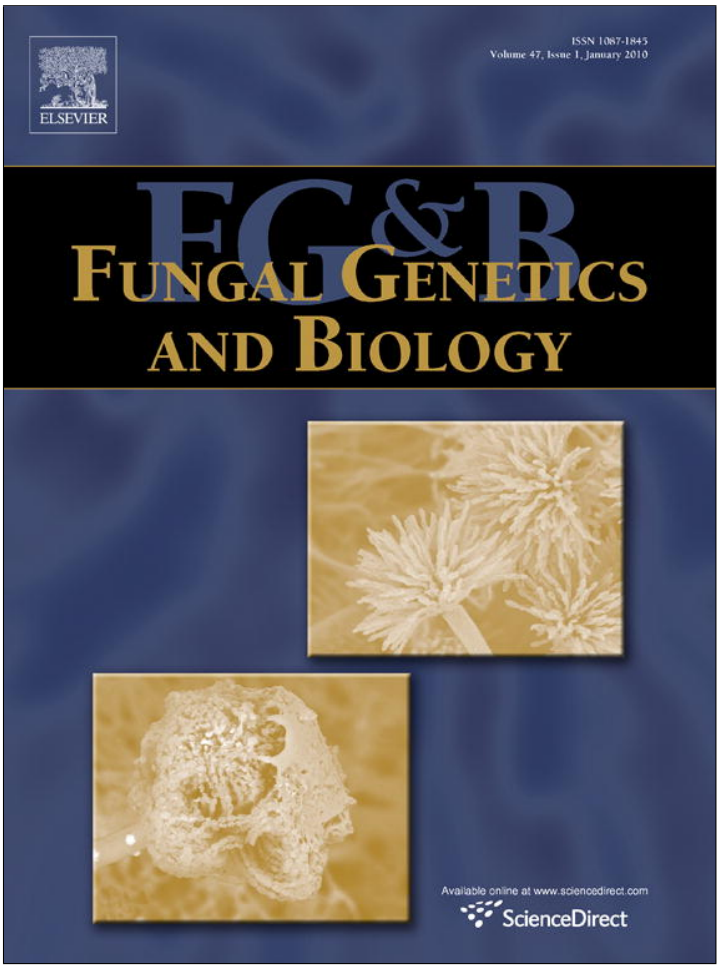

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The *Botrytis cinerea* aspartic proteinase family

Arjen ten Have [a,b,*], José J. Espino [c], Ester Dekkers [b], Steven C. Van Sluyter [d,e], Nélida Brito [c], John Kay [f], Celedonio González [c], Jan A.L. van Kan [b]

[a] *Instituto de Investigaciones Biológicas-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, cc1245, 7600 Mar del Plata, Argentina*
[b] *Laboratory of Phytopathology, Wageningen University, PO Box 8025, 6700EE Wageningen, The Netherlands*
[c] *Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, 38206 La Laguna, Tenerife, Spain*
[d] *The Australian Wine Research Institute, Urrbrae, SA 5064, Australia*
[e] *School of Botany, The University of Melbourne, VIC 3010, Australia*
[f] *School of Biosciences, Cardiff University, Cardiff CF10 3AX, Wales, UK*

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ABSTRACT

The ascomycete plant pathogen *Botrytis cinerea* secretes aspartic proteinase (AP) activity. Functional analysis was carried out on five aspartic proteinase genes (*Bcap*1-5) reported previously. Single and double mutants lacking these five genes showed neither a reduced secreted proteolytic activity, nor a reduction in virulence and they showed no alteration in sensitivity to antifungal proteins purified from grape juice. Scrutiny of the *B. cinerea* genome revealed the presence of nine additional *Bcap* genes, denoted *Bcap*6-14. The product of the *Bcap*8 gene was found to constitute up to 23% of the total protein secreted by *B. cinerea*. *Bcap*8-deficient mutants secreted ~70% less AP activity but were just as virulent as the wild-type strain. Phylogenetic analysis showed that *Bcap*8 has orthologs in many basidiomycetes but only few ascomycetes including the biocontrol fungus *Trichoderma harzanium*. Potential functions of the 14 APs in *B. cinerea* are discussed based on their sequence characteristics, phylogeny and predicted localization.

1. Introduction

The gray mould fungus *Botrytis cinerea* (*Ascomycota*; *Pezizomycotina*; *Leotiomycetes*; *Helotiales*; *Sclerotiniaceae*; *Botryotinia)* is an important plant pathogen that can infect at least 235 plant species (Jarvis, 1977), on various organs and during different stages of development. *B. cinerea* is a typical necrotroph that kills plant cells which subsequently serve as nutrient source (Williamson et al., 2007). *B. cinerea* secretes many enzymes and metabolites that are presumed to enable the fungus to kill and subsequently feed on plant cells (van Kan, 2006). *B. cinerea* enzymes that have been studied in this context are mostly enzymes that can degrade plant cuticle and cell wall components such as cutin (van der Vlugt-Bergmans et al., 1997; van Kan et al., 1997), pectin (Kars et al., 2005a,b; ten Have et al., 1998), hemicellulose (Brito et al., 2006) or cellulose (Espino et al., 2005).

Only a few studies have examined proteinases of plant pathogenic fungi and their possible role(s) in pathogenesis (Jia et al., 2000; Murphy and Walton, 1996; Plummer et al., 2004). In addition to providing the pathogen with nutrient amino acids, proteolytic activity could conceivably be linked to pathogenesis for example by destabilization of the plant cell wall through attack on structural proteins that are involved in wall assembly and integrity (Hall and Cannon, 2002). Plant cell wall degradation has been shown to occur during infection by *B. cinerea* (Kars et al., 2005b; ten Have et al., 1998, 2002). Furthermore, plants secrete pathogenesis-related (PR) proteins, some of which have antimicrobial activity and thereby protect the plant from pathogen attack (for review see van Loon et al., 2006). In turn, the effect of such PR proteins could be counteracted by proteinases released by the pathogen. Also, the avirulence gene AVR-Pita of *Magnaporthe grisea* has been demonstrated to encode a proteinase that directly interacts with the Pita resistance gene product of rice, resulting in a hypersensitive response (Jia et al., 2000).

For *B. cinerea*, we have shown previously that aspartic proteinase (AP) activity was the only class of proteolytic activity detectable in medium of axenic cultures (ten Have et al., 2004). Five AP-encoding genes were cloned, denominated *Bcap*1–*Bcap*5. BcAP2 was unquestionably the vacuolar aspartic proteinase of this fungus while BcAP1 and BcAP5 were considered to be secreted enzymes; BcAP3 and BcAP4 were predicted to remain attached to the plasma membrane by means of a GPI-anchor (ten Have et al., 2004). All five genes were shown to be expressed in axenic cultures and during infection of *B. cinerea* on various plant tissues (ten Have et al., 2004). Infected plant tissues contained high AP activity but it was not readily apparent whether this activity originated from the pathogen or the plant (ten Have et al., 2004). Movahedi and Heale

* Corresponding author. Fax: +54 223 4724143.
E-mail addresses: atenhave@mdp.edu.ar, tenhave@gmail.com (A.ten Have).

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(1990) had also demonstrated earlier that the specific AP inhibitor pepstatin could reduce disease development of *B. cinerea* on carrot disks but whether this effect was due to inhibition of fungal or plant APs was unresolved. Collectively, these results suggest that APs may be involved in pathogenesis of *B. cinerea*. Here we describe the functional analysis of the five previously-cloned AP genes and an additional gene, *Bcap*8, one of a further nine APs that were identified within the *B. cinerea* genome.

2. Materials and methods

2.1. Construction of ΔBcap mutant strains

Mutants in the genes *Bcap*1–*Bcap*5 were generated essentially as described by Kars et al. (2005a) using primers listed in Supplementary Table S1. Briefly, initial 5′- and 3′-flanking gene fragments of approximately 500 bp were amplified using primer combinations 5.1–5.3 and 3.1–3.3, respectively. Overlap extensions were performed using the 5′- and 3′-fragments obtained for each gene, a selection cassette for either hygromycin or nourseothricin and primers 5.2 and 3.2, which are nested with respect to 5.1 and 3.1. The resulting fragment was transformed to *B. cinerea* strain B05.10 and transformants were screened by PCR using primer combinations 5.1-Screen 5 and 3.1-Screen 3 for positive identification of replacement mutants. Positives were further characterized by Southern blot analysis. For generation of double mutants, a well-characterized single-gene mutant was used as recipient for a second transformation with a construct comprising the other selectable marker.

For mutation of the *Bcap*8 gene, plasmid pBSAP833.14 was constructed containing a 2.7-kb genomic fragment carrying the *Bcap*8 coding region plus 1.1 Kb and 0.5 kb of 5′- and 3′-non-coding regions, amplified by PCR from *B. cinerea* genomic DNA with primers AP8FW and AP8RV (Supplementary Table S1). A 1044 bp *Nar*I-*Eco*RI fragment comprising most of the *Bcap8* coding region was excised from this plasmid and substituted by a hygromycin resistance cassette obtained from plasmid pLOB1 (GenBank accession AJ439603) with the same restriction enzymes, yielding plasmid pBSAP8HYGR38. From the latter plasmid, an *Xho*I-*Apa*I fragment carrying the antibiotic resistance cassette plus *Bcap*8-flanking regions was obtained and used to transform *B. cinerea* strain B05.10 as described by Noda et al. (2007). Transformants were subjected to one round of single spore isolation and screened by PCR with primers AP8FW and CHECKRV (Supplementary Table S1), to check for homologous integration. They were also checked with primers AP8FW-AP8RV to ensure the absence of intact copies of *Bcap*8 in heterokaryons. Further characterization was done by Southern blot with a probe generated by PCR from plasmid pBSAP8HYGR38 with primers AP8RV and HYG5FSal (Supplementary Table S1) to ensure that no additional copies of DNA had integrated ectopically.

2.2. Phenotypic characterization of mutants

Infection assays and quantification of AP activity in culture fluid of wild-type strain B05.10 and BcAP-deficient mutants were performed essentially as described by ten Have et al. (2004). Proteinase activity in skimmed milk agar plates was determined as described by Schulze Gronover et al. (2001).

2.3. Analysis of the secretome

Conidia were germinated for 16 h in 250 ml Gamborg's B5 medium containing 10 mM KH_2PO_4, 2 mM sucrose and 0.05% Tween-80. In each flask, a dialysis bag was included that contained 30 ml of a 50% (w/v) strawberry fruit extract made in the same medium present in the flask. The medium was collected by filtration, precipitated with 6% TCA, resuspended in 8 M urea, reprecipitated with methanol–chloroform (Wessel and Flügge, 1984), and resuspended in SDS–PAGE loading buffer.

2.4. SDS–PAGE and identification of BcAP8

Protein electrophoresis was carried out in a Bio-Rad (Hercules, CA, USA) Mini-PROTEAN 3 system, according to the manufacturer's instructions and stained with colloidal Coomassie Brilliant blue G250 (Neuhoff et al., 1985). Band intensity was quantified using the image analysis software Quantity One (Bio-Rad). The most intense band was excised from the gel and sent to the proteomics facility at the Centro Nacional de Biotecnología (Madrid, Spain) for identification via tryptic digestion and MALDI-TOF mass spectrometry. A non-curated database of *B. cinerea* proteins (http://www.broad.mit.edu/annotation/genome/botrytis-cinerea/Home.html) was searched with the software Mascot (Matrix Science Inc. Boston, MA). BcAP8 was the only protein identified in that band, having a Mascot score of 170.

2.5. Quantitative real-time PCR (Q-RT-PCR)

Mycelium was grown as described above for the analysis of the secretome but with different plant extracts inside the dialysis bag. For the *in planta* expression studies, tomato leaves were inoculated with 5-μL droplets of 4.3×10^8 conidia/ml in water. At various time points the infected area was excised and RNA was extracted from it, using the RNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). RNA was converted to cDNA with the First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche). PCR amplifications were carried out in a Bio-Rad iCycler iQ Real-Time PCR Detection System with the Bio-Rad iQ SYBR Green Supermix, using primers RTAP8FW and RTAP8RV (Supplementary Table S1) for the *Bcap*8 gene and primers ACTINAFW and ACTINARV (Supplementary Table S1) for the actin gene *Bcact*1. For both genes, one of the primers used spanned an exon–exon junction to avoid amplification from contaminant genomic DNA. The relative *Bcap*8 mRNA amounts were calculated by the ΔΔCt method from the mean of three independent determinations of the threshold cycle, as described (Applied Biosystems, 2004).

2.6. Fungal growth inhibition assays with grape PR proteins

B. cinerea wild-type strain B05.10 and individual single and double *Bcap*-deficient mutants were grown in the dark at 20 °C on 90 mm plates of synthetic grape agar inoculated with 3 μL of 10^6 conidia/mL. Synthetic grape agar was a modification of Henschke and Jiranek (1993) containing 100 g/L glucose, 100 g/L fructose, 0.01% Tween 20, 5 mM alanyl-glutamine, and 1X each of Kao and Michayluk vitamins, MEM amino acids, and MEM non-essential amino acids (Sigma, St. Louis MO). A solution of the tartrate and phosphate salts with 20 g/L agar was adjusted to pH 6.9 with KOH, autoclaved, and combined with a pH 2.7 (KOH) solution of the remaining components such that the resulting pH was 3.4.

Two thaumatin-like proteins and two chitinases, arbitrarily designated A through D were purified from Semillon grape juice according to Van Sluyter et al. (2009). Crude grape protein was prepared from Semillon grape juice by ammonium sulfate precipitation at 80% saturation. Grape proteins in 0.1 M potassium malate, pH 3.5 were added to Miracloth disks surrounding growing mycelium at ca. 72 hpi and again at 96 hpi. Plates were imaged with a digital camera at 6 and 7 dpi.

2.7. Genome sequence data mining

The genome sequence of *B. cinerea* strain B05.10 (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html) was mined for additional AP-encoding genes by Pfam profile alignment (Finn et al., 2006) using domain description PF09668 for eukaryotic aspartic proteinases. Twelve sequences with two D[T/S]G motifs were detected, including those of the previously described *Bcap*1–*Bcap*5 (ten Have et al., 2004). One sequence, Bc1G_08815, was highly similar to APs but lacked the hallmark D[T/S]G motif and was therefore excluded from further analyses. Further searches were performed including PSI-BLASTP (Altschul et al., 1997) and PHI-BLASTP (Zhang et al., 1998) using BcAP1 as query and Prosite pattern PS00141 (Hulo et al., 2008) as PHI pattern. Each output was checked manually. This analysis identified two partial AP genes. BLASTN using the two identified partial sequences against the genome sequence of *B. cinerea* strain T4 (http://urgi.versailles.inra.fr/projects/Botrytis/index.php) identified two loci encoding an AP gene, which were incorrectly predicted in the annotation of strain B05.10. PSI-PHI-BLAST identified seven sequences with similarity but lacking one or more D[T/S]G motifs. We annotated all nine newly discovered sequences manually. Large stretches of gaps detected in BLASTP analysis using as queries sequences predicted from automatically generated gene models provided by the Broad Institute (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html) were considered as indicative for incorrect gene modeling. Incorrect models were corrected with splice site conservation and amino acid sequence similarity optimization as criteria. Sequences corresponding to manually annotated gene models have been deposited in NCBI (see Table 3 for accession numbers).

2.8. Phylogenetic analysis

AP sequences of *Sclerotinia sclerotiorum, M. grisea, Aspergillus niger, Aspergillus fumigatus, Trichoderma reesei* (Martinez et al., 2008) and *Laccaria bicolor* (Martin et al., 2008) were identified by MEROPS batch BLAST (http://merops.sanger.ac.uk/, Rawlings and Morton, 2008) using all protein sequences predicted by each genome sequence project. The sequences were downloaded using their NCBI accession numbers, except for *T. reesei* sequences that were downloaded from the JGI website (http://genome.jgi-psf.org/Trire2/Trire2.home.html). The *B. cinerea* AP sequences correspond to the annotation and NCBI accession numbers listed in Table 3. Putative orthologs of BcAP8 and BcAP14 were identified using BLASTP at various sequence databases, with BcAP8 and BcAP14 as query, respectively. Sequences of putative hits were downloaded from NCBI and checked for being Best Bidirectional Hit. Further *Trichoderma harzianum* APs were found by Entrez (http://www.ncbi.nlm.nih.gov/sites/gquery). All sequences were screened for the presence of AP hallmark motifs and the absence of large gaps (>10 aa residues) in the first 10 alignments produced by BLASTP against Fungal Refseq sequences at NCBI. Sequences that lacked the AP hallmark motifs and/or produced large gaps, were analyzed for possible incorrect automatic gene modeling. The first intron position in the automatically generated gene model XP_364869.1 from *M. grisea* was modified to Mg_364869A to correct for a large gap that was present as compared to other fungal APs. The corrected model is the only other possible model and consistently yields better pairwise alignments in BLAST analyses. All sequences were collected in a single Fasta archive and subjected to alignment. T-Coffee (Notredame et al., 2000) was used to obtain a guide tree. Sequences were divided in groups based on the guide tree and the groups were aligned in T-Coffee. Alignment was then adjusted manually by aligning the sequences groupwise against the BcAP orthologs (as aligned in Fig. 1), using the clade-specific T-Coffee alignments for fine tuning. The C-termini were trimmed according to Fig. 1 and 3 additional residues were excluded from the N-termini (with reference to pig pepsin) due to the lack of similarity. We aligned all sequences and removed putative recent paralogs. The resulting multiple sequence alignment, shown in Supplementary Fig. S4, contains two poorly aligned blocks. The first of these lies in the BcAP14 clade and BcAP7 and introduces an approximate 20 residue gap between Met199 and Asp200 (numbering system of pig pepsin). The second is a non-homologous segment which corresponds to a region known as the polyproline loop (contained between Glu287-Trp299 in pig pepsin) that makes a major contribution to define the specificity of the active site of individual aspartic proteinases (Dunn, 2002; Ng et al., 2000). The corresponding columns were removed prior to phylogeny. A Maximum Likelihood tree was constructed using PhyML (Guindon and Gascuel, 2003). Bootstrap analysis was performed using PhyML. The obtained trees were rooted and reorganized using PHYLIP v. 3.67 (Felsenstein, 1989), with the node connecting pig pepsin and Nothepsin from antarctic fish *Chionodraco hamatus* serving as outgroup.

3. Results

3.1. Construction and phenotypic characterization of mutants in *Bcap1–Bcap5*

Loss-of-function mutations in the previously described genes *Bcap*1–*Bcap*5 (ten Have et al., 2004) were made by gene replacement in wild-type strain B05.10 as described by Kars et al. (2005b) using nourseothricin or hygromycin resistance as selection. Following selection and several rounds of single spore isolation, all transformants were screened for homologous recombination by PCR using the primers listed in Supplementary Table S1 (data not shown). The occurrence of the desired homologous recombination and the absence of additional ectopic inserts were confirmed by Southern blot analysis (Supplementary Fig. S1).

Mutants for each gene – two or three independent mutants, if available – were subjected to phenotypic analysis. Virulence assays on detached tomato leaves showed that none of the mutants displayed a reduction in virulence (Table 1). Mutants were also just as virulent as the wild type when tested on either bell pepper or apple fruit. In the few cases where a reduction in virulence was observed, this effect was not consistent when the assays were repeated with the same mutant or with an independent mutant in the same gene (Table 1). Cell-free filtrates of axenic cultures of each of the mutants contained comparable levels of proteolytic activity to the wild-type strain B05.10 (Table 2). Further analyses using plate assays containing skimmed milk powder as sole nutrient source (Schulze Gronover et al., 2001), did not indicate any reduction in growth rate. No differences were observed between the mutants and wild-type strain B05.10 in clarification of the turbid skimmed milk powder underneath and around fungal colonies (Supplementary Table S2).

Since *B. cinerea* was shown previously to contain at least five APs (ten Have et al., 2004), it is conceivable that functional redundancy might have obscured effects of single-gene replacements on virulence and proteolytic activity. Therefore double mutants were made for the non-vacuolar enzymes (BcAP1, BcAP3, BcAP4 and BcAP5), using a second antibiotic selection marker. Five double mutant combinations were generated: Δ*Bcap*1/Δ*Bcap*3, Δ*Bcap*1/Δ*Bcap*4, Δ*Bcap*1/Δ*Bcap*5, ΔBcap3/Δ*Bcap*4 and Δ*Bcap*3/Δ*Bcap*5. No Δ*Bcap*4/Δ*Bcap*5 double mutant was obtained. None of the double mutants (two independent mutants, if available were subjected to analysis) showed reduction in virulence (Table 1). No obvious differences could be observed between the double mutants and

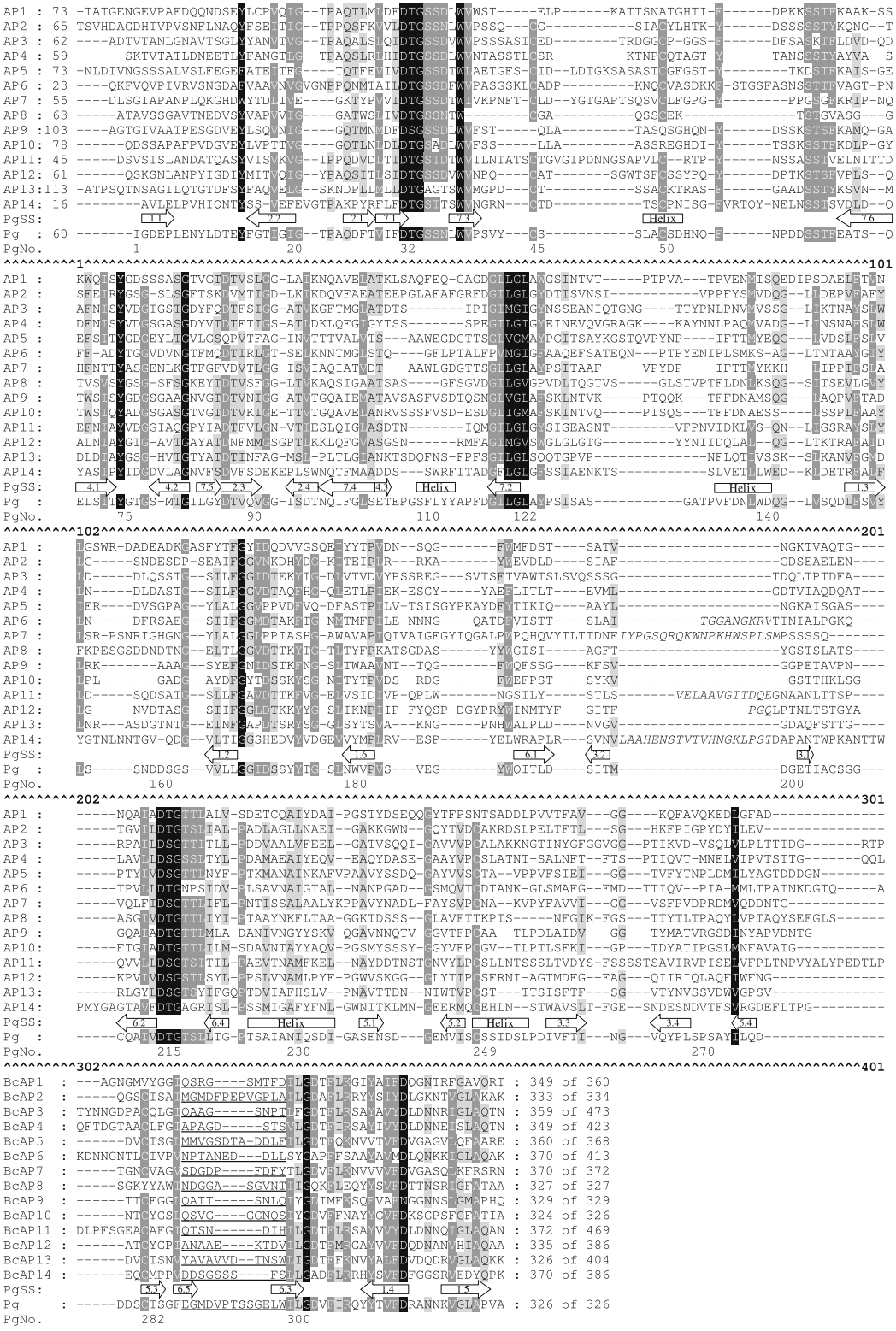

Fig. 1. *Botrytis cinerea* aspartic proteinase multiple sequence alignment. Partial alignment of aspartic proteinase sequences from *B. cinerea* (AP1-AP14) with that of pig pepsin (Pp), excluding the non-homologous N- and C-terminal regions (see Supplementary Figs. S2 and S3). Gaps in the alignment are indicated by a hyphen (-). Black, dark-gray and light-gray shading of residues correspond to 100%, 80% and 60% overall similarity between the 15 sequences aligned. The standard numbering for the mature region of pig pepsin (PpNo.) is included underneath the sequence blocks. The number preceding each sequence indicates the position of the first residue of the predicted mature proteinase with respect to the start codon, whereas the number following the sequence corresponds to the number of the last homologous residue of the predicted mature proteinase. The number in brackets corresponds to the length of the protein as determined from the first residue of the mature proteinase. The numbering in the block separator refers to the numbering of the alignment. PpSS indicates the position of secondary structures in pig pepsin (3pep). BcAP sequences were first aligned based on conserved hallmark sequences and disulfide bridges, and subsequently on structurally conserved secondary structures as determined by PHYRE loop modeling. Amino acids in the polyproline loop are underlined, a stretch of amino acids that are poorly aligned due to a ~20 amino acid insertion in BcAP7 and BcAP14 are marked in italics.

Table 1
Virulence of Δ*Bcap*1-5 mutants and double mutants. Radial lesion growth of mutants deficient in the respective *Bcap* genes and double mutants lacking two of these five genes, as compared to lesions of wild-type *B. cinerea* strain B05.10. The growth of each lesion (measured as cm ± standard error of mean) was monitored between 48 h and 72 h post inoculation. The amount of growing lesions monitored is indicated between brackets. Independent mutants in the same gene are indicated by hyphenated numerals. The results of replicate, independent assays with a particular mutant on the same host are indicated on separate rows. Values that differ significantly from those of the wild-type strain are marked with one asterisk (∗; *t*-test, $P < 0.05$) or two asterisks (∗∗; *t*-test, $P < 0.01$).

	Tomato		Bell pepper		Apple	
	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant
Δ*Bcap*1-1	4.6 ± 1.2 (22)	4.7 ± 1.1 (25)	5.7 ± 2.1 (40)	6.0 ± 2.0 (37)	7.8 ± 0.9 (39)	7.7 ± 0.9 (38)
	5.9 ± 0.4 (16)	6.2 ± 0.4 (16)	3.9 ± 2.4 (15)	5.3 ± 2.8 (16)	11.4 ± 2.4 (22)	3.9 ± 3.0 (8)
Δ*Bcap*1-3	5.0 ± 0.7 (28)	5.4 ± 0.6 (28)	7.1 ± 2.6 (16)	6.4 ± 1.7 (16)		
Δ*Bcap*1-7	5.0 ± 0.7 (30)	5.1 ± 0.6 (30)	7.5 ± 2.5 (16)	6.9 ± 2.4 (16)		
Δ*Bcap*2-4	4.8 ± 0.3 (26)	5.1 ± 0.4 (25)	6.3 ± 2.3 (16)	6.8 ± 2.1 (16)	9.1 ± 0.8 (40)	9.2 ± 0.9 (38)
					10.1 ± 4.4 (12)	8.7 ± 3.2 (23)
Δ*Bcap*3-5	4.9 ± 0.8 (28)	4.9 ± 0.6 (29)	7.8 ± 1.7 (16)	6.9 ± 2.0 (16)	9.8 ± 0.9 (40)	8.5 ± 1.2 (39)∗∗
	6.1 ± 0.3 (16)	6.3 ± 0.3 (16)	3.1 ± 1.2 (15)	4.8 ± 1.5 (16)∗	6.1 ± 2.7 (25)	6.0 ± 2.2 (27)
Δ*Bcap*4-2	4.3 ± 0.6 (30)	4.3 ± 0.5 (30)	6.0 ± 2.6 (16)	6.1 ± 1.8 (16)		
Δ*Bcap*4-5	4.7 ± 0.4 (30)	4.7 ± 0.3 (30)	5.7 ± 2.3 (16)	5.9 ± 2.5 (16)		
	4.3 ± 0.5 (16)	3.7 ± 0.6 (16)∗				
Δ*Bcap*5-6	4.3 ± 0.7 (29)	4.6 ± 0.4 (30)	5.8 ± 1.3 (16)	6.1 ± 1.7 (16)	8.2 ± 1.0 (40)	8.1 ± 0.9 (40)
	5.0 ± 0.2 (29)	4.8 ± 0.3 (28)	6.3 ± 2.2 (15)	6.1 ± 2.7 (14)	8.1 ± 4.0 (16)	7.3 ± 2.8 (26)
Δ*Bcap*1/Δ*Bcap*3-1	5.1 ± 0.8 (23)	4.7 ± 0.7 (24)	5.1 ± 1.3 (16)	5.8 ± 2.0 (16)		
Δ*Bcap*1/Δ*Bcap*3-2	4.5 ± 0.9 (18)	4.3 ± 0.9 (22)	6.2 ± 2.9 (13)	5.1 ± 2.1 (16)		
Δ*Bcap*1/Δ*Bcap*4-7	5.2 ± 0.5 (30)	5.0 ± 0.7 (29)	6.6 ± 2.1 (16)	6.9 ± 2.9 (16)		
	4.6 ± 0.7 (17)	4.8 ± 0.7 (19)				
Δ*Bcap*1/Δ*Bcap*4-16	5.2 ± 0.6 (26)	5.3 ± 0.9 (25)	6.8 ± 2.2 (16)	6.9 ± 1.7 (16)		
	4.1 ± 1.0 (26)	4.0 ± 0.9 (25)				
Δ*Bcap*1/Δ*Bcap*5-4	5.4 ± 0.9 (27)	5.6 ± 1.0 (25)	4.8 ± 1.6 (16)	5.1 ± 2.2 (15)		
Δ*Bcap*1/Δ*Bcap*5-7	4.2 ± 0.8 (27)	4.5 ± 0.8 (26)	4.3 ± 3.6 (13)	6.4 ± 2.5 (16)		
Δ*Bcap*3/Δ*Bcap*4-1	4.6 ± 0.9 (28)	4.7 ± 0.7 (27)	5.3 ± 1.9 (16)	6.4 ± 2.7 (16)		
	4.7 ± 1.1 (26)	5.0 ± 1.1 (26)				
Δ*Bcap*3/Δ*Bcap*5-1	4.1 ± 0.4 (30)	4.6 ± 0.4 (30)	6.4 ± 2.2 (16)	7.0 ± 2.3 (16)		
	4.1 ± 1.1 (20)	4.1 ± 0.9 (26)				

Table 2
Secreted proteolytic activity. In experiment 1, each culture was grown for 41 h in basal salt medium amended with 1% glucose (G) and 1% bovine serum albumin (BSA). In experiment 2, each culture was grown for 24 h in medium amended with only 1% glucose (1% G) or 1% Glucose plus 1% BSA. Proteinase activities are in arbitrary units ± standard error of mean ($n = 3$).

	Experiment 1	Experiment 2	
	1% G + 1% BSA	1% G	1% G + 1% BSA
B05.10	450 ± 40	400 ± 30	360 ± 20
Δ*Bcap*1-1	470 ± 80	270 ± 30	470 ± 10
Δ*Bcap*1-3	590 ± 18		
Δ*Bcap*1-7	810 ± 100		
Δ*Bcap*3-5	430 ± 20	350 ± 30	350 ± 20
Δ*Bcap*4-2	530 ± 10	240 ± 40	390 ± 20
Δ*Bcap*4-5	660 ± 30		
Δ*Bcap*5-4	470 ± 100	380 ± 40	500 ± 10
Δ*Bcap*1-1/Δ*Bcap*3-1	370 ± 10	230 ± 60	200 ± 10
Δ*Bcap*1-1/Δ*Bcap*3-2	570 ± 14		
Δ*Bcap*1-1/Δ*Bcap*4-7	510 ± 10	120 ± 10	380 ± 10
Δ*Bcap*1-1/Δ*Bcap*4-16	520 ± 10		
Δ*Bcap*1-1/Δ*Bcap*5-4	460 ± 20	200 ± 20	430 ± 50
Δ*Bcap*1-1/Δ*Bcap*5-7	450 ± 80		
Δ*Bcap*3-5/Δ*Bcap*4-1	520 ± 20	220 ± 20	370 ± 10
Δ*Bcap*3-5/Δ*Bcap*5-1	440 ± 60	240 ± 10	390 ± 20

wild-type strain B05.10 in secreted proteinase activity in axenic culture, nor did growth of mutants on skimmed milk plates differ from the wild type with respect to colony diameter and the formation of a transparent zone accompanying the colony (Tables 1 and 2, Supplementary Table S2).

3.2. The Botrytis cinerea genome contains nine additional AP-encoding genes

Since none of the tested single or double mutants in the *Bcap*1–*Bcap*5 genes displayed a detectable reduction in secreted AP activity, additional APs were possibly responsible for the measured proteolytic activity. Upon public availability, the genome sequence of *B. cinerea* strain B05.10 was analyzed for the presence of additional putative AP-encoding genes using Pfam profile alignment and PSI- and PHI-BLAST. A total of 21 sequences with similarity to APs were identified, seven of which were discarded because they lacked one or both D[T/S]G active site motifs. The remaining 14 sequences included the *Bcap*1–*Bcap*5 genes that had been described earlier (ten Have et al., 2004). The newly identified sequences were denoted as *Bcap*6–*Bcap*14 (Table 3). All 14 genes were also present in the genome of *B. cinerea* strain T4, which recently became publicly available. Numbers of the gene models of the *Bcap*1-14 genes in strain T4 are also listed in Table 3. In order to corroborate each of these new sequences as a bona fide AP, a detailed analysis was undertaken *in silico*.

Eukaryotic APs are typically produced in the form of a precursor. Each polypeptide consists of a signal peptide and a pro-peptide region of varying length preceding the mature enzyme itself. In some APs, the polypeptide continues beyond the conventional location of the C-terminus, as defined by the established reference standard pig pepsin (Blundell et al., 1998; Khan and James, 1998; Dunn, 2002) so that a C-terminal extension or "tail" is present. The mature enzyme regions of the nine new BcAP sequences all showed considerable similarity to one another, to the previously described BcAP1–BcAP5 polypeptides and to pig pepsin. Alignment of this region from all 14 BcAP sequences with that of pig pepsin is shown in Fig. 1. Since the signal peptide and pro-peptide regions, as well as the C-terminal tails (where present) are not homologous, these regions have been omitted from Fig. 1 but are depicted in Supplementary Figs. S2 and S3, respectively. The mature enzyme regions of BcAP1–BcAP14 were manually aligned with the sequence of pig pepsin. Alignment was first performed on conserved AP hallmark motifs and, subsequently, on secondary structure elements (Fig. 1; residue numbering in both text and figure is that of pig pepsin (Pp)). Eukaryotic APs typically consist of two

Table 3
Aspartic proteinase encoding genes in the genome of *B. cinerea* strain B05.10. Entries in the gene model column correspond to the identifiers allocated at http://www.broad.mit.edu. NCBI indicates the accession number of the nucleotide sequence deposited at NCBI. Prosite indicates the location of the PS00141 pattern for APs with respect to the start of the zymogen. ESTs lists the number of ESTs represented in 15 different EST libraries generated and analyzed by the *Botrytis* genome annotation consortium.

Protein	T4 model	B05.10 model	NCBI	Prosite	Predicted location	ESTs
BcAP1	BofuT4_P054370	Bc1G_07068	AF121229	107-18	Cytosol/mucleus	27
BcAP2	BofuT4_P088640	Bc1G_06849	AY361913	100-11; 283-94	Vacuole	94
BcAP3	BofuT4_P145970	Bc1G_05845	AY507155	294-305	Cell membrane	40
BcAP4	BofuT4_P052010	Bc1G_06540	AY507156	281-92	Cell membrane	9
BcAP5	BofuT4_P135550	Bc1G_01794	AJ617485	106-17	Secreted	2
BcAP6	BofuT4_P091730	Bc1G_13520*	XP_001548014.1	56-67	Secretory pathway	3
BcAP7	BofuT4_P021760	Bc1G_00545	–	86-97	Secreted	2
BcAP8	BofuT4_P134040	Bc1G_03070	XP_001558038.1	271-82	Secreted	82
BcaAP9	BofuT4_P120760	Bc1G_03579	XP_001554261.1	132-43; 318-29	Secreted	16
BcAP10	BofuT4_P083940	Bc1G_07521	XP_001553961.1	105-16; 288-99	Secreted	0
BcAP11	BofuT4_P029480	Bc1G_01218	XP_001560386.1	75-86; 281-92	Cell membrane	0
BcAP12	BofuT4_P040820*	Bc1G_01397*	–	284-95	Extracellular matrix	0
BcAP13	BofuT4_P145020*	Bc1G_04573/4*	GQ336874	Not detected	Cell membrane	3
BcAP14	BofuT4_P010710	Bc1G_08393	XP_001552528.1	44-55	Secretory pathway	11

* Gene models incomplete or incorrect.

homologous domains, each of which provides a catalytic Asp residue to the active site (Khan and James, 1998). Each Asp residue is present in the hallmark motif – Asp-Thr/Ser-Gly – (D[T/S]G), which is followed further downstream by a – Hydrophobic–Hydrophobic-Gly – sequence. Together, these motifs form a structural feature known as a psi loop (Blundell et al., 1998). In all 14 BcAP sequences the first and second psi loops are fully conserved with three minor exceptions in the second psi loop. Here, the residue Asp303, highly conserved among eukaryotic APs (Yamauchi et al., 1988) is replaced by Ala in BcAP6 and BcAP14, and by Gln in BcAP8. All mature BcAP sequences have a Tyr residue at position 75, which is another strictly conserved residue positioned near the tip of the β-hairpin loop or "flap" that overlies the active site (Khan and James, 1998; Dunn, 2002). Furthermore, with the exception of BcAP5 and BcAP7, all mature BcAP enzymes contain an Asp residue at a position equivalent to the highly-conserved Asp87 residue. The presence of all of these motifs that are known to be critical for AP activity suggests that all 14 of the *Bcap* genes encode functional enzymes.

Conservation of structure was studied using 3D models of the mature enzyme region of each BcAP, predicted by PHYRE loop modeling (Bennett-Lovsey et al., 2008), using as reference the pig pepsin structure 3PEP (Abad-Zapatero et al., 1990). BcAP14 has a 20 aa residue insertion corresponding to pig pepsin position 200 (Fig. 1) which could not be modeled by PHYRE. After verifying that the above mentioned hallmarks were in the correct position in the 3D models of the 14 BcAPs, the secondary structures suggested by the models were aligned to the structure of pig pepsin (Fig. 1). All BcAPs showed significant correspondence of structure which further corroborates that they are all likely to be functional enzymes. Most sequences also contain four cysteine residues in locations that coincide with residues known to form disulfide bonds in many eukaryotic APs including pig pepsin (between residues 45–50 and 249–282 respectively, Fig. 1). The exceptions are BcAP9 and BcAP10 which lack the 45–50 disulfide bridge, BcAP8 which lacks the one between residues 249–282 and BcAP1 which does not contain any disulfide bridge, as discussed previously (ten Have et al., 2004).

The sequences at the N-terminal end of each BcAP polypeptide are shown in Supplementary Fig. S2. With the exception of BcAP1, all of the BcAP proteins are predicted to have a signal peptide and so, as with most AP precursors, appear destined for import into the lumen of the endoplasmic reticulum where the typically inactive zymogens can undergo post-translational modification prior to onward trafficking or secretion. AP zymogens are converted into the mature enzymes by proteolytic cleavage(s) that removes the pro-peptide segment. This process can occur by autoactivation or can be mediated by the action of an extrinsic proteinase (Dunn, 2002), sometimes by cleavage behind a single or two basic residue(s). The existence of possible ($P > 0.1$) and probable ($P > 0.5$) dibasic or monobasic cleavage sites for pro-peptide removal was tested using ProP (Duckert et al., 2004). In most cases, such sites were observed (Supplementary Fig. S2); the precursor forms of BcAP9 and BcAP13, however, most likely are converted into the mature enzyme forms shown in Fig. 1, by autoactivation analogous to pig pepsinogen (Dunn, 2002). Notably, the BcAP6 and BcAP14 polypeptides appear to lack a pro-peptide completely; each (predicted) mature enzyme region appears to follow on directly from the signal peptide (Fig. 1; Supplementary Fig. S2). The absence of a pro-peptide region in these two polypeptides suggests that each of these two mature enzymes must be generated directly in its active conformation after removal of the signal peptide upon entering the endoplasmic reticulum. The absence of a pro-peptide region was also observed in orthologs of BcAP14 in *S. sclerotiorum* and *Phaeosphaeria nodorum* (see below). Notably, the 20 residue insert in BcAP14, in the position equivalent to Asp200 in pig pepsin (see above) also appears to be conserved among these two sequences.

Fungal APs are generally considered to be either targeted to the vacuole (Parr et al., 2007) or secreted (Monod et al., 2002). The latter class may subsequently attach to the cell membrane by means of a GPI-anchor (Gagnon-Arsenault et al., 2006). Table 3 summarizes the cellular locations predicted for the BcAPs based on combined bioinformatic analyses (SignalP (Nielsen et al., 1997) WoLF PSORT (Horton et al., 2007), ProP (Duckert et al., 2004) and biGPI (Eisenhaber et al., 2004)). BcAP2 is homologous to APs from other fungal species that are known experimentally to be located in the vacuole. BcAP5, BcAP7, BcAP8, BcAP9 and BcAP10 are all predicted to be secreted proteins. BcAP3, BcAP4, BcAP11 and BcAP13 contain a GPI-anchor motif (Eisenhaber et al., 2004, Supplementary Fig. S3). Moreover, these same four proteins, as well as BcAP12 contain in their C-terminus one or more stretches with more than 50% Ser/Thr residues (Supplementary Fig. S3). These offer potential sites for O-glycosylation which might contribute to retention of the enzymes within the extracellular matrix.

3.3. The most abundant secreted protein, BcAP8, provides the majority of secreted AP activity but is not essential for pathogenesis

In 15 EST libraries analyzed by the *Botrytis* genome annotation consortium (Lebrun et al., in preparation), a total of 289 ESTs were identified as being derived from 11 of the 14 *Bcap* genes (Table 3). BcAP10, BcAP11 and BcAP12 were not represented by any EST in

these libraries. From their representation in EST libraries and the predictions for their cellular location, BcAP5, BcAP8 and BcAP9 emerged as the best candidates for the secreted AP activity. The *B. cinerea* secretome was analyzed to identify the enzyme(s) contributing the secreted AP activity. The total secreted protein fraction of *B. cinerea* strain B05.10, grown in liquid culture, was analyzed by SDS–PAGE (Fig. 2, lane WT). The pattern was relatively simple and one protein band with an apparent molecular mass of approximately 35 kDa was predominant. Quantitative examination of the gel indicated that this band comprised 23% of the total protein secreted. The band was excised from the gel and identified by peptide mass fingerprint using MALDI-TOF mass spectrometry as the BcAP8 protein. BcAP5, BcAP9 and BcAP10 were also identified as being present in the secretome, albeit at low abundance (not shown).

The expression of the *Bcap*8 gene was monitored by Q-RT-PCR in different liquid media (Fig. 3A) and *in planta* (Fig. 3B). In all of the liquid media tested, the ratio of *Bcap*8 mRNA versus actin mRNA increased rapidly during germination, reaching a peak at 12 h where an increase of 1000–5000-fold was observed relative to ungerminated conidia. The ratio dropped to a level close to baseline levels thereafter (Fig. 3A). A comparable increase in expression for *Bcap*8 was also observed in *B. cinerea*-inoculated tomato leaves, with a peak around 3–4 days (Fig. 3B). Attempts to follow expression of the *Bcap*8 gene during the early stages of infection *in planta*, were unsuccessful.

Since *Bcap*8 had such high expression during conidia germination this gene was also functionally analyzed by gene replacement. Two transformants were identified, Δ*Bcap*8-9 and Δ*Bcap*8-19, which carried a single integration of a hygromycin cassette that replaced the wild-type gene (Supplementary Fig. S1). No *Bcap*8 mRNA was detectable by Q-RT-PCR in either of these mutants (not shown) and the BcAP8 protein was not detectable in the pool of secreted proteins of either mutant (Fig. 2). As BcAP8 accounted for 23% of the total secreted protein in wild-type strain B05.10, a *Bcap*8-deficient mutant might be expected to show reduced proteolytic activity, even with other proteinases continuing to be produced at normal levels. The two Δ*Bcap*8 mutants showed a significant reduction in secreted proteinase activity, both in synthetic liquid media (>71% reduction) and in plates (Fig. 4). Upon growth in liquid culture in the presence of a strawberry extract, a similar reduction in activity was observed in the *Bcap*8-deficient mutants (not shown). Interestingly, both mutants showed additional changes in protein profiles as judged from SDS–PAGE (Fig. 2). Several bands disappeared in addition to that of BcAP8 and a number of bands appeared. This suggests that some of the bands in the secreted protein profile of strain B05.10 may be proteolytic products of larger proteins that serve as substrates for BcAP8. The absence of BcAP8 in the extracellular medium may have resulted in prolonging the lifetime of (as yet uncharacterised) proteins that would not be detected in wild-type strains.

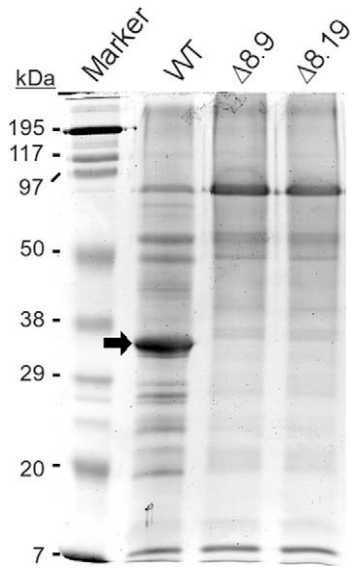

Fig. 2. SDS–PAGE of the *Botrytis cinerea* early secretome. Media aliquots (83 ml) were harvested 16 h after inoculation of wild-type strain B05.10 (WT) and the two *Bcap*8 mutants, Δ*Bcap*8-9 and Δ*Bcap*8-19. After concentration, the respective samples were analyzed by SDS–PAGE using a 12%-acrylamide gel. The respective mass (kDa) of each of the proteins in the marker lane is indicated. The BcAP8 band is high-lit by an arrow.

The virulence of Δ*Bcap*8 mutants was tested on tomato leaves. In six independent experiments, the mutants were found to exhibit wild type virulence (Table 4) Furthermore, the virulence of these Δ*Bcap*8 mutants was also tested on tobacco leaves, strawberries, grape berries and tomato fruit. No difference in virulence was observed for either mutant on any plant tested, as compared to the wild-type strain B05.10 (data not shown). Filtration fluid obtained from eggplant tissue infected with mutant Δ*Bcap*8-9 did contain AP activity, but the level was about 50% of that in eggplant tissue infected with the wild-type strain B05.10 (not shown). Thus, despite being by far the most abundant protein in the secretome, BcAP8 appears to be dispensable for virulence, at least under the conditions used in these experiments.

3.4. Contribution of extracellular proteinases in sensitivity to antifungal PR proteins

If the function of a fungal enzyme should be to counteract the antifungal activity of plant PR proteins, then PR proteins might be predicted to be more effective in restricting the growth of proteinase-deficient mutant fungal strains. Four antifungal PR proteins originating from grape were separately purified to homogeneity, of which two were chitinases and two were thaumatin-like proteins. All four purified proteins were able to inhibit growth of *B. cinerea* wild-type strain B05.10, albeit with different efficiency (Fig. 5). These proteins were also tested for their effect on multiple independent transformants of each of the 11 different *Bcap*-deficient mutants that were generated as described above. None of the mutants displayed an increased sensitivity to any of the four PR proteins. A representative growth inhibition comparison for the Δ*Bcap*3/Δ*Bcap*5 double mutant and the wild-type strain B05.10 is shown in Fig. 5.

3.5. Classification of BcAPs

Plant cell wall degradation is important for *B. cinerea* virulence (van Kan, 2006) but the studies described above suggest that BcAP1–BcAP5 as well as BcAP8, are not essential in this process. Deletion of each of the remaining genes, either individually or in combinations, in order to study the role of BcAPs in virulence would be prohibitively time-consuming. We considered that a bioinformatics/phylogenetic analysis might give important insights into possible alternative roles for the 14 APs in *B. cinerea.* A phylogenetic classification, for example, might identify APs that are specific for *B. cinerea*, or are shared only between *B. cinerea* and closely related species such as *S. sclerotiorum*, or are conserved among fungi that are plant pathogens.

The genomes of a number of fungi were mined for their AP gene content by performing a MEROPS batch BLAST (Rawlings and Morton, 2008). The species, analyzed included *S. sclerotiorum*, two sordariomycetes – the pathogen *M. grisea* and the saprophyte *T. reesei*, two eurotiomycetes – the saprophyte *A. niger* and the human pathogen *A. fumigatus;* as well as the Basidiomycete *L. bicolor.*

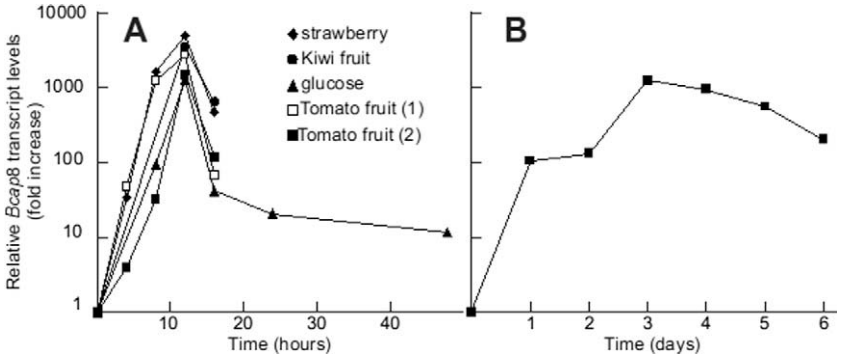

Fig. 3. Expression of *Bcap8* in culture and in planta. Wild-type strain B05.10 mycelium was grown in basal salt medium amended with 2 mM glucose in the presence or absence of extracts of the indicated fruits (panel A) or on tomato leaves (panel B). At the indicated times, RNA was extracted and the levels of *Bcap*8 mRNA were determined by Q-RT-PCR. Values are expressed as the increase in the ratio of *Bcap*8 mRNA versus actin mRNA, with respect to the same ratio in non-germinated conidia.

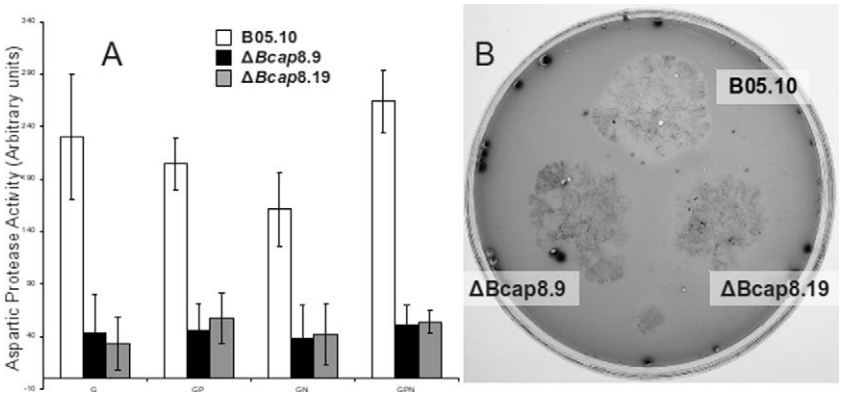

Fig. 4. BcAP8 mutants show reduced proteinase activity. Panel A: total proteinase activity was measured in cell-free culture filtrates from *B. cinerea* strain B05.10 and mutants Δ*Bcap*8-9 and Δ*Bcap*8-19 after 24 h of growth on basal salt medium with 1% glucose (G) amended with 1% peptone (GP), 100 mM NH4Cl (GN) or 1% peptone and 100 mM NH4Cl (GPN). Proteinase activity on hemoglobin as substrate was quantified by measuring change of absorbance at 280 nm. Error bars indicate standard error of means ($n = 3$). Panel B: Degradation of proteins in plates amended with 1% bovine serum albumin and 2 mM sucrose. Proteins were stained with colloidal Coomassie brilliant blue G250 after two days of growth.

Table 4
Virulence of Δ*Bcap*8 mutants on tomato leaves. Wild-type *Botrytis cinerea* strain B05.10 and two independent mutants deficient in the *Bcap*8 gene were inoculated onto tomato leafs. The growth of successfully expanding lesions (measured as cm ± standard error of mean) was monitored over a 48 h period (assays 1 and 2) or a 24 h period (assays 3–6).

Assay	Wild type	Δ*Bcap*8–9	Wild type	Δ*Bcap*8–19
1	11.1 ± 0.2	10.9 ± 0.2	10.6 ± 0.2	11.1 ± 0.2
2	9.6 ± 0.2	10.5 ± 0.2	9.4 ± 0.3	10.0 ± 0.3
3	3.2 ± 0.2	3.2 ± 0.1	3.0 ± 0.1	3.3 ± 0.1
4	5.7 ± 0.4	5.4 ± 0.2	2.8 ± 0.2	3.4 ± 0.1
5	5.3 ± 0.3	5.0 ± 0.5	4.1 ± 0.3	5.1 ± 0.2
6	3.8 ± 0.2	3.8 ± 0.2	4.4 ± 0.2	4.5 ± 0.2

These species are hereafter referred to as 'core species of this phylogeny'. The resultant sequences together with those of the 14 mature BcAPs were subjected to Maximum Likelihood (ML) phylogeny. A preliminary ML tree (not shown) indicated that all BcAP sequences except BcAP8 have a single ortholog in one or more ascomycete core species of this phylogeny or form part of a clade containing at least one *B. cinerea* paralog and several sequences from other species. BcAP8 appeared in a branch with a number of *L. bicolor* sequences. Furthermore, BcAP14 appeared in a branch with only SsAP14. Public sequence databanks were further mined to identify BcAP8 and BcAP14 orthologs in other species (see Section 2). We included in the final phylogenetic analysis several sequences orthologous to BcAP8, one sequence orthologous to BcAP14 and a number of additional outlier sequences. The multiple sequence alignment is shown in Supplementary Fig. S4 and the resulting phylogenetic tree is shown in Fig. 6. Supplementary Fig. S5 shows the highly similar consensus tree that was obtained upon bootstrap analysis.

The tree was rooted at the node between pig pepsin (Pp) and icefish nothepsin (ChNot, Capasso et al., 1998) and shows a large number of primary bifurcations indicated by roman numerals. Clades are typically named after a BcAP sequence that they contain. Outlier sequences other than the outgroup are found in the first and third monophyletic clade that appear, all fungal APs occur in other clades. Ascomycetes and Basidiomycetes are phyla of the subkingdom Dikarya, and orthologs from both phyla are found in the BcAP2, BcAP8 and BcAP13 clades. The sequences in these clades can therefore be considered to have evolved from an ancestral family of AP paralogs formed prior to the ascomycete–basidiomycete divergence. All other clades appear to be specific for ascomycetes.

BcAP2 was previously shown to be homologous to APs, several of which were experimentally shown to be targeted to vacuoles (ten Have et al., 2004). The BcAP2 clade contains a single AP from all included fungal species. The fourth major clade that diverges is the BcAP8 clade. No orthologs of BcAP8 were found in the ascomycete core species of this phylogeny. Extensive BLAST analyses identified three ascomycete orthologous sequences among which a sequence in the ascomycete *T. harzianum*, hereafter referred to as ThCAI91181 (Suárez et al., 2005). The next best BLAST hits obtained with BcAP8 originated from basidiomycetes, including an

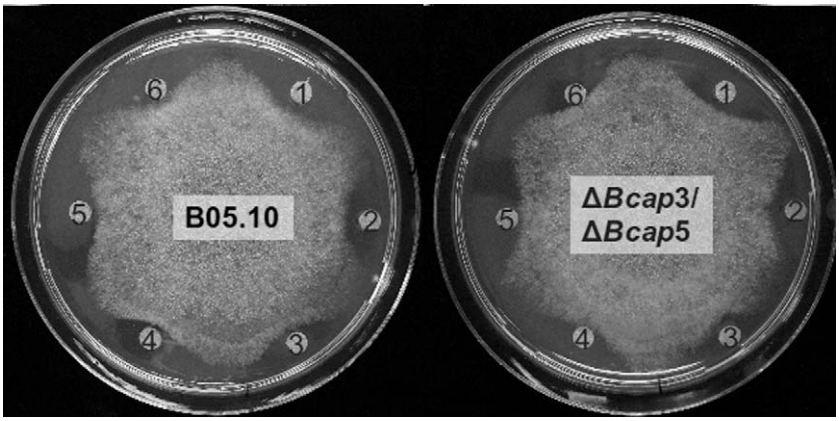

Fig. 5. Growth inhibition by grape PR proteins. Miracloth disks were saturated with 0.58 mg crude grape protein (1), 0.58 mg chitinase A (2), 0.17 mg thaumatin-like protein B (3), 0.44 mg thaumatin-like protein C (4), 0.25 mg chitinase D (5), and 0.1 mg thaumatin-like protein C (6). The left plate contains wild-type strain B05.10; the right plate contains a Δ*Bcap*3/Δ*Bcap*5 double mutant and shows a representative result for all the mutants tested. All mutants were indistinguishable from the wild type ($n \geqslant 3$).

AP from *Irpex lacteus*, of which the tertiary structure has been determined (Fujimoto et al., 2004). All these basidiomycete sequences appear in a single subclade (Fig. 6). Other hits corresponded to APs from cucumber and from housefly and were of low significance. A subsequent bifurcation (Fig. 6, V) results in the BcAP13 clade containing a single sequence from each ascomycete genome and two from *L. bicolor*. All AP sequences in the genome of *L. bicolor* have been included as an example to demonstrate how basidiomycete APs relate to BcAPs and other ascomycete APs included in the phylogeny. All APs from *L. bicolor,* except Lb_1876254, as well as all the other basidiomycete APs included, are found in the BcAP2, BcAP8 and BcAP13 clades. The sixth bifurcation results in two large, complex clades (Fig. 6) containing only ascomycetes sequences and orphan Lb_1876254. All these ascomycete sequences can be considered to have evolved following gene duplication after the ascomycete–basidiomycete divergence.

The top clade resulting from bifurcation VI consists of the BcAP5/7 clade and a complex clade that subsequently splits into the BcAP12 and the BcAP4 superclade. The BcAP5/7 clade contains sequences from all three *Pezizomycotina* classes but not from the plant pathogens *M. grisea* and *S. sclerotiorum*. The BcAP12 clade lacks sequences from *S. sclerotiorum* and from eurotiomycetes. All BcAPs present in the BcAP4 superclade as well as the BcAP12 clade have C-terminal extensions that were not included in the multiple sequence alignment used for the phylogeny. The C-terminal extensions of BcAP3 and BcAP4 both contain a Ser/Thr-rich subsequence preceding a GPI-anchoring motif (ten Have et al., 2004). All sequences found in the BcAP4 and BcAP12 clade have a C-terminal extension, albeit that the extension of BcAP6 is relatively short. BiGPI analysis (Eisenhaber et al., 2004) indicated that most but not all proteins in both the BcAP4 superclade and the BcAP12 clade are predicted to have a GPI-anchor, in addition many sequences contain Ser/Thr-rich stretches (Fig 6).

The lower clade resulting from bifurcation VI splits into the BcAP14 clade and a complex clade containing all APs that lack the first of the conserved disulfide bridges (See Supplementary Fig. S4). Orthologs of BcAP14 were detected only in *S. sclerotiorum* and in *P. nodorum* (a wheat pathogen from the *Pezizomycotina* class of dothideomycetes). The large clade with APs that lack the first disulfide bridge shows, besides some orphans, three subclades with a *B. cinerea* AP (BcAP1, BcAP9 and BcAP10, Fig. 6), their orthologs in *S. sclerotiorum* and at least one ortholog from both an eurotiomycete and a sordariomycete.

The bootstrap analysis largely confirms the Maximum Likelihood tree. The consensus tree obtained after 500 bootstraps (Supplementary Fig. S5) shows two differences with the Maximum Likelihood tree. Firstly, the BcAP14 clade, resulting from bifuraction VII in the Maximum Likelihood tree, is found as a subclade in the BcAP5-4 superclade (Supplementary Fig. S5), however with very low bootstrap support (64 out of 500). Secondly the BcAP10 clade, which comprises sequences from all ascomycete species included in this study, is not supported by bootstrap analysis. Bootstrap analysis suggests that BcAP10 and SsAP10 are more closely related to their BcAP9 and SsAP9 paralogs. Bootstrap support for many bifurcations in this clade is low. In an attempt to minimize possible negative side-effects administered by other clades we performed a new Maximum Likelihood analysis based on a subset of sequences (all sequences of the BcAP1 superclade and the outgroup). Both the Maximum Likelihood and the bootstrap-consensus tree were topologically identical to the branching patterns observed in full trees shown in Fig. 6 and Supplementary Fig. S5, respectively (data not shown).

4. Discussion

Initially, this study set out to examine by systematic generation of single and double knock-out mutants whether any of the five previously-identified aspartic proteinases are involved in virulence of *B. cinerea*. However, the sheer size of the BcAP gene family that was unraveled during the course of these investigations increased the scale of this task beyond technical limits (by lack of sufficient selection markers) and beyond logistic feasibility. Consequently, a combination of approaches was employed to tackle this challenge and attempt to establish which individual members of this gene family significantly contribute to secreted AP activity and to virulence. Particular attention was given to enzymes that might be specific to *B. cinerea,* or be produced only by *B. cinerea* and close relatives such as *S. sclerotorium,* or be shared only among plant pathogenic ascomycetes.

Deletion of the previously-identified *Bcap* genes (ten Have et al., 2004) singly and in various double combinations indicated that BcAP1–BcAP5 are not essential for virulence and do not contribute substantially to secreted AP activity. Certain molecular facets of these enzymes elucidated by bioinformatic and phylogenetic analyses, further substantiated this conclusion. The BcAP1 polypeptide is unique as a pepsin-like AP in that it does not contain a signal peptide. It has orthologs in the genomes of necrotrophic and saprophytic ascomycetes (although *A. fumigatus*, for example, does not have an ortholog). None of these orthologs (Fig. 6) has a signal peptide either and the most probable sub-cellular location of the sequences in the BcAP1 clade was predicted by WoLFPSORT to be

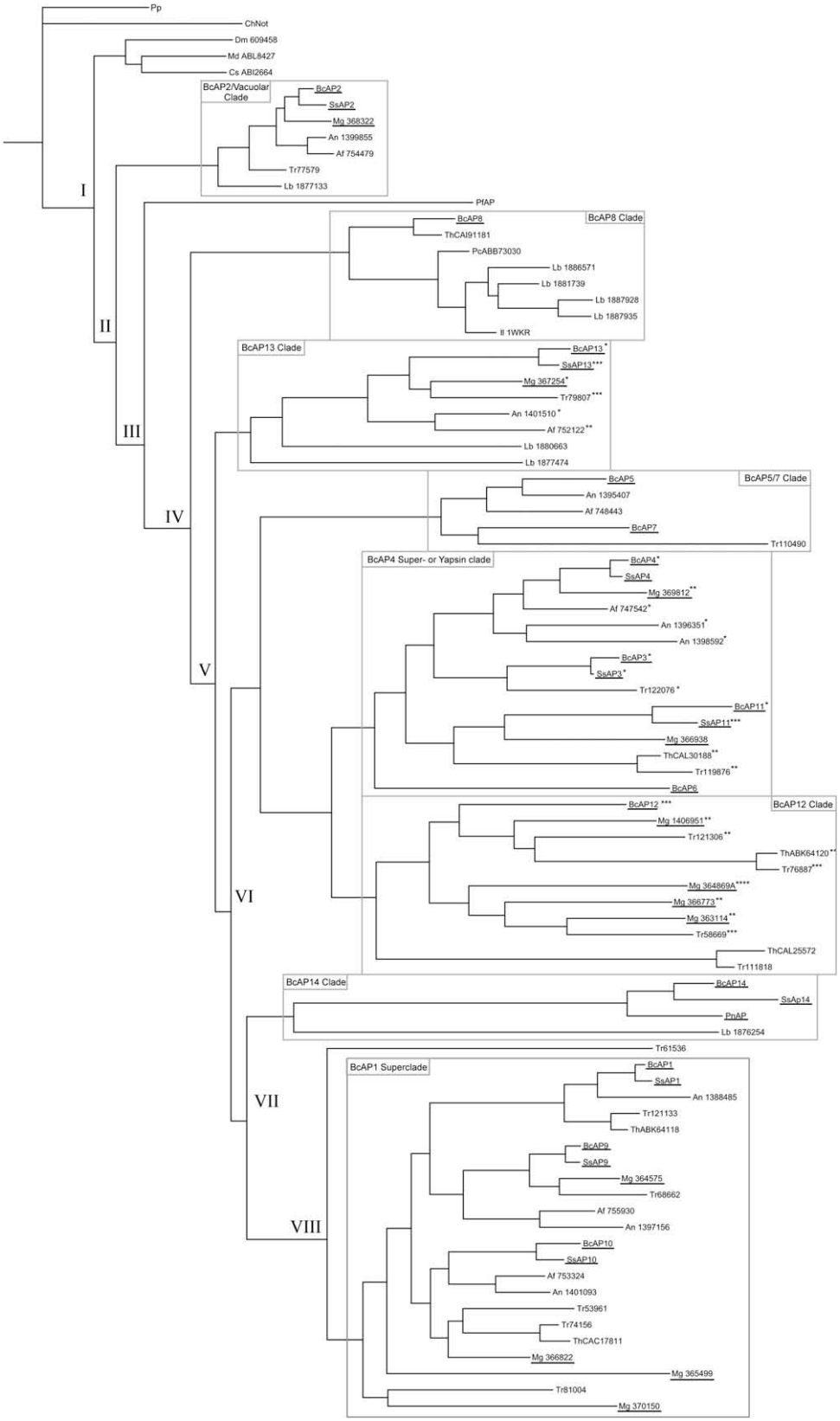

Fig. 6. Maximum likelihood tree of *Botrytis cinerea* and fungal aspartic proteases. The alignment shown in Supplementary Fig. S4 was used for phylogenetic analysis by Maximum Likelihood using PHYML as described in Section 2. The tree was rooted at the node connecting pig pepsin (Pp) and icefish nothepsin (ChNot). Clades are named after a BcAP present in the clade. APs from plant pathogens are underlined. Main bifurcations are indicated by roman numerals. The consensus tree obtained after of 500 bootstraps is shown in Supplementary Fig. S5. ∗: Has predicted GPI-anchor motif and Ser/Thr-rich stretch (at least 5 in 10 residues) in C-terminal extension. ∗∗: Has predicted GPI-anchor motif in C-terminal extension. ∗∗∗: Has Ser/Thr-rich stretch (at least 5 in 10 residues) in C-terminal extension. ∗∗∗∗: C-terminal extension likely incorrect.

in the cytosol or nucleus, with entry into the secretory pathway as the least probable option (not shown). If in contrast to all other pepsin-like APs, BcAP1 does not enter the lumen of the ER, post-translational modifications that are performed therein, such as disulfide bond formation and glycosylation, are unlikely to occur on this polypeptide. Although the sequences in the BcAP1 clade do contain cysteine residues (Supplementary Fig. S4), none of these correspond in their location in the polypeptide to any of the conserved residues that have been extensively documented to participate in the three hallmark disulfide bonds of archetypal pepsin-like APs (see e.g. Dunn, 2002), nor are they in a correctly juxtaposed position in the 3D structure to permit alternative disulfide bonds to be formed. Similarly, none of the N-glycosylation motifs in BcAP1 are conserved throughout the BcAP1 clade. If BcAP1 is not able to gain access to the ER, then this, together with the knock-out mutant data, establishes that BcAP1 cannot be considered to contribute to secreted AP activity or virulence.

BcAP2 showed extensive sequence identity to the APs from other fungal species that have been demonstrated experimentally to reside in the vacuole, of which the AP from *Saccharomyces cerevisiae* is the most extensively characterized representative (Winther et al., 2004). The phylogenetic analysis confirmed that the BcAP2 clade contains an ortholog in every fungus for which the genome sequence was included in this study, including saprophytes. On this basis, BcAP2 was identified early in our investigations as the vacuolar AP from *B. cinerea.* Since its fate is clearly not to be secreted from the cell, this enzyme cannot contribute to secreted AP activity or to virulence.

Based on the sequence features, BcAP5, BcAP7, BcAP8, BcAP9 and BcAP10 were predicted to be secreted. Secretome analysis confirmed that BcAP8 was a major component of the secretome, whereas BcAP5, BcAP9 and BcAP10 were detected in low amounts. BcAP8 will be discussed in detail further below. Deletion of the *Bcap5* gene alone and in combination with the *Bcap*1 and *Bcap*3 genes did not significantly lower the secreted AP activity nor did it affect virulence on the plants examined. The phylogenetic analysis revealed that BcAP5, BcAP9 and BcAP10 have orthologs in saprophytes and in *A. fumigatus* and thus are unlikely to be specific for virulence on plants. Bootstrap analysis puts BcAP10 and its ortholog SsAP10 as a separate subclade related to BcAP9. BcAP9 and BcAP10 are highly likely therefore to possess similar properties as an enzyme.

BcAP3, BcAP4, BcAP11, BcAP12 and BcAP13 all have C-terminal extensions which include a Ser/Thr-rich region and a predicted GPI-anchor motif (the latter is absent in BcAP12). These features are also found in yapsins and yapsin-like APs which were first characterized in *S. cerevisiae* (Cawley and Loh, 2004). Previously we showed that BcAP3 and BcAP4 cluster with APs from yeasts, among which some contain a GPI-anchor motif (ten Have et al., 2004). Yapsins have a high substrate specificity and functionally overlap with Kex-like serine proteinases (Loh and Cawley, 1995; Cawley and Loh, 2004) that act as proprotein convertases and are involved in fungal cell wall integrity (Gagnon-Arsenault et al., 2006). Furthermore, the yapsin-like APs SAP9 and SAP10 from the human pathogen *Candida albicans* have been shown to be involved in pathogenesis (Monod et al., 2002). However, the *B. cinerea* mutants lacking BcAP3 and BcAP4 did not display any reduction in virulence, possibly as the result of redundancy caused by some or all of the other C-terminally modified BcAPs. Additional analysis of the BcAP13 clade, the BcAP4 superclade or the BcAP12 clade (Fig. 6) indicated that these clades contain mostly sequences with a predicted GPI-anchor and/or Ser/Thr-rich subsequences. Hence it is likely that these APs are retained to the cell membrane or the extracellular matrix, however, their physiological functions and/or enzymatic characteristics remain to be studied.

BcAP6 is also related to the yapsin-like APs, but it has only a short C-terminal extension and appears to lack motifs that enable this protein to be retained in the cell membrane or extracellular matrix. No orthologs of BcAP6 were detected in the genomes of the other fungal species examined. In contrast, BcAP14 orthologs were detected in *S. sclerotiorum* and *P. nodorum.* In common with BcAP6, these proteins all lack a pro-peptide region which is unprecedented in an AP from any species so far reported. All have a signal peptide and so each nascent polypeptide, after translocation into the lumen of the ER, may be active directly without any need for prior activation by removal of a pro-peptide region. The Asp303-Ala substitution in BcAP6 and BcAP14 appears to be conserved in the sequences of the BcAP14 orthologs in *S. sclerotiorum* and *P. nodorum*. This replacement has previously been observed in renin which is active at pH values close to neutrality, and this property requires the Asp303-Ala replacement (Yamauchi et al., 1988).

BcAP8 contributed approximately one-quarter of the total protein mass secreted by *B. cinerea* cultures in early growth stages. The expression analysis (Fig. 3) showed a strong *Bcap*8 transcript accumulation during early stages of fungal growth in axenic cultures with a drop after 16 h. This explains why the gene was not represented in an EST library that was based on RNA extracted from nitrogen-starved cultures (Viaud et al., 2006). In contrast, EST analysis of *B. cinerea* germlings grown in rich medium revealed that 33 ESTs (out of 11,482) in this library were derived from *Bcap*8 (Silva et al., 2006). BcAP8 was also detected in a proteome analysis performed on solid nutrient media (Shah et al., 2009). The abundance of BcAP8 in the secretome of germlings clarifies why the single and double deletion mutants in *Bcap*1–*Bcap*5 did not show any notable reduction in proteinase activity or virulence. In contrast, two independent *Bcap*8-deficient mutants in axenic culture produced only about 20–30% of the AP activity detected in wild-type strains. Notwithstanding this clear reduction in protease activity, deletion of the *Bcap*8 gene had no significant effect on virulence. The observation that plant material infected with the Δ*Bcap*8-deficient mutants still showed ~50% of the AP activity that was detected in plants infected with wild-type *B. cinerea,* suggests that other *Bcap* genes may be induced *in planta* (although the possibility that the additional activity originates from the plant itself cannot be excluded). Proteome analysis suggests BcAP5, BcAP9 and BcAP10 might confer redundancy. Here we note that *S. sclerotiorum* lacks an ortolog of BcAP5 (besides orthologs of BcAP6, BcAP7, BcAP8 and BcAP12). In adittion, bioinformatics analyses of the genome sequence has revealed the existence of various other, predicted secreted acidic proteases (ten Have, unpublished) as is also shown by the recent identification of a glutamic peptidase in *B. cinerea* (Rolland and Bruel, 2008).

There are two aspects to BcAP8 that suggest that it is likely to have another function. Firstly, its expression level and contribution to total secreted AP activity in *B. cinerea* are likely to be subject to strong functional constraint. Secondly, BcAP8 is in a distinct and ancient monophyletic clade with high conservation among its member sequences (including Basidiomycete sequences) which indicates strong negative selection. Thus, BcAP8 is likely to have a function similar to that of its orthologs. Expression of the ortholog in *T. harzianum* (ThCAI91181) is induced by growth in the presence of cell walls from other fungi, including *B. cinerea* (Suárez et al., 2007) which suggests that the ThCAI91181-derived AP has a role in mycoparasitism, as proposed by Suárez et al. (2005, 2007), or in competition with fungi. It might be interesting to study whether BcAP8 has a role in competition with fungi.

5. Concluding remarks

These studies have provided evidence for the existence of a family of 14 aspartic proteinases in *B. cinerea.* The proteins are predicted to be distributed among several cellular compartments,

including the cytosol (BcAP1), which has not been known previously to contain such activity. BcAP8 is by far the most abundant enzyme in the secretome of *B. cinerea* germlings and the *Bcap*8 knock-out mutant showed a 70–80% reduction in total secreted proteinase activity, despite the remaining 13 *Bcap* genes being intact. The virulence of the *Bcap*8 knock-out mutant was not notably reduced however as compared to the wild type. A similar outcome was observed for the single and double knock-out mutants in the *Bcap*1–*Bcap*5 genes. The expansion of the *B. cinerea* aspartic proteinase family and evolution of paralogs with such diverse characteristics and cellular locations suggests that functional diversification has occurred. It will be important to perform more elaborate function predictions before embarking again on a strategy of testing knock-out mutants. It may be interesting to study the role of APs in secreted protein maturation (Cawley and Loh, 2004), pheromone proteolysis and sexual reproduction (Alby et al., 2009) or microbial competition (Suarez et al., 2007).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2009.10.008.

References

Abad-Zapatero, C., Rydel, T.J., Erickson, J., 1990. Revised 2.3 a structure of porcine pepsin: evidence for a flexible subdomain. Proteins 8, 62–81.

Alby, K., Schaefer, D., Bennett, R.J., 2009. Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*. Nature 460, 890–894.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Applied Biosystems, 2004. Guide to Performing Relative Quantitation of Gene Expression Using Real-time Quantitative PCR. Applied Biosystems, Foster City, CA, USA.

Bennett-Lovsey, R.M., Herbert, A.D., Sternberg, M.J.E., Kelley, L.A., 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins: Struct., Funct., Bioinform. 70, 611–625.

Blundell, T.L., Guruprasad, K., Albert, A., Williams, M., Sibanda, B.L., Dhanaraj, V., 1998. The aspartic proteinases. An historical overview. Adv. Exp. Med. Biol. 436, 1–13.

Brito, N., Espino, J.J., Gonzalez, C., 2006. The endo-β-1, 4-xylanase Xyn11A is required for virulence in Botrytis cinerea. Mol. Plant–Microbe Interact. 19, 25–32.

Capasso, C., Riggio, M., Scudiero, R., Carginale, V., di Prisco, G., Kay, J., Kille, P., Parisi, E., 1998. Molecular cloning and sequence determination of a novel aspartic proteinase from Antarctic fish. Biochim. Biophys. Acta 1387, 457–461.

Cawley, N.X., Loh, Y.P., 2004. Yapsin1. In: Barrett, A.J., Rawlings, N.D., Woessner, J.F. (Eds.), Handbook of Proteolytic Enzymes. Elsevier Academic Press, Amsterdam, pp. 128–131.

Duckert, P., Brunak, S., Blom, N., 2004. Prediction of proprotein convertase cleavage sites. Protein Eng. Des. Sel. 17, 107–112.

Dunn, B.M., 2002. Structure and mechanism of the pepsin-like family of aspartic peptidases. Chem. Rev. 102, 4431–4458.

Eisenhaber, B., Schneider, G., Wildpaner, M., Eisenhaber, F., 2004. A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. J. Mol. Biol. 337, 243–253.

Espino, J.J., Brito, N., Noda, J., Gonzalez, C., 2005. Botrytis cinerea endo-β-1, 4-glucanase Cel5A is expressed during infection but is not required for pathogenesis. Physiol. Mol. Plant Pathol. 66, 213–221.

Felsenstein, J., 1989. PHYLIP – phylogeny inference package (version 3.2). Cladistics 5, 164–166.

Finn, R.D., Mistry, J., Schuster-Böckler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A., Durbin, R., Eddy, S.R., Sonnhammer, E.L.L., Bateman, A., 2006. Pfam: clans, web tools and services. Nucleic Acids Res. 34, D247–D251.

Fujimoto, Z., Fujii, Y., Kaneko, S., Kobayashi, H., Mizuno, H., 2004. Crystal structure of aspartic proteinase from *Irpex lacteus* in complex with inhibitor pepstatin. J. Mol. Biol. 341, 1227–1235.

Gagnon-Arsenault, I., Tremblay, J., Bourbonnais, Y., 2006. Fungal yapsins and cell wall: a unique family of aspartic peptidases for a distinctive cellular function. FEMS Yeast Res. 6, 966–978.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Syst. Biol. 52, 696–704.

Hall, Q., Cannon, M.C., 2002. The cell wall hydroxyproline-rich glycoprotein RSH is essential for normal embryo development in Arabidopsis. Plant Cell 14, 1161–1172.

Henschke, P.A., Jiranek, V., 1993. Yeasts—metabolism of nitrogen compounds. In: Fleet, G.H. (Ed.), Wine microbiology and biotechnology. Harwood Academic, Chur, Switzerland, pp. 77–164.

Horton, P., Park, K.-J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J., Nakai, K., 2007. WoLF PSORT: protein localization predictor. Nucleic Acids Res. 35, W585–W587.

Hulo, N., Bairoch, A., Bulliard, V., Cerutti, L., Cuche, B.A., de Castro, E., Lachaize, C., Langendijk-Genevaux, P.S., Sigrist, C.J., 2008. The 20 years of PROSITE. Nucleic Acids Res. 36, D245–D249.

Jarvis, W. R. 1977. *Botryotinia* and *Botrytis* species: taxonomy, physiology, and pathogenicity. Monogr. Res. Branch Can. Dept. Agric. 15.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., Valent, B., 2000. Direct interaction of resistance gene and avirulence gene products confers rice Blast resistance. EMBO J. 19, 4004–4014.

Kars, I., Krooshof, G., Wagemakers, C.A.M., Joosten, R., Benen, J.A.E., van Kan, J.A.L., 2005a. Necrotising activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 43, 213–225.

Kars, I., Wagemakers, C.A.M., McCalman, M., van Kan, J.A.L., 2005b. Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. Mol. Plant Pathol. 6, 641–652.

Khan, A.R., James, M.N., 1998. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci. 7, 815–836.

Loh, Y.P., Cawley, N.X., 1995. Processing enzymes of the pepsin family: yeast aspartic proteinase3 and pro-opiomelanocortin converting enzyme. Methods Enzymol. 248, 136–146.

Martin, F., Aerts, A., Ahrén, D., Brun, A., Danchin, E.G., Duchaussoy, F., Gibon, J., Kohler, A., Lindquist, E., Pereda, V., Salamov, A., Shapiro, H.J., Wuyts, J., Blaudez, D., Buée, M., Brokstein, P., Canbäck, B., Cohen, D., Courty, P.E., Coutinho, P.M., Delaruelle, C., Detter, J.C., Deveau, A., DiFazio, S., Duplessis, S., Fraissinet-Tachet, L., Lucic, E., Frey-Klett, P., Fourrey, C., Feussner, I., Gay, G., Grimwood, J., Hoegger, P.J., Jain, P., Kilaru, S., Labbé, J., Lin, Y.C., Legué, V., Le Tacon, F., Marmeisse, R., Melayah, D., Montanini, B., Muratet, M., Nehls, U., Niculita-Hirzel, H., Oudot-Le Secq, M.P., Peter, M., Quesneville, H., Rajashekar, B., Reich, M., Rouhier, N., Schmutz, J., Yin, T., Chalot, M., Henrissat, B., Kües, U., Lucas, S., Van de Peer, Y., Podila, G.K., Polle, A., Pukkila, P.J., Richardson, P.M., Rouzé, P., Sanders, I.R., Stajich, J.E., Tunlid, A., Tuskan, G., Grigoriev, I.V., 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature 452, 88–92.

Martinez, D., Berka, R.M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S.E., Chapman, J., Chertkov, O., Coutinho, P.M., Cullen, D., Danchin, E.G., Grigoriev, I.V., Harris, P., Jackson, M., Kubicek, C.P., Han, C.S., Ho, I., Larrondo, L.F., de Leon, A.L., Magnuson, J.K., Merino, S., Misra, M., Nelson, B., Putnam, N., Robbertse, B., Salamov, A.A., Schmoll, M., Terry, A., Thayer, N., Westerholm-Parvinen, A., Schoch, C.L., Yao, J., Barbote, R., Nelson, M.A., Detter, C., Bruce, D., Kuske, C.R., Xie, G., Richardson, P., Rokhsar, D.S., Lucas, S.M., Rubin, E.M., Dunn-Coleman, N., Ward, M., Brettin, T.S., 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei*. Nat. Biotechnol. 26, 553–560.

Monod, M., Capoccia, S., Léchenne, B., Zaugg, C., Holdom, M., Jousson, O., 2002. Secreted proteases from pathogenic fungi. Int. J. Med. Microbiol. 292, 405–419.

Movahedi, S., Heale, J.B., 1990. The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of various host tissues by different isolates of *Botrytis cinerea* Pers ex. Pers. Physiol. Mol. Plant Pathol. 36, 303–324.

Murphy, J.M., Walton, J.D., 1996. Three extracellular proteases from *Cochliobolus carbonum*: cloning and targeted disruption of ALP1. Mol. Plant–Microbe Interact. 9, 290–297.

Neuhoff, V., Stamm, R., Eibl, H., 1985. Clear background and highly sensitive protein staining with Coomassie blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis 6, 427–448.

Ng, K., Petersen, J.F.W., Cherney, M.M., Garen, C., Zalatoris, J.J., Rao-Naik, C., Dunn, B.M., Martzen, M.R., Peanasky, R.J., James, M.N.G., 2000. Structural basis for the inhibition of porcine pepsin by Ascaris pepsin inhibitor-3. Nat. Struct. Biol. 7, 653–657.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Noda, J., Brito, N., Espino, J.J., Gonzalez, C., 2007. Methodological improvements in the expression of foreign genes and in gene replacement in the phytopathogenic fungus *Botrytis cinerea*. Mol. Plant Pathol. 8, 811–816.

Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: a novel method for multiple sequence alignments. J. Mol. Biol. 302, 205–217.

Parr, C.L., Keates, R.A., Bryksa, B.C., Ogawa, M., Yada, R.Y., 2007. The structure and function of *Saccharomyces cerevisiae* proteinase A. Yeast 24, 467–480.

Plummer, K.M., Clark, S.J., Ellis, L.M., Loganathan, A., Al-Samarrai, T.H., Rikkerink, E.H.A., Sullivan, P.A., Templeton, M.D., Farley, P.C., 2004. Analysis of a secreted aspartic peptidase disruption mutant of *Glomerella cingulata*. Eur. J. Plant Pathol. 110, 265–274.

Rawlings, N.D., Morton, F.R., 2008. The MEROPS batch BLAST: a tool to detect peptidases and their non-peptidase homologues in a genome. Biochimie 90, 243–259.

Rolland, S.G., Bruel, C.A., 2008. Sulphur and nitrogen regulation of the protease-encoding *ACP1* gene in the fungus *Botrytis cinerea*: correlation with a phospholipase D activity. Microbiology 154, 1464–1473.

Schulze Gronover, C., Kasulke, D., Tudzynski, P., Tudzynski, B., 2001. The role of G protein alpha subunits in the infection process of the gray mold fungus *Botrytis cinerea*. Mol. Plant–Microbe Interact. 14, 1293–1302.

Shah, P., Atwood, J.A., Orlando, R., El Mubarek, H., Podila, G.K., Davis, M.R., 2009. Comparative proteomic analysis of *Botrytis cinerea* secretome. J. Proteome Res. 8, 1123–1130.

Silva, E., Valdés, J., Holmes, D., Shmaryahu, A., Valenzuela, P.D.T., 2006. Generation and analysis of expressed sequence tags from *Botrytis cinerea*. Biol. Res. 39, 367–376.

Suárez, M.B., Sanz, L., Chamorro, M.I., Rey, M., González, F.J., Llobell, A., Monte, E., 2005. Proteomic analysis of secreted proteins from *Trichoderma harzianum*: identification of a fungal cell wall-induced aspartic protease. Fungal Genet. Biol. 42, 924–934.

Suárez, M.B., Vizcaíno, J.A., Llobell, A., Monte, E., 2007. Characterization of genes encoding novel peptidasesin the biocontrol fungus *Trichoderma harzianum* CECT 2413 using the TrichoEST functional genomics approach. Curr. Genet. 51, 331–342.

ten Have, A., Mulder, W., Visser, J., van Kan, J.A.L., 1998. The endopolygalacturonase gene *Bcpg*1 is required for full virulence of *Botrytis cinerea*. Mol. Plant–Microbe Interact. 11, 1009–1016.

ten Have, A., Tenberge, K., Benen, J.A.E., Tudzynski, P., Visser, J., van Kan, J.A.L., 2002. The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens (review). In: Kempken, F. (Ed.), The Mycota XI Agricultural Applications, vol. 20. Springer-Verlag, Berlin, Heidelberg, pp. 341–358 (Chapter 17).

ten Have, A., Dekkers, E., Kay, J., Phylip, L.H., van Kan, J.A.L., 2004. An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. Microbiology 150, 2475–2489.

Van der Vlugt-Bergmans, C.J.B., Wagemakers, C.A.M., Van Kan, J.A.L., 1997. Cloning and expression of the cutinase A gene of *Botrytis cinerea*. Mol. Plant–Microbe Interact. 10, 21–29.

van Kan, J.A.L., 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11, 247–253.

van Kan, J.A.L., Van't Klooster, J.W., Wagemakers, C.A.M., Dees, D.C.T., van der Vlugt-Bergmans, C.J.B., 1997. Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. Mol. Plant–Microbe Interact. 10, 30–38.

van Loon, L.C., Rep, M., Pieterse, C.M., 2006. Significance of inducible defense-related proteins in infected plants. Ann. Rev. Phytopathol. 44, 135–162.

Van Sluyter, S.C., Marangon, M., Stranks, S.D., Neilson, K.A., Hayasaka, Y., Haynes, P.A., Menz, R.I., Waters, E.J., 2009. Two-step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. J. Agric. Food Chem., in press, doi:10.1021/jf902365r.

Viaud, M., Legeai, F., Pradier, J.-M., Brygoo, Y., Bitton, F., Weissenbach, J., Brunet-Simon, A., Duclert, A., Fillinger, S., Fortini, D., Gioti, A., Giraud, C., Halary, S., Lebrun, I., Pêcheur, P., Samson, D., Levis, C., 2006. Expressed sequence tags from the phytopathogenic fungus *Botrytis cinerea*. Eur. J. Plant Pathol. 111, 139–146.

Wessel, D., Flügge, U.I., 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141–143.

Williamson, B., Tudzynski, B., Tudzynsi, P., van Kan, J.A.L., 2007. *Botrytis cinerea*: the cause of grey mould disease. Mol. Plant Pathol. 8, 561–580.

Winther, J.R., Phylip, L.H., Kay, J., 2004. Saccharopepsin. In: Barrett, A.J., Rawlings, N.D., Woessner, J.F. (Eds.), Handbook of Proteolytic Enzymes. Elsevier Academic Press, Amsterdam, pp. 87–90.

Yamauchi, T., Nagahama, M., Hori, H., Murakami, K., 1988. Functional characterization of Asp-317 mutant of human renin expressed in COS cells. FEBS Lett. 230, 205–208.

Zhang, Z., Schäffer, A.A., Miller, W., Madden, T.L., Lipman, D.J., Koonin, E.V., Altschul, S.F., 1998. Protein sequence similarity searches using patterns as seeds. Nucleic Acids Res. 26, 3986–3990.