Isolation of a Polygalacturonase-Inhibiting Protein (PGIP) from Wheat

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Evidence for the presence of a polygalacturonase-inhibiting protein (PGIP) from a monocotyledonous cereal is presented. A 40.3-kDa PGIP that was closely associated with the cell wall was acetone-extracted and purified from wheat (*Triticum aestivum* L.) leaves and stems. Wheat PGIP exhibited a highly selective inhibitory activity against endopolygalacturonase (EPG) from various fungi. Of nine EPG tested, wheat PGIP only inhibited EPG from *Cochliobolus sativus*, a pathogen of the tribe Poaceae. A short N-terminal amino acid sequence of wheat PGIP shows no similarity to any other characterized PGIP.

Plants, unlike humans, have no immune system to protect them from invading pathogens but have evolved alternative defense mechanisms for protection. One of these mechanisms involves the production of antimicrobial compounds (Fritig et al. 1998). These stress compounds, which include pathogenesis-related (PR) proteins such as β -1,3-glucanases, chitinases, and polygalacturonase inhibiting proteins (PGIP), are designed to restrict or halt pathogen invasion. Endopolygalacturonases (EPG) (EC 3.2.1.15) are typically among the first polysaccharide-degrading enzymes secreted by phytopathogenic fungi during plant infection (Cooper 1984). According to the Henrissat structural classification scheme (Coutinho and Henrissat 1999), EPG are members of family 28 of the glycosyl hydrolases in the pectinases group of cell wall-degrading enzymes. EPG degrade unesterified regions of homogalacturonan, the 1,4 linked α -D-galactosyluronic acid polymer found as a component of pectin in the plant cell wall (Peretto et al. 1992).

In 1971, Albersheim and Anderson observed that proteins extracted from the plant cell walls of bean cv. Red Kidney inhibited polygalacturonase activity. Since then, the occurrence of PGIP has been widely reported, and they have been purified from a variety of dicotyledonous plants. These inhibitors range in size from about 37 kDa in bean to a 54-kDa inhibitor purified from citrus (Barmore and Nguyen 1985). In general, the primary structure of mature PGIP is characterized by the presence of repeats derived from a 24-amino acid leucine-rich peptide (De Lorenzo et al. 1994). This leucine-rich repeat (LRR) element matches the extracytoplasmic consensus found in several *R* genes that participate in gene-for-gene resistance (Staskawicz et al. 1995). EPG inhibition appears to be the result of EPG-PGIP complex formation (Cervone et al. 1987). A recent paper suggests the binding of PGIP to EPG at a region opposite the substrate binding cleft,

leading to a conformational change in EPG and resulting in the observed decrease in EPG activity (King et al. 2002). Evidence has also been presented to support an alternative suggestion that PGIP binds at the substrate binding cleft (Cervone 2003).

PGIP from different plant sources differ in their inhibitory activities. For example, PGIP from bean was able to successfully inhibit EPG from six pathogenic fungi, while PGIP from pear and tomato could only inhibit four of the same EPG (Cook et al. 1999). Furthermore, the purified pear PGIP completely inhibits EPG from *Aspergillus niger*, *Botrytis cinerea*, and *Dothiorella gregaria*, slightly inhibits EPG from *Penicillium expansum*, and does not inhibit EPG from *Fusarium oxysporum* at all (Abu-Goukh et al. 1983). In addition, PGIP from a single plant source are selective in their ability to inhibit EPG from different fungi or different EPG from the same fungus. This high level of specificity toward EPG is currently attributed to the composition and three-dimensional motif of the LRR in the structure of PGIP (Leckie et al. 1999).

Shortly after the discovery of PGIP, the small amounts of pectins in the cell walls of monocotyledons led to the conclusion that the presence of the inhibitor is unlikely in this group of plants (Albersheim and Anderson-Prouty 1975). In 1997, two inhibitor isoforms of 39 and 42 kDa were isolated from the nonpoaceous monocot, *Allium porrum* (Favaron et al. 1997). Since the isolation of these PGIP, little work has been published on the presence of PGIP in monocotyledonous plants, and few references have been made to its presence in the economically important cereals from the family Poaceae (formerly known as the Gramineae).

As is the case with most agronomic crops, poaceous crops suffer substantial yield and quality reductions due to fungal disease. Wheat in particular is no exception, with an estimated annual loss of 20% due to diseases (Wiese 1987). The demonstration in 1995 that the initial attack of *Cochliobolus carbo-nium* on barley (a monocot) involves pectin-degrading enzymes (Clay et al. 1997) caused us to rethink the plausibility of the presence of PGIP in the family Poaceae. With this work, we intend to expand the body of knowledge associated with pectin-degrading enzymes in the cereals and their involvement in plant pathogenesis by demonstrating that PGIP exists in wheat. Its characteristics, specificity for EPG, and the location of PGIP expression in the plant will also be discussed.

RESULTS

Purification.

Following acetone extraction from healthy adult wheat plants, followed by Con-A affinity chromatography, both the bound and

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unbound fractions were fractionated by cation exchange (CEX) chromatography (Fig. 1) on a HiTrap-S (Hi-S) column. CEX fractions were assayed for inhibitor activity against seven EPG from the following fungi: *Aspergillus niger* (EPG I and II), *Cochliobolus sativus*, *Colletotrichum lindemuthianum*, *Postia placenta*, *Fusarium moniliforme*, and *Cryphonectria parasitica* against *A. niger* exopolysaccharuronase and against the bacterial EPG from *Ralstonia solanacearum*. Of the nine polygalacturonases assayed, the partially purified wheat extracted fractions from the Con-A-bound-Hi-S-bound samples were able to successfully inhibit only the EPG from *Cochliobolus sativus* (Fig. 2). Apart from the Con-A-bound and Hi-S-bound fractions, none of the fractions from the other eluents (Con-A-bound-Hi-S-unbound, Con-A-unbound-Hi-S-bound, Con-A-unbound-Hi-S-unbound) exhibited any ability to inhibit the *C. sativus* EPG.

Fraction 38, one of six fractions with inhibiting activity that eluted from the CEX column at around 160 mM (53% buffer B) NaCl (Fig. 1), was used to characterize the crude inhibitor. The extent of EPG inhibition is shown in Fig. 3. When separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), numerous bands appear in this fraction, three of which

Fig. 1. Chromatograms of Hi-S-bound fractions obtained from healthy wheat leaves. The boxed area indicates the fractions that exhibited *in vitro* *Cochliobolus sativus* endopolygalacturonase inhibition, which were subsequently combined and put over the size exclusion column.

Fig. 2. Inhibition of activity (measured in terms of reducing sugar concentration as determined by the *p*-hydroxybenzoic acid hydrazide assay and expressed as a percentage of inhibition) of nine polygalacturonases from various sources treated with fraction 38 from the ion-exchange column.

appear to be dominant (Fig. 4). Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) of the same fraction confirmed the presence of numerous bands, including four dominant proteins with sizes of approximately 20, 22, 33, and 40 kDa (data not shown).

The six fractions (fractions 35 to 40) that exhibited inhibition were pooled and further purified by means of size exclusion chromatography (SEC). Five fractions (fractions 29 to 33, data not shown) eluted from the SEC column and revealed a peak of activity, as defined by inhibition of *C. sativus* EPG (63% inhibition for fraction 31, data not shown). Fraction 31 revealed a single band on the silver-stained polyacrylamide gel (Fig. 4), which was confirmed by mass spectrometry (Fig. 5) to have a mass of approximately 40.3 kDa.

Fig. 3. *Cochliobolus sativus* endopolygalacturonase inhibition by fraction 38 from the Hi-S column. Absorbencies following *p*-hydroxybenzoic acid hydrazide assays of enzyme with and without inhibitor are shown.

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis separated and silver-stained gel showing the proteins present in the fractions. Lane 1, Pinto bean polygalacturonase-inhibiting protein control; lane 2, fraction 38 from the Hi-S column; lane 3, fraction 31 from the size exclusion column.

Due to the low pectin content in cereals, we expected only small amounts of PGIP to be isolated, an expectation that was confirmed when we extracted only 350 ng of PGIP from 1 g of wheat leaf tissue. This represents about 1 to 2% of the amount of PGIP that we were able to extract from pear fruits when using the same extraction method (G. Kemp, *unpublished data*). By increasing the amounts of the purified inhibitor, we were able to assess that wheat PGIP can successfully inhibit at least 80% of *C. sativus* EPG activity (Fig. 6). It is possible that a higher level of inhibition could be obtained; however, the limited amount of purified wheat PGIP restricted our ability to proceed further with the assay.

Separation of active PGIP fractions from the CEX and SEC columns by SDS-PAGE, followed by immunoblotting with the PGIP-11 antibody, revealed a 40-kDa band. Fraction 38 from the CEX column exhibited some less-specific recognition of smaller peptides (Fig. 7). When the extracted apoplast proteins were probed with the same antibody, the presence of the 40-kDa inhibitor was confirmed in this area normally associated with *in vivo* PGIP expression, i.e., the intercellular space (Fig. 7).

N-terminal sequencing (Macromolecular Structure Sequencing and Synthesis Facility, Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A.) of the 40-kDa protein in fraction 38 yielded a 13-amino acid sequence, TXFT TSAARGTTXQS. This sequence showed no significant similarity to any known protein. The closest homology (53%) is to a small region of a hypothetical protein from rice (*Oryza sativa*) (GenBank accession number AAG46138), which also shows no similarity to any other PGIP sequences. Further repeated attempts to generate and identify peptides for MS-MS analysis were unsuccessful.

Immunocytochemistry.

Since PGIP is generally associated with plant cell walls, it was important to ascertain where in the wheat leaf this purified protein is located. The fixed plant material was subsequently probed with PGIP-11, and it exhibited good antigenicity for the antibody after an extended 40 h-incubation period. The immunolocalization data is consistent with the presence of PGIP demonstrated by the purification data, as it revealed increased labeling associated with the plant cell wall or regions close to the wall (Fig. 8A). Results presented here illustrate typical examples from approximately eight grids per leaf segment. The preimmune serum (PIS) control also showed a small amount of plant cell wall labeling at high concentrations of the antibody (Fig. 8B). However, the level of labeling was low enough to easily distinguish between the observed increased labeling frequency of PGIP-11 and the lower PIS labeling in the plant cell wall.

Fig. 5. Matrix-assisted laser desorption-ionization time of flight profile of fraction 31 from the size exclusion column. The 20,330 Da peak can be attributed to doubly charged $[M+2H]^{2+}$ species.

DISCUSSION

PGIP research to date has principally focused on the presence, expression, and action of this proteinaceous inhibitor in dicotyledonous plants. Little information on its role and presence in monocotyledonous plants, including those from the economically important family Poaceae, exists. Recently, Favaron (2001) showed that PGIP is present in a noncereal monocot, leek, consisting of not less than 20 isoforms with varying levels of inhibition. Also, a report on the presence of PGIP in the family Poaceae exists (Zheng et al. 1994); however, the source of substrate (pectin) and EPG (a crude mix of various enzymes) used in these inhibition assays suggest the purification of a general pectinase inhibitor rather than a polygalacturonase inhibitor.

In this study, collective biochemical and immunological evidence of PGIP in a cereal crop is presented. The purification of a 40.3-kDa wheat PGIP was central to this investigation and placed this protein among the sizes of glycosylated PGIP purified from numerous dicotyledonous plants (Abu-Goukh et al. 1983; Barmore and Nguyen 1985; Cervone et al. 1987; Favaron et al. 1994) and the only other characterized monocotyledonous plant (Favaron et al. 1997). Furthermore, the purification attempts confirmed the expectation that wheat PGIP would not only be present at extremely low levels but also, like many other PGIP from dicotyledonous plants, would be highly specific for the EPG it inhibits.

Wheat PGIP demonstrated a high level of specificity for EPG when assayed against the nine polygalacturonases. This

Fig. 6. Inhibition assay with increasing amounts of purified wheat polygalacturonase-inhibiting protein (PGIP). To determine the maximum amount of *Cochliobolus sativus* endopolygalacturonase (EPG) inhibition possible by wheat PGIP, an increasing amount of inhibitor (fraction 31 from size exclusion chromatography) was added to the EPG, and the remaining EPG activity was measured.

Fig. 7. Immunoblot of samples probed with PGIP-11 antibody (1:50) and visualized with the enhanced chemiluminescence detection kit. Nonspecific bands at the top of each lane resulting from keratin have been removed. Lane 1, Pinto bean polygalacturonase-inhibiting protein control; lane 2, fraction 38 from the Hi-S column; lane 3, fraction 31 from the size exclusion column; lane 4, wheat leaf intercellular proteins.

proved to be an essential part of the purification process, since only through the use of a suitable EPG would the presence of wheat PGIP be detected. It is significant that only the EPG from *C. sativus*, a wheat pathogen, was successfully inhibited (Fig. 2). The specificity of wheat PGIP for the *C. sativus* endopolygalacturonase is consistent with the high degree of specificity reported for the PGIP from pear and tomato (Cook et al. 1999) and illustrates the complexity of the recognition mechanism involved in the PGIP-EPG interaction. Furthermore, the inability of the wheat PGIP to inhibit other EPG serves to rule out the presence of an indiscriminate substance such as a protease being mistaken for a PGIP.

The anticipated low wheat PGIP levels were compensated for by using larger amounts of material, as wheat cell walls contain about ten times less pectin than dicotyledonous plant cell walls (Darvill et al. 1980). In addition, only vegetative wheat tissue was used in the extraction process, whereas in dicotyledonous plants, such tissues have been shown to contain substantially less PGIP than the fruit or seed (Salvi et al. 1990). The chromatography fractions confirmed that wheat PGIP levels were 60 times lower (approximately 0.000035% wt/wt from fresh wheat leaves) than those observed in, for instance, fractions from pear. In spite of the low level of PGIP, a reasonable level of characterization was possible, facilitated in part through the use of a polyclonal antibody (PGIP-11) generated against a peptide from bean PGIP (Bergmann et al. 1994). Such an antibody was recently used by James and Dubery (2001) in purifying PGIP from cotton. This antibody has also been demonstrated to recognize tomato PGIP, although it failed to recognize pear PGIP (Stotz et al. 2000). As relatively large amounts (20 to 200 µg) of purified protein are required for the successful generation of polyclonal antibodies (Dunbar and Schwoebel 1990), the small amounts of purified wheat PGIP recovered did not allow for the generation of antibodies specific to wheat PGIP. The bean antibody, raised against a peptide corresponding to residues 10 to 21 of bean PGIP, was shown to be reasonably specific for wheat PGIP.

Confirmation of the effectiveness of PGIP-11 in recognizing wheat PGIP was demonstrated when crude intercellular proteins extracted from wheat leaves were immunoblotted. The primary antibody recognized a single protein band with a size corresponding to the molecular weight of the purified wheat PGIP. The existence of a protein recognized by PGIP-11 in the intercellular space, a region where PGIP expression has been established (Salvi et al. 1990), was considered significant. This not only strengthened the confirmation of PGIP in wheat but also demonstrated the suitability of this antibody for immunolocalization of the particular PGIP.

During immunolocalization, labeling of the cell wall was demonstrated. While a low level of nonspecificity was ob-

served between PGIP-11 and fixed leaf material, the observed labeling was judged significant in light of its consistency with the purification data. Its location in the cell walls of uninfected wheat plants is in agreement with previous findings that PGIP is present at low levels in the cell walls of healthy plants (Bergmann et al. 1994; Cervone et al. 1989).

Limited success was obtained with the N-terminal sequencing of the purified wheat PGIP sample, identifying a 13-amino acid sequence. Although no similarity to other characterized PGIP from the dicots was observed, it does show some homology to a small region of a hypothetical protein from rice (*Oryza sativa*), another poaceous monocotyledon.

According to Janick (2001), cereals contribute 69% of the world production of edible dry matter. In this regard, wheat, corn, rice, and barley constitute the dominant human food staples and emphasize the importance of this commodity group. Disease control forms an integral part of sustained crop production, and since the role of the pectin degrading enzymes in fungal diseases of small grain cereals has been demonstrated (Clay et al. 1997), the wheat PGIP described here should contribute to new avenues of investigation in cereal pathogen defense.

MATERIALS AND METHODS

Plant material.

For the purification of PGIP from wheat (*Triticum aestivum*), plants of cv. Thatcher were grown under greenhouse conditions for 4 weeks, at which time foliage and stems were harvested. Thatcher plants for immunocytochemistry were grown in a sterilized soil/peatmoss mixture (1:1 vol/vol) at 20 to 25°C in a greenhouse. Cool-white fluorescent tubes, emitting 120 µEm⁻²s⁻¹, provided a 14-h day length.

Antibodies.

A polyclonal primary antibody (PGIP-11) generated in rabbit against a peptide corresponding to residues 10 to 21 of *Phaseolus vulgaris* PGIP was used (Bergmann et al. 1994). Immunoblot investigations used a goat anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Biosciences, Buckinghamshire, England) with the enhanced chemiluminescence (ECL) detection kit. A 15-nm gold-labeled goat-anti-rabbit IgG (GAR) (British Biocell International Ltd, Cardiff, England) secondary antibody was used for immunocytochemical work.

PGIP extraction and purification.

The inhibitor was extracted from the wheat leaves using a modified method from Favaron and associates (1994). Healthy unstressed wheat leaf and stem tissue (800 g) were homogenized in a blender with 1,200 ml of acetone. The pulp was fil-

Fig. 8. Transmission electron micrographs of healthy wheat leaf sections. **A**, Polygalacturonase-inhibiting protein (PGIP) localized in leaves of healthy seedlings labeled with PGIP-11. **B**, Lower labeling levels by preimmune serum. C = cell, CW = cell wall. Scale: Bar = 1 micrometer.

tered through a nylon cloth, was squeeze-dried, was reblended with 1,000 ml of acetone (repeated three times), and was allowed to completely dry out. The dried pulp was soaked in 2 liters of extraction buffer (20 mM sodium acetate, pH 6.0, 1 M NaCl) and was allowed to stir for 72 h at 4°C.

The material was centrifuged at $14,000 \times g$ for 60 min. The supernatant was recovered and filtered through Whatmann GF/A glass filter paper. The filtered supernatant was concentrated to 800 ml in an Amicon stirred cell with a PM10 membrane and, subsequently, was dialyzed against 20 mM sodium acetate (pH 6.0) over a period of 2 days in standard cellulose dialysis tubing (12,000 to 14,000 molecular weight cut-off [MWCO]). The dialyzed sample was centrifuged for 45 min at $22,000 \times g$, and the supernatant was recovered and concentrated to 200 ml on an Amicon stirred cell with a PM10 membrane. Following concentration, the sample was diluted 1:1 with an equal amount of 2 $\times$ Con A buffer (200 mM sodium acetate, pH 6.0, 2 mM NaCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 2 mM MnCl_2). The diluted sample was applied to a 4-ml Con-A Sepharose column (Amersham Biosciences), and the bound proteins were eluted with 25 ml of 250 mM α -methyl-D-manno-pyranoside at a flow rate of 1 ml per min. This step was repeated twice more with the Con-A unbound fraction to ensure the maximum recovery of protein. Following recovery, the sample was dialyzed against 20 mM sodium acetate (pH 6.0) and concentrated on an Amicon stirred cell with a PM 10 membrane. Before proceeding to the chromatography, the concentrated sample was diluted 1:3 with 20 mM sodium acetate, pH 5.0.

The PGIP was purified from the extract by cation exchange chromatography using a HiTrap-S column (Amersham Biosciences) at a flow rate of 0.5 ml per min. The column was equilibrated with buffer A (20 mM sodium acetate, pH 5.0) and eluted with buffer B (20 mM HEPES, pH 7.5, 300 mM NaCl), using a linear gradient of 1% buffer B per min. The 0.5-ml fractions (Con-A-bound-Hi-S-bound) were analyzed by both an inhibition assay and immunoblotting for the presence of PGIP.

Fractions exhibiting high levels of inhibition were pooled and concentrated down to 300 μl on a Centricon YM10. The pooled fractions were subsequently separated by size on a Superdex 75 size exclusion column, by loading 250 μl onto the column and eluting it at a flow rate of 0.4 ml per min with 50 mM sodium acetate (pH 5.0) containing 300 mM NaCl buffer. Fractions were collected and analyzed for EPG inhibition.

Endo- and exopolygalacturonases.

EPG from different fungal sources were used. EPG from *C. sativus* and *F. moniliforme* were produced and purified to homogeneity as described (Clay et al. 1997; Caprari et al. 1993). *A. niger* PGI, PGII, and exopolygalacturonase were obtained from J. Visser (Wageningen, The Netherlands). *C. lindemuthianum* EPG was produced and purified to homogeneity as described (English et al. 1972) and modified by Cook and associates (1999). *C. parasitica* EPG was obtained from L. Shain (University of Kentucky, Lexington, U.S.A.). *P. placenta* EPG was obtained from C. Clausen (U.S. Forestry Service Laboratory, Madison, WI, U.S.A.). Bacterial (*Ralstonia solanacearum*) polygalacturonase was a gift from M. Schell (University of Georgia, Athens, U.S.A.). All polygalacturonases that were present in sufficient quantity were analyzed for purity as described by Cook and associates (1999).

EPG inhibition and determination of appropriate EPG.

Aliquots from chromatographic fractions were incubated for 1 h at room temperature in 50 mM sodium acetate (pH 5.0) with the EPG and PGA (0.025% polygalacturonic acid in 50

mM sodium acetate) as substrate. The inhibition was quantified following incubation by spectrophotometrically (410 nm) measuring the decrease in the release of reducing sugars by the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985). Controls were included to compensate for possible reducing sugars present in the extract as well as any potential influence of the chromatography buffer on inhibition or enzyme activity.

MALDI-TOF MS.

Samples of interest were analyzed on a Hewlett Packard MALDI-TOF mass spectrometer. The matrix used was a saturated sinapinic acid (3,5 dimethoxy-4-hydroxy-*trans*-cinnamic acid) solution in 50% acetonitrile. Equal volumes of sample and matrix (approximately 0.5 μl) were cocrystallized onto the probe and were dried under vacuum. The laser energy ranged from 10 to 14 μJ .

Infiltration of the leaves and preparation of apoplastic fluid.

Proteins in the intercellular spaces of wheat leaves were extracted through vacuum infiltration (Rohringer et al. 1983; Salvi et al. 1990). The cut ends of approximately 100 seedling leaves were washed in water to remove intracellular contamination due to mechanical wounding by the scissors. The leaf pieces were then subjected to vacuum-infiltration for 5 min with 50 mM sodium acetate buffer (pH 5.0) containing 300 mM NaCl (Salvi et al. 1990). To obtain the apoplastic fluid, leaves were centrifuged at $500 \times g$ with their tips pointing upwards. This method of apoplastic fluid extraction routinely gives a clean intercellular fraction with a very small amount (<2%) of cytoplasmic protein contamination, as determined by the marker enzyme nicotinamide adenine dinucleotide malate dehydrogenase (NAD MDH) (EC 1.1.1.37) (Kemp et al. 1999).

Protein determination and gel electrophoresis.

The concentration of intercellular proteins was determined using the protein assay reagent from Bio-Rad (Bio-Rad Laboratories, Hercules CA, U.S.A.) with gamma globulin as standard (Bradford 1976). Intercellular proteins (50 μg) were precipitated with 9 vol acetone for use in immunoblotting of the intercellular proteins. Purified fractions exhibiting inhibitory activity as well as the precipitated intercellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (1970) and silver-stained as described by Shevchenko and associates (1996).

Immunoblotting.

Separated polypeptides were transferred to nitrocellulose membranes using a mini trans-blot electrophoretic transfer cell with transfer buffer (25 mM Tris, pH 8.3, 190 mM glycine, and 10% [vol/vol] methanol) at a constant amperage of 350 mA (Towbin et al. 1979).

Following transfer, the nitrocellulose membrane was blocked in TBST (20 mM Tris, pH 7.6, 135 mM NaCl, 0.1% Tween-20) containing 5% (wt/vol) ECL blocking agent (Amersham Biosciences), followed by incubation in PGIP-11 primary antibody (24 h, 1:50 dilution) in TBST containing 5% (wt/vol) blocking agent. The membrane was washed with TBST and was incubated in a 1:7,500 dilution of the secondary antibody in 5% (wt/vol) blocking agent. The antigens in the purified fractions were visualized using the ECL detection kit, as described by the manufacturer (Amersham Biosciences).

Tissue preparation for electron microscopy.

Replicate tissue pieces (two of each), approximately 1 cm long, were cut from three-week-old wheat plants. The samples

were fixed in 3% (vol/vol) glutaraldehyde in 100 mM phosphate buffer (pH 7.4) with constant agitation for 1 h at room temperature and were washed five times in phosphate buffer, followed by three washes in distilled water. The samples were subsequently dehydrated in an ethanol series (30 to 100% ethanol, 20% increments, three times at 100%, 15 min each), were infiltrated with LR-White acrylic resin (1:1 LR-White/100% ethanol for 60 min; 3× 100% LR-White for 8 h), and were cured for 40 h at 50°C.

Sections (60 nm thick) of selected material were cut using a LKB III ultramicrotome and collected on Formvar coated (0.5% wt/vol in chloroform) 200-mesh gold grids (Agar Scientific Ltd., Essex, England).

Immunocytochemistry.

Wheat plants were prepared for immunogold labeling, to gain an indication of the distribution of PGIP within a leaf. Grids bearing sections were floated with the section side down on drops of phosphate buffered saline (PBS) containing 1% (wt/vol) bovine serum albumin (BSA) (2 times for 15 min) to block unwanted active sites. Grids were subsequently incubated for 18 to 40 h at 4°C under humid conditions with PGIP-11 (1:5) in PBS-BSA and were washed in PBS-BSA (3 times for 15 min). This procedure was followed by incubation in the secondary antibody diluted (1:100) in PBS-BSA for 1 h at room temperature and washing in PBS-BSA (3 times for 15 min) and double-distilled H₂O (3 times for 15 min). Samples were finally stained with 6% uranyl acetate and lead citrate (Reynolds 1963) prior to examination with a Philips CM100 transmission electron microscope at 60 kV. Two additional controls were included whereby the influence of the secondary gold anti-rabbit antibody as well as the preimmune serum on the recorded results were intensively scrutinized.

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LITERATURE CITED

Abu-Goukh, A. A., Greve, L. C., and Labavitch, J. M. 1983. Purification and partial characterization of 'Bartlett' pear fruit polygalacturonase inhibitors. *Physiol. Plant Pathol.* 23:111-122.

Albersheim, P., and Anderson, A. J. 1971. Proteins from plant cell walls inhibit polygalacturonase secreted by plant pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 68:1815-1819.

Albersheim, P., and Anderson-Prouty, A. J. 1975. Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. *Ann. Rev. Plant Physiol.* 26:31-52.

Barmore, C. R., and Nguyen, T. K. 1985. Polygalacturonase inhibition in rind of Valencia oranges infected with *Diplodia natalensis*. *Phytopathology* 75:446-449.

Bergmann, C. W., Ito, Y., Singer, D., Albersheim, P., and Darvill, A. G. 1994. Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. *Plant J.* 5:625-634.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-253.

Caprari, C., Richter, A., Bergmann, C. W., Lo Cicero, S., Salvi, G., Cervone, F. and De Lorenzo, G. 1993. Cloning and characterization of a gene encoding the endopolygalacturonase of *Fusarium moniliforme*. *Mycol. Res.* 97:497-505.

Cervone, F. 2003. Structure-function and molecular studies on fungal polygalacturonases and their inhibitors PGIPs. Pages 293-297 in: *Advances in Pectin and Pectinases Research*. F. Voragen, H. Schols, and R. Visser, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Cervone, F., De Lorenzo, G., Degrà, L., Salvi, G., and Bergami, M. 1987. Purification and characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. *Plant Physiol.* 85:631-637.

Cervone, F., De Lorenzo, G., Pressey, R., Darvill, A. G., and Albersheim, P. 1989. Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiol.* 90:542-545.

Clay, R. P., Bergmann, C. W., and Fuller, M. S. 1997. Isolation and characterization of an endopolygalacturonase from *Cochliobolus sativus* and a cytological study of fungal penetration of barley. *Phytopathology* 87:1148-1159.

Cook, B. J., Clay, R. P., Bergmann, C. W., Albersheim, P., and Darvill, A. G. 1999. Fungal polygalacturonases exhibit different substrate degradation patterns and differ in their susceptibilities to polygalacturonase-inhibiting proteins. *Mol. Plant-Microbe Interact.* 12:703-711.

Cooper, R. M. 1984. The role of cell wall degrading enzymes in infection and damage. Pages 13-28 in: *Plant Disease, Infection, Damage and Loss*. R. K. S. Wood and G. J. Jellis, eds., Blackwell Scientific, Oxford.

Coutinho, P. M., and Henrissat, B. 1999. The modular structure of cellulases and other carbohydrate-active enzymes: An integrated database approach. Pages 15-23 in: *Genetics Biochemistry and Ecology of Cellulose Degradation*. K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita, and T. Kimura, eds. Uni Publishers, Tokyo.

Darvill, A. G., McNeil, M., Albersheim, P., and Delmer, D. P. 1980. The primary cell walls of flowering plants. Pages 91-162 in: *The Biochemistry of Plants*. Vol. 1. The Plant Cell. N. E. Tolbert, ed. Academic Press, New York.

De Lorenzo, G., Cervone, F., Bellincampi, D., Caprari, C., Clark, A. J., Desiderio, A., Devoto, A., Forrest, R., Leckie, F., Nuss, L., and Salvi, G. 1994. Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. *Biochem. Soc. Trans.* 22:396-399.

Dunbar, B. S., and Schwoebel, E. D. 1990. Preparation of polyclonal antibodies. Pages 633-670 in: *Guide to Protein Purification, Methods in Enzymology*. Vol. 182. M. P. Deutscher, ed. Academic Press, New York.

English, P. D., Maglothlin, A., Keegstra, K., and Albersheim, P. 1972. A cell wall-degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. *Plant Physiol.* 49:293-297.

Favaron, F. 2001. Gel detection of *Allium porrum* polygalacturonase-inhibiting protein reveals a high number of isoforms. *Physiol. Mol. Plant Pathol.* 58:239-245.

Favaron, F., D'Ovidio, R., Porceddu, E., and Alghisi, P. 1994. Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. *Planta* 195:80-87.

Favaron, F., Castiglioni, C., D'Ovidio, R., and Alghisi, P. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. *Physiol. Mol. Plant Pathol.* 50:403-417.

Fritig, B., Heitz, T., and Legrand, M. 1998. Antimicrobials proteins in induced plant defense. *Curr. Opin. Immunol.* 10:16-22.

James, J. T., and Dubery, I. A. 2001. Inhibition of polygalacturonase from *Verticillium dahliae* by a polygalacturonase inhibiting protein from cotton. *Phytochemistry* 57:149-156.

Janick, J. 2001. New crops for the 21st century. Pages 307-327 in: *Crop Science: Progress and Prospects*. J. Nösberger, H. H. Geiger, and P. C. Struik, eds. CABI Publishing, Wallingford, U.K.

Kemp, G., Botha, A.-M., Kloppers, F. J., and Pretorius, Z. A. 1999. Disease development and β -1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings. *Physiol. Mol. Plant Pathol.* 55:45-92.

King, D., Bergmann, C., Orlando, R., Benen, J. A. E., Kester, H. C., and Visser, J. 2002. Use of amide exchange mass spectrometry to study conformational changes within the endopolygalacturonase II-homogalacturonan-polygalacturonase inhibiting protein system. *Biochemistry* 41:10225-10233.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680-685.

Leckie, F., Mattei, B., Capodicasa, C., Hemmings, A., Nuss, L., Aracri, B., De Lorenzo, G., and Cervone, F. 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): A single amino acid substitution in the solvent-exposed β -strand/ β -turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:2352-2363.

Peretto, R., Favaron, F., Bettini, V., De Lorenzo, G., Marini, S., Alghisi, P., Cervone, F., and Bonfante, P. 1992. Expression and localization of polygalacturonase during the outgrowth of lateral roots in *Allium porrum* L. *Planta* 188:164-172.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208-210.

- Rohringer, R., Ebrahim-Nesbat, F., and Wolf, G. 1983. Proteins in inter-cellular washing fluids from leaves of barley (*Hordeum vulgare* L.). J. Exp. Bot. 34:1589-1605.
- Salvi, G., Giarrizzo, F., De Lorenzo, G., and Cervone, F. 1990. A polygalacturonase-inhibiting protein in the flowers of *Phaseolus vulgaris* L. J. Plant Physiol. 136:513-518.
- Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68:850-858.
- Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. Science 268:661-667.
- Stotz, H. U., Bishop, J. G., Bergmann, C. W., Koch, M., Albersheim, P., Darvill, A. G., and Labavitch, J. M. 2000. Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. Physiol. Mol. Plant Pathol. 56:117-130.
- Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76:4350-4354.
- Wiese, M. V. 1987. Compendium of Wheat Diseases. 2nd ed. The American Phytopathological Society, St. Paul, MN, U.S.A.
- York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., and Albersheim, P. 1985. Isolation and characterization of plant cell walls and plant cell wall components. Methods Enzymol. 118:3-40.
- Zheng, Y., Yang, Z., Li, J., Yu, L., and Wu, W. 1994. Purification of polygalacturonase inhibiting protein from *Triticum aestivum* L. Acta Biochim. Biophys. Sinica 26:555-558.