Regulation of acp1, encoding a non-aspartyl acid protease expressed during pathogenesis of Sclerotinia sclerotiorum

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When grown in the presence of sunflower cell walls, Sclerotinia sclerotiorum, an ubiquitous necrotrophic fungus, secretes several acid proteases including a non-aspartyl protease. The gene acp1, encoding an acid protease, has been cloned and sequenced. The intronless ORF encodes a preproprotein of 252 aa and a mature protein of 200 residues. In vitro expression of acp1 is subject to several transcriptional regulatory mechanisms. Expression induced by plant cell-wall proteins is controlled by both carbon and nitrogen catabolite repression. Glucose on its own represses acp1 expression while ammonium repression requires the simultaneous presence of a carbon source. Ambient pH higher than pH 5 overrides induction resulting in full repression of acp1. These transcriptional regulatory mechanisms and the presence of several motifs in the promoter of acp1 that may encode binding sites for the regulators CREA, AREA and PacC suggest the involvement of these regulators in the control of acp1 expression. acp1 is expressed in planta during sunflower cotyledon infection. Expression is low at the beginning of infection but increases suddenly at the stage of necrosis spreading. Comparison of in vitro and in planta acp1 expression suggests that glucose and nitrogen starvation together with acidification can be considered as key factors controlling Scl. sclerotiorum gene expression during pathogenesis.

Keywords: acid protease, gene expression, pH regulation, carbon and nitrogen repression, plant pathogenesis

INTRODUCTION

During interaction of a plant parasitic fungus with its host, the pathogen secretes extracellular enzymes which can degrade the host cell-wall components. These enzymes not only contribute to generate an important assimilable nutrient source but also facilitate penetration, colonization and maceration of the host tissues. Biochemical analysis of the infection process revealed that pathogenic fungi produce a wide range of cell-wall-degrading enzymes able to attack all the cell-wall polymers i.e. carbohydrates and proteins. These enzymes include cellulases, pectinases, xylanases, a range of oligosaccharide-degrading enzymes and proteases (for reviews see Oliver & Osbourn, 1995; Annis & Goodwin, 1997; Walton, 1994). Among the cell-wall degrading enzymes produced by phytopathogenic fungi, pectinolytic enzymes have been well studied as they are typically produced in large amounts and are able to macerate plant tissues (Alghisi & Favaron, 1995). Conversely, other enzymes such as proteases have received limited attention. However, based on the cell-wall structure and on the fact that up to 10% of the plant cell wall consists of proteins (Carpita & Gibeaut, 1993), proteolytic enzymes probably play an important role in pathogenesis. Fungal proteases would facilitate localized penetration of the pathogen by breaking down the structural fibrous glycoproteins of the plant cell wall (Rauscher et al., 1995). They could also provide nitrogenous nutrients to the fungus during infection.

The possible roles of fungal proteases in plant pathogenicity have only been investigated in a few systems and contradictory results have been obtained.
aspartyl protease has been identified as an important virulence factor in infections by Botrytis cinerea and as one of the factors required for establishing fungal growth within the host (Mohavedi & Heale, 1990). In Pyrenopeziza brassicae, evidence that a cysteine protease may represent a pathogenicity determinant has been presented by complementation of a protease-negative, non-pathogenic mutant to obtain a fully pathogenic protease-positive transformant (Ball et al., 1991). In contrast, disruption of two alkaline protease encoding genes in Cochliobolus carbonum reduced the proteolytic activity without affecting virulence of the mutants. This indicates that these two enzymes are not required for pathogenicity of C. carbonum (Murphy & Walton, 1996). The importance of proteases in pathogenicity is also suggested by the fact that plants have evolved mechanisms to counter pathogen-secreted enzymes by producing protease inhibitors (for review, see Ryan, 1990). These inhibitors may act as regulators of endogenous enzymes. They could also contribute to plant defense and host resistance by inhibiting fungal proteases and by exhibiting a strong antifungal activity (Pernas et al., 1999; Chen et al., 1999).

Extracellular proteases are produced by many pathogenic fungi but the profiles of proteases these organisms produce have often not been thoroughly investigated. It has been proposed that the range of proteases produced by a fungus reflects the adaptation of saprophytic or pathogenic fungi to the requirements of their ecological niches (St Leger et al., 1997; Bidochka et al., 1999a, b). One can imagine that in response to environmental signals, protease production during pathogenesis must be regulated by the structural cell-wall proteins, nitrogen and/or nutrient limitation, and the ambient pH.

Sclerotinia sclerotiorum is an ubiquitous necrotrophic fungus that is able to infect a wide range of cultivated plants resulting in important economic losses. Extracellular proteins secreted by this fungus are able to degrade host cell-wall components and to macerate the plant tissues. They contain the glycoside hydrolase activities that complement polysaccharidase enzymes to release monomers from each plant cell-wall polymer (Riou et al., 1991). Each enzymic system contains several endo- and exo-enzymes and, like the pectinolytic system, must be encoded by a multigene family (Martel et al., 1996; Fraissinet-Tachet et al., 1995). With respect to their potential contribution to cell-wall degradation and matrix-protein hydrolysis, characterization of proteases and isolation of all the genes for a given enzyme are of importance in order to test the role of proteases in plant pathogenicity. During saprophytic growth and pathogenesis, Scl. sclerotiorum secretes large amounts of oxalic acid which accumulates in infected tissues and decreases rapidly the ambient pH (Magro et al., 1984). As ambient pH regulates gene expression (Caddick et al., 1986; Tilburn et al., 1995), we have focused our attention on acid proteases and we describe here the characterization and regulation of acpI, a gene that encodes a non-aspartyl acid protease expressed during the infection process.

**METHODS**

**Biological material and culture conditions.** Sclerotinia sclerotiorum S5 was maintained on potato-dextrose agar. It was grown in minimal liquid medium containing (per litre) 1 g KH$_2$PO$_4$, 0.1 g MgSO$_4$, 0.05 mg biotin, 250 mg citric acid, 230 mg ZnSO$_4$, 50 mg Fe(SO$_4$)$_2$, 15 mg Cu(SO$_4$)$_2$, 25 mg each of MnSO$_4$, H$_2$BO$_3$, Na$_2$MoO$_4$, and supplemented with 1% sunflower extract, or 2% glucose and 100 mM NH$_4$Cl. Sunflower extracts were prepared from cotyledons of 1-week-old germlings. Cotyledonal leaves were cut into very small pieces and were frozen until use. The culture medium was buffered at different pH values in 0.15 M citrate/phosphate (MacIlvaine) buffer. The cultures were inoculated with mycellal disks cut from 4-d-old colonies and incubated at 24°C under constant agitation.

Escherichia coli strain Sure R (Stratagene) was the host for recombinant plasmds and was grown in LB broth medium supplemented with ampicillin (50 μg ml$^{-1}$). E. coli P2392 was used for bacteriophage lambda EMBL3 screening and was grown in NZY medium (Sambrook et al., 1989). The plasmid pUC18 was used for cloning experiments.

**Pathogenicity tests.** Phytopathogenicity assays were performed on sunflower cotyledons as hosts. Sunflower seeds were sown in a peat/pouzzolane mix. Germlings were grown at 25°C (95% humidity) with a 14 h light period per day. Cotyledonal leaves from 1-week-old germlings were infected by depositing a 4 mm mycelial disk on the upper face of the cotyledons. At various times after inoculation (corresponding to different stages of disease development), infected cotyledons were harvested and frozen at −80°C. Each assay was carried out three times on separate plants. Controls were performed using mycellal disks previously heated for 30 min at 65°C. All experiments were performed twice and the same pattern of symptom development was found.

**Acid protease assays.** The culture medium (200 ml) was filtered to eliminate the mycelia, dialysed against distilled water and then freeze-dried. Thirty infected sunflower cotyledons, collected at different stages of symptom development, were ground in a mortar and pestle in cold Tris–HCl buffer. The cultures were inoculated with mycelial disks cut from 4-d-old colonies and incubated at 25°C, pH 7.5. After 10 min centrifugation at 10000 g, supernatants were collected, dialysed against distilled water then freeze-dried. Lyophilized proteins (i.e. proteins secreted in the culture medium or extracted from infected cotyledons) were dissolved in 3 ml water and assayed for protease activity according to Griffen et al. (1997). ACP1 was assayed in a reaction mixture containing 200 μl enzyme solution, 900 μl 100 mM KCl/HCl buffer, pH 2, and 100 μl 15% (w/v) azocasein dissolved in the buffer. The following buffers were used to determine the pH profile of the secreted proteases: 100 mM KCl/HCl (pH 1.5–2.5); 100 mM citrate/phosphate (pH 2.5–7.5); and 100 mM Tris/HCl (pH 7.5–9). Following a 2 h incubation at 37°C, the reaction was stopped by the addition of 300 μl 30% (w/v) TCA. After centrifugation of the assay mixtures for 7 min at 13000 g, 500 μl supernatant was withdrawn and the colour reaction was developed by the addition of 500 μl 1 M NaOH and measured at 450 nm. Appropriate controls without either enzyme or substrate were run simultaneously. One arbitrary unit of protease activity was defined as the amount of enzyme necessary to develop an absorbance of 0.5. Protein determinations were carried out by the method of Bradford (1976). Specific active-site inhibitors were incubated with the enzymes for 30 min prior to assaying the protease activity. The final concentrations for protease inhibitors (Sigma) used were 0.04 mM E-64 (a cysteine protease inhibitor), 0.08 mM PMSF (a serine protease inhibitor), 0.04 mM phosphoramidon.
(a metalloprotease inhibitor) and 0.04 mM pepstatin A (an aspartyl protease inhibitor). All protease assays were run in triplicate and data are shown as mean and standard deviations. The results of one representative experiment are presented.

**DNA isolation and Southern blot analysis.** DNA was prepared as described by Brownlee et al. (1988) from freeze-dried mycelium grown on potato-dextrose agar. DNA was digested to completion, electrophoresed on a 0.8% agarose gel and blotted onto Nytran membranes (Schleicher and Schuell). Membranes were hybridized (50%, w/v, formamide, 5× SSC, 0.2%, w/v, SDS, 1× Denhardt’s solution, 100 μg denatured salmon sperm DNA ml⁻¹) at 42 °C with a 32P-labelled PCR fragment or acp1 coding region. The probes were radiolabelled using a random-primed DNA labelling kit (Promega). Following overnight hybridization, membranes were washed with 2× SSC, 0.1% (w/v) SDS then with 0.2× SSC, 0.1% (w/v) SDS.

**RNA isolation and Northern analysis.** Total RNA was isolated from freeze-dried mycelium or infected cotyledons after lysis in a buffer containing 50% (w/v) guanidinium thiocyanate, followed by centrifugation in caesium chloride solutions (Sambrook et al., 1989). For Northern blotting, 15 or 30 μg total RNA was loaded per lane onto 1.5% (w/v) formaldehyde agarose gels, transferred to Nytran membranes after electrophoresis and UV cross-linked. Hybridization and washing of the membranes were done as described above. The membranes were stripped between hybridizations with different probes by washing in 5 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, pH 8.5, 0.05% (w/v) pyrophosphate, 0.1× Denhardt’s solution for 1 h at 65 °C (Sambrook et al., 1989).

**Preparation of a probe by PCR.** For amplification of an acp1-specific fragment, *Scl. sclerotiorum* genomic DNA was used as the template in a PCR reaction with two degenerate oligonucleotide primers. They were respectively a 16-mer and a 14-mer of 8- and 96-fold degeneracy and were synthesized as the template in a PCR reaction with two degenerate oligo-

**RESULTS**

**Detection of a secreted acid protease activity**

Mycelium from *Scl. sclerotiorum*, grown for 48 h in a minimal medium containing glucose and NH₄Cl, was harvested by filtration, washed in sterile water then transferred to minimal medium supplemented with sunflower extracts used as the sole carbon and nitrogen sources. Eight hours after transfer, the cell-free culture medium was collected and assayed for protease activity. At this time, this enzymic activity was maximal and remained stable for several hours. The pH optimum of the protease activity was determined. The highest level of activity was detected at pH 4.5 and a minor peak was repeatedly found at pH 2. At least two families of proteases are therefore likely to contribute to the total proteolytic activity measured under these culture conditions. Pepstatin, a specific inhibitor of aspartyl proteases, was tested in order to identify the type of proteases responsible for this acidic enzymic activity. The activity at pH 4.5 was strongly inhibited (75%) by pepstatin, while the activity at pH 2 was only weakly inhibited (15%). These data indicate that the main protease activity secreted to the medium corresponds to an aspartyl protease. On the other hand, the enzyme responsible for the activity at pH 2 seems to belong to another class of acid proteases. PMSF, E-64 and phosphoramidon, inhibitors of serine, cysteine and metallo-proteases, respectively, did not affect this latter activity, indicating that the acid activity secreted in the medium containing cell walls consists of overlapping activities of aspartyl and non-aspartyl proteases (G. Billon-Grand, N. Poussereau & M. Fève, unpublished). The acidic pepstatin-non-inhibitable enzymic activity exhibits properties similar to those of the non-aspartyl acid proteases from *Aspergillus niger* (Iio & Yamasaki, 1976), *Scytalidium lignicolum* (Maita et al., 1984) and *Cryphonectria parasitica* (Jara et al., 1996). It was therefore of interest to isolate and characterize the gene encoding this enzymic activity to understand the regulation of its expression during pathogenesis.

**Isolation and characterization of acp1**

Acid proteases have been isolated and characterized for only three filamentous fungi: *A. niger* (Takahashi et al., 1991), *Scy. lignicolum* (Maita et al., 1984) and *C. parasitica* (Jara et al., 1996). Comparison of the amino acid sequences of these proteases revealed several conserved regions which could be used for designing primers for PCR amplification. Oligonucleotides corresponding to the conserved regions WYEYW and NAEWI allowed amplification of a single fragment of 204 bp from *Scl. sclerotiorum* genomic DNA. Its identity was confirmed by sequencing and by comparison with the sequences of known acid proteases.

The PCR fragment, cloned in pUC18, was used as a probe to screen a genomic library of *Scl. sclerotiorum* constructed in the lambda phage EMBL3 (Riou, 1991). Four hybridizing clones were selected out of 10⁴ individual recombinant bacteriophage plaques. The phages were isolated, purified, and their DNA digested with several restriction enzymes and subjected to Southern analysis. A DNA fragment of 1.3 kb, present in all the phages hybridizing to the probe, was cloned into the *PstI* site of pBluescript, yielding the plasmid pAC1.

Southern blot analysis of genomic DNA of *Scl. sclerotiorum* was performed using the 204 bp PCR fragment described above as a probe. Genomic DNA was digested with different restriction enzymes for which no internal site was present in the amplified fragment, transferred to a Nylon membrane and hybridized under low stringency. Southern analysis showed that the PCR fragment hybridized to a single band (data not shown), indicating that the acid protease activity is encoded by a single
gene, as described for \textit{A. niger} and \textit{Scy. lignicolum} (Takahashi \textit{et al.}, 1991; Oda \textit{et al.}, 1998).

The nucleotide sequence of the cloned fragment determined by sequencing both strands revealed that it contains the entire nucleotide sequence of the \textit{acp1} gene (GenBank accession no. AF221843). The \textit{acp1} gene consists of an intronless ORF of 739 bases, which encodes a polypeptide of 252 aa. By comparison with the N-terminal region of secreted acid proteases of \textit{C. parasitica} (Jara \textit{et al.}, 1996) and \textit{A. niger} (Takahashi \textit{et al.}, 1991), it may be assumed that \textit{acp1} is synthesized as a zymogen following the general rule for fungal acid proteases (aspartyl or non-aspartyl proteases) and contains an N-terminal preproregion of 52 aa (Fig. 1). The mature form of the enzyme would be a 200 residue protein with a calculated molecular mass of 20.7 kDa and a calculated pI of 3.9. The deduced primary sequence of the mature protease contains one potential N-linked glycosylation site.

The mature ACP1 protein exhibits 62\% identity with protease A (PRTA) of \textit{A. niger} (Takahashi \textit{et al.}, 1991), 65\% and 55\% identity with EAPC and EAPB of \textit{C. parasitica} (Jara \textit{et al.}, 1996), respectively, and 50\% identity with SLB of \textit{Scy. lignicolum} (Maita \textit{et al.}, 1984). In \textit{A. niger}, the enzyme has a two-chain structure with a light chain non-covalently linked to the heavy chain.

![Fig. 1. Comparison of the predicted amino acid sequence of ACP1 of \textit{Scl. sclerotiorum} with the corresponding sequences of the fungal acid proteases PRTA from \textit{A. niger}, SLB from \textit{Scy. lignicolum}, EAPB and EAPC from \textit{C. parasitica}. Amino acids that are conserved in all proteins are marked by asterisks; dots indicate conservative replacements. Dashes indicate gaps introduced by the CLUSTAL program to optimize the sequence alignments. The N-terminal residue of mature PRTA, EAPC and SLB is underlined. The putative N-terminal residue of ACP1 and EAPB is also underlined. The intervening sequence separating the two chains of PRTA is boxed.](image-url)
Regular amino acid sequences are found in the deduced polypeptide sequences of the other genes encoding acid proteases (Fig. 1). By comparison with the enzymes from Scytalidium lignicolum and Cladosporium parasitica, the acid protease from Sclerotinia sclerotiorum appears to be a single-chain enzyme.

**acp1 expression during saprophytic growth of Sclerotinia sclerotiorum**

Transfer experiments were performed to investigate acp1 expression during growth in the presence of plant extracts. *Sclerotinia sclerotiorum* was grown for 48 h in a medium containing glucose and NH$_4$ then collected, washed with distilled water and transferred to a minimal medium containing sunflower extracts. Total RNA isolated at different times after transfer was hybridized with acp1 then with 16S rDNA (Fig. 2). No acp1 signal was detected at the time of transfer to minimal medium, indicating that the protease gene is not constitutively expressed. Four hours after transfer, a strong signal was observed and 8 h after transfer the hybridization signal decreased and then appeared to remain at a constant level. On the other hand, the enzymatic activity measured in the culture medium, which seems likely to be attributable to the acp1 gene product, continued to increase up to 8 h after transfer then remained high. *Sclerotinia sclerotiorum* secretes oxalic acid during growth (Magro et al., 1984). Consequently, the pH of the culture medium decreased rapidly and 6 h after transfer stabilized to pH 2.5–3 (Fig. 2). Constant levels of pH and of acp1 transcripts were observed at the same time following transfer, suggesting that the ambient pH may affect acp1 expression.

**acp1 expression is pH regulated**

The effect of the ambient pH of the medium was studied by transferring mycelia cultivated for 48 h in a minimal medium supplemented with glucose and NH$_4$ to unbuffered or buffered media at pH 3, 4, 5 and 6 containing sunflower extracts as the carbon and nitrogen sources. Eight hours after transfer, total RNA was extracted for Northern analysis and blots were incubated with the acp1 and rDNA probes. The data presented in Fig. 3 show that acp1 expression is strictly dependent on the ambient pH. Expression was restricted to a very narrow range of pH, being maximal at pH 4, weakly expressed at pH 3 and 5, and not detected at pH 6. In the unbuffered medium, the pH progressively decreased from 4.4 to 2.6, allowing the strong expression of acp1.
during this decrease. These data show that pH regulation overrides induction by the plant extracts and that an acidic pH is not sufficient to allow acp1 induction as no transcripts were detected in the glucose-NH₄ medium.

Carbon and nitrogen regulation of acp1

The effect of carbon and nitrogen sources on acp1 expression was examined by Northern analysis (Fig. 4). Mycelia were grown for 48 h in a glucose-NH₄ medium then transferred to a medium containing sunflower extracts (lane 2) supplemented with ammonium (lanes 3–5), ammonium and 2% v/v glycerol (lanes 6–8), 2%, v/v, glycerol (lane 9) or 2% glucose (lane 10). Eight hours after transfer, the pH of the culture medium was measured. Total RNA was extracted, electrophoresed on agarose gels and hybridized with acp1. As a control for RNA loading, filters were stripped and re-probed with the rDNA probe.

**Fig. 4.** Effects of carbon and nitrogen sources on acp1 expression. Mycelia were grown for 48 h in a glucose-NH₄ medium (lane 1) then transferred to a medium containing sunflower extracts (lane 2) supplemented with ammonium (lanes 3–5), ammonium and 2%, v/v glycerol (lanes 6–8), 2%, v/v, glycerol (lane 9) or 2% glucose (lane 10). Eight hours after transfer, the pH of the culture medium was measured. Total RNA was extracted, electrophoresed on agarose gels and hybridized with acp1. As a control for RNA loading, filters were stripped and re-probed with the rDNA probe.

Carbon source, is provided, nitrogen repression of protease expression can occur. This pattern of transcriptional regulation is observed for the genes involved in the utilization of metabolites such as proline or aminobutyric acid which can serve as both carbon and nitrogen sources (Arst & Bailey, 1977; Marzluf, 1997).

Analysis of the acp1 promoter sequence

The experiments described above show that acp1 expression is controlled in a complex manner by several environmental factors. It was of interest to examine the 5’ non-coding sequence for the presence of motifs involved in the regulation of fungal gene expression (Fig. 5). The sequence contains 19 copies, in each orientation, of the GATA motif which is the recognition sequence of the nitrogen regulatory proteins AreA from *A. nidulans* (Kulmburg et al., 1993) and NIT2 from *Neurospora crassa* (Fu & Marzluf, 1990). However, only eight of these motifs at the positions –912 and –903, –867 and –856, –522 and –498, –131 and –111 are in an inverted-repeat orientation separated by less than 30 bp, corresponding to the functional organization of the GATA binding sequences in *N. crassa* (De Bernardis et al., 1998; Marzluf, 1997; Caddick et al., 1994). There are three degenerate copies of the recognition sequence 5’-SYGGRG-3’ of CREA, a factor involved in glucose-mediated carbon catabolite repression (Kulmburg et al., 1993). Only one copy of the motif 5’-GCCARG-3’, the recognition site for the PACC protein mediating pH regulation in *A. nidulans* (Tilburn et al., 1995), is present. The presence of these motifs, together with the data obtained from Northern blot analyses, suggest
that acp1 is under the control of homologous wide-domain regulatory genes in *Scl. sclerotiorum*.

**Expression of acp1 during sunflower infection**

In planta expression of the *acp1* gene of *Scl. sclerotiorum* was analysed in a time-course experiment. Sunflower cotyledons were infected with mycelial disks and harvested at different times after inoculation. Total RNA was extracted from healthy and infected cotyledons, and analysed using Northern blots incubated successively with *acp1*, *gpd* and 16S rDNA probes (Fig. 6). To appreciate the proportion of the fungal RNA extracted from infected cotyledons, the phosphoglyceraldehyde dehydrogenase gene of *Scl. sclerotiorum*, *gpd*, was used as a reference; *gpd* is constitutively expressed during mycelial growth (P. Cotton, personal communication). Hybridization with a 16S rDNA probe which reacts with fungal and plant RNA allowed an estimate of the total RNA (plant and fungal) loaded in each lane.

No hybridization signal was detected when the *acp1* probe was incubated with total RNA extracted from cotyledons inoculated with heated mycelial disks, indicating that cotyledons do not express an *acp1*-related gene (not shown). During the early stages of the infection following inoculation with non-heated mycelial disks, a low hybridization signal with the *acp1* probe was detected (Fig. 6). The level of *acp1* transcripts remained low and constant during the first 16 h and then increased rapidly between 20 and 24 h of infection. This increase of the hybridization signal coincided with the phase of symptom development in which intensive colonization of the cotyledonary leaves by the fungus and maceration of host tissue occurred; at that stage, a third of the cotyledon was infected by the fungus (results not shown). This strong hybridization signal remained stable for 24 h and decreased 56 h after infection when sunflower cotyledons were completely invaded and degraded. While equal amounts of RNA were loaded, as illustrated by the rDNA hybridization, *gpd* hybridization was not detected in the RNA sample of non-infected cotyledons, proving the fungal specificity of the probe. Expression of *gpd* was low at the beginning of infection, increased 24 h after infection and remained high during cotyledon colonization. This indicates that the amount of fungal RNA present in the total RNA extracted from infected plant tissues increased suddenly at the start of leaf necrosis. The enzymic activity likely to have been attributable to ACP1, detected during the early stages of infection, increased to reach a maximal level 24 h after infection then decreased during spreading of the cotyledon necrosis. This enzymic activity appeared therefore to be transient.

**DISCUSSION**

When grown in vitro in the presence of a crude sunflower extract, or during plant infection, *Scl. sclerotiorum* secretes a pool of acidic proteases which contains at least two types of enzymes: the aspartyl protease and the non-aspartyl protease. The first enzymic activity is characterized by an optimal pH of 4.5 and is sensitive to pepstatin, a specific aspartyl protease inhibitor. The latter enzymic activity is defined by a pH optimum of pH 2 and is not inhibited markedly by pepstatin; such properties are similar to those of PRTA of *A. niger* (Iio & Yamasaki, 1976), SLB of *C. lignicolum* (Maita et al., 1984) and EAPB and EAPC of *C. parasitica* (Jara et al., 1996). We have cloned the *acp1* gene encoding a non-aspartyl acid protease of *Scl. sclerotiorum* and investigated its pattern of expression. Previous studies have shown that expression of aspartyl- and metallo-proteases is regulated in response to environmental signals (Jarai & Buxton, 1994; Hensel et al., 1995; Gente et al., 1997; St Leger et al., 1997). It was of interest to determine the factors which influence, in vitro, the expression of the *acp1* gene to relate these to the environmental conditions encountered in planta by the fungus during symptom development. In this study, we show that *acp1* expression is tightly regulated at the level of transcription by the availability of carbon and nitrogen sources, and by the ambient pH.
acp1 expression was induced when the mycelium was cultivated in the presence of sunflower extracts. The presence of glucose prevented this induction, indicating that production of this protease is regulated by carbon catabolite repression. These data are compatible with the presence, in the Scl. sclerotiorum acp1 promoter, of several binding sites for the glucose repressor CREA, and with the synthesis and nuclear compartmentation of the homologous protein CRE1 in the presence of glucose (Vautard-Mey et al., 1999). The addition of ammonium to the plant-cell-extract medium did not repress acp1 expression, indicating that this preferred nitrogen source is, on its own, unable to induce nitrogen repression. However, the addition of glycerol, a non-repressive carbon source on its own, to media which contained plant-cell extracts and an increasing concentration of ammonium, reduced markedly acp1 expression leading to full repression at an ammonium concentration of 100 mM. This control, induced by the simultaneous presence of carbon and nitrogen sources, indicates that the sunflower cell-wall preparation did not contain available single carbon sources, as acp1 was expressed in the presence of ammonium and the cell-wall preparation. The products released by cell-wall-protein proteolysis must serve as both carbon and nitrogen sources with nitrogen repression by a convenient nitrogen source (i.e. NH4) only occurring when the requirement for a carbon source is fulfilled i.e. by the addition of glycerol. This situation is reminiscent of the situation for a set of proline-specific genes in A. nidulans: repression of these genes requires the presence of both ammonium and glucose (Gonzalez et al., 1997). Inducer exclusion appears to be responsible for the carbon and nitrogen repression of the genes of the proline utilization gene cluster of A. nidulans (Marzluf, 1997; Cubero et al., 2000). It will be of interest to determine whether protease inducers are released in the presence of nitrogen and carbon sources in sclerotiorum; their absence would signify that nitrogen and carbon metabolite repression of acp1 expression is indirect by prevention of inducer production or by inducer exclusion.

Expression of acp1 is also tightly regulated by ambient pH. Following transfer of mycelium into inducing medium, acp1 transcripts were only detected within a very narrow range of ambient pH, between pH 3 and 5. Transcription of acp1 requires acidic environmental conditions; the presence of a proteinaceous inducer is not sufficient for expression of this gene. pH control overrides induction. Moreover, synthesis of the acidic ACP1 is prevented at neutral or alkaline pH values at which the enzyme is inactive. pH regulation is an adaptive response for the production of secreted enzymes at their optimal pH of activity (Jarai & Buxton, 1994; Gente et al., 1997). In A. nidulans, the PacC transcription factor has been shown to activate transcription of alkaline genes and to repress transcription of acidic genes (Tilburn et al., 1995). Identification of a pacC homologue in Scl. sclerotiorum (S. Creton & N. Poussereau, unpublished data) together with the presence of a PacC binding site in the promoter region of acp1 (Fig. 5), suggest the involvement of a pH regulator in the control of acp1.

We have investigated the pattern of acp1 expression in response to changes in the availability of carbon and/or nitrogen sources, and alteration of the ambient pH demonstrating that pH controls the circuit of acp1 induction and repression prevails over specific induction. acp1 is strongly expressed in planta during the course of symptom development of infected sunflower cotyledons. The ambient conditions which impose a very tight control of acp1 expression in vitro must be encountered in planta to allow acp1 expression during infection. The pH-conditional expression observed in vitro suggests that pH during infection must rapidly become acidic to reach pH 4, the value allowing acp1 expression. In vitro, glucose imposes full repression but carbon catabolite repression must not occur during infection as acp1 is expressed at the early stages of infection. Similarly, nitrogen limitation may also allow gene expression during infection as illustrated in other systems (Talbot et al., 1997; Coleman et al., 1997).

In summary, using acp1 as a reporter system and by comparing in vitro and in planta gene expression it was possible to define conditions which mirror growth conditions in plant tissues. Our data suggest that glucose and nitrogen starvation together with acidification are key factors which control Scl. sclerotiorum gene expression during pathogenesis.

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