Structure and Expression of Two Polygalacturonase Genes of *Claviceps purpurea* Oriented in Tandem and Cytological Evidence for Pectinolytic Enzyme Activity During Infection of Rye

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ABSTRACT


Two putative polygalacturonase (PG) genes were isolated from strain T5 of *Claviceps purpurea*, using the *pgall* gene of *Aspergillus niger* as a probe. The two genes (*pg1* and *pg2*) are closely linked and arranged head-to-tail. They are highly homologous even in the upstream noncoding sequences, possess one intron each in the same position, and have cleavage sites for processing enzymes. They probably code for mature proteins of 343 and 344 amino acids, respectively, and share significant homology with endo-PGs of other filamentous fungi. Expression of *pg1* and *pg2* in axenic culture and during various stages of infection of rye was demonstrated using reverse transcription-polymerase chain reaction.

The potential substrate of the putative products of *pg1* and *pg2* (polygalacturonic acid), for the first time, was shown to be a component of the host cell walls in rye ovaries. This was achieved by immunogold transmission electron microscopy with the monoclonal antibody (MAB) JIM 5, specific for nonmethyl-esterified epitopes of pectin. This homogalacturonan was localized along the usual infection path in healthy carpels together with its methyl-esterified galacturonan type in the same cell walls with another MAB, JIM 7. At the interface of the penetrating hyphae and the host ovary epidermis, JIM 5 label density was locally enhanced and very high above hyphal sheaths. In the vicinity of intercellularly growing hyphae, label density was highly increased, and gold label occurred not only above the middle lamella area but also throughout the entire host cell wall. Chemical demethylation and immunogold labeling indicated a high total content of galacturonan and a conversion of pectic compounds at the host-parasite interface. During late infection phases, the lack of any JIM label, which previously occurred at the interface of intracellular hyphae, emphasized the complete utilization of homogalacturonan together with other plant polysaccharides. The observed host wall alterations provide evidence for secretion and activity of extracellular pectinolytic enzymes in planta. Both the expression of the two genes during infection of rye and the modification and degradation of homogalacturonan detected only at fungal sites indicate the fungal origin of pectinolytic enzymes, the activities of which have been documented previously in infected ovaries by B. I. Shaw and P. G. Mantle.

Additional keywords: host-parasite interaction.

The ubiquitous ascomycete *Claviceps purpurea* (Fr.) Tul. is the causal agent of ergot of cereals and grasses. The fungus is highly organ-specific; it exclusively attacks young ovaries, replacing the plant ovarian tissue, and establishes a stable host-parasite interface after having tapped the host vascular system (Fig. 1). *Claviceps* is not expected to secrete vast amounts of hydrolytic enzymes due to its hemibiotrophic, balanced interaction with the host plant during most of its life cycle. Nevertheless, cell wall-degrading enzymes might play an important role during the first stages of infection and in the process of establishing and maintaining open contact with the host phloem. For this latter process, a β-1,3-glucanase could be important in degrading callose, which normally protects the host phloem. An extracellular β-1,3-glucanase of *C. purpurea* has been purified from axenic culture, characterized in detail (7), and shown to be secreted in planta (149); K. B. Tenberge, B. Brockmann, and P. Tudzynski, *unpublished data*. Several other hydrolytic enzyme activities have been detected in axenic culture, e.g., xylanolytic, cellulolytic, and pectinolytic enzymes (51).

To elucidate the role of these enzymes during the parasitic cycle of *C. purpurea*, we designed a molecular approach that covers genetics, enzymology, and structural cytology, using cloning and characterization of corresponding genes, expression studies (reverse transcription-polymerase chain reaction [RT-PCR]), and secretion studies (immunogold transmission electron microscopy [TEM]) in planta. Unequivocal evaluation of the function of these enzymes in pathogenicity should be obtained from gene-disruption analysis if these experiments are feasible. Recently, we cloned a putative cellobiohydrolase gene (*cell*), studied its expression in planta, and deleted it by gene disruption ([33]; U. Müller, K. B. Tenberge, B. Oeser, and P. Tudzynski, *unpublished data*). In addition, two xylanase isoforms have been purified and characterized, and one putative xylanase gene (*xyl*) has been cloned, sequenced, and deleted (S. Giesbert and P. Tudzynski, *unpublished data*). Fungal enzymes might not only be able to break down the major cell wall components, cellulose and xylans, but in addition, enzymes degrading pectic components might be of special importance for ergot pathogenicity. This was assumed because the fungus shows an intercellular mode of growth. Pectinolytic activity detected in colonized host tissue has been documented (44), but there is no unequivocal evidence for the fungal origin of pectinolytic enzymes in infected ovaries or for their operation on pectin components in host cell walls.
In this article, we report on the cloning and characterization of two polygalacturonase (PG) genes and their expression in planta as well as in axenic culture. We were able to verify the presence of homogalacturonases, the potential substrate of PGs in ovary walls, and demonstrate their demethylation, structural modification, and degradation during infection of rye. Finally, the function of PGs in pathogenicity with respect to observed cell wall alterations is discussed.

MATERIALS AND METHODS

Strains and culture conditions. A standard field isolate of C. purpurea strain T5 isolated from rye (Secale cereale L.) in Hofenheim, Germany, was used for all studies. Mycelia were cultivated in 100 ml of modified complete medium according to Mantle and Nisbet (32) with reduced sugar content (1% [wt/vol] glucose) in 300-ml Erlenmeyer flasks at 28°C on a rotary shaker at 150 rpm. For enzymatic analyses, 0.5% (wt/vol) pectin and varying amounts of N-source (wt/vol asparagine) were used. Escherichia coli DH5α (22) was used routinely for cloning and propagation of plasmids. Lambda clones were propagated in E. coli strain K 803 (54).

Parasitic culture on rye plants. Rye plants were cultivated in growth chambers as described by Smit and Tuzdyski (45). To obtain the most “natural” inoculation, small drops of a conidial suspension in 10 mM sodium phosphate buffer, pH 6.0, containing 0.8% NaCl, were transferred with a fine brush onto the stigmas of rye flowers at anthesis. Ears remained noninoculated for control. Conidia were taken from axenic cultures of C. purpurea T5 grown on complete medium, according to Mantle and Nisbet (32), containing 2% (wt/vol) agar.

Enzymatic analyses. Determination of PG activity was done as described by Kombrik et al. (29), using 0.5% (wt/vol) sodium polygalacturonate (Sigma Chemical Company, Deisenhofen, Germany) as substrate in sodium succinate buffer, pH 5.0.

Immunogold electron microscopy. To process specimens for on-grid immunolabeling, inoculated florets of defined infection stages of ergot were selected from parasitic culture on rye plants; corresponding noninoculated controls were selected as well. The selected stages were as follows: infected ovaries before honeydew production, 1 to 7 days postinoculation (dpi); young spachelia with honeydew production, 8 to 10 dpi; old spachelia, about 15 dpi; and young sclerotia, about 20 dpi. The ovary axes with whole pistils or developing sclerotia were fixed in sectioned parts of appropriate size for 1.5 h at room temperature in a mixture of 5% (wt/vol) formaldehyde and 1% (vol/vol) glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.0. Further processing of the non-osmicated samples via dehydration in ethanol, embedding in LR white (TAAB Laboratories, Munich) at 60°C, ultramicrotomy with diamond knives, handling of nickel grids during immunolabeling, and electron microscopy was performed as described previously (47).

For immunogold localization of homogalacturonase oligomers in pectin, previously described protocols were modified (4,5,34,40). Sections were treated with normal goat serum diluted 1:30 in 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol-Tris-HCl buffer, pH 7.5, and 150 mM NaCl (TBS) containing 0.5% bovine serum albumin (BSA; Sigma) for 30 min. Sections were either incubated with the cell culture supernatant of JIM 5, an antimonomethylated galacturonan monoclonal antibody (MAB) (52) or with the cell culture supernatant of JIM 7, an antimethylated galacturonan MAB (28), diluted 1:5 in TBS plus 0.5% BSA for 2 h at 37°C. Grids were washed thoroughly in TBS and treated with goat anti-rat immunoglobulin linked to 15 nm colloidal gold particles (EM.GAT IgG 15, BioCell, Cardiff, England), diluted 1:30 in TBS plus 0.1% BSA for 1 h at room temperature. The grids were washed thoroughly in TBS and double-distilled water and air-dried and contrasted with uranyl acetate and lead citrate. For on-grid chemical deesterification, some sections were incubated with 0.1 M sodium carbonate for 15 h at 4°C, washed thoroughly in double-distilled water, and processed according to the labeling protocol with JIM 5 to label all homogalacturonan. Controls for labeling specificity were run as a parallel set of sections incubated either without a primary MAB or with an antibody solution immune-precipitated with a corresponding antigen, 1 mg of polygalacturonate (Sigma) per ml or 1 mg of pectin from citrus (Sigma) per ml, for 17 h at 4°C before section treatment. For light microscopic overview, carboxylated polysaccharides, including pectic components, were localized with 0.05% toluidine blue O, pH 4.4, in semithin section as described elsewhere (46).

Molecular techniques. PG genes were isolated by screening a genomic EMBL 3 library from strain T5 (45) by plaque filter hybridization according to Sambrook et al. (41). A fragment of the pgaI gene (a 1.25-kbp BamHI/BglII fragment of pgW 1800) from Aspergillus niger Tiegh. (9) was used as a probe at moderately stringent conditions (61°C). Plaques (40,000) were screened in a first round. Positive clones were plated again and purified as single plaques. Phage DNA was extracted and characterized by restriction analysis and Southern hybridization with the pgaI probe.

For RT-PCR, 1 µg of total RNA and 200 ng of either primer PG1rev (CCCTAGGTAGTCGCCCCCGGCGCGG) or PG2rev (CTGGGATCACCGGG) were heated for 10 min at 70°C and cooled on ice. For the RT reaction, cDNAs (1 µM for each cDNA) 1x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.01% [wt/vol] gelatin, and 0.1% Triton X-100), and 100 units of

![Fig. 1. Illustration of different primary infection sites (arrows) and infection paths of Claviceps purpurea in a rye pistil, as described in Tuzdyski et al. (51): dotted lines show spore germination and infection via stigma (si) and style (sy), corresponding to the pollen tube path up to the microsporangial region (m); plus line shows infection at the base of the ovary wall (ow); dashed line shows spore germination on the stigma. The infection point is at the base of the ovary wall; double-arrow head indicates indusium or filament base. ad = adaxial; fr = furrow region. The routes in the histologically heterogeneous pistil are all terminated at the vascular tissue in the rachila (ra) tip and usually lead through several different tissues: the cuticularized epidermis of the ovary (1) or rachilla (2), the ovary wall mesophyll (3), the transmitting tissue (4), the integuments (5) surrounding the ovule (ov), the rachilla cortical parenchyma (6), and the vascular tissue (7) with xylem and phloem elements and parenchyma.](image-url)
RNA guard (Pharmacia Biotech, Freiburg, Germany) were added. After a preincubation step (2 min at 42°C), 200 units of M-MLV reverse transcriptase (Gibco BRL Life Technologies, Eggenstein, Germany) were added. The reaction was stopped after 1 h (95°C for 5 to 10 min, cooled on ice). At least one-quarter of the RT reaction was used for PCR amplification of pg1 (PG1rev and PG3 [GGGT-AGCTTTTGTTGTCGG]) or pg2 (PG2rev and PG3). The PCR run began with a hot start for 75 s at 94°C, followed by 36 cycles of 45 s at 94°C for denaturation, 30 s at 55°C for primer annealing, and 1 min at 72°C for polymerization. In the thirty-seventh cycle of 45 s at 94°C, 45 s at 55°C, and 6 min at 72°C, the incubation times for primer annealing and polymerization were extended.

Isolation of total DNA for Southern and PCR analyses was performed according to Doyle and Doyle (18). For DNA sequencing, the T7-sequencing and the nested-deletion kits (Pharmacia) were used. Computer analysis of the sequence was done by HUSAR (EMBL, Heidelberg, Germany) and PC Gene (IntelliGenetics, Inc., Mountain View, CA). RNA was prepared according to Chambers and Russo (13); RNA was collected from infected ovaries at various stages and immediately frozen as well as lyophilized. Standard molecular methods (cloning, DNA preparation from E. coli, gel electrophoresis, DNA and RNA blotting, labeling of DNA, and screening of a lambda library) were performed according to Sambrook et al. (41) and Ausubel et al. (2).

RESULTS

Cloning of putative PGs. A genomic EMBL 3 library of C. purpurea strain T5 was screened, using a fragment of the pgalla gene of A. niger as a probe (described above). Twenty-nine positive clones analyzed by restriction digest and Southern hybridization contained pgalla-homologous sequences. A homologous 4.8-kbp HindIII/SalI fragment was subcloned in pUC19, yielding plasmid pPG4.8. Detailed restriction mapping, Southern hybridization, and partial sequencing of this fragment indicated the presence of two closely linked pgalla-homologous regions. Complete sequencing of pPG4.8 confirmed this finding, showing the presence of two open reading frames (pg1 and pg2) in tandem on pPG4.8 (Fig. 2). The putative translation products of pg1 and pg2, derived from sequence data, contain the conserved domains of bacterial and fungal PGs (Fig. 3).

To rule out the possibility that this unusual orientation represents a cloning artifact, analysis of other positive lambda clones showed they cover the same genomic region and comprise the two tandem PG genes (data not shown). Southern blotting experiments, using genomic DNA from strain T5 of C. purpurea and pg1 as a probe (data not shown), confirmed that the spatial orientation of pg1 and pg2 represent the genomic organization. Fragments of the expected sizes, as derived from the map (Fig. 2), hybridized to the probe. These experiments also established that there is no evidence for the presence of another genomic copy of a PG gene in strain T5, because no additional signals occurred. Because pg1 and pg2 are not completely identical on the nucleotide level (described below), it was possible to design specific primers for PCR amplification of either gene (Fig. 2). Using genomic DNA of T5 and nine other field isolates of C. purpurea as template, we obtained PCR fragments of the expected sizes for pg1 and pg2 in all samples, proving the existence of both genes in all of the tested strains (data not shown). Taken together, the data indicate that the insert of pPG4.8 represents a typical genomic region of C. purpurea.

Structure of pg1 and pg2. The nucleotide and obtained amino acid sequences of both genes compared in Figure 4. Both genes are highly homologous at the nucleotide (94.8%) and amino acid level (90.8%). Most changes are neutral, occurring in the third codon position, but in some cases, the nucleotide differences result in different amino acids. Both genes contain one intron that is 93 and 100 bp in length, respectively, at identical positions. The intron is comparable to the first intron of the pgala gene of A. niger and to the second intron of the endo-PG gene of Fusarium moniliforme (9,10). The 5’ and 3′ splice sequences and the internal control sequence match the corresponding fungal consensus sequences. In addition, they are highly homologous to those of other C. purpurea genes (Table 1). The localization and size of introns in pg1 and pg2 have been confirmed by RT-PCR analyses (below).

The nucleotide sequences of both genes predict polypeptide sizes of 368 and 369 amino acids (aa), respectively, for pg1 and pg2; there is a deletion of 1 aa at position 127 in pg1. By analogy to other PGs (10), there is probably a maturation of the preproprotein by a signal peptide (at alanine 19) and a monobasic processing enzyme at arginine 25 (Fig. 4), yielding putative mature proteins of 343 and 344 aa, respectively. Both genes comprise three potential sites for N-glycosylation (asparagine 217/218, 280/281, and 314/315) and six cysteine residues that are conserved in other fungal PGs (8), indicating conserved disulfide bridges.

Sequences of the C. purpurea enzymes and sequences of PGs of other organisms show significant homology. The overall homology, including introns, ranges from about 40% (similarity) in bacterial systems (e.g., Erwinia carotovora [25]) to more than 60% in fungal genes. The highest degree of homology was observed in comparison with fungal endo-PGs, as shown in Figure 3.

Seventy-two amino acids of pg1 and pg2 are conserved within all fungal enzymes listed. The region spanning from glycine 182/183 and serine 274/275 is especially conserved and might contain the catalytic center; histidine 229/230 could correspond to the histidine residue that was identified as essential in the A. niger enzyme (37). The obviously essential carboxyl groups (36) could be provided by the conserved aspartate residues in positions 186/187, 207/208, and 208/209; furthermore, serine 232/233 could be important for catalytic activity (24). Taken together, there is good evidence that pg1 and pg2 code for PGs, most likely for endocutting enzymes, although this can be proven only by biochemical analysis of the purified enzymes.

![Fig. 2](https://example.com/fig2.png)  
Fig. 2. Restriction map of the HindIII SalI fragment of pPG4.8. The coding regions of genes pg1 and pg2 are represented by striped boxes; the introns are represented by black boxes. Position and orientation of the primers used for reverse-transcription polymerase chain reaction are shown by arrowheads.
The striking homology between pg1 and pg2 is not restricted to the coding regions. As shown in Figure 5, the 5' upstream sequences are highly conserved up to about ~300 (from the ATG), although there is a major deletion of 38 bp in the pg1 sequence (all further nucleotide values refer to pg2). Upstream of ~350, there is very little homology between both promoter sequences. The following putative major promoter elements are conserved: the Kozak consensus sequence (20) immediately upstream of the ATG, a CT-rich region (from -229 to -246), two putative TATA boxes (at -276 and -340), and two CAAT sequences (at -266 and -310). In addition, two putative binding sites (GATA) for nitrogen regulatory proteins, such as areA in A. nidulans, are present (30).

Expression studies. It was of interest to test whether the different distances to the translation start, due to the deletion, cause any major differences in promoter activity. PG activity in axenic culture of C. purpurea was rather low. The total amount of activity was neither substrate-inducible nor subject to carbon and nitrogen catabolite repression (data not shown). To study the expression of pg1 and pg2 separately in axenic culture and, far more important, in planta, we chose the RT-PCR approach. Primers were designed (described above; Figs. 2 and 4) that span the intron-containing

![Fig. 3. Comparison of the deduced amino acid sequences from the pg1 and pg2 genes of Claviceps purpurea with polygalacturonases (PGs) of C. carbonum (PG), Aspergillus oryzae (PG), A. niger (PGI, PGC), Sclerotinia sclerotiorum (PGI), and Fusarium moniliforme (PG). Conserved residues of all nine sequences are printed in bold letters and indicated by asterisks. For optimum alignment, gaps were introduced (dots).](image-url)
regions of the genes. This enabled us to discriminate between PCR products derived from either pg1 or pg2 and to distinguish between RNA-derived products (size without intron) and PCR products due to DNA contamination of the RNA preparation. As shown in Figure 6A, primer combinations PG1rev/PG3 and PG2rev/PG3 only yield PCR products with pg1 and pg2 DNA, respectively, confirming the specificity of the primers. In Figure 6b, RT-PCR experiments are documented, using RNA from four stages of rye plant infection by C. purpurea. In all samples, except those of the last stage, a mRNA-derived fragment occurred that was about 100 bp smaller than the DNA-derived fragments (Fig. 6A), because the intron was lacking. This confirmed the expression of both genes during the early stages of infection in planta, i.e., the nonsclerotial (sporangial) phase. In phase IV, the sclerotial phase, only a putative DNA-derived fragment occurred. The corresponding RT-PCR products also were obtained in axenic culture (4 days old, 0.05% asparagine) (data not shown).

In situ localization of galacturonate epitopes in healthy pistil tissues. To prove the presence of the potential substrate of the PGs, homogalacturonan regions in pectin were made visible in

Fig. 4. Comparison of the nucleotide sequences and derived amino acid sequences of the coding regions in the pg1 and pg2 genes of Claviceps purpurea. Amino acid differences are marked by bold letters. The location of the intron in both genes (b, the first amino acid in the mature protein (v), and conserved cysteine residues (underlined) are indicated. Asterisks mark conserved residues in all fungal polygalacturonases. Underlined nucleotide sequences in italics indicate to the binding sites for the polymerase chain reaction primers PG1rev, PG2rev and PG3.
situated by TEM. After incubating ultrathin sections of pistils from noninoculated rye florets with the hybridoma culture supernatant of JIM 5 specific for nonmethyl-esterified homogalacturonan and goat anti-rat gold particles, definite gold labeling occurred above distinctive areas of the ovary tissue. The JIM 5 label pattern observed in healthy pistils was identical with that of noncolonized areas of inoculated pistils. Gold particles exclusively appeared above the plant apoplast of carpels. The middle lamella, which was visible as a narrow zone of contact between neighboring cells, exhibited JIM 5 epitopes precisely marked by a distinct line of gold granules above this electron-dense cell wall layer (Fig. 7A and B). No label was found above the growing cell walls attached inside to the middle lamella. From the middle lamella onward, the labeling extended into stronger labeling of the outer surface of intercellular spaces (Fig. 7A). Intense labeling was present above the intercellular cell wall material filling the three-way junctions (data not shown) and above the cellular junctions adjacent to the intercellular spaces (Fig. 7A). Sporadically, clusters of a few gold particles were seen outside the plasma membrane above the periplasmic space (data not shown). No specific gold label was observed inside the plasma membrane above the plant protoplast.

After incubating ultrathin sections of pistils with the cell culture supernatant of JIM 7 specific for methylesterhomogalacturonan, distinct gold labeling occurred above the growing cell walls (Fig. 7C). All of the cell wall areas—the middle lamella zone, the cell

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**Fig. 5.** Comparison of the 5' upstream sequences of the *pg1* and *pg2* genes of *Claviceps purpurea*. Putative promoter elements are marked as follows: TATA box (---), CAAT box (underlined), pyrimidine-rich regions (>>>), and GATA elements (bold letters). The Kozak consensus sequence is indicated by underlined letters in italics.

**TABLE 1.** Conserved intron sequences in different *Claviceps purpurea* genes and polygalacturonase genes of other filamentous fungi

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>5' Intron splice sequences</th>
<th>Internal splice sequences</th>
<th>3' Intron splice sequences</th>
<th>Intron length (bp)</th>
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<td><em>C. purpurea</em></td>
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<td>GTGAGT CGCTAA CAG</td>
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<td>Mut7</td>
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<td>GuT*</td>
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<td>TAG</td>
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<td>8</td>
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<td></td>
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<td>GTGAGT CGCTAA CAG</td>
<td>TAG</td>
<td>62</td>
<td>Mut7</td>
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<tr>
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<td><em>pgal</em></td>
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<td>GTGAGT CGCTAA CAG</td>
<td>TAG</td>
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<td>42</td>
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<tr>
<td>* Fusarium moniliforme*</td>
<td><em>endop g</em> a</td>
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<td>TAG</td>
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<td>TAG</td>
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Consensus sequence for filamentous fungi: GTANGT PyGCTAAC C TAG

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8 S. Giesbert and P. Tudosynski (unpublished data).
wall layers attached inside, and the cellular junctions—were labeled for methyl-esterified galacturonic acid sequences of pectin. The labeling density, however, was low or absent above the outer surface of the intercellular spaces (data not shown). In some cases, little or no labeling was visible above the innermost wall layer of pistil cells. This layer exhibited a coarse fibrillar ultrastructure and appeared to be produced recently (Fig. 7C compared with Fig. 8D).

Gold labeling specific for homogalacturonan was observed above ovary and rachilla epidermal cell walls, pericarp mesophyll walls, and rachilla cortical walls. In addition, it was detected above cell walls of the integuments and several different wall types of vascular tissue, including phloem sieve plates. Neither electron-transparent cell wall areas, especially in phloem sieve plates, which most likely were composed of callose, nor secondary cell wall thickenings in xylem tissue or the cuticle proper within cuticular membranes exhibited any galacturonan labeling after JIM 5 or JIM 7 treatment. All the performed controls, lacking primary antibodies or application of immune-precipitated antibody solution, were devoid of gold particles (data not shown).

**In situ localization of galacturonan epitopes in colonized pistil tissues.** Although the JIM 5 and JIM 7 labeling patterns in noncolonized areas of inoculated pistils were not altered, modifications of gold labeling became conspicuous in colonized host tissues. Away from the cellular junctions in the epidermis, JIM 5 gold labeling was very weak in the outer epidermal wall of the ovary, typically only a few particles were located beneath the host cuticle (Fig. 8A and B, large arrowheads). However, label density was characteristically enhanced at the interface of hyphae growing subcortically in the outer epidermal wall (Fig. 8A) or penetrating through the anticline walls at suitable junction sites of epidermal cells into the pericarp tissue (Fig. 8C). Gold particles were not uniformly distributed throughout the epidermal cell walls but were they located at a particular layer of the host wall. Instead, particles locally accumulated above certain host areas, extending from the

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**Fig. 6.** Reverse transcription-polymerase chain reaction (RT-PCR) expression analysis of *Claviceps purpurea* genes pg1 and pg2 in planta. A, Proof of primer specificity: DNAs of plasmid pPG2.8HX (pg1) and pPG2.35B (pg2) were used as templates for PCR reactions with primer combinations PG1rev/PG3 and PG2rev/PG3, respectively (Figs. 2 and 4). B, RT-PCR analyses with total RNA (DNase treated) as template from rye ovaries at different stages of infection with *C. purpurea*: I, young infected ovaries, about 5 days postinoculation (dpi); II, honeydew producing phacelacia, about 10 dpi; III, first symptoms of sclerosis development, about 15 dpi; and IV, young sclerosis, about 20 dpi. PG1rev (pg1) and PG2rev (pg2) were used for RT experiments, together with primer PG3 in subsequent PCR amplification.

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**Fig. 7.** A–C, Immunogold electron microscopic localization of homogalacturonan in noncolonized pistil tissues of rye. A and B, Labeling with monoclonal antibody (MAb) JIM 5 specific for nonmethyl-esterified polygalacturonic acid in *Claviceps purpurea*-infected ovaries, 7 days postinoculation (dpi). The middle lamella (ml) between the host cell walls (hcw), the surface of the intercellular space (is), and the cellular junction exhibiting JIM 5 labeling are shown for A, the ovary wall mesophyll and B, the rachilla cortical parenchyma. C, Labeling with MAb JIM 7 specific for methyl-esterified polygalacturonic acid in infected ovaries, 8 dpi. JIM 7 labeling is present throughout the growing cell wall in the rachilla cortical parenchyma. Samples were fixed in formaldehyde and glutaraldehyde; there was no osmication; samples were embedded in LR white; uranyl and lead salts were used for section staining. Scale bars = 1 μm.
Fig. 8. A–D, Immunogold localization of homogalacturonan at penetration sites of the ovary epidermis of rye inoculated with *Claviceps purpurea*. At sites distant from the interface (B, large arrowheads) of hyphae (F) in cell walls of the host epidermis (H), weak monoclonal antibody (MAb) JIM 5 labeling was present above a narrow layer below the cuticular membrane (cm) of the outer epidermal wall (oew). However, JIM 5 labeling was intense after chemical demethylation (D). At the interface in the outer epidermal wall (A and B) or in the anticline wall (C, aw), intense JIM 5 labeling was present locally in the host wall (A, open arrows) or abundantly in hyphal sheaths (s) above electron-dense fibrils (B, small arrowheads). fcw = fungal cell wall. A–C, Labeling with JIM 5 for polygalacturonic acid, 7 days postinoculation (dpi); and D, sodium carbonate treatment for demethylation and JIM 5 labeling, 8 dpi. Scale bars = 1 μm.
hyphal surface into the host wall (Fig. 8A and C, open arrows). In these labeled areas, the cell wall appeared to be loosened, exhibiting a more fibrillar arrangement of wall material (Fig. 8A).

Typically, hyphae were closely attached to the ovary surface with electron-dense fibrillar material. This material surrounded the fungal cells completely, like a sheath that extended into the host’s outer epidermal wall (Fig. 8B). This sheath was highly gold labeled, demonstrating numerous JIM 5 epitopes often located on electron-dense fibrils (Fig. 8B, small arrowheads). The fibrils, which contained polygalacturonic acid, appeared to be connected.

Fig. 9. A–D, Immunogold localization of homogalacturonan in infected mesophyll tissue of rye ovary inoculated with Claviceps purpurea. Distant from intercellular hyphae (F) in the ovary mesophyll (H), weak monoclonal antibody (Mab JIM 5 labeling was limited to the intercellular space (is) surface and to the middle lamella (ml); however, JIM 5 labeling was intense throughout host walls after demethylation (D). At the interface, intense JIM 5 labeling was present above former cellular junctions (A, arrowhead), the entire host wall (A, open arrows), and the altered middle lamella zone (A and B). In highly colonized ovary tissues, Mab JIM 7 labeling was present above fibrillar cell wall remnants (C, hwr). A and B, Labeling with JIM 5, 7 days postinoculation (dpi); C, labeling with JIM 7, 8 dpi; and D, demethylation with sodium carbonate and JIM 5 labeling, 8 dpi. Scale bars = 1 µm.
to the fungal surface, at which they were most clearly separated. The structural integrity of the cell wall and the label density increased toward the host epidermal cell as the distance grew between the fungus and the host (Fig. 8B, open arrows). Below this zone, neither structural wall alterations nor JIM 5 labeling were obvious; the label extended into the normal label pattern in noncolonized tissue that was more distant from the interface. The hyphae seemed to disintegrate the outer epidermal wall structure.

Fig. 10. A–D, Immunogold localization of homogalacturonan in penetrated ovary cells of infected tissue of rye ovaries inoculated with Claviceps purpurea. At the interface of hyphae breaching living ovary parenchyma cells, monoclonal antibody JIM 5 and JIM 7 labeling was limited to a thin host wall layer (A, open arrows, and C, arrow), or the host wall and the JIM labeling were completely lacking between the fungal and the host plasma membrane (B, open arrow, and D, arrow) of hyphae (F) in phloem parenchyma (B, pp) near sieve tubes (B, st). A and B, Demethylation with sodium carbonate and labeling with JIM 5 for polygalacturonate acid, 8 days post inoculation (dpi); and C and D, labeling with JIM 7 for methylated polygalacturonate acid, 8 dpi. Scale bars = 1 μm.
completely during the infection process, whereas host galacturonan was incorporated into the sheaths around the invading fungal cells.

In external mycelia that were more distant from the outer epidermal wall, hyphal sheaths were completely devoid of any JIM 5 gold labeling. When hyphae grew epidermically above an apparently intact cuticular membrane, which forms a barrier between the pathogen and polysaccharide wall layers of the host, no modification of the galacturonan labeling was noticed in the outer epidermal wall (data not shown).

At the interface of host cells and hyphae growing intercellularly in the carpel mesophyll, the JIM 5 labeling pattern was modified. The label density of the outer surface of host cell walls facing the intercellular space was moderate when distant from fungal cells, but labeling was more dense whenever the cell wall was in direct contact with the pathogen. Because the intercellular space is not continuous throughout the pericarp, the fungus enters mesophyll cell walls at the junction sites on a route toward the ovary base (Fig. 9A, arrowhead). Host cells were separated by intercellular hyphae in the middle lamella zone. In front and to the sides of intercellular hyphae growing in the host wall, loosened cell wall material exhibiting a fibrillar structure was commonly observed attached to the fungal wall surface (Fig. 9B). This material was continuous with the middle lamella and stained as more electron-dense with uranyl and lead salts. This material was heavily immunoreactive with JIM 5 for polygalacturonic acid. Its intense gold labeling extended into weaker labeling above the unaffected middle lamella, exhibiting a stippled line of gold particles (Fig. 9B). In addition to the observed modification, infection with ergot resulted in another change in the wall structure, as was concluded from specific, intense JIM 5 labeling detected not only above the middle lamella area, but throughout the entire host cell wall (Fig. 9A and B, open arrows).

Toluidine blue O staining of semithin sections of colonized ovary tissue resulted in a red color for carboxylated polysaccharides. Staining was most intense at the interface of host cells and hyphae splitting adjacent cells by intercellular growth, whereas uncolonized host tissues were only weakly stained (data not shown). A light microscopic overview showed that the whole surface of the hyphae, in contact with host cells, was stained red, which most likely corresponded to the JIM 5 label for polygalacturonic acid. However, immunogold electron microscopy revealed that the red layer was not part of the fungal cell wall but represented host wall areas with an altered polysaccharide structure.

Sodium carbonate treatment and subsequent processing with JIM 5 resulted in intense gold labeling of host cell walls independent of hyphal locations. In colonized tissues far away from a host-parasite interface, the JIM 5 labeling was not limited to a narrow subcuticular layer or to the middle lamella zone but occurred throughout the entire wall (Fig. 8D and 9D). After demethylation experiments, JIM 5 labeling indicated a high total content of galacturonan in the complete cell walls, except for an innermost attached layer, with a coarse fibrillar structure, that sometimes was produced by pericarp cells lacking any galacturonan constituents (Fig. 8D).

In later infection phases, the host ovary tissue above the stable host-parasite boundary in the rachilla tip completely disintegrated. Next to the boundary, host remnants were often located between fungal cells. JIM 5 labeled fibrillar structures, which indicated they contained methylelated pectic substances and were cell wall fragments of host origin (Fig. 9C). After the late sphaecial phase, no JIM 5 or JIM 7 gold labeling was observed in the former ovary area now occupied by fungal structures, indicating the complete elimination of host galacturonans. No specific gold labeling was found above fungal areas, neither above the fungal cell wall nor above its protoplast (Figs. 8 to 10).

At particular sites in the colonized ovary tissue, no gold label was detected after immunolabeling with either of the two MAbs.

Hyphal cells were located in certain mesophyll cells, especially in phloem parenchyma cells next to sieve tubes in the rachilla vascular tissue (Fig. 10). Both the invading hyphae and the invaded host cells were still living, as judged by the well-preserved ultrastructural features found after embedding glutaraldehyde- and osmium tetroxide-fixed samples in epoxy resin (data not shown). At first, intercellular hyphae left the middle lamella zone and grew in the primary cell wall of one phloem parenchyma cell. Finally, the intercellular hyphae breached the host cells (Fig. 10A). The wall of the latter was clearly loosened, exhibiting a wide-meshed fibrillar polysaccharide network that was markedly reduced in thickness and total amount of galacturonan label toward the plant protoplast. However, after demethylation, JIM 5 labeling indicated that galacturonans were still present in the thin plant wall layer (Fig. 10A, open arrows). These galacturonans contained methylated galacturonic acid epitopes, made visible by JIM 7 gold labeling (Fig. 10C, open arrows). At the interface of other sectioned hyphal parts in the same host cell, neither JIM 5 label after deesterification nor JIM 7 label was present (Figs. 10D and B). Therefore, the galacturonans were completely absent between the fungal wall and the host plasma membrane. Because the plant cell wall was completely broken down, hyphae were living intracellularly in these host cells.

DISCUSSION

Using the A. niger pgall1 gene as a probe, we isolated two PG genes (pg1 and pg2) from strain T5 of C. purpurea. The high homology of both genes with other fungal endo-PG genes strongly indicates that they also code for an endo-cutting enzyme, although detailed enzymatic characterization is necessary to prove this assumption. pg1 and pg2 are closely linked (separated by about 1.0 kb) and arranged in tandem—a rather unusual orientation for fungal genes. A comparable situation has been described for two glyceraldehyde-3-phosphate dehydrogenase genes of Agaricus bisporus, in which the upstream copy is inactive (23), and in three planta-induced genes of Phytophthora infestans (35). Comparative Southern blotting and PCR analyses of other field isolates indicated that this special orientation is conserved in this species and that the underlying gene duplication process cannot be a recent event—it must have occurred before or concomitant with species development. It would be interesting to check other species of the genus and other Clavicipitales for gene duplication. The stable existence of such a tandem structure of two obviously active genes indicates that mechanisms such as repeat-induced point mutation and methylation induced premeiosically found in Neurospora (43) and Aspergillus (39) are unlikely to exist in C. purpurea, because these mechanisms strictly inactivate duplicated sequences. Because meiosis is an obligate process in the life cycle of C. purpurea, the hypothesis proposed by Kistler et al. (27), that gene duplications are restricted to asexually propagating fungi, might not be valid here.

The existence of two or more unlinked PG genes is quite common in filamentous fungi. Whereas Cochliobolus carbonum (42) and F. moniliforme (10) seem to contain only one (endo)-PG gene, in A. niger three genes have been identified so far, and several more are suspected (8). A family of PG genes exists in Sclerotinia sclerotiorum (38). In C. purpurea strain T5, Southern analyses (even with low stringency) did not indicate the existence of PG genes other than pg1 and pg2. Both genes are highly conserved, not only with respect to their nucleotide sequence in the coding and even in major parts of the 5′ noncoding region, but also in the position and size of an intron. Most nucleotide differences in the coding region are neutral, but there are 34 aa changes, 17 of them analog; an in-frame deletion of 3 nucleotides in pg1 leads to a deletion of 1 aa. The high degree of homology even of the 5′ noncoding region up to ~350 bp is unexpected, because it should not be under such strict selective pressure. A similar situation has
been reported for the niaD genes of *F. moniliforme* and *F. oxysporum* (50).

The 5′ upstream sequences of the two genes differ mainly in the deletion in pg1 at -45, which did not destroy any putative promoter element but did alter the spatial organization of the elements. This does not seem to cause inactivation of pg1, because RT-PCR analyses showed that both genes are expressed as well in axenic culture as in planta from very early stages of infection up to the beginning of sclerotia formation.

In axenic culture, very low PG activity is detectable, and the activity is not subject to carbon and nitrogen catabolite repression. This is in contrast to the situation in other phytopathogenic fungi (53), in which cell wall-degrading enzymes normally are repressed by catabolites. In *C. purpurea*, xylanase and β-1,3-glucanase activity are under glucose repression, however, cellulase activity is not (S. Griesbert, U. Müller, and P. Tudadzynski, unpublished data; [7]). In many cases, cell wall-degrading enzymes are substrate-inducible, which does not seem to be the case for pg1 and pg2. This might be due to the lack of natural substrate, as has been reported for other pathogens, because pectic substances differ considerably in different plants and tissues (11). However, the extremely low activity of PG in axenic culture of *C. purpurea* also could be due to the more biotrophic mode of growth of this fungus. Biotrophic fungal pathogens often utilize PGs in a well-balanced mode, possibly to avoid cytotoxic effects that are found for PGs (12).

Our RT-PCR experiments indicated that PG activity measured in honeydew and extracts from infected ovariies by Shaw and Mantle (44) is of fungal origin, or at least, fungal PG contributes to the detected PG activity. In general, two functions are attributed to PGs in pathogenic interactions: primarily, they degrade pectic substances in the middle lamella, resulting in tissue maceration and degradation of pectic components in the cell wall, weakening the wall and increasing pore size. Secondarily, PGs release oligosaccharides involved in eliciting or signaling. As far as the interaction system of *C. purpurea* and its host plant is concerned, the ultrastructural and immunocytological investigations provide substantial evidence for a function of PGs during parasitism.

According to the model of molecular cell wall architecture generalized by Carpera and Gibeaut (11), based mainly on coleoptiles and foliage leaves of oat or maize, grasses have developed a special cell wall type that is characterized by low content of pectin components (15). Nevertheless, in oat roots, pectin components are distributed in tissue-specific manner (28), and organ- or tissue-specific diversity of the primary wall structure is apparent, especially in flowers, fruit, and epidermal tissues (11). Therefore, we questioned whether ovary cell walls really contain certain pectic components at all. To establish the role of PGs in pathogenicity, we examined where exactly these pectic components occur along the potential infection route throughout the histologically heterogeneous pistil of rye.

Using immunogold labeling, we discovered antigens reacting with MAb JIM 5, which specifically recognizes homogalacturonic acid sequences in nonesterified or low methyl-esterified pectin molecules, as demonstrated by VandenBosch et al. (52) and Knox et al. (28). In ovarian mesophyll cells of rye, JIM 5 epitopes were abundant in the outer wall surface of intercellular spaces and in cellular junctions. The epitopes were less abundant in the middle lamella and rare at the cell wall surface adjoining the plasma membrane. Therefore, we conclude that homogalacturonic acid is a constituent of cell walls of rye ovaries. In addition, our labeling results showed that JIM 5 epitopes were located in cell walls of ovary and rachilla epidermal cells, carpel mesophyll cells, rachilla cortical cells, and cells of rachilla vascular tissue. These results confirm that potential substrates of PGs, homogalacturonic acid regions in pectin, are present in all ovarian tissues along the potential infection path.

Along with nonmethyl-esterified galacturonic acid, the primary walls of the same host cells contained methyl-esterified homogalacturonic acid epitopes in their pectin wall polysaccharides, demonstrated by specific immunoreactions with MAb JIM 7. In this respect, the rye ovary tissue differed from other leaf types of grasses that contain one unique type of galacturonan (28). Compared with the gold label distribution and density after direct JIM 5 labeling, the deesterification experiments and subsequent JIM 5 treatment demonstrated that the bulk of the cell wall pectin was of the methylated type of galacturonan. Therefore, the major quantity of this pectin is not utilizable by the fungal PGs without preceding modification.

In infected ovaries at the interface of host cells and hyphae growing subcuticularly, penetrating the epidermis, or growing intercellularly, we detected intense labeling of JIM 5 epitopes in the adjacent host cell wall zone, whereas distant from hyphae the labeling density remained weak. After contact with fungal hyphae, the number of immunoreactive sites recognizable by JIM 5 labeling increased drastically. Three explanations, at least, may account for this phenomenon.

(i) PG activity could be involved. *C. purpurea* grows mainly intercellularly in the host pistil, completely separating cells, at which fine structural alterations in the middle lamella zone are detectable by electron microscopy (44, 44, 51). Enzymatic degradation of the cementing material has long been speculated to be the mechanism for this mode of growth. This idea is supported by the finding of pectinase activity in axenic culture and honeydew exudates of parasitic culture (44) and by the evidence presented in this paper that PG genes are expressed in planta. PG activity might cause maceration of host tissue, making the interface zone highly accessible to JIM 5 antibodies in widened and loosened middle lamella areas. On the other hand, because these changes are the result of a local polygalacturonan digest, one could certainly expect a reduction or absence of label. Possibly, the galacturonan fragments remain fixed to the complex polysaccharide network by phenolic cross-links that are common in grasses (11) until the host wall becomes completely removed, as seen in later infection phases. Probably, the action of PGs alone is not sufficient to breach or decompose the host cell walls completely, but it can make them more accessible to other fungal enzymes, such as cellulases and xylanases (26), e.g., by enlarging the pore size of the host walls (11). This could be an important effect of PG activity, because it enables large fungal proteins, such as the 92-kDa β-1,3-glucanase that is supposed to degrade callose deposited in the host periplasmic space, to diffuse through the host wall.

(ii) Other fungal proteins with cell wall-degrading capacity, such as cellulases or xylanases, could result in intensified JIM 5 gold labeling. These enzymes might change the cell wall architecture in such a manner that polygalacturonan is laid open and, consequently, accessibility to JIM 5 increases. Recently, carboxymethyl-cellulose decomposing activity has been found in *C. purpurea*, and a putative celllobiohydrolase gene (*cel1*) has been cloned (U. Müller, and P. Tudadzynski, unpublished data; [33]). We were able to demonstrate the absence of cellulase in situ at exactly those sites marked intensely with JIM 5 gold label (49).

(iii) There could be an increase in pectin methyllesterase (PME) activity. Chemical demethylation experiments resulting in observed intense JIM 5 labeling throughout the host wall emphasizes that the locally intensified label at the interface might be due to fungal PME. It is likely that the activity of PME causes a decrease in the degree of methyl-esterification, and this alteration of the molecular structure of pectin most likely creates additional antigenic sites for JIM 5. This phenomenon was found recently in host walls infected with the rust *Uromyces vignae* (17). Further support comes from the positive JIM 5 labeling visible in the entire host cell wall at the interface, whereas labeling in the primary wall of the same cell was missing when distant from hyphae. Recently, low PME
activity has been found in axenic culture of *C. purpurea* (H. Harling and P. Tuzdynski, unpublished data).

The enhanced labeling by JIM 5 revealed an increased number of anionic sites at the host-parasite interface. Most likely with PME activity causing this negatively charged interface throughout the infection route, the fungus might control the environment for other extracellular enzymes that are presumed to be important for pathogenicity. Secreted proteins with an isoelectric point in the range of the apoplastic pH, which is about 5.0 to 6.5 (19), would freely diffuse in the wall, whereas basic forms would be bound to the wall due to their positive charge. Strongly basic enzymes, e.g., xylanase and β-1,3-glucanase, are secreted by *C. purpurea*. The latter is immunolocalized in the host wall and is confined to the narrow interface (49). This phenomenon of electrostatic interactions, recently discussed by Deising et al. (16), would limit the activity of such basic proteins to the host-pathogen interface, restricting operation and damage to a small area, which is essential for biotrophy (14).

At present, we can only speculate about which one of these three alternatives might be valid. However, apart from the PG activity, we favor simultaneous action of PME. The data clearly show the presence of both substrates, low amounts of nonesterified and higher quantities of methyl-esterified galacturonic acids, in the cell wall material surrounding the growing hyphae. The distribution of the polygalacturonic acid fibrils in hyphal sheaths formed in the outer epidermal wall, especially its local and graded fine structural alteration of pectic components, points to a fungal growth mechanism mediated by enzymatic operation rather than by pressure. PME activity could convert the bulk of the pectic wall components into a suitable substrate for digestion by PCs. Complete homogalacturanan degradation, verified by the absence of any label in late infection phases, in addition to splitting of middle lamellae for intercellular growth, supports a role for PCs in the colonization process.

In addition to this function as putative fungal pathogenicity factors, PCs might play a completely antagonistic role: action of PCs may lead to the formation of elicitors, inducing the plant's defense reactions (1), including synthesis of PGIPs (PG-inhibiting proteins [3,21]). The JIM 5 label of the host epidermal walls and the hyphal sheaths during primary penetration could indicate the formation of polygalacturonic acid fragments, which might be cleaved from pectin molecules and set free from the host wall. These fragments possibly could serve for elicitation; however, during the colonization phase, host defense reactions of a structural type were rarely noticed microscopically. PGIPs have been found only in dicots thus far.

To study the role of pectinases in host-fungus interactions, targeted gene-disruption experiments have been performed in several systems. Disruption of the (single) endo-PG gene of *Colletotrichum carbonum* abolishes endo-PG activity completely (leaving some exo-PG activity) but has no significant influence on pathogenicity (42). The same holds true for the PG gene of *Penicillium oxalotum* (31) and a pectin lyase gene from *Glomerella cingulata* (6). These deal reports deal with necrotrophic fungi that normally have a whole set of pectolytic enzymes, which could complement each other, e.g., pectin/pectate lyases for loss of PGs. In the biotrophic fungus *C. purpurea*, the situation might be different, because very low activity of cell wall-degrading enzymes occurs. The tandem orientation of pg1 and pg2 provides the possibility of disrupting both genes in one step. Therefore, significant proof for the role of PGs in the interaction is conceivable.

**LITERATURE CITED**


