

pH controls both transcription and post-translational processing of the protease BcACP1 in the phytopathogenic fungus *Botrytis cinerea*

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During pathogenesis, the ascomycete *Botrytis cinerea* secretes a range of cell-wall-degrading enzymes such as polygalacturonases, glucanases and proteases. We report the identification of a new member of the G1 family of proteases, BcACP1, which is secreted by *B. cinerea* during infection. The production of BcACP1 correlates with the acidification of the plant tissue, and transcriptional analysis of the *Bcacp1* gene showed that it is only expressed under acidic growth conditions. Using a transcriptional reporter system, we showed that pH regulation of *Bcacp1* is not mediated by the canonical PacC transcription factor binding site. Like other G1 proteases, BcACP1 is produced as a pro-enzyme. Trapping of the zymogen form allowed investigation of its maturation process. Evidence is presented for an autocatalytic proteolysis of the enzyme that is triggered by acidic pH. Environmental pH therefore controls Bcacp1 production at both the transcriptional and post-translational level.

INTRODUCTION

The first barrier encountered by a phytopathogenic fungus during infection is the host cuticle and the primary plant cell wall. Successful infection depends not only on the pathogen's ability to penetrate and colonize the host tissues, but also on its ability to degrade the plant cell wall polymers (polysaccharides and several classes of structural proteins: Carpita & Gibeau, 1993) into nutrients required for its development. Enzymic degradation of the parietal polymers is therefore considered an important aspect of plant fungal infections. Phytopathogenic fungi secrete a wide range of cell-wall-degrading enzymes that act at different stages of the infection. Among these enzymes are pectinases, cellulases and xylanases that are involved in polysaccharide degradation (St Leger *et al.*, 1997; Cotton *et al.*, 2003; Olivieri *et al.*, 2004; Brito *et al.*, 2006). Several classes of proteases have also been described (Billon-Grand *et al.*, 2002; Bindschedler *et al.*, 2003; Ten Have *et al.*, 2004), whose proposed role would be to facilitate host tissue penetration and colonization by degrading structural plant cell wall proteins, and by releasing amino acids, which represent the pathogen's main source of nitrogen and sulfur during infection (Rauscher *et al.*, 1995).

Fungi can secrete several proteases, each characterized by an optimal pH for activity, and many produced in correlation with the environmental pH. In *Sclerotinia sclerotiorum* for example, secreted proteases with an acidic optimum pH can be detected when the environment has been acidified via oxalic acid production by this phytopathogenic fungus (Marciano *et al.*, 1983; Magro *et al.*, 1984; Poussereau *et al.*, 2001; Cotton *et al.*, 2003). Similarly, in the insect pathogen *Metarhizium anisopliae*, a secreted subtilisin with an alkaline optimum pH can be detected when the ambient pH has become alkaline following production of ammonia by the fungus (St Leger *et al.*, 1999). In order to ensure the secretion of these proteases in their optimal environment, their production needs to be controlled in response to the environmental pH. Although the effect of ambient pH on fungal growth, physiology and differentiation is well established, the molecular responses to environmental pH are only now being elucidated. In *Aspergillus nidulans*, alkaline pH is perceived and transmitted via the *pal* signalling pathway, which activates the zinc finger transcription factor PacC (Penalva & Arst, 2002). PacC is a transcriptional activator of alkaline-induced genes and a transcriptional repressor of acid-induced genes (Tilburn *et al.*, 1995). PacC and its regulatory pathway are conserved in fungi, and it has been demonstrated that this regulator participates in the virulence of some pathogenic fungi (Rollins, 2003; Caracuel *et al.*, 2003; You & Chung, 2007; Miyara *et al.*, 2008).

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Abbreviations: GUS, β -glucuronidase; p.i., post-inoculation.

The ascomycete *Botrytis cinerea* is a pathogen of more than 200 plant species (Elad *et al.*, 2004). It can infect different organs including cotyledons, leaves, stems, roots, flowers and fruits at different stages of their development. *B. cinerea*, like the closely related necrotrophic fungus *S. sclerotiorum*, uses an infection strategy based on the acidification of the ambient environment via the production of organic acids (oxalic or citric acids) (Verhoeff *et al.*, 1988) and on the secretion of numerous cell-wall-degrading enzymes, including proteases (Manteau *et al.*, 2003). It has been reported that *B. cinerea* secretes several acidic proteases during pathogenesis. Movahedi & Heale (1990) purified and characterized one aspartic protease from infected carrots, while Ten Have *et al.* (2004) described a family of five aspartic proteases which they detected during the infection of several plant species.

In this paper, we report on an unusual acidic protease of *B. cinerea*, BcACP1, which belongs to the G1 family of proteases, a newly discovered class of endopeptidases found exclusively in fungi, and more specifically in ascomycete species (Sims *et al.*, 2004; Pillai *et al.*, 2007). Members of this family of proteases are characterized by an optimal activity at acidic pH and by their insensitivity to pepstatin A (inhibitor of the aspartic proteases). The environmental cues that control the enzyme production are still unknown. We show herein that BcACP1 is regulated by the environmental pH at both the transcriptional and post-translational level.

METHODS

Biological material and culture conditions. *Botrytis cinerea* strain BO5.10 was maintained at 21 °C on solid sporulation medium containing 1.5% malt extract, 0.5% glucose, 0.1% tryptone, 0.1% casein hydrolysate, 0.1% yeast extract and 0.02% tRNA, with 8 h black light illumination per day. *B. cinerea* transformant strains were maintained on malt agar medium (2% malt extract, 1.5% agar) supplemented with 100 µg hygromycin B ml⁻¹ (InvivoGen) and 200 µM cefotaxim (Sigma). For *BcACP1* expression, *B. cinerea* was grown on a rich solid medium containing 1.5% agar, 2% malt extract, 2% glucose, and 100 mM NH₄Cl on the surface of cellophane sheets. After 3 days of incubation at 22 °C, the mycelium was transferred onto the surface of 2 ml Gamborg medium (Gamborg *et al.*, 1968) supplemented with 2% glucose (minimal medium) but lacking nitrogen and sulfur, and buffered at different pHs as indicated in the text. At 8–16 h after the transfer (as indicated in the text), the mycelia were harvested by filtration, frozen in liquid nitrogen and kept at -70 °C for protein or RNA extraction as described previously (Rolland *et al.*, 2003). The pH values after fungal growth were measured using a pH electrode. *Escherichia coli* strain Sure S (Stratagene) was used for cloning and plasmid propagation and was grown in LB broth, supplemented with ampicillin (50 µg ml⁻¹) (Sigma) when required (Sambrook *et al.*, 1989).

Phytopathogenicity tests. Phytopathogenicity assays were performed on apple fruits. *B. cinerea* strains were grown for 15 days on sporulation medium. Harvested conidia (2 × 10⁶ conidia ml⁻¹) were germinated in liquid sporulation medium with agitation (150 r.p.m.) for 12 h at 22 °C. The surface of apple fruits was disinfected with 70% ethanol prior to the infection. Slices of apple (approx. 1 cm thick) were inoculated with 10 µl of the germinated conidia

suspension and kept in a Petri dish under high-humidity conditions at 22 °C.

Preparation of protein extracts from infected fruits. Slices of apple collected at different times after inoculation were suspended in sterile deionized water, sonicated for 5 min at 4 °C (Vibra Cells, Sonics & Materials Inc.) and centrifuged at 4 °C for 15 min at 8000 g. The proteins present in the supernatant were precipitated overnight with (NH₄)₂SO₄ (85% saturation) and centrifuged at 10 000 g for 20 min. The precipitate was solubilized in 3 ml water, dialysed for 6 h against 1 l distilled water twice at 4 °C and stored at -20 °C before use.

BcACP1 immunodetection. Detection of BcACP1 by Western blotting was performed using rabbit anti-SsACP1 polyclonal antibodies at 1/1000 dilution as described previously (Rolland *et al.*, 2003). Proteins from concentrated filtrates were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for 16 h with anti-SsACP1 antibodies, washed and incubated for 45 min in the presence of rabbit anti-IgG secondary antibodies as described in the manufacturer's instructions (ECL Western detection kit, Pierce Super Signal Substrate; Perbio). Chemiluminescent reaction was used to reveal the immunoreactive proteins.

RNA isolation and Q-PCR. The mycelium of interest (100 mg) was frozen in liquid nitrogen, ground, and total RNA was extracted by phenol/chloroform separation and lithium chloride precipitation (Verwoerd *et al.*, 1989). For Q-PCR experiments, 20 µg total RNA of each sample were treated with DNase I (Ambion) to remove genomic DNA. Absence of genomic DNA was controlled by PCR using primers (actA-F, 5'-CCGTGCTCCAGAAGCTTTGT-3', and actA-R, 5'-GTGGATACCACCGCTCTCAAG-3') specific for the actin-encoding gene *BcactA* (accession number AJ000335) and a *Taq* DNA polymerase (MP Biomedicals). Quality of total RNA was verified using an Agilent 2100 Bioanalyser, Agilent RNA 6000 Nano reagents and Agilent RNA Chips from Agilent. Total cDNA was produced by treating 5 µg DNase I-treated total RNA with ThermoScript RT (Invitrogen) as described by the manufacturer. Q-PCR experiments were performed on an ABI PRISM 7900HT (Applied Biosystems) using primers specific for the *BcACP1* gene (acp1-F, 5'-TGACGG-AGACCTTTGCGAGTA-3', and acp1-R, 5'-TGCCCTCTCGAAA-TCTTCA-3'), using the Power SYBR Green PCR Master Mix (Applied Biosystems) and according to the manufacturer's instructions. Following examination of the primer efficiencies, the amplification reaction was carried out as follows: 95 °C 10 min, 95 °C 15 s and 60 °C 1 min (50 cycles), 95 °C 15 s, 60 °C 15 s and 95 °C 15 s. Relative quantification was based on the 2^{-ΔΔC_t} method using *BcactA* as reference; the accuracy of the method could be expected from the high and similar amplification efficiencies of *BcACP1* and *BcactA*. Three independent biological replicates were analysed.

Northern analysis. Samples (15 µg) of total RNAs were separated on 1.5% formaldehyde agarose gel, transferred to Nytran membrane (Schleicher and Schuell) and UV cross-linked. RNA gel blot hybridizations were performed using standard protocols (Sambrook *et al.*, 1989). The RNA hybridization probe was ³²P-labelled using a random-primed DNA labelling kit (Promega) and purified by exclusion chromatography (Spin column, Amersham Pharmacia Biotech). The membrane was hybridized overnight, in the presence of the α-³²P-labelled probe, at 42 °C in hybridization buffer [50% (w/v) formamide, 5 × SSC, 0.2% (w/v) SDS, 1 × Denhardt's solution and 100 µg denatured salmon sperm DNA ml⁻¹] and washed sequentially with 2 × SSC, 0.1% (w/v) SDS and 0.2 × SSC, 0.1% (w/v) SDS. The signals were detected using a phosphorimager (Cyclone, Packard).

Construction of the reporter gene vectors. The β -glucuronidase (GUS)-encoding gene was fused to a 0.4 kb fragment of the promoter of the *BcACP1* gene of *B. cinerea*. Genomic DNA from *B. cinerea* and two specific primers, ACP4BC (5'-ACACCGCGGTGATCTGTATGGGTCTATACGGGT-3') and ACP7BC (5'-GCGCCATGGTTT-GATTGATTGATTGAA-3') were used in a PCR to amplify a 400 bp fragment upstream of the start codon of the *BcACP1* gene. The PCR product was cloned upstream of the *gus* gene. The resulting plasmid was digested with *Xba*I and *Eco*RI to excise the *gus* cassette and subclone it into the *Agrobacterium*-mediated transformation vector pBHt2 (Mullins *et al.*, 2001) to yield pBc400. Plasmid p18, corresponding to an *Agrobacterium*-mediated transformation vector containing the *gus* reporter gene under the control of 1.1 kb of the promoter of the *SsACP1* gene of *S. sclerotiorum*, has been previously described (Rolland *et al.*, 2003). In this study, we refer to it as pSs1100. Another vector containing a promoter region of 400 bp upstream of the start codon of the *SsACP1* gene was also constructed. The reporter cassette was amplified by PCR from the vector pSs1100 using the primers ACPD2 (5'-CTGTCTAGATGAAGTCCCGTTCTGT-3') and TERNOS (5'-CTTCTAGACTAGTAACATAGATGACA-3'). This fragment was digested with *Xba*I and cloned into plasmid pBHt2 (Mullins *et al.*, 2001) to yield pSs400.

Fungal transformation. Transformation of *B. cinerea* BO5.10 was performed as described by Rolland *et al.* (2003). *Agrobacterium tumefaciens* LBA1126 carrying pBc400 or pSs400 was used to transform conidia of *B. cinerea*. The transformants arose after 15 days of incubation at 21 °C on solid malt agar medium containing hygromycin B (70 μ g ml⁻¹) and 200 μ M cefotaxim (Sigma).

GUS extraction and GUS assays. GUS activity was measured essentially as described by Rolland *et al.* (2003). Mycelia were harvested, frozen in liquid nitrogen and ground. After adding 1 ml GUS extraction buffer (50 mM sodium phosphate pH 7.0, 0.1% Triton X-100, 0.1% *N*-lauroyl sarcosine, 10 mM EDTA, 10 mM β -mercaptoethanol) to 100 mg mycelium, the cells were disrupted and centrifuged at 13 000 g at 4 °C for 10 min. Samples (100 μ l) of supernate were incubated at 37 °C in 1 ml GUS extraction buffer containing 1 mM *p*-nitrophenyl β -D-glucuronide (Sigma-Aldrich). At various time points, 100 μ l samples of the reaction were mixed with 900 μ l stop buffer (0.2 M Na₂CO₃, pH 9.5). Absorbance was measured at 405 nm, and the protein concentration was determined according to Bradford (1976).

BcACP1 maturation assay. *B. cinerea* was grown on solid medium containing 2% malt extract on the surface of cellophane sheets. After 3 days incubation at 21 °C, the cellophane sheets were transferred onto the surface of 2 ml Gamborg medium supplemented with 2% glucose and buffered to pH 7. Sixteen hours after transfer, the medium was recovered, put on ice and its pH was adjusted as indicated in the text by addition of HCl. The mixture was then incubated at different temperatures, in the presence or absence of protease inhibitors, and an aliquot of 1.4 ml was recovered at different times. The reaction was stopped by precipitating the proteins (Rolland *et al.*, 2003) and analysed by Western blotting.

RESULTS

Isolation and characterization of the *BcACP1* gene

Two degenerate oligonucleotides corresponding to two conserved regions of the G1 proteases (WYEWY and NAEWI) were previously used successfully to clone the *ACP1* gene of *S. sclerotiorum*. This gene encodes the protease SsACP1, whose characteristics are an acidic optimal pH for

activity (pH 2.0) and an insensitivity to pepstatin A (inhibitor of aspartic proteases) (Poussereau *et al.*, 2001). Since *B. cinerea* and *S. sclerotiorum* are closely related necrotrophic fungi, we used the same strategy to amplify a 200 bp fragment from the genomic DNA of *B. cinerea*. A SON (single oligonucleotide nested) PCR approach (Antal *et al.*, 2004) was then used to clone the entire coding sequence flanked by 1 kb and 200 bp of the promoter and the terminator sequences, respectively (GenBank accession no. DQ151453). The gene was named *BcACP1* while the *ACP1* gene of *S. sclerotiorum* was renamed *SsACP1* to avoid confusion. Analysis of the recently released *B. cinerea* (strain BO5.10) genome sequence on the website of the Broad Institute confirmed the sequence of *BcACP1* and revealed that it is the only G1 protease-encoding gene in this fungus.

BcACP1 encodes a protein of 257 amino acids sharing 89% identity with SsACP1, 55% identity with PrtA of *Aspergillus niger* (Takahashi *et al.*, 1991) and 45% identity with SGP of *Scytalidium lignicolum* (Maita *et al.*, 1984). Despite high conservation between the sequences of SsACP1 and BcACP1, two notable differences exist. BcACP1 contains four extra amino acids in its putative pro-peptide sequence and two cysteines in its carboxyl-terminal region (Fig. 1A). By comparison with the other G1 proteases, BcACP1 is likely to be secreted as a zymogen and to contain an N-terminal pre-pro-sequence of 57 amino acids. The unprocessed BcACP1 is predicted to contain 239 amino acids and to have a molecular mass of 24.75 kDa. The mature BcACP1 is predicted to contain 200 amino acids and to have a molecular mass of 20.57 kDa. As previously described (Rolland *et al.*, 2003), the secreted BcACP1 can be detected in *B. cinerea* culture filtrate as a single protein of 28 kDa by using antibodies raised against SsACP1 of *S. sclerotiorum*. The apparent molecular mass of BcACP1 is therefore 7–8 kDa higher than that predicted from its sequence. This difference could be due to post-translational modifications.

Secretion of BcACP1 during plant infection

We have shown that SsACP1 is secreted by *S. sclerotiorum* during pathogenesis (Poussereau *et al.*, 2001). To test whether this was also true for *B. cinerea*, apple fruits were infected with conidia and the production of BcACP1 was followed by Western blotting of the proteins collected from the infected tissues (Fig. 2). Primary infection occurred within 16 h post-inoculation (p.i.), a time at which necrotic areas appeared around the inoculation point. The infection then progressed to near-complete maceration of the fruit disc in 96 h. BcACP1 was detected at 96 h p.i., but a faint band could already be observed at 72 h p.i. These results indicate that BcACP1 is indeed produced when *B. cinerea* attacks plants. In parallel, the pH of the infection sites was monitored by using a flat contact electrode placed directly onto the necrotic area. A decrease in pH from 4.2 to 3.8 was recorded, suggesting the secretion of acids by *B. cinerea*. When bean leaves were used as hosts, the pH also dropped (from 6 to 4.5) during

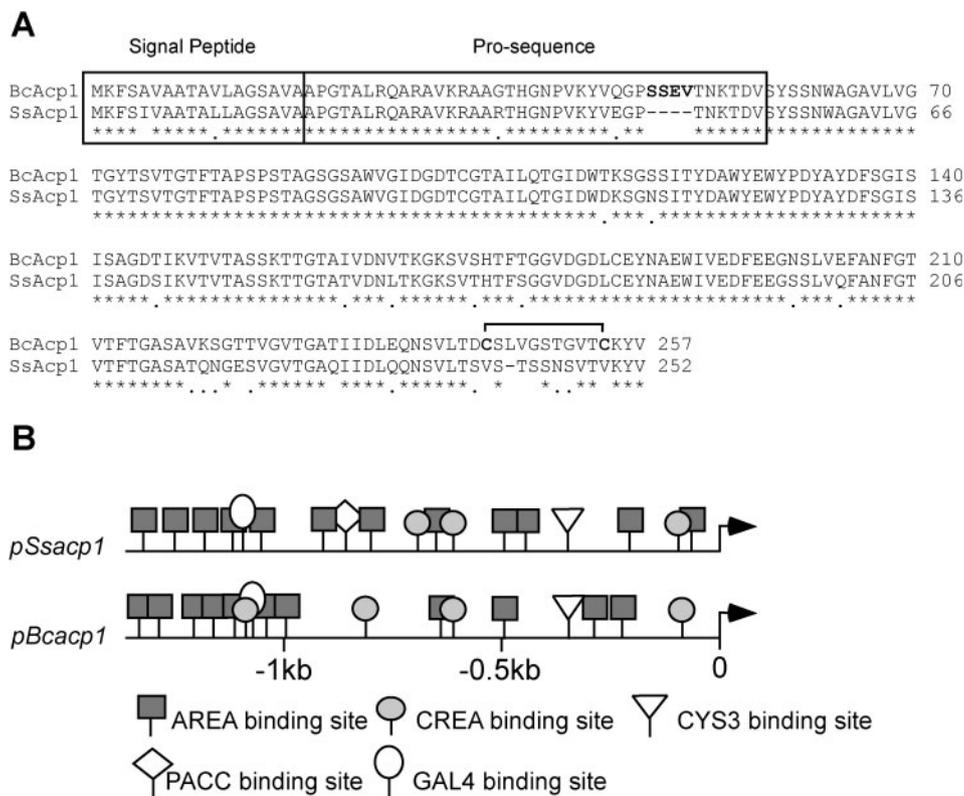


Fig. 1. BcACP1 and SsACP1 share high similarity at both the protein and the promoter level. (A) Alignment of BcACP1 and SsACP1. The predicted signal peptide sequence and the pro-sequence are indicated. The two major differences between the BcACP1 and SsACP1 sequences are highlighted in bold. (B) Schematic of the *BcACP1* and *SsACP1* promoters. The positions of the AREA, CREA, CYS3, PacC and GAL4 consensus binding sites are indicated.

infection over 3 days, and again secretion of BcACP1 could be observed (data not shown).

pH regulation of *BcACP1* transcription

We investigated *in vitro* the role of ambient pH in the control of *BcACP1* expression. *B. cinerea* was grown for

3 days on solid rich medium under conditions where the presence of sulfur and nitrogen repressed *BcACP1* expression (Rolland & Bruel, 2008). The mycelium was then transferred to minimal medium buffered to different pHs. Eight hours after transfer, the mycelium was harvested and total RNA was extracted for quantitative PCR analysis. The actin-encoding gene *BcactA* was used as reference, and we

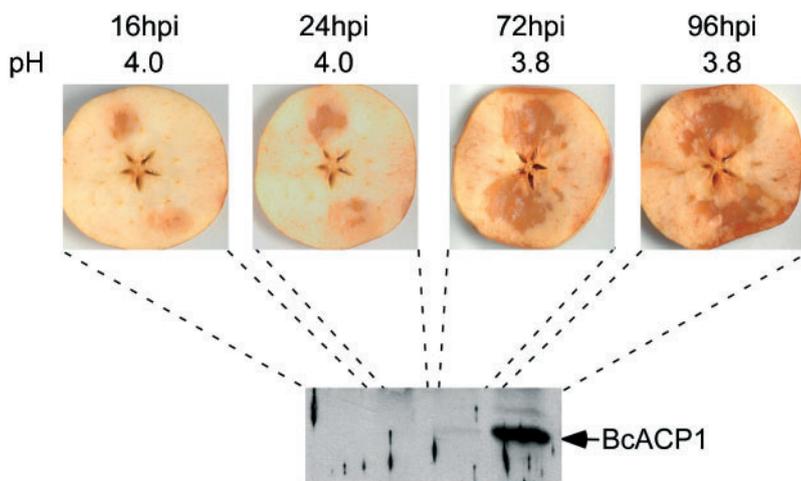


Fig. 2. BcACP1 is produced during pathogenesis. (A) Pathogenicity assay. Apple fruit discs were inoculated with germinated conidia of *B. cinerea*. The infection was followed for 96 h post-inoculation (hpi) and the pH at the centre of the necrotic area was monitored. (B) Detection of BcACP1. Proteins collected from the infected tissues were analysed by Western blotting using anti-SsACP1 antibodies.

verified that its expression did not change with pH (C_t value of 17.96 at pH 3 and 18.3 at pH 7). In contrast, expression of *Bcacp1* varied significantly with pH: expression could be observed at pH 3, 4 and 5 while barely any expression occurred at pH 6 or 7 (Fig. 3A). Similarly to what was found for *Ssacp1* in *S. sclerotiorum* (Poussereau *et al.*, 2001), these results indicate that *Bcacp1* transcription is pH dependent, requires acidic conditions to proceed, and could be maximal at pH 4.

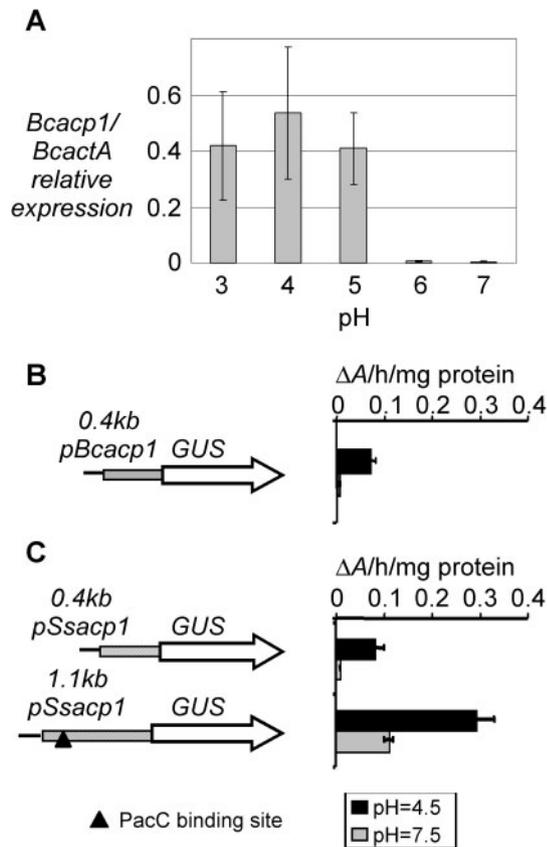


Fig. 3. *Bcacp1* transcription is controlled by the environmental pH independently of the canonical PacC consensus binding site. (A) Q-PCR analysis of *Bcacp1* regulation. Following growth under nitrogen and sulfur conditions that repressed *Bcacp1* expression, *B. cinerea* was transferred (8 h) to minimal medium buffered to pH 3 to pH 7. Total RNA was extracted and submitted to quantitative PCR analysis. *Bcacp1* expression is reported relative to that of the actin-encoding reference gene (*BcactA*). (B) Functional activity of the *Bcacp1* promoter. *B. cinerea* transformants carrying the *GUS* reporter gene under the control of a 0.4 kb promoter region of *Bcacp1* were grown on rich solid medium and then transferred (16 h) to minimal medium buffered to pH 4.5 or 7.5. The mycelium was harvested and *GUS* specific activity was measured. (C) Functional activity of the *Ssacp1* promoter. The same approach was adopted with the *Ssacp1* promoter, using fragments of 0.4 kb or 1.1 kb. Means \pm SD are plotted ($n=3$).

It is well established in fungi that the regulation of gene expression in response to changes in ambient pH is mediated by a conserved signal transduction pathway acting through the transcription factor PacC and its 5'-GCCARG-3' consensus DNA-binding sequence (Penalva & Arst, 2002). PacC is present in *B. cinerea* (accession number AY647218), but, curiously, analysis of 1.5 kb of the *Bcacp1* promoter failed to reveal any putative PacC binding site in this DNA sequence. To further investigate the regulation of *Bcacp1* by pH, we constructed a reporter system by fusing 0.4 kb of the *Bcacp1* promoter to the β -glucuronidase-encoding gene. This *pBcacp1-GUS* cassette was introduced into *B. cinerea* by *Agrobacterium*-mediated transformation (Rolland *et al.*, 2003). Several transformants were obtained and Southern-blot analysis revealed the presence of only one copy of the transgene in each transformant's genome (data not shown). Five transformants were grown, transferred for 16 h to buffered conditions (pH 4.5 or pH 7.5), and *GUS* activity was measured. In all strains, a higher activity was detected when the fungus was transferred to acidic rather than neutral conditions (Fig. 3B). This result correlates with the expression profile of the endogenous *Bcacp1* gene (Fig. 3A), and it indicates that pH regulation of the *Bcacp1* promoter activity can occur in the absence of a consensus PacC binding site and in the presence of only 0.4 kb of the promoter sequence.

The *Bcacp1* and *Ssacp1* promoters share 75% identity, and the two genes might hence share some regulators (Fig. 1B). However, one major difference is the presence of one PacC consensus binding site in position -834 bp of the *Ssacp1* promoter, and the role of this site in pH regulation was examined. We first compared the capacity of 0.4 kb of the *Ssacp1* promoter to support pH regulation. *B. cinerea* transformants carrying *pSsacp1(0.4 kb)-gus* constructs were generated and *GUS* activity was measured as described above. As shown in Fig. 3(C), the results obtained were identical to those obtained with *pBcacp1-gus*. Transformants carrying the *gus* gene under the control of 1.1 kb of *pSsacp1* were next generated and studied. Placed in either an acidic or neutral environment, these transformants always exhibited a threefold higher *GUS* production than those carrying the short version of the promoter (Fig. 3C); this suggests the existence of sequences enhancing *Ssacp1* expression within the -1.1 to -0.4 kb region of the promoter. Disregarding this difference, pH regulation still occurred with this construct and was not different from that observed with the shorter version of the promoter. pH regulation of *Ssacp1* hence appears to be independent of the presence of a consensus PacC binding site, similar to what was observed with *Bcacp1*.

pH-dependent maturation of BcACP1

Besides being controlled by ambient pH, *Bcacp1* expression is also controlled by nitrogen and sulfur catabolic repression (Rolland & Bruel, 2008). Indeed, *Bcacp1*

expression is activated in the absence of readily metabolizable sulfur or nitrogen sources such as sulfate or ammonia. Conversely, the presence of such sources of both nitrogen and sulfur abolishes expression. In accordance with these known types of regulation, culture of *B. cinerea* in malt extract medium supplemented with $MgSO_4$ and NH_4Cl led to no expression of *BcACP1* while culture in non-supplemented medium led to accumulation of *BcACP1* mRNA (Fig. 4, top). When the mycelia that were collected from these two different cultures were transferred to minimal medium lacking nitrogen and sulfur sources, and buffered to pH 4, secreted BcACP1 could be immunodetected in the culture broth in both cases (Fig. 4, bottom). Under acidic conditions, and in the absence of nitrogen and sulfur, this result confirmed at the protein level the *BcACP1* gene expression results shown in Fig. 3(A). When the mycelium grown in the presence of sulfur and nitrogen was transferred to minimal medium buffered to pH 7, BcACP1 could not be detected among the secreted proteins (Fig. 4, bottom left), and this again confirmed the results presented in Fig. 3(A). Lastly, and interestingly, a protein of apparent molecular mass 35 kDa was immunodetected among the secreted proteins when the mycelium grown in the absence of nitrogen and sulfur was transferred to minimal medium buffered to pH 7 (Fig. 4, bottom right). In the absence of *BcACP1* expression at this pH (Fig. 3A), this result could be explained by the translation of the mRNA that had accumulated during pre-culture and the artificial trapping of a pro-protein form of BcACP1 at this

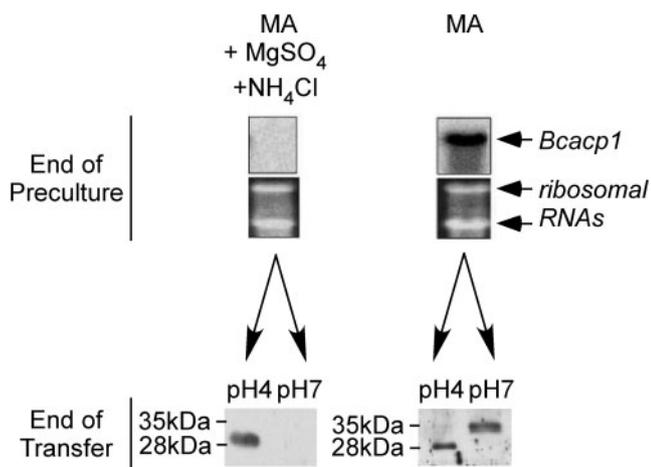


Fig. 4. BcACP1 is produced as a pro-enzyme. *B. cinerea* was grown on solid malt extract (MA) medium supplemented or not with $MgSO_4$ (1 mM) and NH_4Cl (50 mM). Using ethidium bromide staining of rRNA to approximate the amount of total RNA, the *BcACP1* mRNA level was monitored at the end of the preculture by Northern blotting (top panel). The mycelium was then transferred to minimal medium lacking nitrogen and sulfur, and buffered to pH 4 or pH 7. After 16 h of culture under these conditions, the secreted proteins were analysed by Western blotting using anti-ACP1 antibodies (bottom panel).

neutral pH. To test this hypothesis, the pH 7 culture broth was collected and acidified *in vitro* to pH 4 with HCl, incubated at 4 °C for 1–50 min, and analysed by Western blotting. As shown in Fig. 5(A), the 35 kDa band progressively disappeared over time while a band corresponding to a 28 kDa protein appeared; 50% of the shift occurred in 30–40 min. We next investigated whether the processing was sensitive to temperature. The same experiment was performed at 10 °C and 20 °C, and 50% of the shift occurred in 15 and 5 min, respectively, indicating that the processing was indeed temperature dependent (data not shown). The pH dependence of the observed processing was then investigated. The pH 7 culture broth was acidified to different pH values and the processing was monitored after 1 h of incubation at 4 °C (Fig. 5B). Whereas the 28 kDa band did not appear at a pH value of 5.8 or higher, it did appear at a pH value of 5.6 or lower. At pH 5.6, only about 15% of the protein was processed under the conditions used.

Transformation of the 35 kDa protein into its 28 kDa counterpart most likely resulted from proteolysis. Whether this proteolysis relied on proteases present in the culture broth or on BcACP1 itself was finally explored. The *in vitro* processing experiment was performed in the presence of pepstatin A (aspartic protease inhibitor), leupeptin (serine and cysteine protease inhibitor), E64 (cysteine protease inhibitor), NEM (*N*-ethylmaleimide, cysteine protease inhibitor) or EDTA (metalloprotease inhibitor), a series of inhibitors that block most if not all the main families of proteases, except the G1 family of proteases. None of these inhibitors prevented the appearance of the 28 kDa band (data not shown). Dilution of the sample before acidification was also carried out, and no effect on the maturation

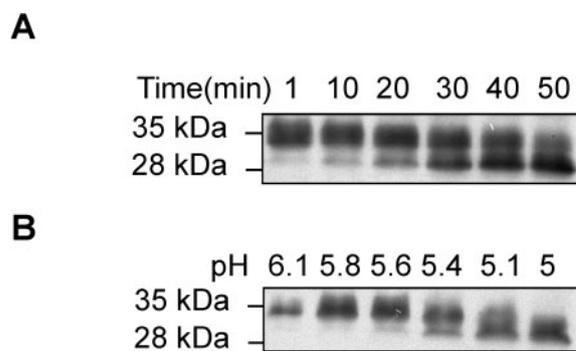


Fig. 5. The maturation of BcACP1 is pH dependent. (A) Kinetics of BcACP1 pH-dependent maturation. The pH 7 culture medium containing the secreted 35 kDa form of BcACP1 was acidified *in vitro* with HCl to pH 4 and incubated at 4 °C for 1 to 50 min. Precipitation and Western-blot analysis of the proteins using anti-ACP1 antibodies were performed on six aliquots over time. (B) pH dependence of the maturation process. The same culture medium was acidified to different pH values (as indicated) and incubated for 1 h at 4 °C. The proteins were precipitated and analysed by Western blotting.

kinetics was observed (data not shown). Taken together, these results suggest first that the 35 kDa protein detected by the anti-SsACP1 antibodies corresponds to a pro-protein form of BcACP1. Second, the results suggest that this form can mature to BcACP1 (28 kDa) via a temperature- and pH-dependent maturation process which probably relies on autocatalytic proteolysis.

DISCUSSION

We have cloned the *BcACP1* gene encoding a member of the G1 family of proteases in *B. cinerea*. This gene is orthologous to the *S. sclerotiorum* *SsACP1* gene previously studied (Poussereau *et al.*, 2001). The G1 proteases are endopeptidases found exclusively in ascomycete species (Sims *et al.*, 2004). Comparison of *BcACP1* and *SsACP1* revealed that the coding regions of these two genes exhibit high DNA similarity. The BcACP1 and SsACP1 proteins are 89% identical, allowing the polyclonal anti-SsACP1 antibodies to recognize the BcACP1 protein. Remarkably, high conservation is also observed in the promoter regions of the two genes, suggesting possible binding of conserved regulators. Comparison of the now available genomes of *B. cinerea* and *S. sclerotiorum* (Broad Institute) revealed that the two genes encode a unique G1 protease in each fungus. Using apple fruit or bean leaves as hosts, we showed that BcACP1 is produced during infection concomitantly with acidification of plant tissues. This is reminiscent of what has been observed with SsACP1 in *S. sclerotiorum* (Poussereau *et al.*, 2001) and suggests that these two closely related fungi behave similarly during infection with respect to the production of this G1 protease.

We investigated the role of environmental pH in the regulation of *BcACP1*. Q-PCR analysis of *B. cinerea* transferred to different pH conditions showed expression of *BcACP1* at pH 3, 4 and 5 but little or no expression at pH 6 and pH 7. *BcACP1* is therefore an acid-expressed gene and its expression profile is similar to that of *SsACP1* (Poussereau *et al.*, 2001). Based on this result, BcACP1 production would be expected during plant infection as soon as the fungus met an acidic pH environment. Surprisingly, BcACP1 is not detected at the early time points of apple fruit infection while pH is permissive to its production. This could be explained by a shortage of fungal biomass and the production of subdetectable amounts of protease. Alternatively, other regulations besides pH could also occur *in planta* that could prevent *BcACP1* expression in the early phases of infection.

In fungi, the zinc finger transcription factor PacC plays an important role in the cell's response to ambient pH. Through binding to a 5'-GCCARG-3' consensus DNA sequence, PacC is considered an activator of alkaline-expressed genes (Penalva & Arst, 2002) and it was shown to repress one acid-expressed gene (Espeso & Arst, 2000). Other acid-expressed genes, however, lack the consensus PacC binding site upstream of their coding sequence

(Sarkar *et al.*, 1996). The absence of such a site in the *BcACP1* promoter and its presence at position -834 in the *SsACP1* promoter prompted us to perform a functional analysis of these two promoters. Transcriptional fusions using the GUS reporter gene were introduced in *B. cinerea*, and GUS activity measurements showed that pH regulation of *SsACP1* and *BcACP1* is independent of the presence of a consensus PacC binding site. We cannot rule out that the results on *SsACP1* regulation could differ when investigated in *S. sclerotiorum*, but our results would suggest that *BcACP1* and *SsACP1* add to previously described acid-expressed genes whose pH regulation does not operate through a consensus PacC binding site. Our results could indicate that PacC regulation of *BcACP1* and *SsACP1* proceeds through another DNA motif. One such motif exists in *Candida albicans* (5'-CCAAGAA-3'; Ramon & Fonzi, 2003), but it is absent in the *BcACP1* and *SsACP1* promoters. Alternatively, the results could indicate that *BcACP1* and *SsACP1* are not regulated via PacC, as shown for several genes in *C. albicans* (Davis *et al.*, 2000). However, a change in *SsACP1* transcription in a strain of *S. sclerotiorum* carrying an activating allele of the PacC orthologue (Kim *et al.*, 2007) suggests that PacC plays a role in *SsACP1* regulation, but the molecular details of this regulation remain unknown and an indirect intervention of PacC cannot be ruled out.

In the course of this work, we found experimental conditions that allowed trapping and visualization of the pro-protein form of BcACP1. When grown at acidic pH in the absence of simple sulfur and nitrogen sources, *B. cinerea* produces high levels of transcripts, and subsequent transfer of the fungus to minimal medium buffered to pH 7.0 results in the secretion of a 35 kDa form of BcACP1. Transfer of this protein to acidic pH results in its conversion into the 28 kDa form that is detected in the fungus secretome during plant infection or growth *in vitro* under acidic conditions. We showed that this conversion is pH dependent and most likely autocatalytic. Indeed, inhibition of most if not all known protease families did not block the 35 kDa to 28 kDa processing of BcACP1, and dilution had no effect on the maturation kinetics. This result is consistent with previous studies performed with other members of the G1 family of proteases (Kataoka *et al.*, 2005; Huang *et al.*, 2007), but we cannot exclude the existence of an unknown family of proteases involved in the processing of BcACP1. We tried to use the same experimental approach to reveal the pro-protein form of SsACP1 of *S. sclerotiorum* but without success. This could be due to the difference in the stability of *SsACP1* mRNA or SsACP1 protein compared to their *BcACP1* counterparts.

In conclusion, the present study highlights the importance of pH in the control of the production of a new member of the G1 family of proteases, BcACP1. Ambient pH regulates both transcription and post-translational processing of this secreted enzyme, and such a tuning system probably participates in the adaptation of *B. cinerea* to a dynamic pH environment.

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