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; 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. An
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 defence 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, 1990). In contrast, disruption of two alkaline protease encoding genes in *Sclerotinia sclerotiorum* indicates that these two enzymes are not required for pathogenesis, *S. sclerotiorum* S5 was maintained on potato-dextrose agar. It was grown in minimal liquid medium containing (per litre) 1 g KH₂PO₄, 0·1 g MgSO₄, 0·05 mg biotin, 250 mg citric acid, 230 mg ZnSO₄, 50 mg Fe(SO₄)₂, 15 mg Cu(SO₄)₂, 25 mg each of MnSO₄, H₂BO₃, NaMoO₄, and supplemented with 1% sunflower extract, or 2% glucose and 100 mM NH₄Cl. Sunflower extracts were prepared from cotyledons of 1-week-old germlings. Cotyledonary 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 (Mackivaine) buffer. The cultures were inoculated with mycelial disks cut from 4-old colonies and incubated at 24 °C under constant agitation.

*Escherichia coli* strain Sure R (Stratagene) was the host for recombinant plasmids 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/rouzolane mix. Germlings were grown at 25 °C (95% humidity) with a 14 h light period per day. Cotyledonary 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 mycelial 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 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 (0·05 M), 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). ACPI was assayed in a reaction mixture containing 200 μl enzyme solution, 900 μl 100 mM KCl/HCl buffer, pH 2, and 100 μl 15% (v/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 follows: primer mixture A, 5′-TGGTAYGARTGGTAYC-3′; primer mixture B, 5′-ATVMAYCTNGCRTT-3′ where N is A/C/G/T, R is G/A, Y is T/C, V is A/C/G and M is A/C.

The amplification was initiated with a 5 min denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min; annealing at 56 °C for 1 min; and primer extension at 72 °C for 3 min; the final elongation step was 7 min at 72 °C. The amplified products were analysed by agarose gel electrophoresis, isolated by adsorption to glass silica beads (GeneCLean II; Bio101), digested and cloned into the HindIII/XhoI-digested pUC18 vector. The cloned PCR fragment was sequenced and then used as a probe to screen a genomic library.

**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 into 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 protease. PMSF, E-64 and phosphoramidon, inhibitors of serine, cysteine and metalloproteases, respectively, did not affect this latter activity, indicating that the acidic activity secreted in the medium containing cell walls consists of overlapping activities of aspartyl and non-aspartyl proteases (G. Billon-Grand, N. Poussereau & M. Févre, 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* (Maia 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* (Maia 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 WYEWY 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
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Fig. 1. Comparison of the predicted amino acid sequence of ACP1 of Scl. sclerotiorum with the corresponding sequences of the fungal acid proteases PRTA from A. niger, SLB from Scy. lignicolum, EAPB and EAPC from 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.

gene, as described for A. niger and Scy. lignicolum (Takahashi et al., 1991; Oda 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 acp1 gene (GenBank accession no. AF221843). The 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 C. parasitica (Jara et al., 1996) and A. niger (Takahashi et al., 1991), it may be assumed that 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.

In A. niger, the enzyme has a two-chain structure with a light chain non-covalently linked to the heavy chain.
Scl extracts. Expression during growth in the presence of plant

Transfer experiments were performed to investigate Scl. sclerotiorum acp1 expression during saprophytic growth of Scl. sclerotiorum (Inoue et al., 1991; Takahashi et al., 1991). In the deduced amino acid sequence of the precursor of that protein, the two chains are separated by an intervening sequence of 11 residues. Interestingly, this intervening sequence appears to be specific to the sequence of 11 residues. Interestingly, this intervening sequence appears to be specific to the A. niger enzyme and is not found in the deduced polypeptide sequences of the other genes encoding acid proteases (Fig. 1). By comparison with the enzymes from Scy. lignicolum and C. parasitica, the acid protease from Scl. sclerotiorum appears to be a single-chain enzyme.

**acp1 expression during saprophytic growth of Scl. sclerotiorum**

Transfer experiments were performed to investigate acp1 expression during growth in the presence of plant extracts. Scl. sclerotiorum was grown for 48 h in a medium containing glucose and NH₄ 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 enzymic 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. Scl. 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 2–3 (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 NH₄ 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.](image)

The presence of these motifs, together with the GATA motif which is the recognition sequence of the nitrogen regulatory proteins AreA from *N. crassa* (Fu & Marzluf, 1990). However, only eight of these motifs at the positions 5’, 858, 111, 498, 131 and 867 and 903, fi 126 are in an inverted-repeat orientation separated by less than 30 bp, of CREA, a factor involved in glucose-carbohydrate repression (Kulmburg et al, 1993) and NIT2 from *N. crassa* (De Bernardis et al, 1993), is 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 the simultaneous presence of ammonium and glycerol (Fig. 4, lane 6, 7 and 8). Proteases release compounds which can be used as carbon sources. When a compound such as glycerol, which can fulfill the requirement for a 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).

![Fig. 5. Sequence of the acp1 promoter region. Nucleotide numbering begins at the putative translational start of acp1. Putative functional AreA binding sites (GATA) are underlined. The putative PacC recognition sequence (GCCARG) is boxed and CREA (SYGGRG) binding sites are double underlined (R, A or G; S, G or C; Y, C or T).](image)
Non-aspartyl acid protease of Scl. sclerotiorum

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 Scy. 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.

![Fig. 6. Expression of acp1 of Scl. sclerotiorum during infection of sunflower cotyledons. Mycelial disks were deposited on the upper face of the cotyledonary leaves of 1-week-old germlings. Cotyledons were collected at different times after inoculation. Total RNA was extracted, electrophoresed on agarose gels and Northern blots hybridized with acp1 (a). As controls for fungal RNA and total RNA loading, filters were stripped and then successively probed with gpd and rDNA probes, respectively. Proteins were extracted and assayed for acid protease activity (b). Protease activity was assayed in triplicate; values shown represent the mean and bars show standard deviation.](image-url)
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 inhibitor 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.

**ACKNOWLEDGEMENTS**

This work was supported by grants from the CNRS and the Université Claude Bernard Lyon1.

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lation of both acid and alkaline expressed genes by ambient pH. 
EMBO J 14, 779–790.


Received 30 May 2000; revised 2 November 2000; accepted 16 November 2000.