Isolation of Fungal Cell Wall Degrading Proteins from Barley (*Hordeum vulgare* L.) Leaves Infected with *Rhynchosporium secalis*

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Proteins with antifungal activity towards *Rhynchosporium secalis* conidia were isolated from the intercellular washing fluid (IWF) of barley leaves. The active components were purified by high-performance liquid chromatography under conditions that maintained biological activity. Five major barley IWF proteins deleterious to the cell wall of viable *R. secalis* conidia were isolated and identified by a combination of N-terminal amino acid sequencing, peptide mapping, and determination of mass and isoelectric point. They were a 32-kDa β -1,3-glucanase (Pr32), a 25-kDa chitinase (Pr25), and three 22-kDa thaumatin-like (TL) proteins (Pr22-1, Pr22-2, and Pr22-3). Pr22-1 and Pr22-2 were similar to the protein R class of TL proteins, whereas Pr22-3 was more similar to the S class. Pr22-3 was shown to digest laminarin, indicating that this TL protein has glucanase activity. In addition, Pr22-3 was more active in the spore bioassay than Pr22-2. Various combinations of the five proteins had a greater effect on *R. secalis* spores than did the individual proteins. The extraction of proteins with antifungal activity from the IWF of barley leaves indicates their possible role in defense against leaf pathogens. A similar bioassay may be developed for other systems to identify particular isoforms of pathogenicity-related proteins that might have a role in plant disease resistance.

Additional keywords: antifungal proteins, apoplast, PR proteins.

The imperfect fungus *Rhynchosporium secalis* (Oudem.) J. J. Davies is the causal agent of scald, one of the major leaf diseases of barley (*Hordeum vulgare* L.) and other grasses. The disease has been reported to cause grain losses of up to 65% under experimental conditions in the field (Ender et al. 1983). *R. secalis* conidia germinate on the surface of leaves and penetrate the cuticle. The fungus grows into a substantial stroma between the cuticle and the epidermis and, at approximately 8 days, degradation of the underlying epidermal cells becomes evident. In resistant cultivars, the fungus may germinate and penetrate the cuticle but forms sparse subcuticular hyphae that cause little damage to the plant cells (Jones and Ayres 1974; Jorgensen et al. 1993). No hypersensitive response has been reported in this system, but the plant resistance response includes the biosynthesis of pathogenicity-related (PR) proteins

(Ayesu-Offei and Clare 1970; Hahn et al. 1993; Lehnackers and Knogge 1989; Roulin et al. 1997).

In many plants, PR proteins have been shown to play significant roles in defense responses against pathogens. The level of PR proteins is frequently higher in incompatible interactions than in compatible interactions (Van Loon 1999). Moreover, many PR proteins show potential antimicrobial activities. Notably, PR proteins have been reported to deactivate proteases (Heitz et al. 1999), digest pathogen cell walls (Boller 1987; Trudel et al. 1998a), digest proteins (Vidhyasekaran 1997), interfere with reproduction (Moiseyev et al. 1994) deactivate ribosomes (Jensen et al. 1999), or reduce pathogen penetration (Rauscher et al. 1999). Inoculation of barley plants with *R. secalis* has been shown to induce a number of β -1,3-glucanase isoforms (Roulin et al. 1997), as well as genes for a peroxidase and the thaumatin-like (TL) protein, Hv-1 (Hahn et al. 1993). Induction of the antimicrobial peptide thionin also has been reported in barley infected by *R. secalis* (Bohlmann et al. 1988). However, the effect of barley PR proteins on *R. secalis* has not been investigated previously. The plant apoplast is believed to be the main site of interaction between barley and *R. secalis* during the pivotal subcuticular phase. In the present study, the resistance mechanism was studied by examining the in vitro effect of various components of intercellular washing fluid from infected barley leaves on *R. secalis* conidia.

RESULTS

Effects of intercellular washing fluid on *R. secalis* conidia.

Intercellular washing fluid (IWF) was isolated from infected barley leaves and concentrated by ultrafiltration. *R. secalis* conidia incubated in 5× IWF were found to undergo significant changes (Fig. 1A) compared with the water control (Fig. 1B). Normal *R. secalis* conidia are hyaline with two cells, often with a short lateral beak on the apical cell (Fig. 1F). However, IWF-treated spores displayed three different types of deformities, designated as internal granulation (IG), fractured cell walls (FW), and doublets (DB). On average, 20% of spores had IG following incubation in IWF (Fig. 1E) compared with an average of 8% for those incubated in water. Approximately 45% of spores incubated in IWF had FW or ruptured cell walls (Fig. 1C), compared with approximately 2% of water-incubated spores. The rupture generally occurred next to the septum, releasing the cell contents. In addition, in the presence of IWF, 17% of the conidia formed doublets, defined as spores

that appeared broken at the septum, loosely adhering to each other and with apparently intact cell walls (Fig. 1D). No doublets were observed in water-incubated spores. In some cases, there was an overlap of the phenotypes; for example, with spores having both the IG and DB phenotypes. Monitoring of the spores indicated that IG, DB, and FW spores started to form as early as 0.5 h after incubation in 5× IWF.

IWF-active compounds.

Preliminary experiments indicated that boiling the IWF resulted in almost complete loss of deforming activity toward *R. secalis* conidia (data not shown). A bioassay of the ultrafiltered IWF indicated that approximately 90% of the IG and 100% of the DB and FW activity remained in the retentate, suggesting that the active compound or compounds had molecular weights larger than 10 kDa. The results were consistent with the premise that the IWF-active compounds were proteins.

Preliminary fractionation of IWF by gel permeation chromatography (GPC) resulted in the separation of IG-active compounds from FW- and DB-causing compounds, which comigrated. In view of its specificity and reproducibility, DB activity was selected as the criterion upon which to base subsequent protein purification.

Protein purification.

IWF was fractionated by anion exchange (SAX), cation exchange (WCX), hydrophobic interaction (HIC), and GPC chromatography in succession. After each procedure, the DB-active fractions, detected using the bioassay, were collected and applied to the next chromatographic step. Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) determined that five proteins (Pr22-1, Pr22-2, Pr23-3, Pr25, and Pr32), named according to their molecular mass, were associated with DB activity. A synopsis of the purification process is shown in Figure 2. To assess purity and quantify the amount of protein, 100 µl of each purified sample was chromatographed on a reverse phase (RP) column (Vydac C18). Only the Pr22-3 sample was found to contain more than one compound (approximately 5% wt/wt of Pr25), whereas the remaining proteins had no detectable contamination (data not shown). After estimating the amount of each purified protein, the volumes were reduced by ultrafiltration, and the concentration adjusted to levels used in the bioassay. A number of factors were found to suppress the activity of proteins (i.e., the generation of the

doublet spores). First, in the absence of dithiothreitol (DTT), none of the purified proteins had detectable effects on *R. secalis* conidia. The purified samples contained 1 mM DTT and retained their activities for a minimum of 1 week at 4°C. Samples that were deactivated after this period could be partially recovered by again adding DTT to a concentration of 1 mM. In addition, salts used in many of the chromatographic steps interfered with the bioassay; therefore, prior dialysis of the protein solutions was required. Drying of the proteins also caused loss of activity and this was more pronounced in the purified samples.

Protein analysis.

The TL proteins, Pr22-1 and Pr22-2, eluted at different points in WCX, HIC, and RP chromatography, and peptide mapping by RP high-performance liquid chromatography (HPLC) confirmed that they were not the same protein (Fig. 3A). However, sequencing revealed that the 17 N-terminal amino acids of the proteins were identical to each other and to a number of barley TL proteins, including protein R (Hejgaard et al. 1991), Barperm1 (Skadsen et al. 2000), IFW 19 (Trudel et al. 1998b), and the deduced sequences of HVPATHPR5 (AJ001268), TLP6 (AAK55324), and TLP7 (AAK55325) (Fig. 4A). In addition, a peptide peak specific to Pr22-1 ("A" in Fig. 3A) was sequenced, and the first 17 amino acids (aa) of this internal 20-aa sequence were identical to amino acids 25 to 41 of Barperm1, HVPATHPR5, TLP6, and TLP7. There was a single change at position 30 for barley protein R. An attempt was also made to sequence a peak specific to Pr22-2 ("B" in Fig. 3A) but it contained more than one peptide and sequencing was unsuccessful. Peptide mapping also showed that Pr22-3 was different from the other two TL proteins (Fig. 3B). Sequencing of the N-terminus of Pr22-3 determined that 15 residues were identical to the amino-terminal sequence of barley TL protein S and deduced sequences of TLP8 (Fig. 4B). There was less homology with barley TL proteins IFW15 and HV-1a (Fig. 4B). Both protein R and protein S were previously isolated from barley seeds (Hejgaard et al. 1991), whereas the TL proteins studied here were isolated from IWF.

Pr25 was blocked at the N-terminus, and reductive alkylation and tryptic digestion followed by RP HPLC were used to obtain an internal segment for sequencing. A symmetrical peak that eluted from the RP column at approximately 29% acetonitrile (data not shown) was selected and sequenced. In all, 23

Fig. 1. Effects of intercellular washing fluid (IWF) on *Rhynchosporium secalis* conidia. *R. secalis* conidia after 12 h of incubation in **A**, 5× IWF and **B**, water. Different forms of conidia following incubation in IWF or water: **C**, spore with fractured cell wall (FW), **D**, doublet (DB) spore, **E**, spore with internal granulation (IG), and **F**, normal bicellular spore. Bars represent 10 µm.

residues were identified (Fig. 4C) and found to be identical with part of the deduced amino acid sequences of barley chitinases 2a and 2b. These are homologues (>95%) of chitinase 2, originally described as a leaf-specific extracellular protein with an apparent molecular weight of 24.8 kDa and an isoelectric point (pI) of ≥ 9.8 (Kragh et al. 1993).

Pr32 was N-terminally sequenced and 15 aa were shown to be identical to the N-terminal sequence of the barley β -1,3-glucanase isoform GII (Fig. 4D). This enzyme was previously detected in seed (Hoj et al. 1989) and leaf apoplast of barley (Roulin et al. 1997).

The mass of each protein was determined by mass spectrometry and the pIs were estimated by 2D gel electrophoresis of the proteins (Fig. 4).

Effects of purified IWF proteins on *R. secalis* conidia.

The activities of the individual purified proteins towards *R. secalis* conidia were bioassayed at a final concentration of 1 μ M protein and 10^7 conidia/ml. The results indicate that Pr25 (chitinase) and Pr32 (β -1,3-glucanase) have similar activities, with approximately 50% of the spores forming doublets; no FW activity was observed (Fig. 5). At a higher concentration

(5 μ M), Pr32 also caused FW activity (data not shown). The TL proteins Pr22-1, Pr22-2, and Pr22-3 had more pronounced effects, with 50 to 77% DB formation and 13 to 33% FW activity. It also was shown that, by decreasing the protein concentration, first FW activity and then DB formation gradually disappeared. An assessment of viability using fluorescein diacetate indicated that FW spores were nonviable, whereas the DB spores appeared to have an intact cell wall and were viable.

Combinations of Pr22-1, Pr22-2, Pr22-3, and Pr32 were bioassayed for their effect on spores of *R. secalis* at concentrations approximately 10-fold less than the amounts used above. Not enough Pr25 was available for this study. The results (Table 1) illustrate the synergistic activity of the proteins tested. In order to provide a base level of activity, each protein was used individually at 120 nM. No FW was detected, and only Pr22-2 and Pr22-3 caused DB formation in 15 and 20% of the treated spores, respectively (group 1).

A comparison of combinations of two proteins (group 2) revealed that mixtures containing the β -1,3-glucanase protein, Pr32, were more effective than those without it. This was most pronounced when Pr32 was combined with either Pr22-2 or Pr22-3. The synergism between the Pr22-1,2,3 TL proteins is

Fig. 2. Summary of the nondenaturing purification procedure of intercellular washing fluid (IWF) proteins that display antifungal activity. IWF (left-most profile) was concentrated and subjected to anion exchange chromatography (SAX). The SAX-unbound fractions were collected and fractionated on a cation exchange column (WCX), which generated two biologically active regions along the chromatogram profile: one included fractions 26–30 and the other fractions 22 to 24. These were separately pooled and subjected to hydrophobic interaction chromatography (HIC) and then gel permeation chromatography (GPC). WCX fractions 26 to 30 contained only one active protein, Pr22-1, whereas WCX fractions 22 to 24 contained four active proteins that were separated by HIC and GPC. Bands occasionally observed at approximately 55 kDa are artifacts of silver staining.

demonstrated in group 3, where mixtures involving three proteins were compared. The mixture of 30 nM each of Pr22-1, Pr22-2, and Pr22-3 generated more DB and FW activity than any of the proteins individually at 120 nM (group 1). The strongest overall effects in group 3 involved mixtures that contained Pr32 or Pr22-3. When both of these proteins were present, 33 and 51% DB formation was recorded and FW, which was normally low or absent, reached approximately 30%. When all four proteins were present at 30 nM each (group 4), 50% of the spores formed FW and 40% formed DB.

Digestion of the polysaccharide laminarin.

Pr22-2, Pr22-3, and Pr32 were compared with laminarinase isolated from *Trichoderma* spp. for their ability to digest laminarin, an oligosaccharide containing β -1,3-glucan linkages. The results (Table 2) indicate that Pr32, purported to be a β -1,3-glucanase from the sequencing data, was more than twice as effective as laminarinase in digesting laminarin and three times as effective as the TL protein Pr22-3. No digestion by Pr22-2 was detected.

DISCUSSION

This study resulted in the isolation and purification of proteins from the IWF, originating from the apoplast, of barley leaves that are active against *R. secalis* conidia. Unlike previous studies that used digestion of (Grenier et al. 1999; Trudel et al. 1998a) or affinity to (Kragh et al. 1990) fungal cell wall components (i.e., pachyman or chitin) to assess defense mechanisms, biological activity against viable spores was the only criterion used here. Our strategy has enabled the isolation and purification of a number of proteins and their specific isoforms that digest fungal spore cell wall.

A number of effects on spores (FW, DB, and IG) were observed with the addition of IWF. The first two appeared to be associated with an adverse effect on conidial cell walls. DB formation was used initially as the criterion to identify protein fractions from the IWF with antifungal activity. Subsequently, the purified proteins were tested for their ability to degrade *R. secalis* conidia, using the criteria identified. To purify the active components, IWF was purified sequentially through a number of HPLC columns under conditions that maintained

biological activity. Five major barley IWF proteins deleterious to the cell walls of *R. secalis* spores were isolated, and shown to be a 32-kDa β -1,3-glucanase (Pr32), a 25-kDa chitinase (Pr25), and three 22-kDa TL proteins (Pr22-1, Pr22-2, and Pr22-3). At 1 μ M, all five proteins caused DB formation (ranging from 45 to 77%), with the TL proteins showing the highest activity. At this protein concentration, only the TL proteins had FW activity (13 to 33%) and only this phenotype produced nonviable spores. Microscopic studies suggest that DB formation results from breakdown of the septum between the two spore cells, whereas FW appears to involve more extensive damage to the septum, resulting in cell rupture. It is likely that the two phenotypes are related and the concentration and type of protein used in the bioassay may determine which one occurs. The phenomenon of FW activity is possibly analogous to the previously reported cytoplasmic burst of hyphal tips caused by other TL proteins (Cheong et al. 1996; Roberts and Selitrennikoff 1990).

To characterize the proteins, a combination of amino acid sequencing, peptide mapping, and determination of mass and pI was employed. The mass and pI of Pr25 (24,860 Da and 10, respectively) and the sequence of an internal 23-aa fragment of the protein is similar to that predicted for chitinase 2a (24,719 Da and 8.6) (Fig. 4), suggesting that they are closely related proteins. Similarly, the N-terminus of Pr32 and the mass and pI (32,352 Da and 9, respectively) are almost identical to that for barley β -1,3-glucanase isoform GII (32,353 Da and 8.8). Sequence, mass, and pI data indicate that the three remaining proteins, Pr22-1, Pr22-2, and Pr22-3, are similar to TL proteins, which have extensive (approximately 65%) sequence homology to the sweet-tasting 22-kDa protein thaumatin (Cutt and Klessig 1992).

Most barley TL proteins can be classified as either protein R-homologues or protein S-homologues based on amino acid homology (Fig. 4). There is only approximately 60% amino acid homology between protein R and protein S (Hejgaard et al. 1991). Pr22-1 and Pr22-2 are similar to the protein R class of TL proteins. Although the N-terminal sequences of these proteins are identical, peptide mapping indicates that there are some differences. An internal fragment of Pr22-1 has 3 aa distinct from other protein R-type TL proteins, indicating that it is a new isoform. Pr22-3 is most similar to the protein S class of TL proteins. Recently, eight isoforms of TL proteins were isolated from barley leaves infected with *Drechslera teres* (Reiss and Horstmann 2001) using 2-D electrophoresis followed by N-terminal microsequencing. Of these, TLP6, TLP7, and TLP8 (Fig. 4) were the most similar to the proteins reported here. The role of these isoforms in resistance was not reported. The N-terminal sequences of Barperm1, HVPATHPR5, TLP6, and TLP7 (Fig. 4) are identical; however, the different molecular masses and pI indicate they are different isoforms.

Many TL proteins are known to be antifungal (Hejgaard et al. 1991; Vigers et al. 1991), some so potent that they can lyse fungal hyphae in minutes (Cheong et al. 1996; Roberts and Selitrennikoff 1990). However, early studies did not find any enzyme activity against fungal cell wall components. Consequently, it was suggested that these proteins may have membrane permeabilizing (Abad et al. 1996) or membrane pore-forming activity (Roberts and Selitrennikoff 1990), an effect that in turn increases the turgor pressure and causes the cells to burst (Roberts and Selitrennikoff 1990). Later studies suggested that TL proteins react with the cell wall rather than with the membrane (Grenier et al. 1999). Many TL proteins were shown to bind or endohydrolyse β -1,3-glucans, such as pachyman (Grenier et al. 1999; Trudel et al. 1998b). Baker's yeast cell wall, a rich source of complex β -1,3-glucans with smaller amounts of β -1,6-glucans and mannan, was also

Fig. 3. Comparative peptide mapping of the thaumatin-like proteins. **A**, Peptide maps of Pr22-1 (light) and Pr22-2 (dark). Peak A, specific to Pr22-1, and peak B, specific to Pr22-2, were collected for sequencing. The peptide fragments were resolved using reverse-phase high-performance liquid chromatography (RP HPLC) and detected at 214 nm absorbance. **B**, Peptide maps of Pr22-2 (dark) and Pr22-3 (light). The peptide fragments were resolved using RP HPLC and detected at 214 nm absorbance.

strongly digested (Grenier et al. 1993, 1999; Trudel et al. 1998a). In the present study, Pr22-3, but not Pr22-2, was shown to digest laminarin. Pr22-3 was also more active in the spore bioassay than Pr22-2, both by itself and in combination with the other proteins, although it is not known whether the increased antifungal activity is a consequence of the increased laminarinase activity. These findings are consistent with protein S (Pr22-3 homologue) having more antifungal activity than protein R (Pr22-2 homologue) (Hejgaard et al. 1991). Similarly, IFW19 (Pr22-2 homologue) could not digest laminarin (Grenier et al. 1999).

Our studies support the recent finding that TL proteins have glucanase activity. Where TL proteins have been shown to have glucanase activity (Grenier et al. 1999; Trudel et al. 1998a), the studies have involved a one-step in-gel assay using pachyman as a substrate, proteins likely avoiding oxidation by fewer purification steps. In earlier reports (Hejgaard et al. 1991; Roberts and Selitrennikoff, 1990), isolated TL proteins did not show glucanase activity, but this could be due to oxidizing conditions, which inactivated the proteins during chromatographic isolation. In vitro loss of activity following exposure to atmospheric oxygen generally is caused by oxidation of thiol-containing residues, especially cysteine (Edelstein and Bollag 1992). Cysteine residues may participate in disulfide (S-S) bonds that help form the tertiary and quaternary structures of proteins or can be present as free thiol groups that may form part of active sites (Carlsson et al. 1998). Thaumatin and many TL proteins have 16 cysteine residues believed to be arranged in eight S-S bonds (Cutt and Klessig 1992; Grenier et al. 1999; Hejgaard et al. 1991; Van der Wel and Bel 1980). Chitinase 2 has five cysteine residues, whereas β -1,3-glucanase isoform GII and most other β -1,3-glucanases of barley have only one cysteine residue. Millimolar amounts of DTT

reduce the cysteine residues and can reactivate the proteins by altering the tertiary structure or active site. DTT was used throughout the isolation of proteins in this study. It also was observed that proteins stored at 4°C that had lost activity in the bioassay could be partially reactivated by adding DTT, suggesting that the protein had been oxidized. Reactivation of the TL proteins in the presence of DTT is in agreement with the findings of Van der Wel and Bel (1980) for thaumatin.

We have shown the need for nonoxidizing conditions to maintain biologically active enzymes. This suggests that, for these proteins to play a role in plant disease resistance by lysing fungi, the apoplast should be a nonoxidizing environment. The presence of various reducing agents in the apoplast of plants (Flury et al. 1996; Polle et al. 1990), including barley (Vallelian-Bindschedler et al. 1998; Vanacker et al. 1998), and in fungal intercellular spaces (Rebbeer et al. 1998; Sollod et al. 1992) has been shown previously. It is noted that, although oxidation of the proteins is the most likely cause of loss of lytic activity of the isolated proteins, it is possible that the effect of DTT in restoring this activity could be due to an effect on fungal cell walls.

Antifungal proteins have been reported to have synergistic effects. In barley, combinations such as protein S and chitinase C (Hejgaard et al. 1991), chitinase C and glucanase GII (Leah et al. 1991), and protein R and PR-4 proteins (Hejgaard et al. 1992) result in enhanced antimicrobial activity. In the present study, all combinations of the proteins tested had a synergistic effect on *R. secalis* conidia (Table 1). The maximum effect was recorded for those interactions that involved different proteins; that is, the β -1,3-glucanase and any of the TL proteins. However, there was also a slight but noticeable synergism between different TL proteins. The detected synergism between isoforms of one protein is in agreement with the finding of

Fig. 4. Comparison of amino acid sequences, mass, and isoelectric points (pIs) of the antifungal proteins to other barley pathogenicity-related proteins. **A**, N-terminal amino acids of Pr22-1 and Pr22-2. Barley thaumatin-like (TL) proteins; protein R (P33044), Barperm1 (AAB71680), HVPATHPR5 (AJ001268), TLP6 (AAK55324), TLP7 (AAK55325), and IFW19 (Trudel et al. 1998b). Amino acids 25 to 44 of Pr22-1 were obtained from an internal fragment isolated by peptide mapping. **B**, N-terminal amino acids of Pr22-3. Barley TL-proteins; protein S (P33045), TLP8 (AAK55326), IFW15 (Trudel et al. 1998b), HV-1a (X58564). **C**, Amino acid sequence of an internal fragment from Pr25. Barley leaf chitinases; cht2a (S48847), cht2b (S48848). **D**, The N-terminal amino acid sequence of Pr32. GII (P15737) – (β -1,3-glucanase isoform GII). Black boxes show regions of 100% identity, whereas gray boxes denote significant but less than 100% identity. Numbers at the top refer to the amino acid position in mature proteins. NCBI accession numbers are in brackets; * indicates that mass and pI were computed based on the amino acid sequence of the mature protein using programs from the protein parameter page on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics website.

Kragh and associates (1990) for chitinases. However, no synergism between isoforms of TL proteins was observed by Hejgaard and associates (1991), who tested protein R and protein S for growth inhibition of *Trichoderma*, *Candida*, and *Fusarium* spp.

TL proteins in barley initially were found in the seed. Recently, however, proteins such as IFW-15, -16, and -19 have been detected in the extracellular fluid of barley leaves (Trudel et al. 1998b). In the current study, the TL proteins as well as the chitinase and glucanase were extracted from the apoplast of barley leaves. The fact that these proteins are found in leaves supports a role in defense against leaf pathogens.

The bioassay assesses spore breakdown but it may have wider implications on the ability of the enzymes to break down fungal cell walls. Accordingly, a role in disease resistance may be in inhibiting not just conidia but also hyphae. We found it difficult to quantitatively measure the degradative activity of the proteins on the fungal hyphae, although the proteins appeared to have effects on the hyphae similar to those observed with conidia.

In the initial studies, a 5× concentration of the IWF was used to determine the presence of the spore-degrading proteins. Subsequently, concentrations of purified proteins ranging from 30 nM to 1 μM were used. Our unpublished data indicate that the level of proteins in the IWF is approximately 30 nM, which corresponds with the levels used in the study showing the synergistic effects of the proteins on conidia (Table 1). The physiological concentration of the proteins in the leaf required to inhibit a pathogen is hard to determine for a number of reasons. First, as this study has shown, a protein should not be considered in isolation because the proteins can act in concert enhancing their activity. Second, there may be local regions of concentrated proteins because they likely come from particular cell types. Subcellular localization of the proteins is required to show this as well as indicate whether a physiological role in disease resistance would be to act on spores on the cell surface as well as upon subcuticular hyphae.

TL proteins, for example Hv-1, and β-1,3-glucanases (GI, GII, GIII, and ABG2) were induced in the defense response of barley against *R. secalis* (Hahn et al. 1993; Malehorn et al. 1993; Roulin et al. 1997). However, in these studies, no direct effect of these proteins on *R. secalis* was shown. This may be particularly significant considering that it has been reported that not all isoforms of a cell wall lytic enzyme may exhibit antifungal activity (Sela-Buurlage et al. 1993). By using a bioassay that detects proteins active against *R. secalis* spores, it is

much more likely that the proteins isolated have a role in plant disease resistance. Currently, we are investigating the level of induction of these proteins during infection by the pathogen. Preliminary results indicate constitutive production of the proteins, which may function by restricting colonization of *Rhynchosporium* and perhaps other opportunistic pathogenic fungi, such as is the case with thionins (Garcia-Olmedo et al 1992). Additionally, the proteins are induced upon fungal infection.

MATERIALS AND METHODS

Inoculation of barley with *R. secalis*.

R. secalis isolate H2.5 was maintained on lima bean agar (LiBA) plates at 15°C in the dark and subcultured weekly. Periodically, a fresh culture was established from isolates maintained in long-term storage on porcelain beads (Lange and Boyd 1968). Seed of barley cv. Atlas 46, containing the resistance genes *Rrs1* and *Rrs2*, were surface sterilized following the method of Hoj and associates (1989) and planted in pasteurized University of California (UC) soil mix. Plants (8 per 15-cm pot) were grown in a growth cabinet with a 12-h light and dark cycle at 20 and 15°C, respectively, inside containment cylinders made of clear PVC with the top covered with greaseproof paper, so as to prevent contamination. *R. secalis* conidia used for inoculation were harvested from fresh cultures (approximately 10 days old) and suspended in a solution of 0.05% Tween-20 to a concentration of 1×10^6 spores/ml. Plants at the two-leaf stage were inoculated by spraying 2.5 ml of conidial suspension per pot, incubated in the dark for 48 h, and harvested on day 3 post inoculation for extraction of IWF.

IWF extraction and protein purification.

IWF was extracted by infiltrating the leaves with water under vacuum, followed by centrifugation according to the method of Jutidamrongphan and associates (1991). Typically, 35 g of leaves (approximately 130 plants) was harvested 3 days post inoculation and approximately 5 ml of IWF was extracted. This was filter sterilized using a 0.4-μm Ultrafree-MC or -CL filter (Millipore) and then concentrated by ultrafiltration through Microcon or Centricon-10 centrifugal concentrators (Millipore) with exclusion limits of 10 kDa. A volume of 5 ml was concentrated to 500 μl, representing 10× IWF. IWF and subsequent purified fractions were stored at 4°C if used in the bioassay within 1 to 2 days, or frozen at -20°C for longer-term storage.

Concentrated IWF was adjusted to 10 mM ammonium formate, pH 9.0, and subjected to anion exchange chromatography (Polymer Labs SAX column) with a gradient of 10 to 1,000 mM ammonium formate, pH 9.0, at 400 μl/min. Fractions, collected at 400-μl intervals were bioassayed (see below). Active fractions were pooled, adjusted to pH 7.5 (with formic acid) and 1 mM DTT, and loaded onto a cation exchange column (Macrosphere WCX). Proteins were eluted at 400 μl/min using a gradient of 10 to 400 mM ammonium formate, containing 1 mM DTT, pH 7.5, and each 400-μl fraction was bioassayed. Adjacent active fractions were pooled, adjusted to 1 mM DTT, 1.2 M ammonium sulfate, and 150 mM sodium acetate, pH 6.8, and injected onto a hydrophobic-interaction column (Brownlee HIC-300 Aquapore). A gradient of 1.2 M ammonium sulfate, 150 mM sodium acetate, 1 mM DTT, to 0.5 M ammonium sulfate, 62.5 mM sodium acetate, 1 mM DTT at an elution speed of 200 μl/min was used, and each 200-μl fraction was bioassayed. Adjacent active fractions were pooled and concentrated to 30 μl in a Centricon-10 filter unit before loading onto a gel permeation column (Macrosphere GPC 60A). An isocratic gradient comprising 50 mM ammo-

Fig. 5. Effect of the cell wall lytic proteins isolated from intercellular washing fluid on *Rhynchosporium secalis* conidia. Proteins were bioassayed at a final concentrations of 1 μM, and data are an average of two measurements. Dark bars represent the percentage of spores with doublet cell wall activity and clear bars represent the percentage of spores with fractured cell wall activity.

nium formate, pH 6.0, and 1 mM DTT was applied at a flow rate of 200 µl/min and 200-µl fractions were collected and bioassayed. All the chromatographic steps were conducted using a high-performance liquid chromatograph (Hewlett Packard 1090) at room temperature and monitored at an absorbance of 280 nm unless indicated otherwise. The protein composition of all fractions was analyzed by SDS-PAGE.

Protein analysis.

SDS-PAGE and 2D gel electrophoresis of the protein samples was conducted in a BioRad Mini-protein II cell as described by the manufacturer. For SDS-PAGE, an amount equivalent to 10 µl of IWF was dried in a Speed Vac centrifuge and reconstituted in 10 µl of SDS-loading buffer (Laemmli 1970) containing 50 mM DTT. Samples were boiled for 2 min, loaded onto 12.5% polyacrylamide gels, and electrophoresed at 175 V until the dye front had reached the end of the gel. For 2D gel electrophoresis, 60 µl of IWF was similarly dried, reconstituted in 50 µl of IEF-loading buffer (8 mM urea, 50 mM DTT, 2% Triton X-100, and 1.2% Pharmalyte pH3-10) and electrophoresed stepwise at 200 V (30 min), 500 V (30 min), and 1,000 V (3 h). Proteins were visualized by silver staining (Dunn and Crisp 1994).

To estimate the concentrations (wt/vol), purified proteins were subjected to RP chromatography (A: 0.05% trifluoroacetic acid [TFA]; B: 70% acetonitrile containing 0.04% TFA) and the peak areas at 214 nm absorbance were compared with that of known amounts of ovalbumin. Each protein (100 pmol) was sequenced by the Edman method at the Nucleic Acid and Protein Chemistry Unit at the Department of Plant Science, Adelaide University, using a Hewlett Packard G1000A Protein Sequencer. For analysis of terminally blocked proteins or peptide mapping, a 20-µg amount of each protein was dried in a Speed Vac centrifuge and subjected to reductive alkylation and tryptic digestion as described by Rosenberg (1996). Following 18 h of incubation, peptide fragments were resolved using RP HPLC (Vydac C18 column). Protein sequences were matched against the nonredundant databases using BLAST at the NCBI internet site. Theoretical masses and pIs were calculated using ProtParam software at the EXPASY internet site. Electrospray ionization mass spectrometry to determine the molecular mass of proteins was carried out using a Sciex API-100 or Micro-mass QTOF-2. Generally, an amount equivalent to approximately 7 pmol of purified protein was loaded onto a C8 column, eluted by a 1-to-70% step gradient of acetonitrile containing 0.1% TFA and the molecular mass determined.

Bioassay.

Protein samples were microdialyzed (Overall 1987) against the bioassay buffer (10 mM ammonium acetate, pH 6.0, and 1 mM DTT) and the concentration adjusted to the equivalent of 10× IWF. Each dialyzed sample (3 µl) was mixed with 3 µl of *R. secalis* conidia suspension, previously adjusted to 2×10^7 spores/ml of bioassay buffer in a 1.5-ml tube and incubated for 12 h in the dark at 20°C. The final concentration in the bioassay was equivalent to 5× IWF. The 6 µl of bioassay mixture was transferred to a hemacytometer slide and examined under the microscope. Approximately 50 spores, detected in 20 randomly chosen cells of the hemacytometer, were assessed. The percentage of DB spores, those exhibiting FW, and those with IG were calculated. For the viability assay, 3 µl of the bioassay mix was mixed with 3 µl of fluorescein diacetate at 0.1 mg/ml and observed under an Olympus microscope equipped with a UV light source and a barrier filter 47. Spores that fluoresced were considered to be viable (Widholm 1972). Data presented are an average of at least two independent measurements.

Laminarin hydrolysis assay.

One microliter of each purified protein, or laminarinase adjusted to 1 µM, was mixed with 5 µl of the β-1,3-glucan substrate laminarin (Sigma-Aldrich) (2.5% wt/vol) in 10 mM ammonium acetate, pH 6.0, and 1 mM DTT. Following 12 h of incubation at 20°C, samples were assayed for the release of reducing sugars according to the Somogyi-Nelson method explained elsewhere (Paley 1959). Absorbance was measured at

Table 2. Digestion of the polysaccharide laminarin^a

Treatment	Absorbance
Laminarinase + laminarin	1.40
Pr22-2 + laminarin	0
Pr22-3 + laminarin	1.06
Pr32 + laminarin	2.98
Laminarin	0.05
Laminarinase	0
Pr22-2	0
Pr22-3	0
Pr32	0
Control (buffer)	0

^a The ability of the thaumatin-like proteins Pr22-2, Pr22-3, and Pr32 to hydrolyze the β-1,3-glucan substrate laminarin was measured by the Somogyi-Nelson method of detecting the release of reducing sugars. All proteins were assayed at 1 µM concentration. The increase in absorbance at 660 nm corresponds to the amount of reducing sugars in the samples.

Table 1. Synergistic effects of intercellular washing fluid cell wall lytic proteins on *R. secalis* conidia^a

Group	Concentration of proteins (nM)					DB (%)	FW (%)
	Pr22-1	Pr22-2	Pr22-3	Pr32	Total		
1	120	...	...	...	120	0	0
	...	120	...	...	120	15	0
	...	...	120	...	120	20	0
	...	...	...	120	120	2	0
2	30	30	...	...	60	0	0
	30	...	30	...	60	2	0
	30	...	...	30	60	9	0
	...	30	30	...	60	3	0
	...	30	...	30	60	17	0
	...	...	30	30	60	23	0
3	30	30	30	...	90	27	3
	30	30	...	30	90	37	3
	30	...	30	30	90	51	33
	...	30	30	30	90	33	32
4	30	30	30	30	120	40	50

^a Conidia were treated with different combinations of the cell wall lytic proteins, excluding Pr25. In group 1, conidia were incubated in 120 nM of each protein. Groups 2 and 3 included combinations of 2 and 3 proteins, respectively, at 30 nM each. Group 4 combined the four proteins at 30 nM each. DB = doublets, FW = fractured cell walls, and ... = nil.

660 nm using a Beckman DU-68 spectrophotometer. Data presented are an average of two independent measurements.

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AUTHOR RECOMMENDED INTERNET RESOURCE

The protein parameter page on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics website: <http://au.expasy.org/tools/protparam.html>