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Maize endosperm secretes a novel antifungal protein into adjacent maternal tissue

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Summary

A series of endosperm transfer layer-specific transcripts has been identified in maize by differential screening of a cDNA library of transcripts at 10 days after pollination. Sequence comparisons revealed among this class of cDNAs a novel, small gene family of highly diverged sequences encoding basal layer antifungal proteins (BAPs). The *bap* genes mapped to two loci on chromosomes 4 and 10. So far, *bap*-homologous sequences have been detected only in maize, teosinte and sorghum, and are not present in grasses outside the Andropogoneae tribe. BAP2 is synthesized as a pre-proprotein, and is processed by successive removal of a signal peptide and a 29-residue prodomain. The proprotein can be detected exclusively in microsomal membrane-containing fractions of kernel extracts. Immunolocalization reveals BAP2 to be predominantly located in the placentochalazal cells of the pedicel, adjacent to the basal endosperm transfer layer (BETL) cells, although the BAP2 transcript is found only in the BETL cells. The biological roles of BAP2 propeptide and mature peptide have been investigated by heterologous expression of the proprotein in *Escherichia coli*, and by tests of its fungistatic activity and that of the fully processed form *in vitro*. The mature BAP2 peptide exhibits potent broad-range activity against a range of filamentous fungi, including several plant pathogens.

Keywords: endosperm, transfer layer, antifungal, defensin, protease, transport.

Introduction

The developing cereal grain is adapted for rapid nutrient assimilation by the development of a transfer layer. In maize these cells, which form a disc in the basal endosperm corresponding to the area of attachment to the pedicel, are highly modified by the development of cell-wall projections on the basal cell surface (Davis *et al.*, 1989). A series of basal endosperm transfer layer-specific cDNA clones (termed BETL1-x) have been isolated by differential screening of immature kernel cDNA libraries (Hueros *et al.*, 1995; Hueros *et al.*, 1999). The four most abundant BETL clones, BETL1 to -4, encode small polypeptides with M_r between 8 and 10 kDa. These proteins have no close relatives in database accessions, but two of them, BETL1 and BETL3, show some similarity to the defensin supergene family of antimicrobial peptides (Hueros *et al.*, 1999; Terras *et al.*, 1995). Of the remaining BETL clones, BETL4 has some similarities to the Bowman-Birk family of trypsin inhibitors (Hueros *et al.*, 1999), whereas BETL2 lacks close databank relatives.

In an extended screen for maize endosperm transfer layer-specific clones, we have now identified additional basal endosperm-specific cDNAs related to BETL2, which together define a novel gene family. In the light of their restricted distribution and biological properties, we have called these proteins BAPs (basal layer type antifungal proteins), and the corresponding loci *bap1*, *bap2* and *bap3*. BAP transcripts accumulate between 8 and 20 days after pollination (DAP). BAP2 (previously termed BETL2) protein

follows a similar time-course of appearance (Hueros *et al.*, 1999); it turns over by mid-term development and is absent from mature kernels, suggesting a role in development rather than in maturation or germination. We report investigations of the biological function of BAP2 which show it to be a potent antifungal agent that is secreted into the intercellular matrix of the basal endosperm and accumulates predominantly in the adjacent, thick-walled cell layer of the pedicel, the placentochalaza. BAP2 presumably protects the zygote from pathogen ingress through the nutrient-rich basal endosperm cells. BAP2 is a member of a new family of peptides having a putative antimicrobial role, with a structure and mode of activation distinguishing it from the previously characterized families (Garcia-Olmedo *et al.*, 1998).

Results

Classification of a family of transfer layer-specific transcripts

10358 cDNA clones from a 10 DAP endosperm library were gridded onto nylon filters and screened with ^{32}P-labelled cDNA prepared from 10 DAP top and bottom endosperm half mRNA. Sequences more highly expressed in bottom than in top endosperm RNA were sequenced and compared to existing sequence database accessions. As a result of sequencing 100 clones preferentially or exclusively expressed in bottom, as compared with top, endosperm, six cDNAs were identified that were related to BAP2 using BLAST searches. Pairwise comparisons of the six selected sequences were carried out in BLAST2, using the default conditions (Tatusova and Madden, 1999) (Table 1). These sequences were also aligned using the programme CLUSTALW (Thompson *et al.*, 1994), as shown in Figure 1. The corresponding proteins were termed BAPs, and the loci corresponding to the three subgroups of clones were termed *bap1*, *bap2* (previously BETL2) and *bap3* (Table 2). Inspection of the aligned sequences showed a block of conserved residues in the signal peptide and adjacent prodomain region, with three further conserved residues (Leu, Tyr, Thr) clustered around four cysteines in the COOH-terminal 35 amino acids. An attempt to align BAP sequences with plant defensins indicated, despite the presence of four aligned cysteine residues, that the characteristic defensin motif (Conceicao and Broekaert, 1999), CXXXC-GXC-CXC, was not preserved (not shown). BAPs are also unrelated in sequence

Table 1. Random matching probabilities for pairwise matched BAP amino acid sequences using BLAST2 (Tatusova and Madden, 1999)

	bap2	bap1a	bap1b	bap3a	bap3b
bap2	0	6e-07	1e-07	1e-10	6e-09
bap1a		0	2e-28	2e-07	3e-07
bap1b			0	3e-08	3e-07
bap3a				0	5e-38
bap3b					0

Table 2. Suggested nomenclature for the basal layer-type antifungal protein family

Clone working name	Locus terminology	Locus position
pXa8	bap1-a	Ch. 10L, interval 53–55
pX35	bap1-b	Ch. 10L, interval 53–55
BETL2	bap2	Ch. 4, co-ordinate 62–60
pX68	bap3-a	Ch. 10L, interval 53–55
pX81	bap3-b	Ch. 10L, interval 53–55

Figure 1. Multiple sequence alignment of the primary translation products of BAP proteins.
Analysis performed using CLUSTALW (Thompson *et al.*, 1994). Black shaded letters indicate identical amino acids; grey shaded letters indicate homologous amino acids. The shading sequence fraction corresponds to 50%. Arrowheads denote the first residue C-terminal of cleavage sites determined for protein maturation. The sequences have been deposited in the EMBL nucleotide sequence database.

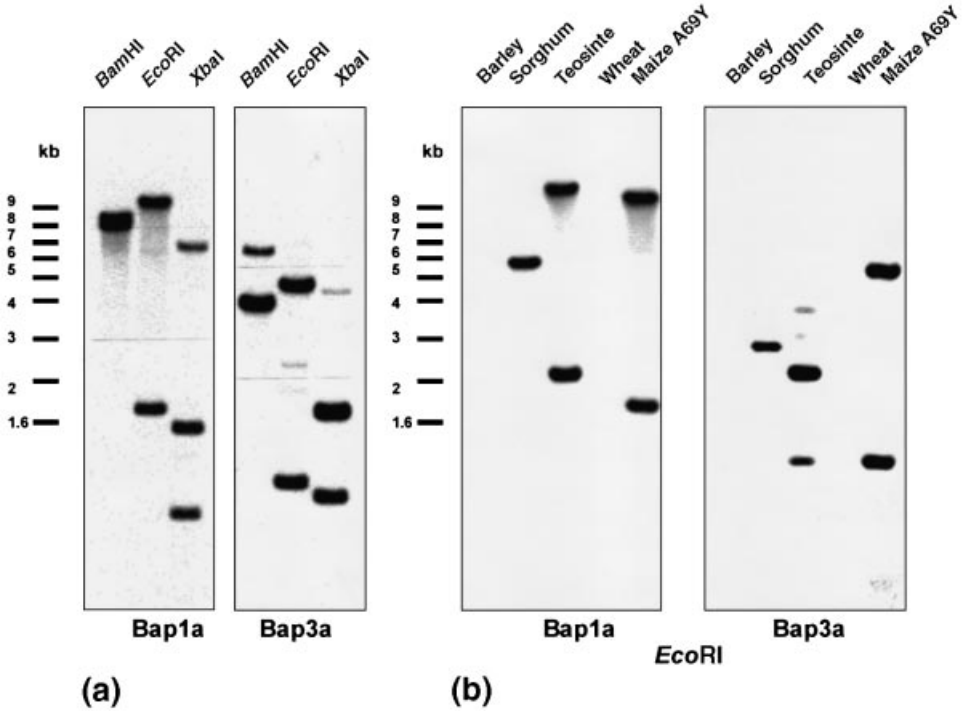

Figure 2. Genomic organization of bap loci.
(a) The BAP genes constitute a small gene family in maize. Maize genomic DNA from the variety A69Y (10 μg per lane) was digested with the enzymes indicated, and fractionated in a 1% agarose gel. Southern blots were hybridized either with Bap1a or Bap3a cDNA inserts, as indicated. The positions of size markers are given in kb.
(b) Presence of BAP-related sequences in other cereals. Genomic DNA from different species (10 μg per lane): barley (*Hordeum vulgare*), Sorghum (*Sorghum bicolor*), Teosinte (*Zea luxurians*), wheat (*Triticum aestivum*) *and* maize (*Zea mays* line A69Y), was digested with *Eco*RI and fractionated on a 1% agarose gel. The resulting Southern blots were hybridized with Bap1a or Bap3a, as indicated. The positions of size markers are given in kb.

to a previously reported antimicrobial peptide from maize kernels (Duvick *et al.*, 1992).

The distribution of BAP family members among members of the Poaceae was investigated by Southern hybridizations with BAP1a and BAP3a probes (Figure 2). As both the (BAP1a and BAP1b) and (BAP3a and BAP3b) pairs have 95% DNA sequence homology with one another and cross-hybridize at high stringency, only BAP1a and BAP3a were used as probes. A single hybridizing band was seen for each probe in Sorghum (*Sorghum bicolor*) DNA, whereas Teosinte (*Zea luxurians*) contained two bands for each probe, as did domesticated maize, *Zea mays* (line A69Y). Neither barley nor wheat DNA gave detectable signals with either probe. Similar results have been obtained for BAP2 (Hueros *et al.*, 1999).

Accumulation of BAP family transcripts during endosperm development.

Samples of mRNA (2 μg) from kernels harvested between 7 and 27 DAP were fractionated by electrophoresis in denaturing formaldehyde gels, blotted onto nylon filters, and hybridized with BAP1a, 1b, 3a, and 3b probes (Figure 3a). Transcripts were evident from 10 to 18 DAP with quantitative variations between the probes. From 20 DAP onwards, transcripts were either undetectable or present only in trace amounts. The specificity of expression of BAP1a, 1b, 3a and 3b genes was monitored by Northern analysis of RNAs from endosperm, leaf, root, shoot and silk tissues (Figure 3b). In all four cases, signal was seen only in the endosperm RNA sample. The timing and specificity of expression of BAP1 and BAP3 transcripts therefore corresponded to that seen previously for BAP2 (Hueros *et al.*, 1999).

Cell-type localization of BAP transcripts and BAP2 protein

All the BAP family members identified are restricted in their expression to the endosperm, and accumulate only during mid-term endosperm development according to Northern blot analyses using RNAs from different tissues (Figure 3). Using one member of each of the newly identified sequence classes (*bap1* and *bap3*) for *in situ* hybridization revealed expression of both to be exclusively in the basal endosperm transfer layer (Figure 4), as also reported for *bap2* (Hueros *et al.*, 1999). Thus both the site

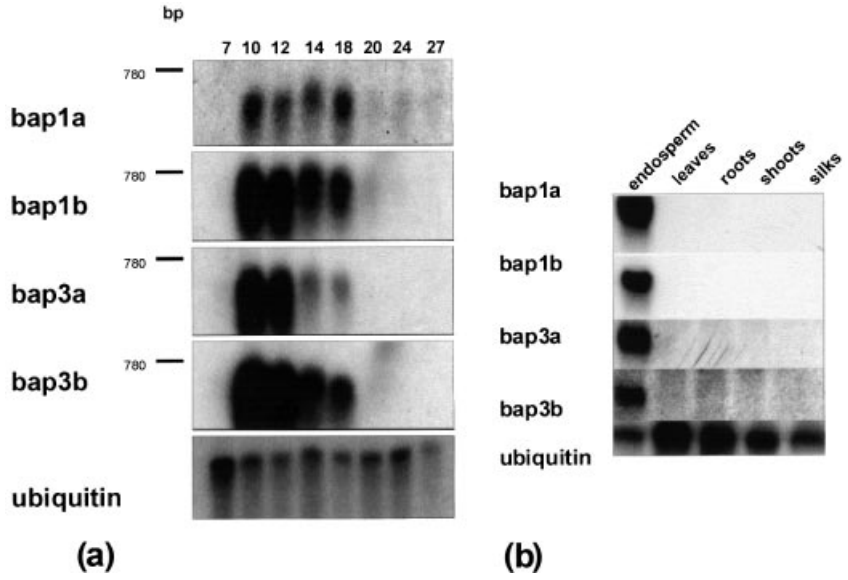

Figure 3. Sites of expression of BAP transcripts.
(a) Accumulation of BAP family transcripts during endosperm development. Samples of mRNA (2 μg) from kernels harvested at 7–27 DAP, as indicated, were fractionated on a denaturing formaldehyde gel. The Northern blot was hybridized with the BAP probes indicated on the left.
(b) Tissue specificity of expression of BAP transcripts. Messenger RNA (2 μg) from different tissues, as indicated, were fractionated on a denaturing formaldehyde gel. The resulting Northern blot was probed sequentially with the indicated BAP probes

and the timing of expression in the kernel are similar for all three BAP-class cDNAs.

Cell-type and subcellular localization of BAP2

The localization of BAP2 protein was carried out to check whether the site of deposition coincides with that of the mRNA. Wax-embedded sections from different developmental stages were immunodecorated with pre-immune serum (Figure 5a) or with anti-BAP2 antibody (Figure 5b), and detected with peroxidase Diaminobenzidene (DAB) staining. The protein was detected as expected in the basal endosperm cells. However, a strong signal was also seen in the placentochalazal layer of the pedicel. Control sections stained with pre-immune serum gave no signal in these cell layers. The results indicate that BAP2 protein is synthesized in the basal endosperm transfer-layer cells and quantitatively deposited in the placentochalaza.

To locate the BAP2 protein more precisely, and to gain insight into the post-translational route taken during deposition, the protein was detected by immunogold labelling and analysed by transmission electron microscopy. Thin (100 nm) sections of 15 DAP kernels embedded in LR-white resin were visualized without OsO4 shadowing (Wells, 1985). BAP2 antibody was detected with a secondary antibody coupled to 15 nm gold particles. The strongest signal was in the pedicel, at the site of the placentochalazal cells, as shown by light microscopy. These cells displayed little intracellular structure, and only rarely was a nucleus seen after 12 DAP. The cell walls were thickened and cytoplasmic structure was absent, that is, the cytosol is probably metabolically inactive. Within the endosperm the first transfer-cell layer, which is largely occupied by cell-wall material by 15 DAP, was relatively weakly labelled. The second layer of endosperm cells labelled much more heavily. These cells possessed a dense cytoplasm lacking large vacuoles. The cells were rich in mitochondria and cell-wall ingrowths, although the latter were less developed than in the first cell layer. The BAP2 protein was located principally at the cell wall/plasma membrane interface of the wall ingrowths (Figure 5, right panel). It was also detected more weakly in membraneous inclusions corresponding to the rough endoplasmic reticulum or golgi vesicles.

Distribution of BAP2 protein forms in basal kernel tissues

The BAP2 open reading frame minus the putative signal peptide (residues 27–95 in Figure 1) was expressed with a COOH-terminal His-tag fusion in pQE60. The construct was used to prepare BAP2 protein to use as an antigen for raising antiserum in rabbits. This antiserum recognizes two polypeptides on immunoblots (arrowed in Figure 6a), of apparent sizes 8 and 4.5 kDa. The 8 kDa band can be resolved into a doublet of two closely migrating polypeptides. Immunoaffinity chromatography-purified peptides were gel-purified and submitted to NH_2-terminal sequencing. The 8 kDa peptide yielded the sequence RTTSGQTK, and the 4.5 kDa peptide possessed the sequence NDDGPCYLDS. From the location of these peptides in the predicted coding sequence (beginning at the arrowheads in Figure 1), we deduced that BAP2 is post-translationally processed in at least two steps: the removal of the signal peptide gives the 8 kDa propeptide; and the cleavage of the 31-residue prodomain from this peptide gives the 4.5 kDa mature peptide.

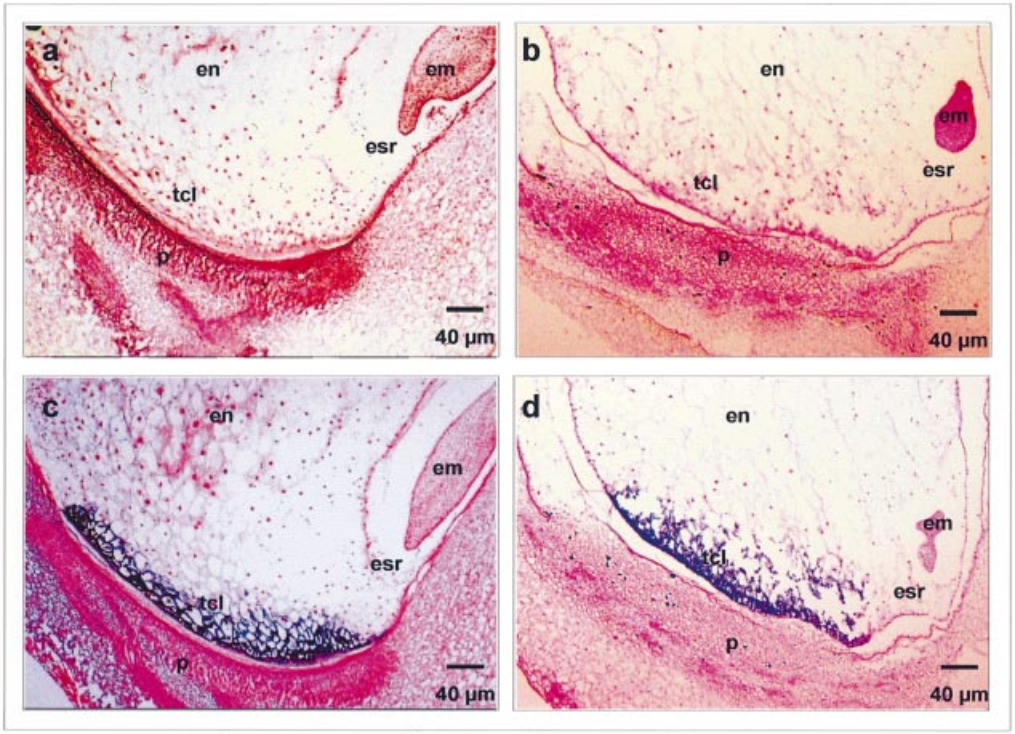

Figure 4. *In situ* hybridization of bap probes to 15 DAP A69Y kernel sections of (a) sense and (c) antisense BAP1a cDNA probes, and (b) sense and (d) antisense BAP3a cDNA probes; en, endosperm; esr, embryo surrounding region; tcl, transfer cell layer; em, embryo; p, pedicel.

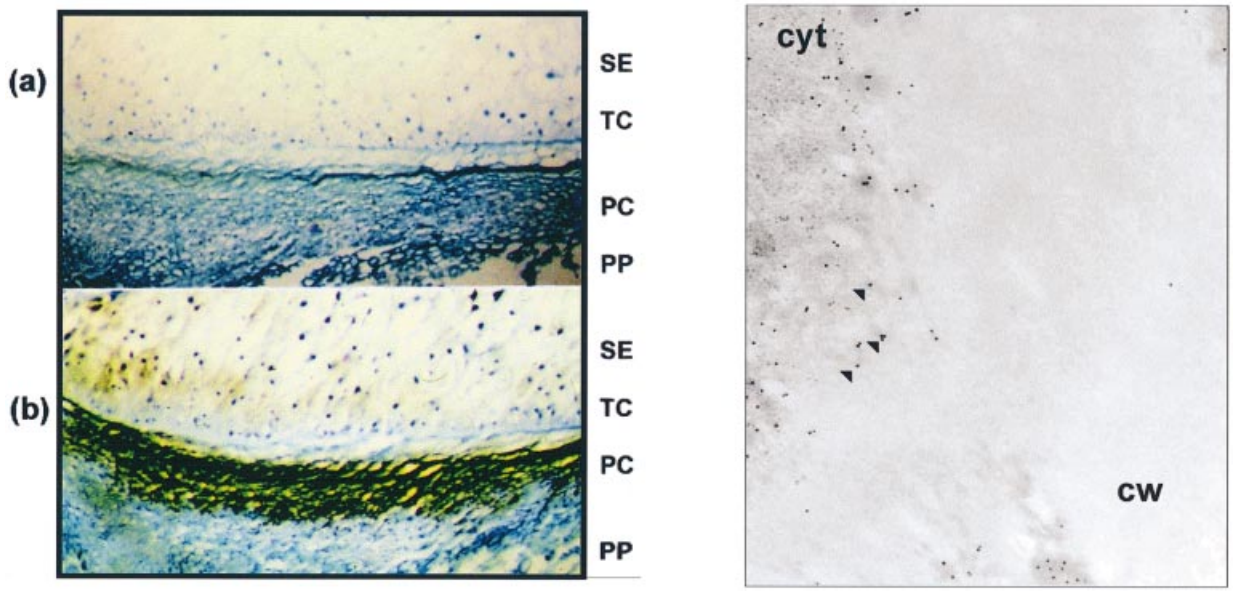

Figure 5. Immunolocalization of BAP2 protein.
Left, immunochemical localization of BAP2 protein on paraffin wax sections of developing maize kernels. (a) Incubation with pre-serum; (b) incubation with anti-BAP2 serum. Black staining indicates the presence of BAP2 antigen. SE, starchy endosperm; TC, transfer cells; PC, placentochalaza; PP, phloem parenchyma.
Right, immunogold localization of BAP2 protein by transmission electron microscopy. Gold particles (15 nm) were located in close proximity to the plasma membrane, not within the matrix of the cell wall (cw). cyt = cytoplasm.

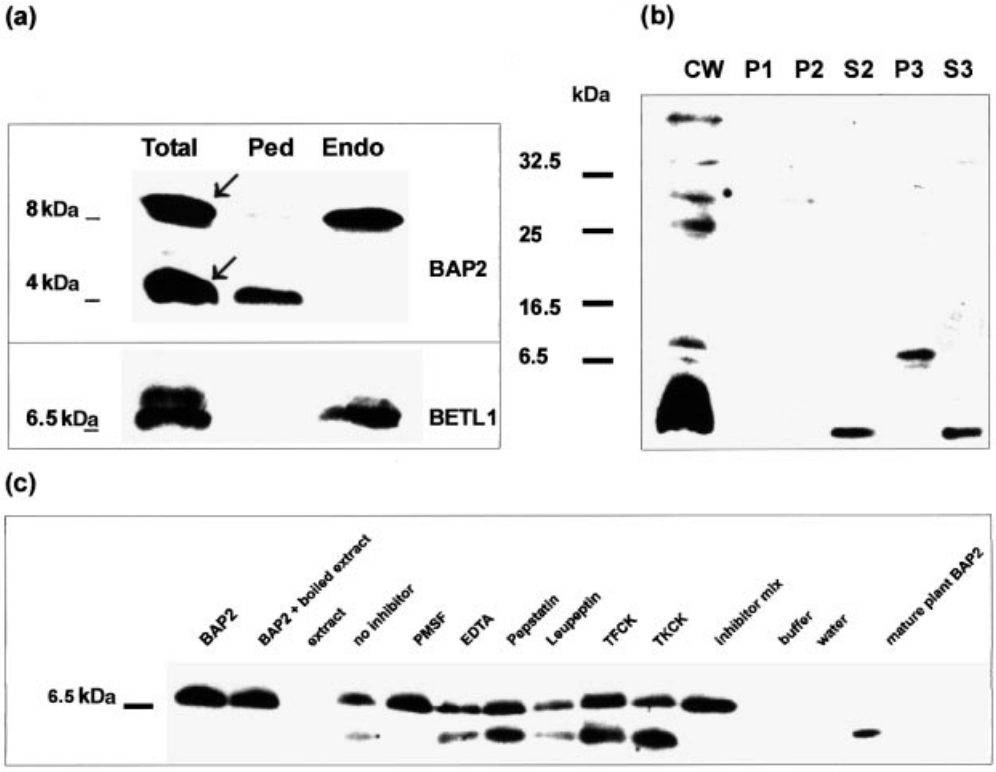

Figure 6. BAP2 exists in propeptide and mature peptide forms.
(a) Distribution of BAP2 protein forms in pedicel and basal endosperm. Immunoblot probe with anti-BAP2. From left to right: 50 μg protein from whole endosperm (total); from pedicel only (ped); and from endosperm tissue only (end).
(b) Localization of BAP2 protein forms by subcellular fractionation. Immunoblot probed with anti-BAP2. From left to right: each 50 μg protein from: cell-wall fraction (cw); 1000 g pellet (P1); 6000 g pellet (P2); 6000 g supernatant (S2); 80 000 g pellet (P3); 80 000 g supernatant (S3).
(c) *In vitro* protease cleavage of BAP2 propeptide: effect of different proteinase inhibitors on propeptide cleavage.

To obtain biochemical evidence for the localization of BAP2 protein in the pedicel, basal endosperm and pedicel tissues were hand-dissected under the binocular microscope, and proteins extracted and analysed by immunoblotting (Figure 6a). As a control for the separation of pedicel and basal endosperm cells, the filter was probed with BETL1 antiserum. BETL1 is synthesized and located in basal endosperm cells where it is tightly cell-wall bound, and is not synthesized or relocated in the pedicel (Hueros *et al.*, 1995). Only traces of BETL1 were detected in the pedicel protein extract (Figure 6a). The 8 kDa BAP2 propeptide is much more abundant in the basal endosperm extract than in the pedicel, whereas the 4.5 kDa mature peptide is more highly represented in the pedicel extract. Presumably the pedicel accumulates only the fully processed form of BAP2, which partitions into the placentochalazal zone after secretion into the apoplast by the basal endosperm cells. This would be consistent with the lack of BAP2 mRNA in pedicel cells.

The predominant location of BAP2 in the cell wall (Figure 5) is supported by analysis of subcellular fractions from cell lysates (Figure 6b). The mature BAP2 peptide was predominantly in the fraction corresponding to a crude cell-wall preparation (CW in Figure 6), and was not recovered in 1000 g (crude nuclear, P1) or 6000 g (crude organellar, P2) pellets. The propeptide, on the other hand, was enriched in the 80 000 *g* microsomal pellet, whereas the supernatant from this centrifugation step (S3) contained only the mature form of BAP2.

Evidence for a BAP2 processing enzyme in maize endosperm

To characterize the protease responsible for removal of the prodomain from the BAP2 propeptide, the protein was synthesized in *Escherichia coli* (with a C-terminal His tag), and purified for *in vitro* protease incubations. As the *E. coli* His-tag fusion protein was deposited in inclusion bodies, the protein was extracted under denaturing conditions with urea, eluted from nickel–agarose columns with a gradient of increasing urea concentration (Shi *et al.*, 1997), and renatured by dialysis.

The recombinant propeptide was incubated in a size-fractionated (>200 kDa) endosperm extract to bring about

Table 3. Antifungal activity of BAP2 and BAP2 propeptide against various fungal strains

	IC_{50} (μg ml^{-1})[a]			
	Medium A[b]		Medium B[c]	
	BAP2	BAP2 propeptide	BAP2	BAP2 propeptide
Alternaria brassicicola	3	>80	20	>80
Aspergillus flavus	2	nd	nd	nd
Botrytis cinerea	20	>80	20	>80
Fusarium culmorum	2	>80	4	>80
Fusarium moniliforme	6	nd	nd	nd
Neurospora crassa	5	80	20	>80
Plectosphaerella cucumerina	3	80	20	>80
Verticillium dahliae	2	>80	10	>80

[a]IC_{50} values (concentration of BAP protein required to inhibit 50% of fungal growth) were determined from dose–response curves with twofold dilution steps. nd = Not determined.
[b]Medium A = 0.5 PDB.
[c]Medium B = 0.5 PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl.

processing to the mature peptide form. By testing pH values in 0.5 pH unit increments, a pH optimum of 7 for protease activity was determined (data not shown). The effect at pH 7 of different classes of protease inhibitors on propeptide cleavage was then investigated (Figure 6c). The protease was inactivated by boiling, but was not affected by most of the inhibitors tested, with the exception of a commercial inhibitor mix containing phenylmethylsulphonylfluoride (PMSF) or this reagent alone. Inhibition by PMSF indicates that a serine-type protease is responsible for the cleavage. The lack of inhibition by Tosyl-L-phenylalanine-chloromethylketone and Tosyl-L-lysine-chloromethylketone, however, suggested the protease was not of the trypsin or chymotrypsin class of serine proteases.

Antifungal properties of the purified BAP2 protein

The antifungal properties of the purified BAP2 protein and its undigested form, BAP2 propeptide, were compared by determining protein concentrations required for 50% inhibition of fungal growth (IC_{50} values). Eight fungi were included in the assay: the saprophytic soil fungus *Neurospora crassa* and the phytopathogenic fungi *Alternaria brassicicola*, *Aspergillus flavus*, *Botrytis cinerea*, *Fusarium culmorum*, *Fusarium moniliforme*, *Plectosphaerella cucumerina and Verticillium dahliae*. *Aspergillus flavus* and *F. moniliforme* are maize pathogens. The antifungal activity assays were carried out in two media: 0.5 potato dextrose broth (PDB), and 0.5 PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl. The results are shown in Table 3. In the medium without the addition of salts, IC_{50} values of BAP2 were generally in the 2–5 μg ml^{-1} range, except for *B. cinerea* which was sensitive to BAP2 at concentrations starting from 20 μg ml^{-1}. In addition, BAP2 propeptide showed no antifungal activity at concentrations up to 80 μg ml^{-1}, except on *N. crassa* and *P. cucumerina*, for which BAP2 propeptide showed a very weak antifungal activity starting from 80 μg ml^{-1}. The effect of salts on the antifungal activity of the BAP2 protein was largely dependent on the test fungus. In general, addition of salts to the medium decreased the activity of the BAP2 protein by a factor of four- to sixfold. Sensitivity of antifungal activity to inorganic cations has been reported for a number of proteins, including the plant defensins (Osborn *et al.*, 1995; Terras *et al.*, 1992).

Membrane permeabilization induced by BAP2 protein

To investigate the mechanism of fungal growth inhibition by BAP2, fungal membrane permeabilization in the presence of BAP2 was studied. To this end, an assay based on the uptake of SYTOX Green was used, as described by Thevissen *et al.* (1999). SYTOX Green is an organic compound that fluoresces upon interaction with nucleic acids, and only penetrates cells with compromised plasma membranes (Matsuzaki *et al.*, 1997; Roth *et al.*, 1997). Membrane permeabilization to SYTOX in the presence of BAP2 and BAP2 propeptide was assessed for *N. crassa* and *F. culmorum*. As can be seen in Figure 7, SYTOX Green uptake in *F. culmorum* and *N. crassa* rose significantly upon treatment with BAP2 at concentrations above 2 and 5 μg ml^{-1}, respectively, which correlates well with the concentrations required for growth inhibition. In contrast, treatment with BAP2 propeptide did not inhibit growth of these fungi, and also failed to cause permeabilization to SYTOX Green.

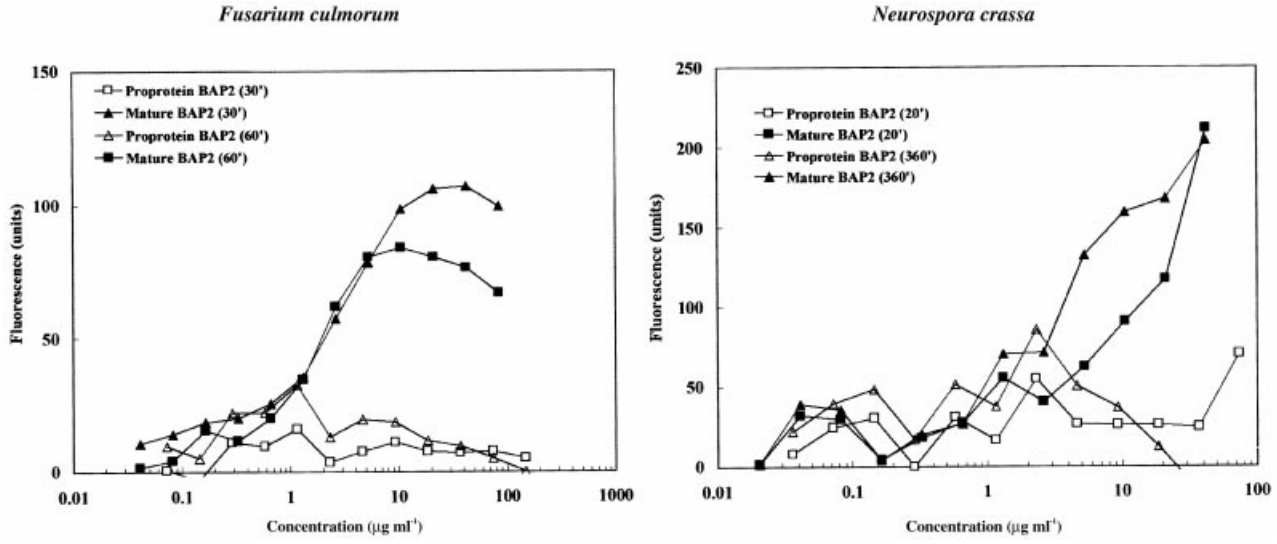

Figure 7. Membrane permeabilization induced by BAP2 and BAP2 propeptide on *Fusarium culmorum* and *Neurospora crassa*. Dose–response curves are presented for membrane permeabilization, measured by SYTOX Green fluorescence, of (a) *F. culmorum* and (b) *N. crassa* incubated with BAP2 (△) and BAP2 propeptide (□). Fungi were suspended in SMF1 supplemented with 0.2 μM SYTOX Green. After incubation in the presence of BAP proteins for 30 min, fluorescence was measured. Data are means of duplicate measurements and correspond to one representative experiment of two.

Discussion

BAP2 belongs to a highly divergent family of basal endosperm-specific proteins

A comparison of the predicted coding sequences of BAP2-related clones indicates that the most extensive homologies lie in the COOH-terminal half of the signal peptide and the prodomain. The mature peptides, extrapolated from the processing site demonstrated for BAP2, contain only six residues (four cysteine, one tyrosine and one threonine) which are present in all the BAP sequences compared. It was not possible to align these residues unambiguously with those of plant defensin sequences, and in particular, the defensin motif was absent. The extensive sequence divergence seen for the *bap* family is commonly seen within families of plant antipathogenic peptides (Broekaert *et al.*, 1997; Conceicao and Broekaert, 1999; Garcia-Olmedo *et al.*, 1998) in which sequence conservation appears to reflect maintenance of a secondary structure without a catalytic function. A comparable extreme sequence divergence exists among families of non-plant cytotoxin genes, for example those encoding conotoxins (Duda and Palumbi, 1999).

The newly isolated *bap1a* and *bap1b*, and *bap3a* and *bap3b*, gene family members were mapped to chromosomal loci using recombinant inbred lines (Burr *et al.*, 1994). All four cDNAs co-segregated to a locus on chromosome 10, unlinked to *bap2* itself, which has previously been mapped to Ch. 4 (maize genome database at http://www.agron.missouri.edu/cgi-bin/sybgw_mdb/mdb3/Map/64506). If we assume *bap1a* and *bap1b* clones are allelic variants at one locus, and that the same applies to *bap3a* and *bap3b*, two co-segregating genes, *bap1* and *bap3*, must be represented. As all four cDNAs arose from an inbred line (B73), however, and two fragments are seen in Southern blots with each probe, it is likely that the cDNAs represent four different cistrons at the locus. Multiple closely related cistrons seem to be typical for defensin-like peptides. The BETL1 locus (Hueros *et al.*, 1999), encoding a maize defensin-like protein, contains three tandem gene copies, and a cluster of five defensin genes is located within 450 kb on human chromosome 8p23 (Linzmeier *et al.*, 1999).

It is of interest to speculate how the BAP gene family has arisen and, in view of the putative role of BAPs in plant defence, to try to establish the overall family size and extent of diversity represented in it. The restriction of BAP sequences to maize, sorghum and teosinte implies that the founder member of the BAP gene class arose in a common progenitor of the *Zea* and *Sorghum* groups in the tribe Andropogoneae. In this connection, it is intriguing that the proximity of *bap1* and *bap3* on chromosome 10 has been maintained, although sequence divergence between the two sequences is extensive (random matching probabilities of $1.e^{-7}$–$1.e^{-10}$). It is possible that the divergence is a consequence of positive selection for resistance to evolving fungal pathogen populations.

BAP2 protein is detected in two cellular locations

An immunocytochemical localization indicated that BAP2 is accumulated at two sites in the developing

caryopsis: the transfer layer and the placentochalaza. Presumably the placentochalazal cell walls preferentially bind apoplastic BAP2 which diffuses from the site of synthesis. It may be that BAP2 can more effectively carry out its role of inhibition of fungal infection if it is located in the maternal tissue, as the orientation of the placentochalazal cell walls perpendicular to the direction of hyphal growth into the endosperm may be a more effective physical barrier and ensure prolonged contact with the cytotoxic proteins. By sequestering the protein in the sieve element-like placentochalazal cells, possible damage to the plasma membranes of other cells may also be reduced. Turnover of the protein, which takes place well before kernel maturity, may also be facilitated by this location.

Cells of the transfer layer contain secretory vesicles, and other proteins are secreted at high concentrations into the cell walls where they may be tightly bound, as seen for BETL1 (Hueros *et al.*, 1995). BAP2 protein, in contrast, is not tightly bound. Although accumulating in the cell-wall fraction, it can be readily eluted from cell-wall preparations under low salt conditions (Figure 6b). Sugar and amino acid uptake into transfer cells occurs via H^+-importing symporters. It is possible that release of high concentrations of BAP2 by these cells contributes to the regulation of extracellular pH, and thereby solute flux.

Processing of BAP2

Antiserum raised to *E. coli*-expressed BAP2 protein detects two peptides, of 8 and 4.5 kDa, in endosperm extracts. Protein sequencing revealed that the 4.5 kDa peptide is derived from the 8 kDa species by proteolytic cleavage. Subcellular fractionation of endosperm homogenates showed the 8 kDa peptide to be restricted to microsomal membrane fractions (Figure 6b). In contrast, the mature peptide was recovered from the 80 000 *g* supernatant, that is, it is not membrane-bound. These findings suggest the processing event giving rise to the mature 4.5 kDa species takes place within the trans-Golgi network (TGN). Similar processing events are seen for other secreted antimicrobial proteins including 4-Cys peptides (Tailor *et al.*, 1997) and proteinase inhibitors (Atkinson *et al.*, 1993). In contrast, vacuolar processing events are involved in the maturation of vicilin-class seed-storage proteins (Jung *et al.*, 1998), although some of the products can also be further cleaved during germination to yield antimicrobial peptides (Chung *et al.*, 1997; Marcus *et al.*, 1999). Thionine precursors were processed in barley leaves by a vacuolar proteinase to give a 5 kDa N-terminal peptide with antifungal properties, and a 6.8 kDa C-terminal peptide which is degraded *in vivo* (Romero *et al.*, 1997). The proteinase responsible differed in its site specificity and inhibitor sensitivity from that reported for vicilin maturation.

Incubation of endosperm extracts with a series of protease inhibitors (Figure 6c) indicates that a serine-type protease is responsible for propeptide cleavage. In contrast to reported substrates for the TGN-localized Kex2-like protease of plants, which is also a serine protease (Jiang and Rogers, 1999), BAP2 does not possess two adjacent basic residues at the predicted cleavage site (Figure 1). There is little conservation of sequence among BAP proteins at the BAP2 proteolytic cleavage site, suggesting that site specificity may be conferred at the tertiary structure level, for example by the formation of an exposed loop prone to cleavage.

Mechanism of action of BAP2

The production of a mature peptide by propeptide processing has been directly demonstrated for BAP2 and BAP1 (A.S., unpublished results) of the BAP proteins analysed to date. Synthesis as a propeptide may protect the host cells from possible cytotoxic action; the processing event may also provide a regulatory step in response to pathogen ingress. A pathogen-induced form of the Kex-like plant protease has been identified (Tornero *et al.*, 1996), which could mediate such a regulation. The effect of the maturation step on the biological activity of BAP2 (Table 3) is dramatic, converting it to a potent plant antifungal protein. We have estimated a BAP2 concentration of 10 μM in the transfer layer at 16 DAP, which is comparable to the IC_{50} values for susceptible fungal species in the bioassay. The biological activity *in vivo* is also likely to be further potentiated by combinatorial effects with other transfer layer-specific antimicrobial proteins such as BETL1 and BETL3.

Evidence that BAP2 acts by disrupting plasma membrane integrity was provided by incubation of BAP2-treated fungal cultures in the presence of SYTOX Green, a dye that is taken up only by cells possessing permeabilized membranes (Roth *et al.*, 1997). The mode of action of BAP2 therefore involves damage to the fungal plasma membrane, as is seen for other antifungal plant proteins such as the plant defensins (Thevissen *et al.*, 1999). Experiments reported here do not allow us to further define a membrane target or mechanism. *Fusarium* species are reported to spread *in planta* by movement of conidia through phloem vessels. Therefore secretion of an antifungal protein into the placentochalaza may prevent the passage of conidia to the endosperm, and may be an effective way of preventing the initiation of an infection there.

Experimental procedures

Molecular biological methods

Standard DNA and RNA methods were carried out as described by Sambrook *et al.* (1989).

Western blotting and immunoblot detection

Proteins were fractionated by SDS–PAGE electrophoresis according to Laemmli (1970), and electroblotted onto polyvinylidine difluoride (PVDF) membranes in a tank blotter. Proteins were detected using enhanced chemiluminescence (ECL, Amersham, UK) and either exposure to NAR-5 X-ray film (Kodak) or, for quantification, image capture using the Lumi-imager (Boehringer, Roche Diagnostics, Mannheim, Germany).

Purification of BAP2 forms by immunoaffinity chromatography and NH2-terminal sequencing

To extract cytoplasmic proteins, 12–15 DAP maize kernels were ground in a Waring blender with a minimum volume of extraction buffer (20 mM sodium phosphate pH 7.1, 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF), and 1 μg ml^{-1} proteinase inhbitors (pepstatin, leupeptin and aprotinin). The debris was removed by centrifugation at 100 000 *g*, 4°C for 1 h.

BAP2 antiserum and the corresponding pre-serum were bound to AffiGel-10 beads (BioRad, München, Germany), according to the supplier's recommendations. 1.5 ml of antiserum was used for 1 ml of resin. For immunoprecipitation, solutions were brought to 200 mM NaCl and 0.2% Triton X-100. Affigel-10-bound antisera were added at a 1 : 50-fold dilution, and proteinase inhibitors to 1 μg ml^{-1}, as before. The mixture was shaken gently at 4°C for 3 h. After incubation, the resin was packed into a column and washed with 80 volumes of buffer I (20 mM sodium phosphate pH 7, 1 mM EDTA, 1 mM PMSF, 200 mM NaCl, 0.2% Triton X-100, 1 μg ml^{-1} proteinase inhibitors as before), followed by 100–200 column volumes of buffer II (20 mM sodium phosphate pH 7, 1 mM EDTA, 1 mM PMSF, 1 μg ml^{-1} proteinase inhibitors), until the A_{280} of the eluant was zero. Bound proteins were then eluted with 1 ml aliquots of ice-cold 200 mM glycine pH 2.7, and immediately neutralized with 1/20th volume of 2 M Tris base. Proteins were separated by electrophoresis on a 16% SDS–PAGE gel, and electroblotted onto Immobilon–PVDF membrane (Millipore, Eschborn, Germany). The membrane was stained with Coomassie Blue, and destained for 10 min with 10% v/v acetic acid before excising bands for sequencing.

BAP2 isolation, refolding and digestion

BAP2 was purified and denatured using the COOH-terminal His tag. The protein in inclusion bodies was redissolved in 6 M guanidine–HCl/0.2 M acetic acid. To refold the protein, the solution was first diluted 1 : 1 with 8 M urea, 0.1 M Na_2HPO_4 and 0.01 M Tris pH 8.0. Then the preparation was dialysed against (4 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris pH 8.0), two changes, for 16 h; (4 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris pH 8.0), two changes for 8 h; 3 × 0.1 M Tris pH 8.0 for 16 h; and 3 × H_2O for 16 h. Digestion was carried out in 0.8 ml 0.2 ml 1 M potassium phosphate pH 7.10, 4 μl 0.25 M EDTA pH 8.0 containing BAP2 at 1–3 mg ml^{-1}. To this was added 40 μl aliquots of a >200 kDa fraction from a non-denaturing extract of 15–16 DAP maize kernels prepared in 20 mM potassium phosphate pH 6.0, 1 mM EDTA, 10 mM 2-mercaptoethanol. The solution was incubated at 37°C until the digestion had reached >90%. The preparation was purified by size-fractionation on a Superose-12 column in the same buffer. Fractions containing BAP2 were located by immunoblotting, pooled, dialysed against water, and concentrated by lyophilization.

Immunolocalization

Maize kernels were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2, for 12–24 h, depending on the tissue volume. Samples were dehydrated and embedded in lowycryl for electron microscopy or fibrowax for light microscopy (Plano GmbH, Marburg, Germany). For light microscopy immunolocalization, sections (8–10 μm) were deparaffinized and blocked with 1% bovine serum albumin in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) for 20 min at room temperature, and incubated overnight with anti-BAP2 antiserum or pre-immune serum (both diluted 1 : 400). The immunoreaction was detected using horseradish peroxidase-coupled second antibody (Sigma A6154; diluted 1 : 800) and 3-3′-diaminobenzidine as the substrate. For immunolocalization by electron microscopy, grids were incubated for 1 h with anti-BAP2 or pre-immune serum (both diluted 1 : 1000). The second antibody was coupled to 10 nm gold particles (Sigma G7402) and sections were counterstained with saturated uranyl acetate for 30 min.

In situ *hybridization*

Immature (15 DAP) maize kernels were fixed in 4% formaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate pH 7.2 for 16 h. Seeds were dehydrated in a graded ethanol–xylene series and embedded in fibrowax (Plano GmbH). Thick sections (10 μm) were cut using a microtome (Reichler-Jung, Nussloch, Germany) and then transferred to glass slides that had been treated with 3-aminopropyltriethoxylane.

Digoxigenin-labelled antisense and sense probes were synthesized using T3 and T7 polymerases (Boehringer-Mannheim, Germany) and partially hydrolysed (200 bases) with sodium carbonate. *In situ* hybridization was carried out essentially as described by De Block and Debrouwer (1993). Before hybridization the sections were deparaffinized and treated with 5 μg ml^{-1} proteinase K (Merck, Darmstadt, Germany). Mounted slides were incubated with 40–80 ng RNA per slide for 16 h at 50°C. After hybridization, the sections were incubated with 40 μg ml^{-1} RNase A at 37°C for 10 min and washed several times in 2 × standard saline phosphate EDTA (SSPE). For immunological detection, the sections were subsequently treated with 0.5% (w/v) blocking reagent (Boehringer-Mannheim) and 1% (w/v) BSA in TNT (100 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.3% (v/v) Triton X-100). Following the blocking reaction, sections were incubated with antidigoxigenin–alkaline phosphatase conjugate (Boehringer-Mannheim, 1 : 3000 dilution) in 1% BSA solution at room temperature for 1 h, washed several times, and rinsed with alkaline phosphatase buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). The colour reaction was performed in alkaline phosphatase buffer containing nitroblue tetrazolium chloride/5-bromo-4-chloro-indolyl phosphate. Sections were then stained with 0.01% safranin, dehydrated in graded ethanol, and mounted with DPX mounting medium (Agar Scientific, Stansted, UK).

Subcellular fractionation

Fractionation of subcellular components was performed according to Nagahashi and Hiraike (1982). B37 endosperm (8 g), harvested 14 DAP, was ground in liquid nitrogen in a pestle and mortar, and suspended in 20 ml extraction buffer (0.5 M sucrose, 5 mM EDTA, 5 mM DTT, 30 mM Tris/MES pH 7.7). The suspension was filtered through one layer of Miracloth (Calbiochem, Novabiochem, Schwalbach, Germany), the retained fraction being treated as crude cell walls, and the filtrate was centrifuged at 1000 *g* for 5 min to yield pellet 1 (P1) and supernatant 1 (S1). S1 was pelleted at 6000 *g* for 20 min to give pellet 2 (P2) and supernatant 2 (S2). S2 was pelleted at 80 000 *g* for 2 h giving rise to pellet 3 (P3) and supernatant 3 (S3). The P3 pellet was resuspended in 1 ml pellet buffer (0.25 M sucrose, 1 mM DTT, 3 mM Tris/MES pH 7.2).

In vitro *antifungal activity assay*

Microorganisms. Fungal strains used in this study are *Alternaria brassicicola* MUCL 20297, *Aspergillus flavus* var. *flavus* CBS 111.45, *Botrytis cinerea* MUCL 30158, *Fusarium moniliforme* CBS 218.76, *Fusarium culmorum* MUCL 30162, *Neurospora crassa* FGSC 2489, *Plectosphaerella cucumerina* (provided by Dr B. Mauch-Mani, Université de Fribourg, Switzerland), and *Verticillium dahliae* MUCL 19210. Filamentous fungi were grown on six-cereal agar, and conidia were harvested as described previously (Broekaert *et al.*, 1990). Conidia spore stocks in 20% (v/v) glycerol were at a final concentration of 2×10^7 conidia ml^{-1}.

Antifungal activity assay. Antifungal activity of BAP proteins against fungi was assayed by microspectrophotometry of liquid cultures grown in microtitre plates as described previously (Broekaert *et al.*, 1990; Terras *et al.*, 1992). Briefly, in a well of a 96-well microplate, 10 µl of the protein sample was mixed with 90 µl of either half-strength potato dextrose broth (0.5 PDB; Difco, Detroit, MI, USA) or 0.5 PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl, containing fungal spores at a concentration of 2×10^4 conidia ml^{-1}. Growth was recorded after 24 h incubation at 22°C. The absorbance at 595 nm served as a measure for microbial growth (Terras *et al.*, 1992). IC_{50} values (the concentration of the antifungal protein required to inhibit 50% of the fungal growth) were calculated from dose–response curves with twofold dilution steps (Terras *et al.*, 1992).

SYTOX Green uptake

Fungal membrane permeabilization was measured by SYTOX Green uptake as described previously (Thevissen *et al.*, 1999). Fungi were grown at an inoculum density of 2×10^5 conidia ml^{-1} in 12 g PDB per litre (Difco) with continuous shaking (200 r.p.m.) at 22°C. After 16 h incubation, hyphae were washed with SMF1 (synthetic medium for fungi containing 50 µM $MgSO_4$, 50 µM $CaCl_2$, 5 µM $FeSO_4$, 0.1 µM $CoCl_2$, 0.1 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.5 µM H_3BO_3, 0.1 µM KI, 0.5 µM $ZnSO_4$, 0.1 µM $MnSO_4$, 10 g l^{-1} glucose, 1 g l^{-1} asparagine, 20 mg l^{-1} methionine, 2 mg l^{-1} myo-inositol, 0.2 mg l^{-1} biotin, 1 mg l^{-1} thiamine-hydrochloride, 0.2 mg l^{-1} pyridoxine-hydrochloride, 0.5 mM K_2PO_4). Aliquots of the fungal suspension (90 µl), supplemented with 0.2 µM SYTOX Green, were mixed with 10 µl of the same medium containing BAP proteins, and incubated in white 96-well microplates (PE white, Perkin Elmer, Norwalk, CT, USA). After incubation for 30 min at 22°C with periodic agitation, fluorescence emitted by the cells in the microplates was measured using a Perkin Elmer fluorescence spectrometer LS 50 B at an excitation wavelength of 488 nm (slit, 10 nm) and an emission wavelength of 540 nm (slit, 5 nm). Fluorescence values of the samples were corrected by subtracting the fluorescence value of a culture in the same medium in absence of the peptides but in the presence of SYTOX Green. Absolute values of fluorescence did not differ more than 50% in independent tests performed under identical conditions.

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