Microbiology (2004), **150**, 2475–2489 DOI 10.1099/mic.0.27058-0

An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features

Arjen ten Have,[1]† Ester Dekkers,[1] John Kay,[2] Lowri H. Phylip[2] and Jan A. L. van Kan[1]

[1]Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, PO Box 8025, 6700 EE Wageningen, The Netherlands

[2]Cardiff School of Biosciences, Cardiff University, PO Box 911, Cardiff CF10 3US, UK

Correspondence
Jan A. L. van Kan
jan.vankan@wur.nl

Received 26 January 2004
Revised 23 March 2004
Accepted 25 March 2004

Botrytis cinerea, an important fungal plant pathogen, secretes aspartic proteinase (AP) activity in axenic cultures. No cysteine, serine or metalloproteinase activity could be detected. Proteinase activity was higher in culture medium containing BSA or wheat germ extract, as compared to minimal medium. A proportion of the enzyme activity remained in the extracellular glucan sheath. AP was also the only type of proteinase activity in fluid obtained from *B. cinerea*-infected tissue of apple, pepper, tomato and zucchini. Five *B. cinerea* genes encoding an AP were cloned and denoted *Bcap1–5*. Features of the encoded proteins are discussed. BcAP1, especially, has novel characteristics. A phylogenetic analysis was performed comprising sequences originating from different kingdoms. BcAP1 and BcAP5 did not cluster in a bootstrap-supported clade. BcAP2 clusters with vacuolar APs. BcAP3 and BcAP4 cluster with secreted APs in a clade that also contains glycosylphosphatidylinositol-anchored proteinases from *Saccharomyces cerevisiae* and *Candida albicans*. All five *Bcap* genes are expressed in liquid cultures. Transcript levels of *Bcap1*, *Bcap2*, *Bcap3* and *Bcap4* are subject to glucose and peptone repression. Transcripts from all five *Bcap* genes were detected in infected plant tissue, indicating that at least part of the AP activity *in planta* originates from the pathogen.

INTRODUCTION

Botrytis cinerea is an important pathogen of at least 235 plant species (Jarvis, 1977). It is an ascomycete that is often regarded as an opportunist, as reflected by its broad host range and the fact that it infects different organs at different stages of plant development. Infection results in rot on fruits and blights on green leaves as well as flower petals. Degradation of the plant cell wall by secreted enzymes is an important aspect in the infection by *B. cinerea*. The action of plant-cell-wall degrading enzymes is believed to facilitate intercellular fungal growth and destabilize host cell integrity (reviewed by ten Have *et al.*, 2002). Plant cell walls are highly organized structures containing as main components cellulose and pectin, along with other polysaccharides and lignins (McCann & Roberts, 1991). Cell walls also comprise proteins such as extensin, which forms a network by intermolecular isodityrosyl bridges (Fry, 1982); expansins, which have a role in wall elongation (Cosgrove, 2000); and arabinogalactan proteins, which are associated with the cell membrane (Albersheim *et al.*, 1996).

Proteinases have been suggested to be involved in many plant–pathogen interactions (Clark *et al.*, 1997; Farley & Sullivan, 1998; Murphy & Walton, 1996; Paris & Lamattina, 1999; Poussereau *et al.*, 2001a) but their precise function has not been elucidated. *B. cinerea* might utilize proteinases to degrade structural plant cell wall proteins or antifungal proteins produced by the host. Treatment of a carrot cell wall preparation with an aspartic proteinase (AP) isolated from a *B. cinerea* culture was shown to reduce the protein content and facilitate the subsequent release of galacturonic acid by pectin lyase (Movahedi & Heale, 1990a). Infection of several fruits and vegetables by *B. cinerea* was reduced by addition of the AP inhibitor pepstatin, suggesting an important role for APs in pathogenesis (Movahedi & Heale, 1990a). However, this study did not permit distinction between proteinases derived from pathogen and host.

†Present address: Instituto de Investigaciones Biologicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, cc 1245 (7600) Mar del Plata, Argentina.

Abbreviations: AP, aspartic proteinase; CF, cell-free culture filtrate; Hb, haemoglobin.

The GenBank accession numbers for the sequences reported in this paper are AF121229 (*Bcap1*), AY361913 (*Bcap2*), AY507155 (*Bcap3*), AY507156 (*Bcap4*) and AJ617485 (*Bcap5*).

Since then, molecular tools have been developed for *B. cinerea* allowing gene function analysis using gene replacement mutants (van Kan *et al.*, 1997). We set out to analyse the proteinases produced by *B. cinerea* and identify the encoding genes in order to capitalize upon the earlier biochemical work (Movahedi & Heale, 1990a, b) with the ultimate aim of evaluating the role of proteinases in the pathogenesis of *B. cinerea*. Here we show that, in agreement with results recently published by Manteau *et al.* (2003), APs contribute the predominant proteolytic activity secreted by *B. cinerea in vitro*, as well as in infected tissue of multiple host plants. Five genes encoding APs were cloned and characterized. The proteins possess different characteristics and may have distinct localizations and functions.

METHODS

Standard culturing and medium shift experiments of *B. cinerea*. *B. cinerea* strain B05.10 was cultured on agar plates and stored as described previously (van der Vlugt-Bergmans *et al.*, 1997). For axenic cultures, conidia were harvested and used to inoculate Gamborg's B5 medium (Duchefa) supplemented with 1 % glucose and 10 mM Na_2HPO_4/NaH_2PO_4, pH 6 (10^5–10^6 conidia ml^{-1}). Several 100 ml standard cultures were grown in this medium in 300 ml flat-bottom flasks at 20 °C on a rotary shaker at 180 r.p.m. for 16 h. Mycelium was then harvested on 22 μm nylon filters, washed with 100 ml Gamborg's B5 medium and resuspended in 25 ml of the same medium. One-tenth of this suspension was transferred to 25 ml of the medium of interest: Gamborg's B5 medium supplemented either with 1 % (w/v) glucose, 0·5 % (w/v) mycological peptone (Oxoid), 100 mM NH_4Cl, 1 % (w/v) BSA or 1 % (w/v) wheat germ extract. The cultures were subsequently incubated at 20 °C on a rotary shaker at 180 r.p.m. for 8–24 h. At the end of the experiment, mycelium was harvested by filtration on miracloth (Calbiochem), blotted dry and stored at −80 °C until RNA or protein extraction. The filtrates were collected, sterilized over 0·22 μm filters (Schleicher & Schuell) and stored at 0 or −20 °C.

Proteinase assays

Standard haemoglobin (Hb) proteinase assay. A dialysed Hb solution (2·5 %, w/v) and the culture filtrate sample were separately adjusted to the desired pH by addition of NaAc (pH 4, 6 or 8, 200 mM final concentration). Upon 10 min preincubation at 37 °C, one aliquot of 50 μl buffered sample was added to 100 μl buffered Hb and incubated 1 h at 37 °C. The reaction was stopped by addition of 100 μl 15 % TCA. As a blank, 100 μl 15 % TCA was first added to 50 μl buffered sample aliquot before adding 100 μl buffered Hb. Inhibitors were always added to the substrate prior to the culture filtrate sample at the following final concentrations: pepstatin A, 1 μM; E64, 20 μM; EDTA, 5 mM; PMSF, 0·1 mM. After TCA addition, samples and blanks were incubated on ice for 30 min and centrifuged at 1000 ***g*** at 4 °C in a Sigma 4K15C centrifuge (Salm and Kipp). Following centrifugation, 100 μl supernatant was removed and the A_{280} determined using a BioPhotometer and UVettes (Eppendorf). The increase of A_{280} was determined by subtracting the blank from the sample value. Each assay was performed in triplicate; values represent the mean of three replicates with an error bar for the standard error of the mean.

BSA (1 %) and casein (0·1 %) degradation assay. *B. cinerea* cell-free culture filtrates (CFs) and substrate solutions were separately adjusted to the desired pH by the addition of NaAc (pH 4, pH 6 or pH 8, 100 mM final concentration), mixed to the desired ratios on ice and subsequently incubated at 37 °C for various periods of time. Up to four 100 μl samples of the reaction mixture were taken at 0 h (blank) and various time points (up to 8 h). The reaction was stopped by the addition of 200 μl 7·5 % TCA and subsequently treated as described above for the Hb assay. Casein-BODIPY proteinase assays (Molecular Probes) were performed according to the manufacturer. Each assay was performed in triplicate. Inhibitors (pepstatin A, E64, EDTA or PMSF) were added to the substrate prior to the sample at final concentrations as described above.

Collection of extracellular fluids from infected plant tissue. Tomato (*Lycopersicon esculentum*) stems derived from hydroponically grown Moneymaker tomato plants were inoculated according to Dik *et al.* (1999). Tomato, bell pepper (*Capsicum annuum*) and apple (*Malus domestica*) fruit from a local grocery were inoculated according to ten Have *et al.* (1998), and zucchini (*Cucurbita pepo* var. *melopepo*) according to ten Have *et al.* (2001). The inoculated plant tissues were incubated at 15–20 °C until clear maceration occurred. The tissue was collected in a tube and centrifuged for 10 min at 3000 ***g*** in a Beckmann J6B. A clear liquid was collected and sterilized over 0·22 μm filters.

BLAST analysis, cloning and sequence analysis. All sequences present in a publicly available database were compared to all sequences present in GenBank using BLASTX. PCR reactions were performed to amplify partial *Bcap2–5* gene fragments from genomic DNA of *B. cinerea* strain SAS56 using the 5′ and 3′ primers indicated in Table 1. The obtained fragments were used to isolate genomic clones from a λEMBL3 phage library of *B. cinerea* strain SAS56 as described (van der Vlugt-Bergmans *et al.*, 1997). Hybridizing fragments of the *Bcap* genes containing coding and flanking regions were subcloned into pBluescript II SK(−) for sequencing. Sequencing was performed at the Wageningen University Laboratory of Molecular Biology or at BaseClear, Leiden, The Netherlands.

Alignments and phylogenetic analysis. The protein sequences predicted by the cDNAs obtained for *Bcap1*, *Bcap2* and *Bcap5* and from the genomic DNA sequences for *Bcap3* and *Bcap4* (after editing to remove the introns) were aligned on the basis of the known sequence and crystallographically determined structure of pig pepsinogen and adjusted manually by visual inspection. The phylogenetic tree was based on a subset of selected AP sequences present in databases. The emphasis was on AP genes from other plant-pathogenic fungi and on the sequences that showed the highest homology to the BcAPs using the TBLASTN program in a BLAST translated database search (Altschul *et al.*, 1997). In addition, some well-characterized APs with typical hallmark features were included. A total of 41 proteinases of fungal origin, and nine non-fungal proteinases (see Table 2) were used to generate a CLUSTALX protein sequence alignment in three steps. The sequences were first aligned manually at the first DTG by introducing gaps before the N-terminal methionine. The sequence stretches that are C- and N-terminal of the first DTG were subsequently aligned in two steps in CLUSTALX.

Phylogenetic trees were obtained using PAUP* version 4.0b10 (Swofford *et al.*, 2001) with a heuristic search using parsimony as the optimality criterion, random stepwise addition and tree-bisection-reconnection (TBR) branch swapping. The topology of the single most parsimonious tree is shown in Fig. 4(a); the horizontal branch lengths are proportional to the number of amino acid changes necessary to explain the differences in protein sequence. Statistical support for this tree was low, therefore parsimonious trees were obtained using PAUP's bootstrap method with 1000 replicates, employing a heuristic search (simple stepwise addition) yielding the tree shown in Fig. 4(b). Numbers adjacent to nodes are the percentages of bootstrap trials supporting the depicted tree topology (50 % majority-rule consensus tree).

Table 1. Identification of *B. cinerea* aspartic proteinase encoding genes

ID indicates the accession number of the EST sequence; Hit indicates the sequence with the highest homology; underlined numbers indicate protein sequences; accession no. indicates the accession number of the genomic sequences. The indicated primers were used for the isolation of *Bcap2–5* genes.

ID	Gene	Hit	Score	E value	5′ Primer	3′ Primer	Accession no.
AL111951	*Bcap1*	U43775	83	2E-15			AF121229
AL113910	*Bcap1*	U43775	125	3E-28			AF121229
AL112055	*Bcap2*	Y15744	264	6E-70	CGGTGGTGTCAACAAGGACC	CCAACAGTGTTCTTACCGAGGTC	AY361913
AL111044	*Bcap2*	Y15744	95	3E-19			AY361913
AL114830	*Bcap3*	P32329	82	4E-15	CGCCCAGCAATCCTCGATTC	CGATATTGGATCCAGTAGCGTC	AY507155
AL111105	*Bcap3*	O42779	68	5E-11			AY507155
AL115034	*Bcap4*	Q12303	79	1E-19	GATGGTTTGATCAATTCAAACGC	GGGCTTCGACTTGTTCGTAG	AY507156
AL116346	*Bcap5*	P22929	66	3E-10	GGATCGCAGCCCGTGACTTT	AAGAAACGACGTACGCGCCC	AJ617485
AL112669	–	P22929	54	7E-7			

RNA blots and hybridization. RNA from mycelia collected from liquid cultures was extracted using Trizol (Gibco-BRL) as prescribed by the manufacturer. RNA from plant tissue was extracted as described by ten Have & Woltering (1997). RNA was separated by denaturing gel electrophoresis (Burnett, 1997) and blotted to Hybond-N$^+$ membranes (Amersham). Hybridization was performed in modified Church buffer at 65 °C as described by van der Vlugt-Bergmans *et al.* (1997) with gene-specific PCR fragments representing the 5′ regions of each gene [*Bcap1*, nt 1–518 (ATG = 201); *Bcap2*, nt 27–407 (ATG = 25); *Bcap3*, nt 1–626 (ATG = 370); *Bcap4*, nt 60–891 (ATG = 544); *Bcap5*, nt 66–516 (ATG = 113)]. For control hybridization, a probe specific for the actin gene *Bcact* (Benito *et al.*, 1998) was used. Blots were subsequently washed for 30 min in 2× SSC/0·5 % SDS, 0·5× SSC/0·5 % SDS and 0·2× SSC/0·5 % SDS at 65 °C.

RESULTS

Characterization of proteinase activity secreted by *B. cinerea*

To characterize the spectrum of proteinases secreted by *B. cinerea*, the fungus was grown on different liquid media. CFs were obtained, dialysed and subsequently assessed for proteinase activity using as substrate Hb, buffered at pH 4, 6 or 8 (see Fig. 1). Proteinase activity in CFs was highest at pH 4, irrespective of the nature of the culture medium. The total secreted activity was twofold higher when *B. cinerea* was grown in the presence of BSA or wheat germ extract, compared to growth on a basal salt or peptone-containing medium. Maximum activity levels were reached after 48–96 h of culture (not shown), but these CFs were more difficult to handle because of the production of a highly viscous glucan polysaccharide. This carbohydrate also hindered determination of the fungal fresh weight in culture and interfered with the determination of total protein content in the CFs.

Specific inhibitors were used to identify the nature of the proteinase activity. When *B. cinerea* was grown in basal salt or peptone media, the activity produced was inhibited 100 % at pH 4 (see Fig. 1) by the addition of pepstatin which has been well-documented as a specific inhibitor of enzymes belonging to the aspartic proteinase family (Valler

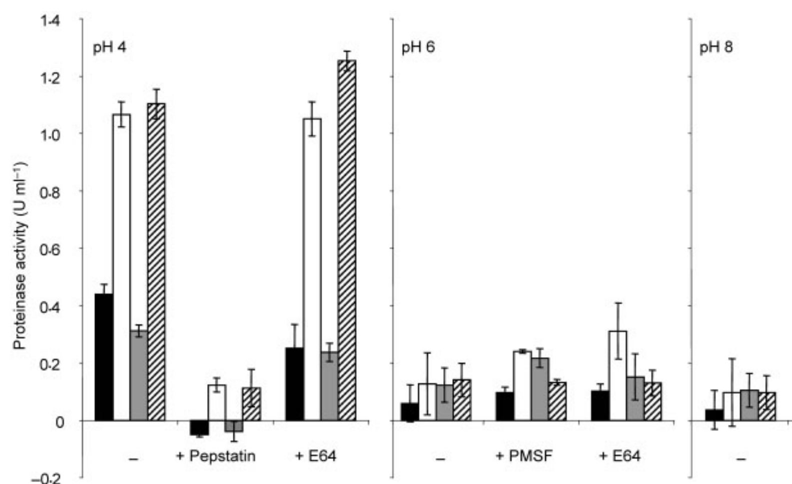


Fig. 1. The effect of pH and inhibitors on the proteinase activity in cell-free culture filtrates from *B. cinerea* grown in different liquid media. Aliquots from CF from fungi grown in Gamborg's B5 basal salt medium (solid bars), supplemented either with BSA (open bars), peptone (shaded bars) or wheat germ (striped bars) were incubated at 37 °C with Hb as substrate at the indicated pH values in the absence of inhibitor (indicated by a hyphen) or in the presence (+) of pepstatin, E64 or PMSF. One unit of activity was defined as an increase in A_{280} in 1 h of 1·0. Values are given as means ± SEM.

Table 2. Proteinases used in the phylogenetic analysis

Protein (best hit)†	Organism	Accession	Length	Reference	Reported localization
Bc_AP1	*Botrytis cinerea*	AF121229	432	Prins (2001)	
Bc_AP2	*Botrytis cinerea*	AY361913	398	This paper	
Bc_AP3	*Botrytis cinerea*	AY507155	529	This paper	
Bc_AP4	*Botrytis cinerea*	AY507156	483	This paper	
Bc_AP5	*Botrytis cinerea*	AJ617485	441	This paper	
Aa_PEPA	*Aspergillus awamori*	AAA78947	394	Berka *et al.* (1990)	Secreted
Af_PEP	*Aspergillus fumigatus*	CAA59419	395	Reichard *et al.* (1995)	Secreted
Af_PEP2	*Aspergillus fumigatus*	CAA75754	398	Reichard *et al.* (2000)	Cellular
Am_1DTG*	*Amanita muscaria*	CAC42132	425	Nehls *et al.* (2001)	Secreted
Anid_PRTB	*Aspergillus nidulans*	AAD04378	386	Unpublished	Extracellular
Anig_PEPE	*Aspergillus niger*	AAA20876	398	Jarai *et al.* (1994b)	Cellular
Anig_PROCB	*Aspergillus niger*	BAA08123	394	Lu *et al.* (1995)	
Ao_PEPA	*Aspergillus oryzae*	BAA02994	404	Gomi *et al.* (1993)	
Ao_opsB (BcAP4)†	*Aspergillus oryzae*	BAC00848	481	Kunihiro *et al.* (2002)	
As_APEPI	*Aspergillus saitoi*	BAA04988	394	Shintani & Ichishima (1994)	
At_AP	*Arabidopsis thaliana*	AAC49730	486	Dhondt *et al.* (1997)	
At_GAP	*Arabidopsis thaliana*	NP_176419	513	Unpublished	
At_GAP2	*Arabidopsis thaliana*	NP_193936	336	Unpublished	
Ca_APR1	*Candida albicans*	AAA79879	419	Unpublished	Vacuolar
Ca_SAP1	*Candida albicans*	P28872	391	Hube *et al.* (1991)	Secreted
Ca_SAP7	*Candida albicans*	CAA82925	588	Monod *et al.* (1994)	Secreted
Ca_SAP8	*Candida albicans*	O42778	405	Monod *et al.* (1998)	Secreted
Ca_SAP9	*Candida albicans*	AAC69996	544	Monod *et al.* (1998)	Secreted
Cc_AP	*Cynara cardunculus*	CAA57510.1	509	Cordeiro *et al.* (1994)	Secreted
Cp_EAPA	*Cryphonectria parasitica*	P11838	419	Barkholt (1987)	Secreted
Ct_SAPT4	*Candida tropicalis*	AAD33218	394	Zaugg *et al.* (2001)	Secreted
Fo_SAP1	*Fusarium oxysporum*	BAA78105	382	Unpublished	Secreted
Fv_AP	*Fusarium venenatum*	AAL69900	407	Unpublished	
Gc_SAP	*Glomerella cingulata*	AAB57763.1	407	Clark *et al.* (1997)	Secreted
Hs_BACE	*Homo sapiens*	NP_036236	501	Vassar (2001)	Membrane
Hs_MEM1	*Homo sapiens*	Q9Y5Z0	518	Yan *et al.* (1999)	Membrane
Il_AP	*Irpex lacteus*	1512141A	340	Kobayashi *et al.* (1989)	Secreted
Le_WIP	*Lycopersicon esculentum*	S71591	506	Schaller & Ryan (1996)	Secreted
Nc_cDNA (BcAP1,3,5)†	*Neurospora crassa*	XP_330143	481	Galagan *et al.* (2003)	
Nc_PEP4	*Neurospora crassa*	AAA79878	396	VasquezLaslop *et al.* (1996)	Vacuolar
Os_cDNA (BcAP2)†	*Oryza sativa*	AK100826.1	400	Kikuchi *et al.* (2003)	
Pa_PAPA	*Podospora anserina*	AAC49997	425	Paoletti *et al.* (1998)	
Pepsin	*Sus scrofa*	p00791	386	Tsukagoshi *et al.* (1988)	Secreted
Pj_PEPA	*Penicillium janthinellum*	P00798	323	James & Sielecki (1983)	
Pp_AP	*Pichia pastoris*	AAB14787	410	Unpublished	
Pr_PR1	*Phaffia rhodozyma*	AAC17105	405	Bang *et al.* (1999)	
Rn_1	*Rhizopus niveus*	P10602	389	Horiuchi *et al.* (1988)	
Rn_2	*Rhizopus niveus*	P43231	391	Unpublished	
Rn_3	*Rhizopus niveus*	Q03699	391	Unpublished	
Rn_4	*Rhizopus niveus*	Q03700	398	Unpublished	
Rn_5	*Rhizopus niveus*	P43232	392	Unpublished	
Rm_II	*Rhizopus microsporus*	AAB59305	393	Unpublished	
Sc_PEP4	*Saccharomyces cerevisiae*	P07267	405	Woolford *et al.* (1986)	Vacuolar
Sc_YPS1	*Saccharomyces cerevisiae*	AAB82367	569	Ash *et al.* (1995)	Secreted GPI
Ss_ASPS	*Sclerotinia sclerotiorum*	AAF76202	435	Poussereau *et al.* (2001b)	Secreted

*Sequence reported as aspartic proteinase but with only 1 DTG motif.
†Best hit in BLASTP or TBLASTN search using the predicted BcAP protein sequences as queries.

et al., 1985). Inhibition by pepstatin was around 90 % in CFs from cultures on BSA or wheat germ media (see Fig. 1). A similar inhibition was obtained when the assays were performed with pepstatin at pH 6 (data not shown). The cysteine proteinase inhibitor E64 had no effect on the total proteinase activity measured at pH 4 or 6 (see Fig. 1). The serine proteinase inhibitor PMSF had no effect on the total activity at pH 4 (not shown) or 6 (see Fig. 1). EDTA, an inhibitor of metalloproteinases, unexpectedly stimulated proteinase activity irrespective of the culture medium and pH (data not shown). EDTA may destabilize the association between the haem group and the globin, thereby making the Hb substrate more accessible to proteolysis, thus enhancing the quantitative response in the proteinase assay.

Comparable results were obtained when Hb was replaced with BSA as the substrate (not shown). The maximum activity was detected at pH 4 and pepstatin lowered the activity by approximately 90 %. E64 and PMSF had no inhibitory effect. With BSA as substrate, EDTA did not affect the total proteinase activity. From these data, it would appear that the activity detected in CFs originating from the four different culture media was only derived from AP activity.

AP activity was not only detected in the culture medium but also in extracts of the mycelium remaining on the filter. *B. cinerea* is renowned for its glucan production (Gil-ad *et al.*, 2001; Masih & Paul, 2002; Stahmann *et al.*, 1995) in which several secreted enzymes may be retained (Gil-ad *et al.*, 2001). The extent to which proteinase activity was retained in the glucan sheath was analysed by washing the mycelium with basal salt medium. This resulted in the elution of the majority of proteinase activity into the washing fluid. After two washes, the remaining mycelium contained only 5 % residual proteinase activity and about 90 % of the initial activity was present in the collected washing fluids (not shown). This indicates that the proteinase activity initially detected in mycelial extract resulted from enzymes loosely attached to the glucan sheath covering the hyphal wall, rather than from truly intracellular enzyme activity.

Infected plant tissue contains high amounts of AP activity

Four different plants (apple, zucchini, bell pepper and tomato) were inoculated with *B. cinerea* and cell-free extracts were prepared by centrifugation of the rotten tissues and filter-sterilizing the supernatant. Proteolytic activity was assessed using Hb as substrate. Substantially higher levels of activity were detected when the assays were performed at pH 4 compared to pH 6 and 8 (see Fig. 2a), just as described above for *B. cinerea* grown in axenic cultures. Higher activity was measured in the stem of infected tomato plants compared to the fruit (see Fig. 2a). The activity in infected zucchini appeared to be substantially higher than that in infected bell pepper or apple (see Fig. 2a), although it should be noted that practical limitations frustrated the attempts to correlate proteinase activity with fungal biomass. All of the activity in each infected plant species and tissue type was inhibited completely by inclusion of pepstatin in the pH 4 assays (see Fig. 2b), while E64 (see Fig. 2b) and PMSF (not shown) had no effect. However, the high levels of activity that were measured in infected zucchini and tomato stem were inhibited 5–10 % by inclusion of 2 mM EDTA when the assays were conducted with BSA as substrate. The activity measured in the extracts from infected plant tissues would thus also appear to result from AP action, just as observed in *B. cinerea* grown in defined media.

Cloning and characterization of five AP-encoding genes

BLAST analysis (Altschul *et al.*, 1997) of sequences in a publicly available *B. cinerea* EST database (www.genoscope.

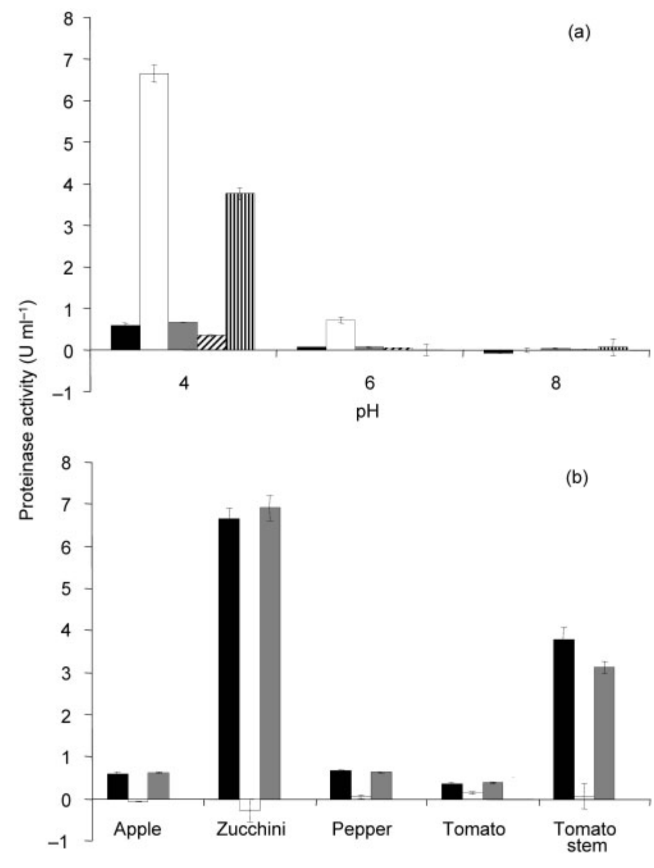
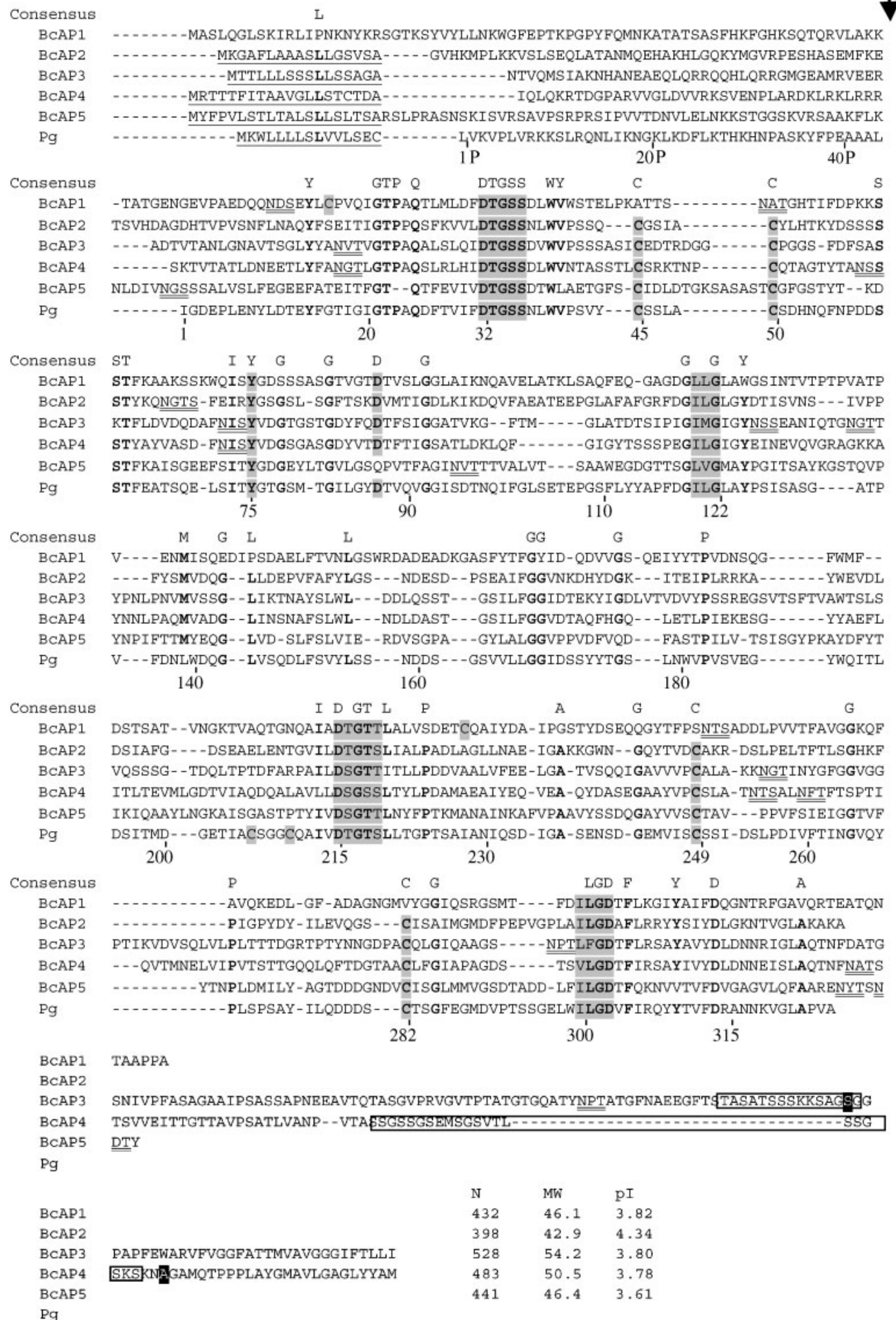


Fig. 2. The effect of pH and inhibitors on the proteinase activity in extracts from different plants infected with *B. cinerea*. Aliquots of macerated tissue from infected apple (solid bars), zucchini (open bars), bell pepper (shaded bars), and tomato fruit (diagonally striped bars) and stems (vertically striped bars) were assayed using Hb as substrate at the indicated pH values (a), as described in the legend to Fig. 1. (b) The inhibition of each activity at pH 4 was assessed by inclusion of pepstatin (open bars) or E64 (shaded bars) in the assay. Solid bars, no inhibitor. Values are given as means ± SEM.

cns.fr/externe/English/Projets/Projet_W/W.html) revealed nine accessions with homology to APs (Table 1). Of these, three contained overlapping sequences so that six unique sequences appeared to be present. Eukaryotic APs characteristically consist of two internally homologous domains, each of which provides a catalytic Asp residue to the active site (Khan & James, 1998). Each Asp residue is present in the hallmark motif ~Hydrophobic-Hydrophobic-Asp-Thr/Ser-Gly~, 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 eukaryotic APs, the domains are contained within one polypeptide so that each motif occurs twice within the molecule and



in the encoding gene (Tang, 1979). The six predicted *B. cinerea* amino acid sequences were scrutinized for the presence of these hallmark motifs. One sequence (AL112669, Table 1) lacked these features and was not analysed further.

The remaining five genes were denoted *Bcap1* to *Bcap5*. A genomic clone of the gene designated *Bcap1* was isolated previously (Prins, 2001). Primers based on the other four selected EST sequences (see Table 1) were used to perform PCR on genomic DNA isolated from *B. cinerea*. Fragments of the expected length were obtained and used to screen a genomic library. Positive phages were selected and used for subcloning and subsequent sequencing. *Bcap1*, *Bcap2*, *Bcap3* and *Bcap5* are each predicted to contain two introns, whereas *Bcap4* is predicted to have a single intron. The location of each intron in *Bcap1*, *Bcap2* and *Bcap5* was confirmed by sequencing two independent cDNA clones generated by RT-PCR from mRNA isolated from *B. cinerea*. No amplicons were generated when RT-PCR was carried out with primers specific for *Bcap3* and *Bcap4*. The position of the second *Bcap3* intron (55 bp) was provided from the sequence of a *B. cinerea* EST (B. Tudzynski, personal communication) and occurs in an equivalent location to the 60 bp intron 2 from the *Neurospora crassa* gene. In *Bcap3* a 61 bp intron 1 was identified by comparison with the 63 bp intron 1 in the DNA encoding one of the APs from *N. crassa* (EAA36107.1 from AABX01000006; Galagan *et al.*, 2003). Splicing presumably occurs at an identical location in both nucleic acids. The location of a single 53 bp intron in *Bcap4* was similarly deduced by comparison with genomic sequences potentially encoding APs from *Aspergillus oryzae* (BAC 00848; Kunihiro *et al.*, 2002) and *Aspergillus fumigatus* (unannotated sequence on contig TIGR.5201).

Fig. 3 presents an alignment of the predicted amino acid sequences of the five *B. cinerea* proteins (BcAP1–BcAP5) with that of the precursor (pepsinogen) of the well-characterized aspartic proteinase, pig pepsin. The predicted BcAP zymogens vary in length between 398 and 529 aa. BcAP2–5 all appear to contain a signal peptide of 16–20 residues, as determined by SignalP (Nielsen *et al.*, 1997a, b), followed by a propeptide region consisting of 37–52 residues (see Fig. 3). The predicted regions of all five mature enzymes were readily identified from their similarity to those in other functionally active APs. All contained the hallmark active site motif ∼Asp32-Thr33-Gly34∼ (pig pepsin numbering in Fig. 3) and its accompanying ∼Hydrophobic 120-Hydrophobic 121-Gly122∼ motif to form the first psi loop; with ∼Asp215-Thr/Ser216-Gly217∼ plus ∼Hydrophobic 300-Hydrophobic 301-Gly302-Asp303∼ forming the second psi loop, in which Asp303 is highly conserved among almost all eukaryotic APs (Yamauchi *et al.*, 1988). All five (predicted) mature BcAP enzymes have a Tyr residue at position 75, which is another totally conserved feature positioned near the tip of the β-hairpin loop that overlies the active site (Khan & James, 1998). In addition, BcAP1–4 all have an Asp residue at a position equivalent to Asp87 in pig pepsin (see Fig. 3), which is a highly conserved, critical residue. BcAP5, however, has a Gln residue at this location; this is consistent with the only replacement (also by a Gln) that has been reported in position 87 in the AP from an icefish (Capasso *et al.*, 1998). Retention of these functionally critical residues suggests that all five *B. cinerea* enzymes are active as APs in their mature form.

Archetypal APs of mammalian origin contain three characteristic disulphide bonds [between residues 45–50 (#1), 206–210 (#2) and 249–282 (#3)]. BcAP2–5 are predicted to include two (#1 and #3) of these disulphide bonds in their sequences (see Fig. 3). Potential *N*-glycosylation sites occur in all five sequences, ranging in number from one (BcAP2) to seven (BcAP3) sites.

BcAP1, BcAP2 and BcAP5 have their C termini at positions closely comparable to that of pig pepsin (see Fig. 3) and other APs from filamentous fungi. In contrast, BcAP3 and BcAP4 have long C-terminal extensions consisting of ∼115 and ∼78 residues, respectively. In both cases, these extensions contain lengthy stretches of hydrophilic, predominantly serine residues, terminating with ∼20 hydrophobic residues (see Fig. 3). The extensions may be modified to facilitate extracellular attachment of the enzymes in two ways: first by *O*-glycosylation of the serine residues, thereby providing a C terminus that may easily

Fig. 3. Alignment of the predicted amino acid sequences of the five *B. cinerea* aspartic proteinases (BcAP1–BcAP5) with that of pig pepsinogen. Gaps in the alignment are indicated by a hyphen (-). Residues conserved in five out of the six sequences are indicated in bold and as a consensus at the top of each block. Underneath each block of sequences the standard numbering system for the mature region of pig pepsin is included, while the numbering of residues in the propart is marked by a suffix P. Signal peptides for all sequences (except BcAP1) are on the left-hand side of the top block and are underlined. The predicted propeptide cleavage sites of all the precursors are aligned at the right-hand side of the top block and are marked by an arrow. Hallmark motifs that are conserved in APs, as discussed in the text, are indicated in grey. The two active site Asp residues in pig pepsin are at positions 32 and 215 respectively. Disulphide bonds in pig pepsin involve residues 45–50 (#1), 206–210 (#2) and 249–282 (#3). Disulphide bonds #1 and #3 are present in BcAP2–5 but are absent in BcAP1. *N*-Glycosylation signals are double underlined. A serine-rich stretch (∼50 %) indicated by a box, is found at the C terminus of both BcAP3 and BcAP4. The predicted GPI modification sites in BcAP3 and BcAP4 are indicated by black shading. N indicates the number of residues of the precursors whereas MW and pI indicate the predicted molecular mass (kDa) and isoelectric point of the predicted mature proteins, respectively.

associate with carbohydrates present in and on the fungal cell wall; second, cell wall association might be mediated by glycosylphosphatidylinositol (GPI) anchoring. The *S. cerevisiae* aspartic proteases yapsin 1, 2 and 3 have all been shown to be GPI-anchored proteins (Ash *et al.*, 1995; Cawley *et al.*, 1995; Komano & Fuller, 1995; Olsen *et al.*, 1999). Recently, rules for fungal GPI modification motifs have been described (Eisenhaber *et al.*, 2004) and an algorithm is available at a website server (fungal BIG-π predictor, http://mendel.imp.univie.ac.at/gpi/fungi/gpi_fungi.html). The sequences corresponding to the C-terminal extensions (BcAP3, 415–529; BcAP4, 406–483) were analysed for the presence of GPI modification motifs using this algorithm. GPI modification was predicted to occur in BcAP3 at Ser408 and in BcAP4 at Ala398 (see Fig. 3), both with high probability scores of $S \geqslant 11$. Thus, it appears that the mature enzyme product from the *Bcap3* and *Bcap4* genes may be attached to the membrane of *B. cinerea* by means of GPI anchors.

Perhaps the most distinctive features in these five *B. cinerea* sequences, however, are those of BcAP1. While it does contain all the residues necessary to function as an active proteolytic enzyme, the sequence of BcAP1 is highly atypical, in that it does not appear to initiate with a signal peptide and it is not predicted to contain any disulphide bonds. In particular, disulphide bond #3 is conserved in virtually all other eukaryotic AP sequences. BcAP1 does contain two cysteine residues (at positions equivalent to residues 16 and 228 of pig pepsin – see Fig. 3), but these can be safely predicted not to form a disulphide bond. Molecular modelling on the basis of numerous AP X-ray crystallography structures (Khan & James, 1998) shows the cysteine residues not to lie near enough to each other in the three-dimensional structure of the mature BcAP1 polypeptide chain to be able to form a disulphide bond. Eukaryotic AP precursors are commonly synthesized on membrane-bound ribosomes and the nascent polypeptide chains are directed into the endoplasmic reticulum where disulphide bonds are introduced by post-translational modification to enhance stability. While this manuscript was in preparation, the genomic sequence of *N. crassa* was reported (Galagan *et al.*, 2003) and from this, the predicted sequence of an AP precursor was mined (accession no. EAA 32024) and found to have 46 % identity to that of BcAP1. Like BcAP1 from *B. cinerea*, this *N. crassa* sequence is predicted to lack disulphide bonds as well as a conventional signal peptide. Both of the (predicted mature) enzymes have sequences with very acidic isoelectric points; indeed, in the case of the *N. crassa* enzyme, the pI value is almost as acidic as that of pig pepsin which operates in a very acidic environment ($pH < 2$) in the stomach. Thus, whatever the mechanism of trafficking of these *B. cinerea* and *N. crassa* proteins, the cellular compartment in which they finally reside can be predicted to be very acidic, otherwise each polypeptide would unfold and denature. In contrast, BcAP2 can be predicted with confidence to be the vacuolar AP from *B. cinerea* as it has 75 % identity in sequence with other fungal APs, e.g. from *S. cerevisiae*, *Pichia pastoris*, which have been shown experimentally to have vacuolar localizations (Winther *et al.*, 1998; Huh *et al.*, 2003).

Phylogeny of APs

A more rigorous analysis of the characterization of the proteinases was carried out by constructing a phylogenetic tree (see Fig. 4). A CLUSTALX alignment of 50 AP protein sequences (Table 2) was made (as described in Methods), and used for phylogenetic analyses using PAUP with pig pepsinogen as outgroup. Fig. 4(a) shows the most parsimonious tree whereas Fig. 4(b) shows one of the most parsimonious trees obtained with 1000 bootstrap replicates. Four monophyletic clades were distinguished with at least five sequences in each clade.

The BcAP1 sequence is contained in clade 1 together with sequences for APs from saprophytic fungi and all previously known sequences for APs from plant-pathogenic fungi. Most of the proteins in clade 1 have been shown experimentally to be secreted (see Fig. 4b and Table 2). The bootstrap support for BcAP1 is, however, not strong and it clusters within the clade of *Rhizopus* APs in the most parsimonious tree (see Fig. 4a).

BcAP2 was found to be contained within clade 3 (see Fig. 4b) which included all the APs that have been demonstrated to be located in the vacuole as well as those referred to as 'cellular' (listed in Table 2). Clade 3 is closely related to clade 4 which contains the plant APs. The latter are known to contain an additional sequence known as a plant-specific insert or saposin domain, involved in maturation of the active proteinase (White *et al.*, 1999).

BcAP3 and BcAP4 are both present within clade 2, which appears to consist of two subclades (see Fig. 4b). The upper subclade contains APs from filamentous fungi including BcAP3 and BcAP4 whilst the lower one contains secreted APs from yeasts. All four sequences from the upper subclade are predicted to contain a GPI modification site, as determined with the BIG-π fungal predictor (Eisenhaber *et al.*, 2004). Ca SAP9 from the lower subclade was also suggested to contain a potential GPI-anchor modification site (Hamada *et al.*, 1998) whereas a GPI anchor has been confirmed experimentally in YPS1 (Ash *et al.*, 1995; Cawley *et al.*, 1995).

BcAP5 seems to cluster in the most parsimonious tree with the protein IIAP from a basidiomycete fungus and the two human APs known as BACE1 and MEM1 (also known as BACE2) (see Fig. 4a). These both have long C-terminal extensions with membrane-spanning hydrophobic sequences; BACE1 is predominantly produced in brain (and pancreas) and has been identified as the β-secretase that fulfils a critical role in the onset of Alzheimer's Disease (Vassar, 2001). However, bootstrap analysis (see Fig. 4b) does not support this cluster, pushing both BcAP5 and

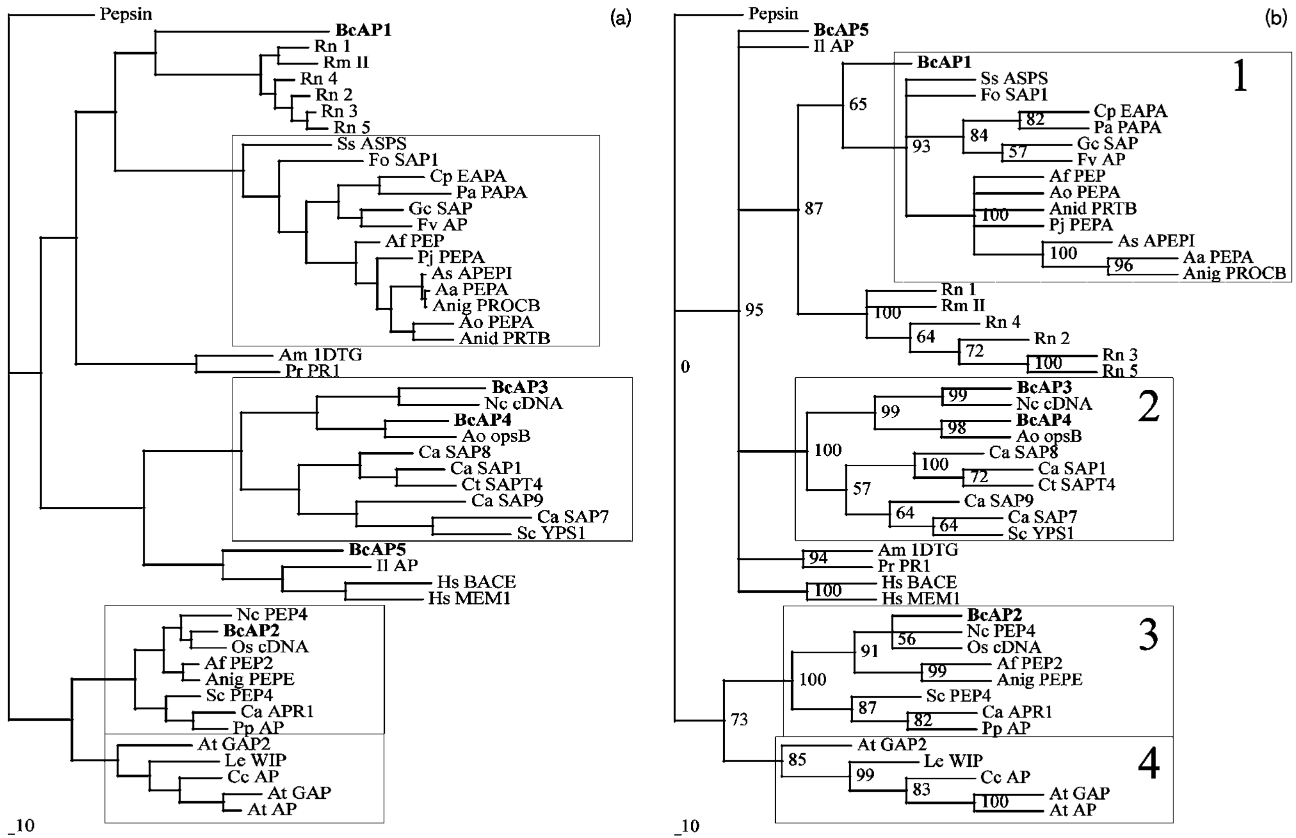


Fig. 4. Phylogenetic analysis of APs. A CLUSTALX alignment of AP sequences listed in Table 2 was used for phylogenetic analysis in PAUP. (a) The most parsimonious tree; (b) the tree resulting from a 1000 × Bootstrap neighbour-joining analysis with parsimony as the optimal criterion. Clades described in the text are indicated in numerals. APs from *B. cinerea* are in bold.

IIAP into separate groups while the two human APs remain clustered.

Expression analysis of the *Bcap* gene family *in vitro* and *in planta*

To examine the expression of the *Bcap1–5* genes, RNA was extracted from *B. cinerea* grown in liquid culture, and from four distinct plant species infected by *B. cinerea*. Hybridization and washing conditions were stringent to ensure that there was no cross-reactivity between the individual cDNA probes used and the different *Bcap* gene transcripts (not shown). The mRNA level of *B. cinerea* actin was analysed in parallel to serve as a reflection of the proportion of mRNA in the total RNA extracted (Benito *et al.*, 1998; Wubben *et al.*, 1999). This proportion fluctuates with growth rates, resulting in different intensities of *Bcact* mRNA even when equal amounts of total RNA are loaded on a gel. Fluctuations in the levels of actin mRNA were reported previously in similar types of analyses in other organisms (Cook *et al.*, 2001; Tatnell *et al.*, 2000).

Fig. 5 shows the hybridization results obtained with RNA extracted from axenic cultures. The hybridization intensities of *Bcap1* and *Bcap2* transcripts were substantially higher than those of *Bcap3*, *Bcap4* and *Bcap5*, essentially in all of the combinations of medium components tested. The levels of *Bcap1* and *Bcap3* transcripts were reduced by supplementing the basal medium with peptone (compare lane B5 to P and lane N to PN) and glucose (compare lane B5 to G, lane PN to PGN and lane N to GN). Repression by glucose seemed to be stronger than that caused by peptone (compare lane G to P and lane PN to PGN). *Bcap2* and *Bcap4* transcript levels were reduced in the presence of glucose (compare lane B5 to G, lane PN to PGN and lane N to GN) and peptone (compare lane B5 to P and lane N to PN) to similar extents. Transcript levels of *Bcap5* were generally low and seemed to largely follow the actin transcript levels, indicative of a constitutive but growth-dependent expression, with one exception. The level of *Bcap5* transcript in lane GN was relatively high as compared to the other samples, and taking into account the relative intensity of *Bcact* mRNA in this sample (see Fig. 5). The experiment was performed twice and yielded essentially similar hybridization patterns.

Four plant tissues (zucchini fruit, bell pepper, tomato fruit and tomato stem) were inoculated with *B. cinerea* and incubated until clear rot symptoms developed. The apparently

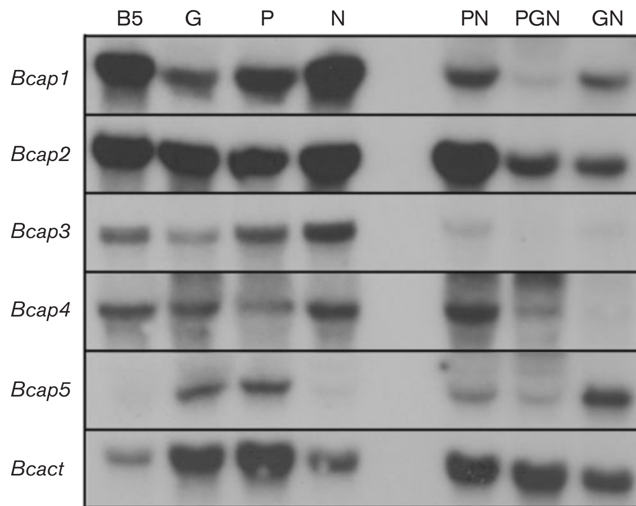


Fig. 5. Expression of the five aspartic proteinase genes in *B. cinerea* grown in different liquid culture media. *B. cinerea* was grown in Gamborg's basal salt medium overnight and then subdivided into fresh culture medium containing basal salt alone (B5), or supplemented with 1 % glucose (G), 1 % peptone (P) or 100 mM NH_4Cl (N) as carbon and nitrogen sources, singly or in combination (GN, PN and PGN). After growth for 8 h, RNA extracts were prepared and analysed on Northern blots. Five gene-specific probes (*Bcap1–5*) were used for each hybridization. Parallel analyses were conducted for the β-actin mRNA (*Bcact*) from *B. cinerea*.

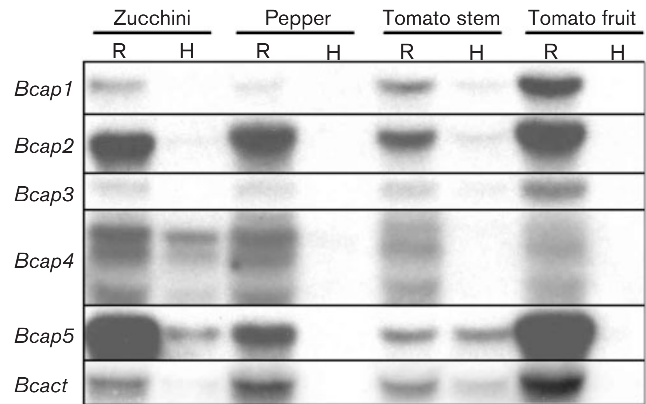


Fig. 6. Expression of the five aspartic proteinase genes in *B. cinerea* grown on different plants. The indicated plants were infected with *B. cinerea* and incubated until significant rot was evident. Samples of rotting (R) and adjacent, apparently healthy (H) tissue were dissected and RNA extracts prepared from each for analysis by Northern blotting. Five gene-specific probes (*Bcap1–5*) were used for each hybridization. Parallel analyses were conducted for the β-actin mRNA (*Bcact*) from *B. cinerea*.

healthy tissue that lay adjacent to the severely macerated tissue was, in each case, dissected carefully and Northern blotting was performed on RNA extracted from each 'rotten' and 'healthy' tissue (see Fig. 6, lanes R and H respectively). Hybridization showed that all five *Bcap* genes were expressed *in planta* albeit at different intensities in different host tissues (see Fig. 6). In some of the apparently healthy tissues, *Bcap* and *Bcact* mRNAs were detected faintly, most likely because fungal growth had presumably preceded symptom development in these cases. This effect was most noticeable in the cases of *Bcap4* and *Bcap5* mRNAs in zucchini and tomato stem (lanes H, Fig. 6), especially when taking into account the relative hybridization intensities of actin transcripts in these samples. The relative intensities of the hybridization signals were different from those in the liquid cultures (compare Fig. 5 with Fig. 6). *Bcap1* transcripts showed a relatively low abundance *in planta* in all tissues except tomato fruit, whilst *Bcap5* transcripts seemed much more abundant *in planta* than *in vitro*, especially in zucchini and tomato fruit. To study the temporal expression pattern of *Bcap* genes during the course of infection, we studied the *Bcap* mRNA levels during a time-course infection of detached tomato leaves over a week, until full maceration and fungal sporulation occurred. All genes were expressed and followed the increase of the actin transcript during the course of infection (data not shown).

DISCUSSION

B. cinerea cultures exclusively contain secreted AP activity

Fungal proteinases have been investigated in saprophytes with industrial applications, like certain aspergilli (Gomi *et al.*, 1993; Jarai *et al.*, 1994a) and penicilli (James & Sielecki, 1983; Durand-Poussereau & Fevre, 1996), but also in fungal pathogens that cause human disease, such as *Candida* spp. (Hube, 1998; Monod *et al.*, 1993), *Rhizopus* spp. and other aspergilli (Reichard *et al.*, 1995). Proteinases have not been studied intensively in plant-pathogenic fungi (Barkholt, 1987; Clark *et al.*, 1997; Murphy & Walton, 1996; Poussereau *et al.*, 2001b). Therefore we set out to obtain a complete view of the proteolytic complex secreted by *B. cinerea*. The pH optimum and inhibitory characteristics indicated that we detected exclusively AP activity in the culture medium. Inhibition by pepstatin was 100 % in some experiments but was incomplete (80–90 %) in other experiments. This residual proteinase activity could not, however, be inhibited by any specific inhibitors of other proteinase types. Pepstatin inhibition was incomplete only in assays that contained either BSA or wheat germ extract. Pepstatin and BSA are both hydrophobic (poly)peptides and wheat germ extract contains hydrophobic lectins. In these experiments the hydrophobic interaction of pepstatin with the excess of BSA or lectin presumably reduced the effective pepstatin concentration. The only proteinase activity detected in *B. cinerea* culture medium therefore represents AP, in agreement with conclusions of Manteau *et al.* (2003) based on similar experiments. Doss (1999) did

not detect any protease activity in conidia and germlings of *B. cinerea*, but this may be due to detection thresholds.

The lack of proteinase activity other than AP is remarkable for two reasons. Other fungi do secrete serine proteinase and/or metalloproteinase activity (Abbas *et al.*, 1989; Ahman *et al.*, 2002; Chou *et al.*, 2002; Jaton-Ogay *et al.*, 1994; Kunert & Kopecek, 2000; Monod *et al.*, 1991, 1993; Moutaouakil *et al.*, 1993; van den Hombergh *et al.*, 1994; Zhu *et al.*, 1990). Second, a public *B. cinerea* EST database contains a number of sequences that presumably encode a non-KEX-like serine proteinase and a carboxypeptidase, respectively. This database was generated from RNA isolated from a liquid culture grown under starvation conditions. However, also in *B. cinerea* cultures grown on minimal medium, we did not detect any activity other than AP over the course of one week (data not shown). It is conceivable that other proteinase types secreted by *B. cinerea* are unstable in the acidic environment or are themselves subject to proteolysis by the AP activity. Even in cell-free mycelial extracts, all proteinase activity was due to AP that was associated with the hyphal extracellular matrix, in which several extracellular enzymes were reported to be retained (Gil-Ad *et al.*, 2001). Only trace amounts of proteinase activity were detected in extracts made from mycelium that was extensively washed. Possibly other proteinase types, if produced, were not solubilized in the extraction or were unstable under the circumstances.

Secreting proteinases with an optimum activity at low pH suits the lifestyle of *B. cinerea*. The fungus acidifies its environment by secreting oxalic and citric acid (Verhoeff *et al.*, 1988; Germeier *et al.*, 1994; Manteau *et al.*, 2003), presumably as part of its infection strategy (Germeier *et al.*, 1994; Manteau *et al.*, 2003; Prins *et al.*, 2000; ten Have *et al.*, 2002). There are numerous examples of the modulation of expression of genes encoding secreted enzymes in plant-pathogenic fungi (Rollins & Dickman, 1998; St Leger *et al.*, 1998; Wubben *et al.*, 2000; Yakoby *et al.*, 2000). Regulation is often such that the genes are expressed only in a pH environment in which their products are effective (Prusky & Yakoby, 2003).

Features of the *Bcap* gene family

Five distinct genes were cloned and characterized, all encoding functional APs. Except for BcAP2, which clearly is a vacuolar AP, all BcAPs have characteristics that are novel among APs from plant-pathogenic fungi and even filamentous ascomycetes. On the basis of phylogeny we predict that BcAP1 is a secreted protein, even though a signal peptide sequence could not be annotated. The absence of a 'typical' signal peptide sequence in a secreted protein is not unprecedented in *B. cinerea*. The superoxide dismutase BcSOD1 also lacks a signal peptide sequence but was unequivocally demonstrated to be a secreted protein (Rolke *et al.*, 2004). BcAP1 has an additional unique characteristic in that it does not contain a disulphide bridge. To remain properly folded and stable, the BcAP1 enzyme presumably resides extracellularly since this is the most acidic 'environment' for *B. cinerea* (Verhoeff *et al.*, 1988; Germeier *et al.*, 1994; Manteau *et al.*, 2003).

Bcap3, *Bcap4* and *Bcap5* encode proteinases that have not thus been described among phytopathogenic fungi. BcAP5 represented an orphan in the phylogenetic tree; no orthologues were mined in fungal genomes of which the entire sequence was established. BcAP3 and BcAP4, however, clustered with secreted candidapepsins, of which many are thought to play a role in human pathogenesis (Hube, 1998). BcAP3 and BcAP4 probably possess a GPI-anchor modification at their C-terminal tail, as predicted by the fungal BIG-π predictor (Eisenhaber *et al.*, 2004). This GPI anchor may facilitate their association with the *B. cinerea* cell membrane. Moreover, BcAP3 and BcAP4 contain serine-rich stretches just upstream of the GPI modification sites. These stretches might be targets for *O*-glycosylation, which may facilitate adherence to the extracellular glucan matrix of *B. cinerea*. The substantial retention of AP activity in the extracellular matrix suggests that indeed the extracellular *B. cinerea* AP isoform(s) are glycoproteins. An AP that is attached to the cell membrane by a GPI anchor, or embedded in the hyphal matrix, might support various functions such as the maturation of other fungal hydrolytic enzymes, the proteolysis of host cell wall proteins in the vicinity of the hyphal tip, or protection of the fungus against plant defence proteins.

The five genes that were cloned may not represent the entire aspartic proteinase gene family in *B. cinerea*. *C. albicans* possesses a family of at least 10 AP genes while *N. crassa* may even contain as many as 14 genes with AP-like features (D. M. Wyatt, personal communication). Nevertheless four of the five *B. cinerea* genes reported here do not cluster in the phylogenetic tree, suggesting they have distinct properties and different physiological functions.

Expression of the *Bcap* gene family *in vitro* and *in planta*

The five *Bcap* genes were expressed under all circumstances tested, both *in vitro* and *in planta* (Figs 5 and 6). The down-regulation of *Bcap1*, *Bcap2*, *Bcap3* and *Bcap4* transcripts by glucose and peptone suggests the involvement of a *creA* type of regulator (Tudzynski *et al.*, 2000) and an as-yet-unidentified regulator responsive to exogenous amino acid levels. A positive regulator may also be involved, since the deletion of a Gα-protein gene in *B. cinerea* resulted in drastically reduced secretion of proteinase activity (Schulze Gronover *et al.*, 2001). The effect of glucose on *Bcap1* and *Bcap3* transcript levels was stronger than the effect of peptone, whereas *Bcap2* and *Bcap4* transcripts were repressed to similar extent by glucose and peptone. Further studies are needed to unravel the mechanisms behind this hierarchy in regulation. *Bcap1* and *Bcap2* transcripts generally showed (much) higher hybridization intensity than *Bcap3–5* transcripts, indicative of a higher abundance of isozymes BcAP1 and BcAP2 when grown in axenic cultures. Hybridization with a *Bcap4* probe often

detected multiple bands of significantly different lengths. It remains to be determined whether these length differences reflect an alternative upstream transcription initiation or a different polyadenylation site.

The expression of *Bcap* genes was also studied *in planta* to detect a possible infection-specific regulation (Fig. 6). All genes were expressed in a range of plant tissues. The relative levels of *Bcap2*, *Bcap3* and *Bcap4* transcripts were mostly comparable to the levels detected *in vitro*, whereas transcript levels of *Bcap1* and *Bcap5* were substantially different from those detected *in vitro*. Moreover, the level of *Bcap4* transcript in symptomless zucchini tissue and of *Bcap5* mRNA in symptomless tomato stem tissue was relatively high, when compared to the levels in severely macerated tissue. The mechanisms responsible for discrepancies between expression levels *in vitro* and *in planta* remain to be unravelled. In a time-course infection experiment, all five *Bcap* genes were expressed in parallel with the actin (*Bcact*) gene, indicating that none of the genes shows a temporal regulation *in planta*, as was observed for a family of genes encoding extracellular endopolygalacturonases (ten Have *et al.*, 2001). Infection of five distinct host tissues from four plants by *B. cinerea* resulted in rot and the decomposed tissue contained high levels of proteinase activity, identified as AP on the basis of pH optimum and inhibitory characteristics. In some host tissues, partial inhibition of proteinase activity by EDTA (5–10 %) was detected. Whether this is due to plant or fungal metalloproteinases remains to be determined. Since the rotten tissue consisted of both plant and fungal tissue, the origin of proteinase activity in these samples remains uncertain. It is even uncertain whether the infected tissue extract exclusively contained truly extracellular proteins secreted by both organisms. The extract was obtained by centrifugation of rotten tissue and contained the contents of dead cells of both plant and fungus, all contributing to the total proteinase activity. At least part of this activity originates from *B. cinerea*, as it secretes proteinase in axenic cultures (Fig. 1) and all five *Bcap* genes are expressed in all interactions studied (Fig. 6). On the other hand, the host plant may also contribute to the AP activity in the infected tissue extract. Tomato activates an AP upon wounding (Schaller & Ryan, 1996) and the infection of tomato leaves by *B. cinerea* induces a wound response (Diaz *et al.*, 2002). This tomato AP is therefore probably present in the infection fluid.

Many APs play crucial functions in human diseases caused by fungi (Monod *et al.*, 2002). A role for APs in pathogenesis on plants has not yet been established. More than a decade ago, Movahedi & Heale (1990a) showed that the addition of pepstatin to the inoculum resulted in a dramatic reduction of infection caused by *B. cinerea* on carrot slices, suggesting a putative role for *B. cinerea* APs in pathogenesis. The best way to evaluate this hypothesis is to make loss-of-function mutants by gene replacement. The construction and analysis of such (single and double) mutants is currently in progress.

ACKNOWLEDGEMENTS

The research was funded by the Dutch Technology Foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs (project WBI.4712). L. H. P. was supported by a grant from the UK BBSRC (Grant Number 72/C13544 to J. K.). The authors acknowledge Dr D. M. Wyatt for assistance in generating the protein alignment in Fig. 3, Dr P. van Baarlen for assistance in phylogenetic analysis, Dr T. W. Prins and Hinse Boonstra for their contribution to the cloning and characterization of the *Bcap1* and *Bcap3* genes and Dr B. Tudzynski for communicating unpublished EST data.

REFERENCES

Abbas, C. A., Groves, S. & Gander, J. E. (1989). Isolation, purification, and properties of *Penicillium charlesii* alkaline protease. *J Bacteriol* **171**, 5630–5637.

Ahman, J., Johansson, T., Olsson, M., Punt, P. J., van den Hondel, C. A. & Tunlid, A. (2002). Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. *Appl Environ Microbiol* **68**, 3408–3415.

Albersheim, P., Darvill, A., O'Neill, M. A., Schols, H. A. & Voragen, A. G. J. (1996). An hypothesis: the same six polysaccharides are components of all higher plants. In *Pectins and Pectinases*, pp. 47–55. Edited by J. Visser & A. G. J. Voragen. Amsterdam: Elsevier.

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.

Ash, J., Dominguez, M., Bergeron, J. J.-M., Thomas, D. Y. & Bourbonnais, Y. (1995). The yeast proprotein convertase encoded by *yap3* is a glycophosphatidylinositol-anchored protein that localizes to the plasma membrane. *J Biol Chem* **270**, 20847–20854.

Bang, M. L., Villadsen, I. & Sandal, T. (1999). Cloning and characterization of an endo-beta-1,3(4)glucanase and an aspartic protease from *Phaffia rhodozyma* CBS 6938. *Appl Microbiol Biotechnol* **51**, 215–222.

Barkholt, V. (1987). Amino acid sequence of endothiapepsin. Complete primary structure of the aspartic protease from *Endothia parasitica*. *Eur J Biochem* **167**, 327–338.

Benito, E. P., ten Have, A., van't Klooster, J. W. & van Kan, J. A. L. (1998). Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. *Eur J Plant Pathol* **104**, 207–220.

Berka, R. M., Ward, M., Wilson, L. J., Hayenga, K. J., Kodama, K. H., Carlomagno, L. P. & Thompson, S. A. (1990). Molecular-cloning and deletion of the gene encoding aspergillopepsin-A from *Aspergillus awamori*. *Gene* **86**, 153–162.

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.

Burnett, W. V. (1997). Northern blotting of RNA denatured in glyoxal without buffer recirculation. *Biotechniques* **22**, 668–671.

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., Wong, M., Pu, L. P., Tam, W. & Loh, Y. P. (1995). Secretion of yeast aspartic protease 3 is regulated by its carboxy-terminal tail: characterization of secreted YAP3p. *Biochemistry* **34**, 7430–7437.

Chou, H., Lai, H. Y., Tam, M. F., Chou, M. Y., Wang, S. R., Han, S. H. & Shen, H. D. (2002). cDNA cloning, biological and immunological characterization of the alkaline serine protease major allergen from *Penicillium chrysogenum*. *Int Arch Allergy Immunol* **127**, 15–26.

Clark, S. J., Templeton, M. D. & Sullivan, P. A. (1997). A secreted aspartic proteinase from *Glomerella cingulata*: purification of the enzyme and molecular cloning of the cDNA. *Microbiology* **143**, 1395–1403.

Cook, M., Caswell, R. C., Richards, R. J., Kay, J. & Tatnell, P. J. (2001). Regulation of human and mouse procathepsin E gene expression. *Eur J Biochem* **268**, 2658–2668.

Cordeiro, M. C., Xue, Z. T., Pietrzak, M., Pais, M. S. & Brodelius, P. E. (1994). Isolation and characterization of a cDNA from flowers of *Cynara cardunculus* encoding cyprosin (an aspartic proteinase) and its use to study the organ-specific expression of cyprosin. *Plant Mol Biol* **24**, 733–741.

Cosgrove, D. J. (2000). Loosening of plant cell walls by expansins. *Nature* **407**, 321–326.

Dhondt, K., Stack, S., Gutteridge, S., Vandekerckhove, J., Krebbers, E. & Gal, S. (1997). Aspartic proteinase genes in the Brassicaceae *Arabidopsis thaliana* and *Brassica napus*. *Plant Mol Biol* **33**, 187–192.

Diaz, J., ten Have, A. & van Kan, J. A. L. (2002). The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. *Plant Physiol* **129**, 1341–1351.

Dik, A. J., Koning, G. & Kohl, J. (1999). Evaluation of microbial anatagonists for biological control of *Botrytis cinerea* stem infection in cucumber and tomato. *Eur J Plant Pathol* **105**, 115–122.

Doss, R. P. (1999). Composition and enzymatic activity of the extracellular matrix secreted by germlings of *Botrytis cinerea*. *Appl Environ Microbiol* **65**, 404–408.

Durand-Poussereau, N. & Fevre, M. (1996). Characterization of a protease deficient strain of *Penicillium roqueforti* generated by heterologous plasmid integration: potential use for protein production. *J Biotechnol* **51**, 97–105.

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.

Farley, P. C. & Sullivan, P. A. (1998). The *Rhizopus oryzae* secreted aspartic proteinase gene family: an analysis of gene expression. *Microbiology* **144**, 2355–2366.

Fry, S. C. (1982). Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. *Biochem J* **204**, 449–455.

Galagan, J. E., Calvo, S. E., Borkovich, K. A. & 74 other authors (2003). The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**, 859–868.

Germeier, C., Hedke, K. & Tiedemann, A. V. (1994). The use of pH-indicators in diagnostic media for acid-producing plant pathogens. *Z Pflanzenkr Pflanzenschutz* **101**, 498–507.

Gil-ad, N. L., Bar-Nun, N. & Mayer, A. M. (2001). The possible function of the glucan sheath of *Botrytis cinerea*: effects on the distribution of enzyme activities. *FEMS Microbiol Lett* **199**, 109–113.

Gomi, K., Arikawa, K., Kamiya, N., Kitamoto, K. & Kumagai, C. (1993). Cloning and nucleotide-sequence of the acid protease-encoding gene (PEPA) from *Aspergillus oryzae*. *Biosci Biotechnol Biochem* **57**, 1095–1100.

Hamada, K., Fukuchi, S., Arisawa, M., Baba, M. & Kitada, K. (1998). Screening for glycosylphosphatidylinositol (GPI)-dependent cell wall proteins in *Saccharomyces cerevisiae*. *Mol Gen Genet* **258**, 53–59.

Horiuchi, H., Yanai, K., Okazaki, T., Takagi, M. & Yano, K. (1988). Isolation and sequencing of a genomic clone encoding aspartic proteinase of *Rhizopus niveus*. *J Bacteriol* **170**, 272–278.

Hube, B. (1998). Possible role of secreted proteinases in *Candida albicans* infections. *Rev Iberoam Micol* **15**, 65–68.

Hube, B., Turver, C. J., Odds, F. C., Eiffert, H., Boulnois, G. J., Kochel, H. & Ruchel, R. (1991). Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. *J Med Vet Mycol* **29**, 129–132.

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S. & O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* **425**, 686–691.

James, M. N. & Sielecki, A. R. (1983). Structure and refinement of penicillopepsin at 1·8 Å resolution. *J Biol Chem* **163**, 299–361.

Jarai, G., Kirchherr, D. & Buxton, F. (1994a). Cloning and characterization of the *pepD* gene of *Aspergillus niger* which codes for a subtilisin-like protease. *Gene* **139**, 51–57.

Jarai, G., Van den Hombergh, J. P. & Buxton, F. P. (1994b). Cloning and characterization of the PEPE gene of *Aspergillus niger* encoding a new aspartic protease and regulation of PEPE and PEPC. *Gene* **145**, 171–178.

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

Jaton-Ogay, K., Paris, S., Huerre, M., Quadroni, M., Falchetto, R., Togni, G., Latge, J. P. & Monod, M. (1994). Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. *Mol Microbiol* **14**, 917–928.

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

Kikuchi, S., Satoh, K., Nagata, T. & 71 other authors (2003). Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. *Science* **301**, 376–379.

Kobayashi, H., Sekibata, S., Shibuya, H., Yoshida, S., Kusakabe, I. & Murakami, K. (1989). Cloning and sequence-analysis of cDNA for *Irpex lacteus* aspartic proteinase. *Agric Biol Chem* **53**, 1927–1933.

Komano, H. & Fuller, R. S. (1995). Shared functions *in vivo* of a glycosyl-phosphatidylinositol-linked aspartyl protease, Mkc7, and the proprotein processing protease Kex2 in yeast. *Proc Natl Acad Sci U S A* **92**, 10752–10756.

Kunert, J. & Kopecek, P. (2000). Multiple forms of the serine protease Alp of *Aspergillus fumigatus*. *Mycoses* **43**, 339–347.

Kunihiro, S., Kawanishi, Y., Sano, M. & 7 other authors (2002). A polymerase chain reaction-based method for cloning novel members of a gene family using a combination of degenerate and inhibitory primers. *Gene* **289**, 177–184.

Lu, J. F., Inoue, H., Kimura, T., Makabe, O. & Takahashi, K. (1995). Molecular-cloning of a cDNA for proctase-B from *Aspergillus niger* var. macrosporas and sequence comparison with other aspergillopepsins I. *Biosci Biotechnol Biochem* **59**, 954–955.

Manteau, S., Abouna, S., Lambert, B. & Legendre, L. (2003). Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. *FEMS Microbiol Ecol* **43**, 359–366.

Masih, E. I. & Paul, B. (2002). Secretion of beta-1,3-glucanases by the yeast *Pichia membranifaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mold disease of the grapevine. *Curr Microbiol* **44**, 391–395.

McCann, M. C. & Roberts, K. (1991). Architecture of the primary cell wall. In *The Cytoskeletal Basis of Plant Growth and Form*, pp. 109–129. Edited by C. W. Lloyd. London: Academic Press.

Monod, M., Togni, G., Rahalison, L. & Frenk, E. (1991). Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. *J Med Microbiol* **35**, 23–28.

Monod, M., Paris, S., Sanglard, D., Jaton-Ogay, K., Bille, J. & Latge, J. P. (1993). Isolation and characterization of a secreted metallo-protease of *Aspergillus fumigatus*. *Infect Immun* **61**, 4099–4104.

Monod, M., Togni, G., Hube, B. & Sanglard, D. (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Mol Microbiol* **13**, 357–368.

Monod, M., Hube, B., Hess, D. & Sanglard, D. (1998). Differential regulation of SAP8 and SAP9, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. *Microbiology* **144**, 2731–2737.

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

Moutaouakil, M., Monod, M., Prevost, M. C., Bouchara, J. P., Paris, S. & Latge, J. P. (1993). Identification of the 33-kDa alkaline protease of *Aspergillus fumigatus in vitro* and *in vivo*. *J Med Microbiol* **39**, 393–399.

Movahedi, S. & Heale, J. B. (1990a). 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.

Movahedi, S. & Heale, J. B. (1990b). Purification and characterization of an aspartic proteinase secreted by *Botrytis cinerea* pers ex. pers in culture and in infected carrots. *Physiol Mol Plant Pathol* **36**, 289–302.

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

Nehls, U., Bock, A., Einig, W. & Hampp, R. (2001). Excretion of two proteases by the ectomycorrhizal fungus *Amanita muscaria*. *Plant Cell Environ* **24**, 741–747.

Nielsen, H., Engebrecht, J., Brunak, S. & von Heijne, G. (1997a). A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int J Neural Syst* **8**, 581–599.

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

Olsen, V., Cawley, N. X., Brandt, J., Egel-Mitani, M. & Loh, Y. P. (1999). Identification and characterization of *Saccharomyces cerevisiae* yapsin 3, a new member of the yapsin family of aspartic proteases encoded by the *YPS3* gene. *Biochem J* **339**, 407–411.

Paoletti, M., Clave, C. & Begueret, J. (1998). Characterization of a gene from the filamentous fungus *Podospora anserina* encoding an aspartyl protease induced upon carbon starvation. *Gene* **210**, 45–52.

Paris, R. & Lamattina, L. (1999). *Phytophthora infestans* secretes extracellular proteases with necrosis inducing activity on potato. *Eur J Plant Pathol* **105**, 753–760.

Poussereau, N., Creton, S., Billon-Grand, G., Rascle, C. & Fevre, M. (2001a). Regulation of *acp1*, encoding a non-aspartyl acid protease expressed during pathogenesis of *Sclerotinia sclerotiorum*. *Microbiology* **147**, 717–726.

Poussereau, N., Gente, S., Rascle, C., Billon-Grand, G. & Fevre, M. (2001b). *aspS* encoding an unusual aspartyl protease from *Sclerotinia sclerotiorum* is expressed during phytopathogenesis. *FEMS Microbiol Lett* **194**, 27–32.

Prins, T. (2001). *Identification and functional analysis of Botrytis cinerea genes induced during infection of tomato*. PhD thesis, Wageningen University, Wageningen, The Netherlands.

Prins, T. W., Tudzynski, P., von Tiedemann, A., Tudzynski, B., ten Have, A., Hansen, M. E., Tenberge, K. & van Kan, J. A. L. (2000). Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. In *Fungal Pathology*, pp. 33–64. Edited by J. W. Kronstad. Dordrecht: Kluwer Academic.

Prusky, D. & Yakoby, N. (2003). Pathogenic fungi: leading or led by ambient pH? *Mol Plant Pathol* **4**, 509–516.

Reichard, U., Monod, M. & Ruchel, R. (1995). Molecular-cloning and sequencing of the gene encoding an extracellular aspartic proteinase from *Aspergillus fumigatus*. *FEMS Microbiol Lett* **130**, 69–74.

Reichard, U., Cole, G. T., Ruchel, R. & Monod, M. (2000). Molecular cloning and targeted deletion of PEP2 which encodes a novel aspartic proteinase from *Aspergillus fumigatus*. *Int J Med Microbiol* **290**, 85–96.

Rolke, Y., Liu, S., Quidde, T., Williamson, B., Schouten, A., Weltring, K.-M., Siewers, V., Tudzynski, B. & Tudzynski, P. (2004). Functional analysis of H_2O_2-generating systems in *Botrytis cinerea*: the major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable. *Mol Plant Pathol* **5**, 17–28.

Rollins, J. A. & Dickman, M. B. (1998). Increase in endogenous and exogenous cyclic AMP levels inhibits sclerotial development in *Sclerotinia sclerotiorum*. *Appl Environ Microbiol* **64**, 2539–2544.

Schaller, A. & Ryan, C. A. (1996). Molecular cloning of a tomato leaf cDNA encoding an aspartic protease, a systemic wound response protein. *Plant Mol Biol* **31**, 1073–1077.

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.

Shintani, T. & Ichishima, E. (1994). Primary structure of aspergillopepsin-I deduced from nucleotide-sequence of the gene and aspartic acid-76 is an essential active-site of the enzyme for trypsinogen activation. *Biochim Biophys Acta* **1204**, 257–264.

St Leger, R. J., Joshi, L. & Roberts, D. (1998). Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hybrophobin by *Metarhizium anisopliae*. *Appl Environ Microbiol* **64**, 709–713.

Stahmann, K. P., Monschau, N., Sahm, H., Koschel, A., Gawronski, M., Conrad, H., Springer, T. & Kopp, F. (1995). Structural properties of native and sonicated cinerean, a beta-(1→3)(1→6)-D-glucan produced by *Botrytis cinerea*. *Carbohydr Res* **266**, 115–128.

Swofford, D. L., Waddell, P. J., Huelsenbeck, J. P., Foster, P. G., Lewis, P. O. & Rogers, J. S. (2001). Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Syst Biol* **50**, 525–539.

Tang, J. (1979). Evolution in the structure and function of carboxyl proteases. *Mol Cell Biochem* **26**, 93–109.

Tatnell, P. J., Cook, M., Peters, C. & Kay, J. (2000). Molecular organization, expression and chromosomal localization of the mouse pronapsin gene. *Eur J Biochem* **267**, 6921–6930.

ten Have, A. & Woltering, E. J. (1997). Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. *Plant Mol Biol* **34**, 89–97.

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., Breuil, W. O., Wubben, J. P., Visser, J. & van Kan, J. A. (2001). *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genet Biol* **33**, 97–105.

ten Have, A., Tenberge, K. B., 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. In *The Mycota, Vol. XI*, pp. 341–358. Edited by F. Kempken. Berlin: Springer.

Tsukagoshi, N., Ando, Y., Tomita, Y. & 7 other authors (1988). Nucleotide sequence and expression in *Escherichia coli* of cDNA of swine pepsinogen: involvement of the amino-terminal portion of the activation peptide segment in restoration of the functional protein. *Gene* **65**, 285–292.

Tudzynski, B., Liu, S. & Kelly, J. M. (2000). Carbon catabolite repression in plant pathogenic fungi: isolation and characterization of the *Gibberella fujikuroi* and *Botrytis cinerea creA* genes. *FEMS Microbiol Lett* **184**, 9–15.

Valler, M. J., Kay, J., Aoyagi, T. & Dunn, B. M. (1985). Interaction of aspartic proteinases with naturally-occurring inhibitors from actinomycetes and *Ascaris lumbricoides*. *J Enzyme Inhib* **1**, 77–82.

van den Hombergh, J. P., Jarai, G., Buxton, F. P. & Visser, J. (1994). Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus niger*. *Gene* **151**, 73–79.

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., 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.

VasquezLaslop, N., Tenney, K. & Bowman, B. J. (1996). Characterization of a vacuolar protease in *Neurospora crassa* and the use of gene RIPing to generate protease-deficient strains. *J Biol Chem* **271**, 21944–21949.

Vassar, R. (2001). The beta-secretase, BACE: a prime drug target for Alzheimer's disease. *J Mol Neurosci* **17**, 157–170.

Verhoeff, K., Leeman, M., van Peer, R., Posthuma, L., Schot, N. & van Eijk, G. W. (1988). Changes in pH and the production of organic acids during colonization of tomato petioles by *Botrytis cinerea*. *J Phytopathol* **122**, 327–333.

White, P. C., Cordeiro, M. C., Arnold, D., Brodelius, P. E. & Kay, J. (1999). Processing, activity, and inhibition of recombinant cyprosin, an aspartic proteinase from cardoon (*Cynara cardunculus*). *J Biol Chem* **274**, 16685–16693.

Winther, J. R., Phylip, L. H. & Kay, J. (1998). Saccharopepsin. In *Handbook of Proteolytic Enzymes*, pp. 848–850. Edited by A. J. Barrett, N. D. Rawlings & J. F. Woessner. New York: Academic Press.

Woolford, C. A., Daniels, L. B., Park, F. J., Jones, E. W., Van Arsdell, J. N. & Innis, M. A. (1986). The PEP4 gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. *Mol Cell Biol* **6**, 2500–2510.

Wubben, J. P., Mulder, W., ten Have, A., van Kan, J. A. L. & Visser, J. (1999). Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. *Appl Environ Microbiol* **65**, 1596–1602.

Wubben, J. P., ten Have, A., van Kan, J. A. & Visser, J. (2000). Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Curr Genet* **37**, 152–157.

Yakoby, N., Kobiler, I., Dinoor, A. & Prusky, D. (2000). pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. *Appl Environ Microbiol* **66**, 1026–1030.

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.

Yan, R., Bienkowski, M. J., Shuck, M. E. & 12 other authors (1999). Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature* **402**, 533–537.

Zaugg, C., Borg-von Zepelin, M., Reichard, U., Sanglard, D. & Monod, M. (2001). Secreted aspartic proteinase family of *Candida tropicalis*. *Infect Immun* **69**, 405–412.

Zhu, W. S., Wojdyla, K., Donlon, K., Thomas, P. A. & Eberle, H. I. (1990). Extracellular proteases of *Aspergillus flavus*. Fungal keratitis, proteases, and pathogenesis. *Diagn Microbiol Infect Dis* **13**, 491–497.