plant which secretes copious amounts of host-encoded proteins, including several PR proteins (Anand et al. 2003a and b). MALDI-TOF/MS and 2-DE gels clearly established that these proteins are pure. Based upon the data collected from N-terminal sequencing, 2-DE gels (anticipated pI values), LC-MS/MS analysis, sequence homology predictions, and the observed molecular masses (MALDI-TOF/MS), we concluded that the purified β -1,3-glucanases are two different leaf-specific inducible glucanases that are different from the ones encoded by cDNA clones previously isolated from scab-infected floral tissues of wheat (Li et al. 2001).

A TLP of approximately 16 kDa also was purified to homogeneity. The peptide mapping of the recovered TLP revealed very low sequence coverage (11%) to other known wheat TLPs and, thus far, only two different TLPs from wheat with sizes of 16 and 22 kDa are reported (Mingeot and Jacquemin 1998; Vigers et al. 1991). However, both the N-terminal sequencing and LC-MS/MS did not identify the purified AFTLP to be either of the two TLPs described earlier (data not shown). The molecular masses of the known TLPs from plants fall into two ranges, one of 26 to 22 kDa and another of <18 kDa. The smaller TLPs supposedly contain internal deletions of 25% or more of the amino acid residues of the higher molecular mass members (Reimann and Dudler 1993). Although Vigers and associates (1992) have reported that the larger TLPs have antifungal activity, evidence for antifungal activity of the smaller group of TLPs from cereals currently is lacking. The purification of the 16-kDa wheat TLP from the AF allowed us to demonstrate that this smaller TLP is indeed antifungal.

The extracellular localization of the chitinases, β -1,3-glucanases, and TLPs in the leaf tissue is consistent with the results observed in other plants (Krishnaveni et al. 1999; Regalado and Ricardo 1996) and their proposed roles as active first line defenses or in recognition processes leading to a signaling cascade (Mauch and Staehelin 1989).

AF proteins have antifungal activity.

Our preliminary screening of the AF proteins using disk assays indicated that the AF proteins at concentrations of $\geq 200 \mu\text{g protein/disk}$ are effective against specific wheat pathogens, including *F. graminearum* and *G. graminis* var. *tritici* (Fig. 3A and B), the causal agents of wheat head scab and take-all diseases, respectively. The AF proteins were found to be ineffective against many other pathogens, including *R. solani*, *S. nodorum*, and *P. tritici-repentis* at similar concentrations. These observations suggest that the AF proteins do not affect the growth of these fungi, perhaps due to inaccessibility of target substrates, or that even higher concentrations may be required in disk assays due to the diffusion effect in agar plates.

The unfractionated AF fraction delayed germination of macroconidial spores of *F. graminearum* for a significant time period (3 h) when compared with AF fractions from nontransgenic plants. These studies were consistent with the results seen in the greenhouse trials, suggesting that the PR proteins, chitinases, glucanases, and TLPs might confer partial resistance to initial disease incidence as seen in type II resistance (resistance to spread in the plant) against scab in wheat (Anand et al. 2003b). All purified protein fractions exhibited high antifungal activity against *F. graminearum* and *P. tritici-repentis* in microtiter plate assays (Figs. 5 and 6), but differed in their potencies. AFGLu was most effective in inhibiting *F. graminearum* spore germination at concentrations of $\geq 150 \mu\text{g ml}^{-1}$. In the assays against *P. tritici-repentis*, the purified barley chitinase was found to be effective at concentrations as low as 40 to 100 $\mu\text{g ml}^{-1}$. These results suggest that different cell wall polysaccharide substrates may be exposed to PR proteins in different fungi (Kauffmann et al. 1987; Legrand et al. 1988), resulting in differential susceptibility to these enzymes (Leah et al. 1991; Schlumbaum et al. 1986). In the present study, a combination of BarChi/AFGLu was most effective against *F. graminearum*, inhibiting spore germination by 62 to 80% at concentrations for each protein ranging from 37.5 to 75 $\mu\text{g ml}^{-1}$. However, the other combinations of either BarChi/AFTLP or AFGLu/AFTLP were no better than AFGLu alone, even at concentrations as high as 200 $\mu\text{g ml}^{-1}$. Our results are in agreement with the findings that chitinases and β -1,3-glucanases have synergistic effects as antifungal agents both in planta and in vitro (Anand et al. 2003b; Mauch et al. 1988).

Individual purified enzymes only delayed the growth of the hyphae of *P. tritici-repentis* for short intervals (3 to 6 h), which probably suggests protein degradation by the fungi or the transient fungistatic activity of these proteins. Assuming the infective hyphae grow and propagate in a similar manner on both the plant epidermis and intracellularly (in the plant tissues), our observations suggest that these enzymes are effective only in delaying the progression of the disease. Maximum transient fungistatic activity against *P. tritici-repentis* was observed with combinations of BarChi/AFGlu /AFTLP that inhibited fungal growth for prolonged periods of up to 9 h. Thus, we found that combinations of PR proteins are much more effective than individual proteins. The above findings are not surprising, given that these enzymes target different cellular components of the fungal cell wall. The chitinases and β -1,3-glucanases hydrolyze the structural components of cell walls, resulting in their weakening and rendering the fungal cells osmotically sensitive. Even though the precise roles of TLPs are yet to be clearly established, they are known to cause permeability changes in fungal cells and cause perturbation in signal transduction pathways (Yun et al. 1998).

The fact that we isolated the chitinases, glucanases, and TLP from the apoplast of a transgenic line mimicking the HR supports a role for their induction as defenses against leaf pathogens. Similarly, the observations on hyphal and conidial inhibition suggest a degradative function of these proteins in vivo. However, it would be very hard to quantify this function under in vitro conditions (Zareie et al. 2002). The observed synergistic effects are consistent with the hypothesis that different cell wall components are exposed sequentially to degradative action of these cell wall-digesting enzymes or that cell walls are reinforced by the crosslinking of different polymers. There are additional reports supporting this view. Jach and associates (1995) reported a synergistic protective action in planta in transgenic tobacco plants expressing a combination of either Chi/Glu or Chi/RIP (ribosomal inactivating protein) against *R. solani*.

Concentration of PR proteins in the apoplast.

The fractions containing chitinases and β -1,3-glucanases as detected by immunoblotting were enzymatically active and had specific activities comparable to those reported in the literature for purified enzymes. Assuming that AF represents 10% of the total fluids (obviously a high value), it is estimated that the minimum concentration of total proteins in the apoplastic fraction is equivalent to 0.47 mg ml⁻¹ (16.5 mg of protein were recovered from 35 g [fresh weight] of leaf samples, and we assume a total volume of 35 ml for the starting material). Based upon the above estimates, we speculate that the purified TLP and β -1,3-glucanase proteins could be present at concentrations of 22 and 36 μ g ml⁻¹, respectively, in the extracellular space of the leaf cells based upon our protein recovery data in Table 1. However, these estimates may be on the lower side if the apoplast occupies less than 5% of the tissue volume of the aerial organs, including leaves, as reported by Grignon and Sentenac (1991). It is estimated that less than half of these proteins actually were recovered after two rounds of column chromatography, suggesting that these PR proteins may very well exist at higher concentrations locally in the AF and especially around lesions. These predicted concentrations are similar to the estimates reported by Vigers and associates (1992) for osmotin and PR-S in maize, and in the range found to be effective in reducing disease incidence in wheat by *F. graminearum* in greenhouse trials (Anand et al. 2003b).

Therefore, the protein concentrations applied in the fungal growth inhibition assays (37.5 to 75 μ g ml⁻¹ in combinations) are likely to be in the physiological range. We speculate that these concentrations can be reached in the AF of nontransgenic

plant cells that react hypersensitively to pathogen attack and in transgenic plants prior to pathogen attack (lesion-mimic phenotype), as is evident from their increased resistance towards diseases (Nishizawa et al. 2003; Pontier et al. 2002). Many fungi penetrate the stomates to tap cellular resources and proliferate predominantly in the extracellular space of plant cells, a physical location consistent with the extracellular targeting of many plant defense enzymes.

In this article, we have successfully purified three members of the PR-protein family from wheat leaf tissues, including a low molecular mass TLP that was not reported earlier. We have also demonstrated that the lower mass TLP (16 kDa) from wheat is antifungal, which, to our knowledge, is the first report in cereals. Our results show that an apoplastic fluid fraction with multiple PR proteins is quite effective in inhibiting the growth of several fungi in vitro. Specific combinations of three PR proteins were found to have strong antifungal activity at relatively lower concentrations that we believe are likely to be reached in either infected plants or in transgenic plants overexpressing combinations of these proteins. Our results strongly suggest that the mixed results obtained previously by our group (Anand et al. 2003b) with transgenic plants may be attributed to either the limited number of transgenic lines that were recovered or the relatively lower concentrations of PR proteins at target sites when challenged with the pathogen. We propose to develop appropriate disease control strategies in the future that will involve identification of the best combinations of PR proteins that are most active in vitro against target pathogens, and achieving the expression of these proteins in transgenic plants at levels which show maximal pathogen growth inhibition.

MATERIAL AND METHODS

Plant material.

Homozygous transgenic wheat plants coexpressing genes encoding PR proteins for a class IV chitinase and an acidic β -1,3-glucanase (line 32A-2-3) driven by the maize ubiquitin promoter-intron that had a developmentally associated lesion-mimic phenotype (Anand et al. 2003a and b) served as the source for PR proteins. More than 100 seed from the line 32A2-3 were germinated and grown to maturity (flowering stage) in growth chambers (16 h, 600 μ E m⁻² s⁻¹, 20°C in the light, 18°C in the dark). Leaves were harvested from mature plants with macroscopic lesions and used for protein extraction and purification.

Protein extraction and localization of the transgene-encoded proteins.

Subcellular fractions were prepared from fresh leaves as previously described (Krishnaveni et al. 1999) with minor modifications. Approximately 150 g (fresh weight) of total leaves were cut into 2.5-cm pieces, thoroughly washed with water, dried on blotting sheets, and vacuum infiltrated for 20 min in 0.1 M sodium phosphate buffer (pH 6.5) containing the mixture of protease inhibitors, Complete Mini EDTA-free (Roche Diagnostic, Mannheim, Germany). After infiltration, leaf strips were blotted gently and rolled to fit into 10-ml centrifuge tubes (without caps) with a 0.5-cm-diameter hole at the bottom. These tubes were placed in 50-ml centrifuge tubes and spun at 2,000 $\times$ g at 4°C for 20 min for recovering the AF. After recovery of AF, the leaves were ground in liquid nitrogen and extracted with 0.1 M sodium phosphate buffer (pH 6.5) for collecting the intracellular protein fraction (total minus AF). To collect total cellular protein, another set of leaves (5 g) was extracted with 0.1 M sodium phosphate buffer (pH 6.5) without vacuum infiltration. The AF, the intracellular fluid, and the total protein extracts were subjected

to Western blot analyses. All protein fractions were stored at -70°C until used.

Enzyme purification and protein quantification.

AF (35 ml, containing approximately 16.5 mg of protein) was centrifuged at $5,000 \times g$ at 4°C for 15 min to remove insoluble material. The soluble protein fraction was dialyzed against 20 mM sodium phosphate buffer (pH 7.8) for 48 h. The extract was applied to a 1-by-15-cm DEAE-Sepharose column (DFF-100; Sigma-Aldrich, St. Louis) equilibrated with 20 mM sodium phosphate buffer (pH 7.8). The unbound protein fraction was collected, dialyzed against 20 mM sodium acetate buffer (pH 4.2), and applied to a 1-by-15-cm CM-cellulose (CCF-100; Sigma-Aldrich) equilibrated with 20 mM sodium acetate buffer (pH 4.2). Bound proteins were eluted with a linear 0- to 0.5-M NaCl (100-ml) gradient in the same buffer, at a flow rate of 500 $\mu\text{l}/\text{min}$. Fractions were collected at 2-ml intervals and aliquots (25 μl) were analyzed by 18% SDS-PAGE and stained with Coomassie Brilliant Blue or electrophoresed in a precast 4 to 20% gradient gel (Invitrogen, Carlsbad, CA, U.S.A.) and stained as per the manufacturer's instructions. Fractions containing proteins were blotted onto Immobilon-P membranes and detected with polyclonal antibodies raised against a barley chitinase, a rice TLP, or a barley glucanase (Anand et al. 2003a and b). Fractions containing a TLP protein (fractions 8 to 15, 16 kDa, >95% purity), different chitinases (fractions 18 to 37, containing a mixture of at least four chitinases with predicted molecular masses ranging from ≈ 26 to 35 kDa, two TLPs [≈ 14 and 24 kDa], and other unknown proteins) and two β -1,3-glucanases (fractions 41 to 51, ≈ 32 kDa, >95% purity) were concentrated (4 $\times$) using the Centrifuplus centrifugal filter devices (Millipore Corp. Bedford, MA, U.S.A.). Protein concentrations of the tissue extracts (AF) and the different column fractions were quantified using the Bradford method (Bradford 1976) and a commercial dye reagent (Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard.

Enzyme assays.

Chitinase and glucanase activities were measured spectrophotometrically by incubating known amounts of the purified protein fractions at 37°C with an appropriate substrate. For chitinase activity, a polymeric substrate, carboxymethyl Remazol Brilliant Violet-chitin (CM-Chitin-RBV; Loewe Biochemica, Munich, Germany) was used; whereas, for the β -1,3-glucanase activity, the protein fractions were incubated with laminarin (Sigma-Aldrich). In the chitinase assay, 200 μl of the enzyme (0.5 μg of protein), 150 μl of 0.1 M sodium phosphate buffer (pH 5.6), and 100 μl of the substrate (CM-Chitin-RBV at 2 mg ml^{-1}) were mixed together and the reaction was carried out for 30 min to 1 h. The enzymatic reaction was stopped by the addition of 500 μl of 2 N HCl and the mixture was centrifuged at $12,000 \times g$ for 15 min. The absorbance of the supernatant was measured at 520 nm. For the β -1,3-glucanase assay, 100 μl of the enzyme (0.5 μg of protein), 100 μl of 0.1 M sodium phosphate buffer (pH 5.6), and 100 μl of the substrate (laminarin at 1 mg ml^{-1}) were mixed and incubated for 15 min. The enzymatic reaction was stopped by adding 1 ml of 1.37 mM potassium ferricyanide followed by boiling for 15 min. The mixture was centrifuged at $2,000 \times g$ and absorbance values of the supernatants were measured at 450 nm (A_{450}). Specific enzyme activity was expressed as $\Delta A/\mu\text{g}$ protein/h, where A is the absorbance at A_{450} or A_{520} .

Electrophoresis of proteins and Western blot analysis.

Purified enzymes or the plant protein extracts were separated in a 12 or 18% gel or in a precast (10 to 20%) gradient gel (Invitrogen). The gradient gels were fixed and stained according to

the manufacturer's instructions, while the 12 or 18% gels were stained with Coomassie Brilliant Blue R-250. For Western blots, proteins were transferred electrophoretically from gels to Immobilon-P transfer membranes (Millipore Corp.) using a BIO-RAD semi-dry transfer system (Bio-Rad) according to the manufacturer's instructions. The filters were blocked with 2.5% (wt/vol) gelatin for at least 1 h and subsequently washed three times with TBST (10 mM Tris, 140 mM NaCl, and 0.05% Tween 20), then incubated overnight with specific polyclonal antibodies against a mung bean TLP (Sehgal et al. 1991), a barley chitinase (Swegle et al. 1992), or a barley β -1,3-glucanase (provided by M. Ballance, University of Manitoba, Winnipeg, Canada) (Anand et al. 2003a and b). Filters were probed with an affinity purified goat anti-rabbit immunoglobulin G (H+L)-horseradish peroxidase (HRP) conjugate (Bio-Rad) for 3 h. The bound antibody was localized using blotting grade HRP color-development reagent (4-chloro-1-naphthol; Bio-Rad).

In-gel trypsin digestion, elution of intact proteins, and 2-D gel electrophoresis.

Protein digestion and LC-MS/MS were performed as previously described (Watson et al. 2003). Briefly, protein bands were excised from the SDS-PAGE gel, destained with 50% acetonitrile (ACN), dehydrated with ACN, and digested with trypsin overnight at 37°C . The supernatant then was recovered and the gel slices were extracted twice with 50% ACN and once with ACN. The extracts were pooled and concentrated under vacuum to 25 μl . LC-MS/MS was performed on an ABI QSTAR Pulsar hybrid Q-TOF mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) equipped with nano-scale HPLC (LC Packings, Sunnyvale, CA, U.S.A.). Peptides were desalted on a C-18 pre-column, separated on a nano C-18 column, and then electrosprayed into the mass spectrometer. The acquired mass spectral data were queried against the NCBI nonredundant protein database using the MASCOT search engine (Creasy and Cottrell 2002; Perkins et al. 1999) with the mass tolerance of 150 ppm.

MALDI-TOF/MS determinations of intact protein masses were performed on a Voyager-DE STR (ABI/PerSeptive Biosystems, Foster City, CA, U.S.A.) equipped with a high-gain detector and externally calibrated with a BSA standard from the instrument manufacturer. Protein bands stained with Electro-Blue (Qbiogene, Carlsbad, CA, U.S.A.) were excised, electro-eluted in ProteoPLUS tubes (PROT0015, Qbiogene) for 2 h at 85 V, and dialyzed overnight against water at 4°C to remove SDS that might interfere with MALDI analyses. The dialyzed protein solutions then were taken to dryness in vacuum, resuspended in 2 μl of water, and mixed 1:1 with matrix (sinapinic acid at 10 mg/ml in 30% ACN). The samples (1 μl) were spotted and analyzed on the Voyager-DE STR operated in a linear detector mode.

For 2-DE gels, purified extracts containing approximately 4 μg (250 μl) of the two β -1,3-glucanases were used to rehydrate 11 cm of immobilized pH gradient strips (linear, pH 3 to 10) from Amersham at 20°C for 16 h. Isoelectric focusing was performed using a Bio-Rad Protean IEF Cell for a total of 20,000 volt hours. After focusing, strips were equilibrated sequentially with reduction and then with alkylation buffers, loaded onto 12.5% Criterion precast SDS-PAGE gels (Bio-Rad). Gels were stained overnight with Coomassie Brilliant Blue R-250 and destained the next day. Experimental pI values were calculated from digitized 2-DE images using the linear calibration option of Genomic Solutions HT Analyzer software (Genomic Solutions, Ann Arbor, MI, U.S.A.).

N-terminal amino acid sequencing.

Purified protein fractions were separated using SDS-PAGE (10 or 18%) and transferred onto Immobilon-P transfer mem-

brane. The filters were stained briefly with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid (20:10) solution. Individual protein bands were cut from the filters and the N-terminal sequence was determined by an automated Edman degradation process in an Applied Biosystem Sequencer at the Biotechnology Core Facility, Kansas State University, Manhattan.

Fungal growth inhibition assay.

The antifungal activities of the different cellular fractions, total plant protein extracts, and AF containing TLPs, chitinases, and β -1,3-glucanases from the transgenic and control plants were tested for their ability to inhibit growth of: *P. tritici-repentis* (isolate-AZ-00, cause of tan spot of wheat), *S. nodorum* (isolate NOD-99, cause of Stagonospora blotch of wheat), *G. graminis* var. *tritici* (isolate RL-01, cause of take-all of wheat), *R. solani* (causes damping off), and *F. graminearum* (isolate Z-3639, cause of head scab of wheat). These fungi are important pathogens of wheat and deployment of cultivars with increased resistance to them is highly desirable (Bockus et al. 2001).

Agar disks (5 mm in diameter) were removed from the growing edges of the above fungal isolates grown at room temperature ($28 \pm 2^\circ\text{C}$) for 3 to 4 days on quarter-strength potato-dextrose agar (PDA) and transferred to petri dishes containing full-strength PDA. Plates were incubated for 48 h at room temperature in the dark. Sterile filter paper disks (5 mm in diameter, three to four disks stacked together) containing an appropriate test fraction were placed on the agar surface at the edge of the mycelial growth and the plates were incubated at room temperature. Experiments were conducted in triplicate and the mycelial growth was recorded after 48 to 64 h.

Suspension cultures of the spore-bearing fungi *P. tritici-repentis* and *F. graminearum* were initiated for in vitro germination assays with the purified proteins. The mycelial cultures of *F. graminearum* were cut into two to three thin cubes (5 mm) and cultured in sterile mung bean broth for 48 h on a rotary shaker (200 rpm) at room temperature for harvesting the conidial suspension. The conidia and conidiophore suspensions of *P. tritici-repentis* were produced on V8 juice agar medium after growth for 3 to 4 days in the dark at $24 \pm 2^\circ\text{C}$ and light exposure for 16 h at $24 \pm 2^\circ\text{C}$, and then kept in the dark for 16 h at $15 \pm 2^\circ\text{C}$. Fungal propagules were harvested with sterile distilled water to produce a suspension containing a majority of conidiophores (90%) and a few conidia (both were infective) (Raymond et al. 1985). The suspension of conidiophores and spores was diluted to a final concentration of 2×10^6 CFU (spores) ml^{-1} . In the second set of experiments, we included a purified, secreted PR protein from barley, barley class II chitinase (Swegle et al. 1992), as the positive control for PR-proteins, AF from the lesion-mimic line 32A-2-3, non-transgenic 'Bobwhite' control plants, and the negative controls as sodium phosphate buffer supplemented with BSA (range, $0.5 \mu\text{g ml}^{-1}$ to 2 mg ml^{-1}) and water (no-protein control) were tested for their ability to inhibit growth of the above suspension cultures. The barley class II chitinase was included in this study for two reasons; first, we were unable to purify to homogeneity any wheat chitinase and, second, barley and wheat are very closely related genomes. In the third set of experiments, barley class II chitinase (Swegle et al. 1992), TLP (16 kDa), and β -1,3-glucanases (32 kDa) from AF were tested for their antifungal activities individually and in combinations against suspension cultures of *P. tritici-repentis* and *F. graminearum*. Antifungal activity assays were conducted in triplicate with 100 μl of the suspension culture incubated with a known concentration of the purified protein, a no-protein control, and BSA control in 96-well microtiter plates. The microtiter plates

were sealed with Parafilm and incubated at room temperature in dark. Spores that exhibited visible germ tube growth under the microscope were counted as positive for germination. Spore germination was recorded at hourly intervals after inoculation. Data on germination was collected by observing a minimum of 100 conidiophores/spores from five distinct zones in each well during each count, and the standard errors were calculated.

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