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A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species

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Out of seeds of 4 Brassicaceae species, 7 antifungal proteins were isolated which are nearly identical to 2 previously characterized radish seed antifungal proteins. These basic proteins, multimers of a 5 kDa polypeptide, specifically inhibit fungal growth. One of the antifungal proteins has decreased antifungal activity and an increased antibacterial activity. In addition, the previously described antifungal activity of the radish seed 2S albumins was extended to the 2S albumins of the seeds of the 4 other Brassicaceae species. A 2S albumin-like trypsin-inhibitor from barley seeds was found to have much less activity against fungi.

Oilseed rape; Mustard; *Arabidopsis*; Radish; Brassicaceae; 2S albumin

1. INTRODUCTION

Apart from the well-known plant antifungal proteins (chitinases, glucanases, thionins, chitin-binding lectins, ribosome-inactivating proteins [1–3]), an increasing number of new types of proteins capable of inhibiting fungal growth in vitro is emerging. Thus, the above list can be completed with the family of thaumatin-like proteins [4–7] and neurotoxin-like antimicrobial peptides from seeds of *Mirabilis jalapa* L. [8]. Recently, we have reported that radish (*Raphanus sativus* L.) seeds also contain two highly potent antifungal proteins (AFPs), called *Rs*-AFP1 and *Rs*-AFP2, homologous to 2 pea pod proteins specifically induced upon fungal infection, and to γ-thionins and sorghum α-amylase inhibitors [9]. Moreover, the 2S albumin fraction from radish seeds was also shown to possess antifungal properties in vitro [9].

In the present paper, we show that the occurrence of *Rs*-AFP-like proteins is not restricted to radish seeds but that nearly identical proteins are found in seeds of at least 4 other Brassicaceae species, *Brassica napus*, *B. rapa*, *Sinapis alba* and *Arabidopsis thaliana*.

Moreover we also demonstrate that the seed 2S albumins of the above mentioned Brassicaceae plants have antifungal activity, as was previously shown for radish.

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Abbreviations: Rs, *Raphanus sativus* L.; AFP, antifungal protein; *Bn*, *Brassica napus*; *Br*, *Brassica rapa*; *Sa*, *Sinapis alba*; *At*, *Arabidopsis thaliana*; MES, *N*-morpholino ethanesulfonic acid; IC_{50}, protein concentration required for 50% fungal growth inhibition; 2S, 2S storage albumins.

2. MATERIALS AND METHODS

2.1. *Materials*

Seeds of *B. napus*, *B. rapa* and *Hordeum vulgare* were obtained from Versele-Laga (Deinze, Belgium). *S. alba* seeds were purchased from Leen De Mos seeds ('s Gravenzande, The Netherlands) and those of *A. thaliana* from Herbiseed (Wokingham, Berkshire, UK). Microplate absorbance measurements were performed with a Bio-Rad 3550 microplate reader. Non-sterile flat-bottom 96-well microplates were used throughout this study.

2.2. *Microorganisms*

Fungi were grown on 6 cereal agar (6 cereal instant flakes from Nestlé 20 g/l, agar 15 g/l) and spores harvested and stored as previously described [10]. The following fungal strains were used: *Alternaria brassicola* (MUCL 20297), *Botrytis cinerea* (MUCL 30158), *Fusarium culmorum* (IMI 180420), *Fusarium oxysporum* f.sp. *lycopersici* (MUCL 909), *Pyricularia oryzae* (MUCL 30166) and *Verticillium dahliae* (MUCL 6963).

Bacteria (Gram-negative: *Agrobacterium tumefaciens* LMG 188, *Alcaligenes eutrophus* LMG 1195, *Azospirillum brasilense* ATCC 29145, *Escherichia coli* strain HB101, *Erwinia carotovora* strain 3912, *Pseudomonas solanacearum* LMG 2293; and Gram-positive: *Bacillus megaterium* ATCC 13632, *Sarcina lutea* ATCC 9342) were pre-cultured overnight in 1% tryptone at 30°C in a rotary shaker. *Saccharomyces cerevisiae* (strain Sp1) was pre-cultured for 24 h in YPD (2% bactopeptone, 1% yeast extract, 2% glucose) medium at 30°C in a rotary shaker.

2.3. *Extraction of the seed proteins*

The purification of AFPs from seeds of Brassicaceae species was performed basically as outlined for *R. sativus* seeds [9]. 100-g amounts of seed flours from the different species was extracted for 2 h (at 4°C) with 200 ml (or 400 ml in the case of *S. alba* and *A. thaliana* seeds) extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, 1.5% polyvinylpolypyrrolidone, 1 mM phenylmeth-

ylsulfonylfluoride, 2 mM thiourea). The precipitates formed between 30 and 70% relative ammonium sulfate saturation were re-dissolved in 40 ml distilled water and heated at 80°C for 15 min. The resulting suspensions were clarified by centrifugation and the supernatants extensively dialyzed against distilled water using dialysis tubing with a molecular mass cut-off of 1 000 Da. The dialyzed solutions were adjusted to 50 mM Tris-HCl (pH 9) and isocratically passed over a Q-Sepharose Fast Flow column (12 × 5 cm, Pharmacia) in equilibrium with 50 mM Tris-HCl (pH 9) at a flow rate of 5 ml/min. The unbound proteins represent the basic protein fractions of the different seeds

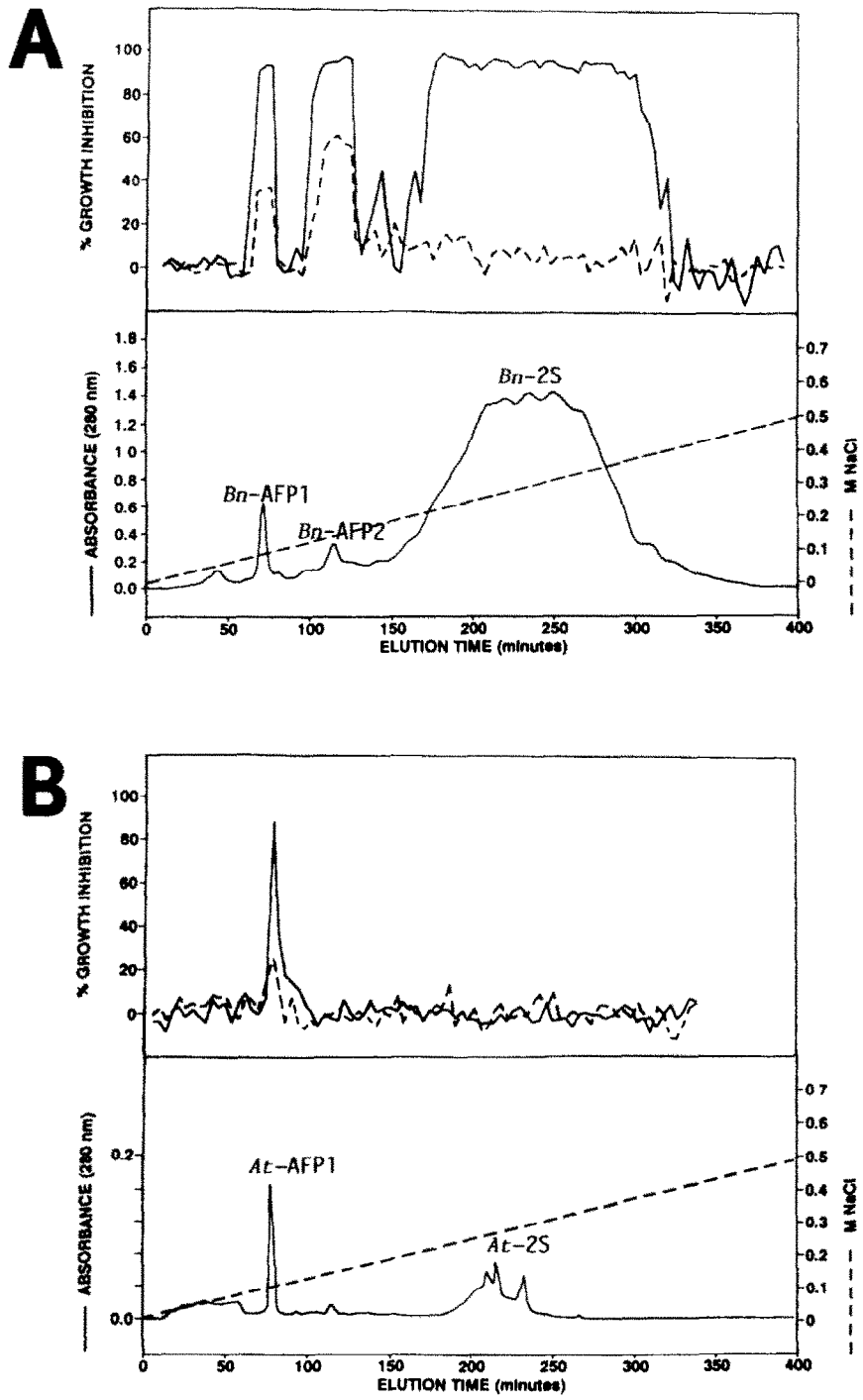


Fig. 1. Separation of the proteins in the basic protein fraction from seeds of *B. napus* (A) and *A. thaliana* (B). The basic protein fractions were loaded on a S-Sepharose high performance cation-exchange column (10 × 1.6 cm; Pharmacia) in equilibrium with 50 mM MES at pH 6. After the A_{280} of the unbound fractions (not shown) dropped below 0.01 absorbance units, the bound fractions were desorbed at 2.5 ml/min with a linear gradient of 1,000 ml from 0 to 500 mM NaCl in 50 mM MES (pH 6). The eluates were monitored for proteins at 280 nm (lower panels) and collected in 10 ml fractions, of which 200 μl was dialyzed overnight in a microdialysis apparatus. After filter-sterilization (0.22 μm), 20 μl of these fractions was tested in a microspectrophotometric antifungal activity assay (upper panels) in half-strength potato dextrose broth both with (dashed line) and without 1 mM $CaCl_2$ and 50 mM KCl (solid line). Chromatography was performed on a Waters 650 HPLC station.

which were further purified by cation-exchange chromatography and reverse-phase chromatography, as described in section 3.

Purification of a trypsin-inhibitor showing homology to Brassicaceae 2S albumins from barley was performed essentialy as described by Mikola and Suolinna [11]. Amino acid sequencing of the first 10 N-terminal amino acids of the purified protein confirmed its identity as the trypsin-inhibitor, CMe, according to the terminology of Garcia-Olmedo et al. [12].

2.4. *Protein concentration determination and amino acid sequencing*

The bicinchoninic acid method [13] adapted to a microplate assay was used for all protein concentration determinations. Bovine serum albumin served as a reference protein.

N-Terminal amino acid sequences were determined by automatic Edman degradation in a 477A Protein Sequenator (Applied Biosystems Inc., Foster City, CA) with on-line detection of the stepwise liberated phenylthiohydanthoin amino acid derivatives in a 120A Analyzer (Applied Biosystems). The Brassicaceae AFPs were digested with pyroglutamate aminopeptidase (sequencing grade, Boehringer, Mannheim, Germany) prior to amino acid sequencing.

2.5. *Electrophoresis*

SDS-PAGE was carried out with a PhastSystem electrophoresis device (Pharmacia, Uppsala, Sweden) using commercial precast High-density gels. Four-fold concentrated sample buffer contained 200 mM Tris-HCl (pH 8.3), 1% (w/v) SDS, 1 mM EDTA, 0.005% (w/v) Bromophenol blue and 1% (w/v) dithioerythritol (DTE). The DTE was omitted for analysis of unreduced proteins. The method of [14] was followed for silver staining of the separated proteins, using 12.5% glutaraldehyde as fixative. Diffusion blotting of proteins on nitrocellulose paper (Hybond C, Amersham) followed by silver staining of the blots was performed as described [15].

2.6. *Antifungal and antibacterial assay*

Antifungal and antibacterial activity and antibiotic effect on yeast were determined by microspectrophotometry [10] under the assay conditions specified in [8]. Briefly, fungal spores or bacteria are suspended in an appropriate growth medium, dispensed in wells of a microplate containing either water or a protein solution, and incubated for an appropriate time period (48 h for fungi, 24 h for bacteria or yeast). Growth of the microorganism is evaluated by measuring the culture absorbance at 595 nm using a microplate reader. Unless otherwise stated, the growth medium used for fungi was a synthetic low ionic strength buffer containing K_2HPO_4 2.5 mM, $MgSO_4$ 50 μM, $CaCl_2$ 50 μM, $FeSO_4$ 5 μM, $CoCl_2$ 0.1 μM, $CuSO_4$ 0.1 μM, Na_2MoO_4 2 μM, H_3BO_3 0.5 μM, KI 0.1 μM, $ZnSO_4$ 0.5 μM, $MnSO_4$ 0.1 μM, glucose 20 g/l, asparagine 2 g/l, methionine 40 mg/l, *myo*-inositol 2 mg/l, biotin 0.2 mg/l, thiamine-HCl 1 mg/l, pyridoxine-HCl 0.2 mg/l. The bacterial growth medium used in the assay consisted of 1% tryptone, 0.5% low melting point agarose, and the yeast growth assay medium of half-strength potato dextrose broth and 0.5% low melting point agarose.

3. RESULTS

3.1. *Purification of the Brassicaceae seed AFPs*

The basic protein fractions from the different seeds were separated on a S-Sepharose high performance cation-exchange column as previously described [9]. Fig. 1A shows the chromatographic profile of the *B. napus* basic protein fraction, which is very similar to those obtained for *B. rapa*, *S. alba* and *R. sativus* [9]. Two minor eluting peaks display antifungal activity when assayed in both half-strength potato dextrose broth or potato dextrose broth supplemented with 1 mM $CaCl_2$ and 50 mM KCl. The AFPs found in these peaks are designated *Bn*-AFP1 and *Bn*-AFP2 for *B. napus* extracts, *Br*-AFP1 and *Br*-AFP2 for *B. rapa* extracts and *Sa*-AFP1 and *Sa*-AFP2 for *S. alba* extracts. Furthermore, a set of major overlapping peaks eluting from 200 to 400 mM NaCl represent the 2S albumin fraction which, as in the case of *R. sativus*, only display antifungal activity in the potato dextrose medium without added salts. The chromatogram of the basic protein fraction from *A. thaliana* (Fig. 1B) differs from those of the above mentioned species in that it displays only 1 early eluting AFP, designated *At*-AFP1, and shows a much lower abundance of the 2S albumin peaks (approximately 5 mg/100 mg seeds compared to 300–500 mg/100 g seeds for the other species). The peaks corresponding to *Bn*-AFP1, *Bn*-AFP2, *Br*-AFP1, *Br*-AFP2, *Sa*-AFP1, *Sa*-AFP2 and *At*-AFP1 were subsequently purified by reverse-phase chromatography. Similar to the *Rs*-AFPs, they all elute at about 30% acetonitrile (in 0.1% trifluoroacetic acid) as is shown for *At*-AFP1 in Fig. 2.

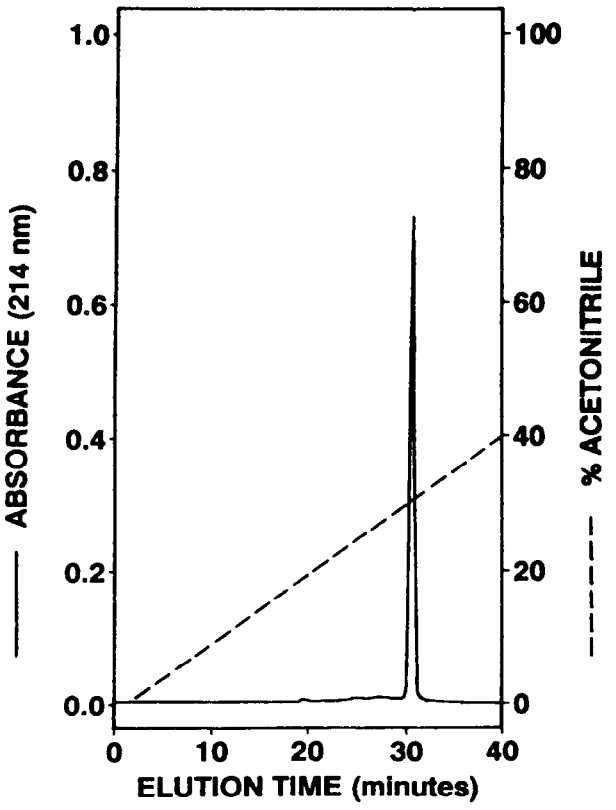


Fig. 2. Purification of *At*-AFP1. 1 mg amounts of the cation-exchange *At*-AFP1 peak were loaded on a reverse-phase Pep-S column (C_2/C_{18} 15 μm porous silica, 25 × 0.93 cm; Pharmacia) in equilibrium with 0.1% trifluoroacetic acid (TFA). The column was eluted at 5 ml/min with the following gradient (solvent B is acetonitrile containing 0.1% TFA): 0–1 min, 0% B; 1–40 min, 0–40% B. The eluate was monitored for protein by measurement of the absorbance at 214 nm. Chromatography was performed on a Waters 600 HPLC station.

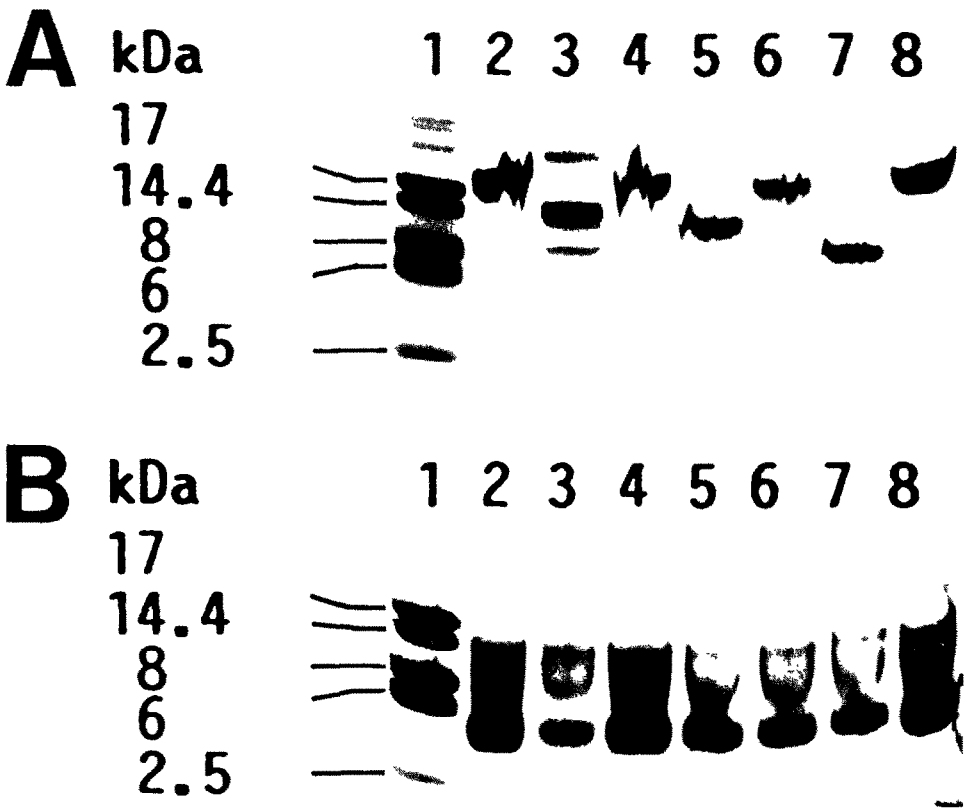
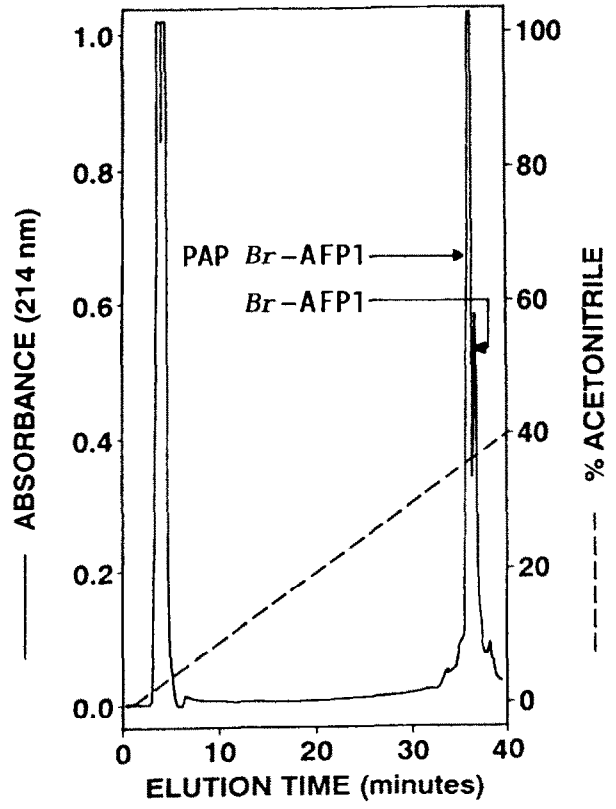


Fig. 3. SDS-PAGE analysis of the purified Brassicaceae AFPs. (A) 200 ng of the different Brassicaceae AFPs were dissolved in sample buffer without DTE, separated on Phastgel high density (Pharmacia) and silver stained. (B) 200 ng of the different Brassicaceae AFPs dissolved in sample buffer with DTE and analyzed as above. Lanes 1, myoglobin fragments with molecular masses indicated in kDa at the left; lanes 2, *Br*-AFP1; lanes 3, *Br*-AFP2; lanes 4, *Bn*-AFP1; lanes 5, *Bn*-AFP2; lanes 6, *Sa*-AFP1; lanes 7, *Sa*-AFP2; lanes 8, *At*-AFP1.



3.2. *Physico-chemical characterization of the Brassicaceae AFPs*

SDS-PAGE analysis of the 7 purified AFPs was performed to estimate their molecular masses. After treatment with disulfide-reducing agents, all AFPs show a major band of 5 kDa and some minor traces of 8–15 kDa which may result from artefactual oligomerization of the 5 kDa protomer by intermolecular formation of cystine bridges (Fig. 3B). When the reducing agents are omitted, all AFPs (except *Br*-AFP2) migrate as single bands of about 20 kDa (*Bn*-AFP1, *Br*-AFP1, *Sa*-AFP1 and *At*-AFP1), 12 kDa (*Bn*-AFP2) or 10 kDa (*Sa*-AFP2). This may indicate that the AFPs form oligomers in their native state. The SDS-PAGE pattern of unreduced *Br*-AFP2 shows a mixture of bands of 5, 10, 15

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Fig. 4. Reverse-phase chromatography of *Br*-AFP1 (100 μg) treated with 7 μg pyroglutamate aminopeptidase (PAP). The position of the digested (PAP *Br*-AFP1) and the remaining undigested (*Br*-AFP1) peaks are indicated by arrows. Chromatography conditions are identical to those described in the legend to Fig. 2 with the exception that an analytical reverse-phase Pep-S column was used (C_2/C_{18} 5 μm porous silica, 25 × 0.4 cm; Pharmacia).

	1				5				10				15				20				25				30
Rs-AFP1	(Q)	K	L	C	E	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N	Q	C	I	N	L	E	K
Rs-AFP2	(Q)	K	L	C	q	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N	Q	C	I	r	L	E	
Br-AFP1	(Q)	K	L	C	E	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N	Q	C	I	N			
Br-AFP2	(Q)	K	L	C	E	R	P	S	G	T	x	S	G	V	C	G	N	N	N	A	C	K	N	Q	C	I	r			
Bn-AFP1	(Q)	K	L	C	E	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N	Q	C	I	N	L	E	K
Bn-AFP2	(Q)	K	L	C	E	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N							
Sa-AFP1	(Q)	K	L	C	E	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	K	N	Q	C					
Sa-AFP2	(Q)	K	L	C	q	R	P	S	G	T	W	S	G	V	C	G	N	N	N	A	C	r	N	Q	C	I				
At-AFP1	(Q)	K	L	C	E	R	P	S	G	T	W	S	G	V	C	G	N	s	N	A	C	K	N	Q	C	I	N			

Fig. 5. Primary structure of the N-terminal regions of the Brassicaceae AFPs. N-Terminal amino acid sequences were determined after treatment with pyroglutamate aminopeptidase. The first residues are suggested to be cyclisized glutamines. Residues differing from those in *Rs*-AFP1 are indicated in lower case. Residue 11 (x) of *Br*-AFP2 does not belong to the group of common amino acids, including hydroxyproline. Amino acid sequences of *Rs*-AFP1 and *Rs*-AFP2 have been reported previously [9].

and 25 kDa, which may represent the protomer and variable oligomers (Fig. 3A).

The radish AFPs (*Rs*-AFP1 and *Rs*-AFP2) were reported to have blocked N-termini which could be removed by pyroglutamate aminopeptidase treatment [9]. Similarly, the here reported AFPs showed a characteristic shift in their chromatographic profile (caused by the removal of the N-terminal cyclisized glutamine) after digestion with pyroglutamate aminopeptidase, as shown in Fig. 4 for *Br*-AFP1. In addition, 2 of the 7

Table I

Antifungal activity of the different Brassicaceae AFPs

Fungus	IC_{50} values (μg/ml)								
	Rs-AFP1	*Rs*-AFP2	*Br*-AFP1	*Br*-AFP2	*Bn*-AFP1	*Bn*-AFP2	*Sa*-AFP1	*Sa*-AFP2	*At*-AFP1
Medium A[a]									
Alternaria brassicola	15	2	3	75	0.6	1.20	1.2	4.5	10
Botrytis cinerea	8	2	1.50	>100	2	2	1.8	3.5	3.90
Fusarium culmorum	5	2	2	38	2.8	2.10	4	2.3	3
Fusarium oxysporum f.sp. *lycopersici*	30	2	1.80	42	1.3	1.50	6	2.3	3
Pyricularia oryzae	0.3	0.4	0.25	3	0.35	0.25	0.5	0.3	0.25
Verticillium dahliae	5	1.5	0.80	15	1.2	1	1.5	1.2	1.50
Medium B[b]									
Alternaria brassicola	>100	20	>100	>100	>100	>100	>100	>100	>100
Botrytis cinerea	>100	>100	>100	>100	>100	>100	>100	>100	>100
Fusarium culmorum	70	5	19	32	33	40	40	32	35
Fusarium oxysporum f.sp. *lycopersici*	>100	>100	>100	>100	>100	>100	>100	>100	>100
Pyricularia oryzae	>100	7	>100	>100	32	8	25	3.8	>100
Verticillium dahliae	>100	50	>100	>100	>100	>100	>100	>100	>100

Protein concentrations required for 50% growth inhibition (IC_{50}) after 48 h of incubation at 25°C were determined from the dose–response curves (percent growth inhibition vs. protein concentration).

[a]Synthetic low ionic strength fungal growth medium [8].

[b]Medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl.

Table II

Sensitivity of the antifungal activity of *Sa*-AFP2 and 2S albumin fractions of *S. alba* to inorganic cations

	Threshold concentration of the cation (μM) for antagonism with	
	Sa-AFP2	*Sa*-2S
Ca^{2+}	500	100
Mg^{2+}	500	500
Ba^{2+}	500	30
Fe^{3+}	30	50
Zn^{2+}	>50	30
Co^{2+}	>30	30
Cu^{2+}	>600	>600
Mn^{2+}	300	60
K^{+}	12,000	10,000
Na^{+}	12,000	10,000

Spores of *Fusarium culmorum* were incubated in synthetic low ionic strength medium containing either 1 μM *Sa*-AFP2 or 3 μM *Sa*-2S, and a serial dilution of the chloride salts of the indicated inorganic cations. The extent of growth inhibition was measured by optical density measurement after 48 h of incubation at 25°C. The threshold concentration for antagonism of antifungal activity by a cation is expressed as the highest cation concentration at which the extent of growth inhibition caused by the AFP is unaffected, relative to protein-treated cultures grown in the synthetic low ionic strengh buffer without added salts. $ZnCl_2$, $CoCl_2$ and $CuCl_2$ were not tested at concentrations higher than 50 μM, 30 μM and 600 μM, respectively because of direct inhibitory effects on fungal growth.

AFPs (*Bn*-AFP1 and *At*-AFP1) were subjected to automated Edman degradation without prior pyroglutamate aminopeptidase treatment but no signals could be recorded. The obtained N-terminal amino acid sequences of the pyroglutamate aminopeptidase-treated AFPs are given in Fig. 5, where they are compared to the previously reported N-terminal regions of the *Rs*-AFPs. With the exception of *At*-AFP1, which has a serine at position 18 instead of an asparagine, all AFP1s have identical N-terminal regions. More variability exists between the N-terminal regions of the AFP2s. The substitution from glutamate (at position 5 in the AFP1s) to glutamine is found in *Rs*-AFP2 and *Sa*-AFP2 and the substition from asparagine (at position 27 in the AFP1s) to arginine is at least found in *Rs*-AFP2 and *Br*-AFP2. Finally, the substitution from lysine (at position 22 in the AFP1s) to arginine is only found in *Sa*-AFP2. Residue 11 of *Br*-AFP2 could not be identified as one of the common amino acids.

3.3. *Antibiotic properties of the Brassicaceae AFPs*

A comparative study of the antifungal activity of the different Brassicaceae AFPs was performed by determining protein concentrations required for 50% inhibition of fungal growth (IC_{50} values). Six fungi were included in this test and the IC_{50} values were measured in a synthetic low ionic strength medium and in the same medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl. The latter medium was also used because we were interested in the extent of sensitivity of the antifungal activity to inorganic cations. The activity of many AFPs is indeed severely decreased in the presence of cations, especially by divalent cations [8,9,16–18]. The results of this test are given in Table I, which also includes IC_{50} values of the *Rs*-AFPs. In the medium without additional salts, IC_{50} values ranging from 0.3 to 30 μg/ml are found for all AFPs, with the exception of *Br*-AFP2, the IC_{50} value of which is at least 10-fold higher. In the medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl an important decrease of the antifungal activity is seen for all AFPs, with *Rs*-AFP2 being the least affected. As was observed earlier for the *Rs*-AFPs [9], the salt sensitivity of the antifungal activity largely depends on the fungus tested. For instance, none of the Brassicaceae AFPs is capable of inhibiting growth of *B. cinerea* and *F. oxysporum* f.sp. *lycopersici* by 50% at concentratrions below 100 μg/ml, whereas all of these AFPs cause 50% inhibition of the growth of *F. culmorum* at concentrations far below 100 μg/ml. The sensitivity to various inorganic cations of the antifungal activity of one of the Brassicaceae AFPs, *Sa*-AFP2, was assessed on *F. culmorum* (Table II). This sensitivity is expressed as the highest cation concentration that does not result in detectable antagonism of the growth inhibitory effect of *Sa*-AFP2. *Sa*-AFP2 seems to be about equally sensitive to different divalent cations, with threshold concentrations for antagonism around 0.5 mM. The monovalent cations, Na^{+} and K^{+}, do not affect the activity of

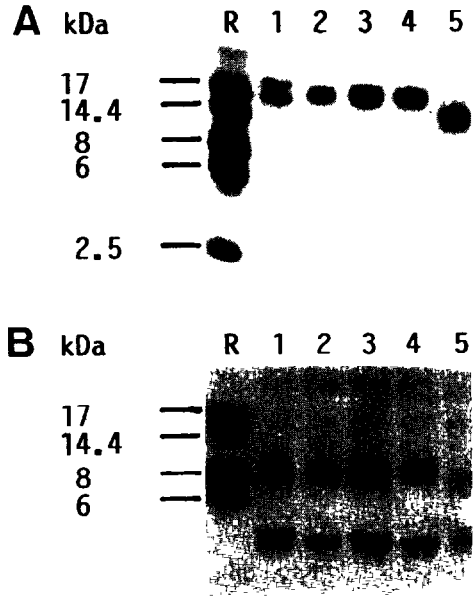

Fig. 6. SDS-PAGE analysis of the different Brassicaceae 2S albumins. (A) 50 ng of the different Brassicaceae 2S albumins were dissolved in sample buffer without DTE, separated on Phastgel high density (Pharmacia) and silver stained in the gel. (B) 50 ng of the different Brassicaceae 2S albumins were dissolved in sample buffer with DTE, separated on Phastgel high density, diffusion blotted and detected by silver staining of the blot. Lanes R, myoglobin fragments with molecular masses indicated in kDa at the left; lanes 1, *Rs*-2S; lanes 2, *Br*-2S; lanes 3, *Bn*-2S; lanes 4, *Sa*-2S; lanes 5, *At*-2S.

Sa-AFP2 at concentrations below 12 mM. The trivalent cation, Fe^{3+}, has a threshold concentration for antagonism of 30 μM.

None of the Brassicaceae AFPs decreases the viability of yeast cells at concentrations below 500 μg/ml, and only *Br*-AFP2 inhibits the growth of *B. megaterium* with an IC_{50} value of 52 μg/ml. It was reported earlier that *Rs*-AFP2 inhibits growth of this bacterium, albeit with a relatively high IC_{50} value (200 μg/ml, [9]). None of the other tested bacteria (see section 2) were affected in their growth by any of the Brassicaceae AFPs.

3.4. *Antifungal activity of the Brassicaceae 2S albumins*

The fractions containing the 2S albumins (obtained by cation-exchange chromatography, see Fig. 1) were pooled, extensively dialyzed against distilled water, and lyophilized. An additional purification step was done by gel filtration (Superose 12 column, 50 × 1.6 cm, Pharmacia; equilibrated with phosphate-buffered saline) and the obtained 2S albumin fractions were dialyzed again. Purity of the different 2S albumin samples was checked by SDS-PAGE (Fig. 6). As expected for 2S albumins [19], the unreduced proteins have apparent molecular masses between 14 and 16 kDa, whereas reduction causes the appearance of large (9 kDa) and small (3 kDa) subunits. The antifungal activity of these samples is given in Table III by means of IC_{50} values. The IC_{50} values of the *R. sativus* 2S albumins [9] are again included, as well as those of CMe, a trypsin-inhibitor from barley showing homology to 2S albumins [12]. In the low ionic strength medium, IC_{50} values ranging from 3 to 200 μg/ml are obtained for the Brassicaceae 2S albumins. However, as was the case for the radish 2S albumins, no antifungal activity was observed in the medium with 1 mM $CaCl_2$ and 50 mM KCl at concentrations as high as 1,000 μg/ml (*Bn*-2S, *Br*-2S and *Sa*-2S) or 200 μg/ml (*At*-2S). The 2S albumin-like protein, CMe, inhibited growth of *Alternaria brassicola* and *Verticillium dahliae* only very weakly, whereas the other fungi were not inhibited at concentrations of CMe below 1,000 μg/ml.

The sensitivity of the 2S albumin fraction of *S. alba* to various inorganic cations was assessed (Table II) as described for *Sa*-AFP2. In general, threshold concentrations for antagonism of multivalent cations with 2S albumins are about 1 order of magnitude lower than for *Sa*-AFP2. It is noteworthy, however, that Mg^{2+} and Ca^{2+} are much weaker antagonists of 2S albumins relative to the other divalent cations tested (Ca^{2+}, Ba^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+}).

4. DISCUSSION

We have shown that seeds of several Brassicaceae species contain highly conserved AFPs. With the exception of *A. thaliana* seeds, from which a single basic AFP was isolated, all examined species contained 2 basic AFPs, one (AFP2) being slightly more basic than the other (AFP1). The antifungal potency of the Brassicaceae AFPs and the sensitivity of their antifungal activity to inorganic cations can be compared with that of the α- and β-thionins [8,9]. In contrast to the α- and β-thionins, however, the Brassicaceae AFPs are devoid of other substantial antibiotic, antimicrobial or cytotoxic activities (this study and [9]). This renders them good candidates for the transfer of their genes to other plants as disease resistance traits.

An interesting observation is the fact that one of the AFPs, namely *Br*-AFP2, not only has a deviating electrophoretic pattern (see Fig. 3) but also shows a different biological activity. While the antifungal activity of *Br*-AFP2 is repressed by at least 1 order of magnitude relative to the other Brassicaceae AFPs, the antibacterial activity against *B. megaterium* is enhanced with by factor of 4 compared to *Rs*-AFP2. It is not known to which structural properties these differences can be attributed, but the alteration of the conserved trypthophane residue at position 11 in *Br*-AFP2 might be of importance in this respect.

We also showed that the observed cation-sensitive antifungal activity of the 2S albumins from radish seeds [9] can be generalized to other Brassicaceae 2S albumins, including napin, the 2S albumin from *B. napus*. However, not all 2S albumin-like proteins appear to have the antifungal properties of the Brassicaceae 2S albumins since CMe, a trypsin-inhibitor from barley which is known to show amino acid sequence homology to napin [12], is devoid of substantial antifungal activity.

It is known that some cysteine-rich proteins display strong affinities for metallic cations, especially divalent cations [20]. Hence, the Brassicaceae AFPs and 2S albu-

Table III

Antifungal activity of the different Brassicaceae 2S albumins and the 2S albumin-like barley trypsin-inhibitor, CMe

Fungus	IC_{50} values (μg/ml)					
	Rs-2S	*Br*-2S	*Bn*-2S	*Sa*-2S	*At*-2S	CMe
Alternaria brassicola	10	25	20	30	170	280
Botrytis cinerea	>500	95	200	200	>200	>1,000
Fusarium culmorum	35	70	40	50	190	>1,000
Fusarium oxysporum f.sp. *lycopersici*	>500	103	100	100	>200	>1,000
Pyricularia oryzae	10	70	42	90	190	>1,000
Verticillium dahliae	3.3	12	8.5	10	200	350

Protein concentrations required for 50% growth inhibition (IC_{50}) after 48 h of incubation at 25°C were determined from the dose– response curves. Tests were performed in the synthetic low ionic strength growth medium [8].

mins may possibly inhibit fungal growth by complexing an essential cation, thereby decreasing its availability to the fungus. However, our results on the effect of inorganic cations on the antifungal activity of *Sa*-AFP2 and *S. alba* 2S albumins argue against such a hypothesis. Indeed, it was found that the antifungal effect of 1 μM of *Sa*-AFP1 or 3 μM of *Sa*-2S was not affected by raising the concentration of any of the metallic cations in the synthetic growth medium to at least 30 μM (Table II). It is unlikely that the proteins can cause significant depletion of a cation if this cation is present in a 10-fold (or higher) molar excess relative to the protein itself. We tend to believe that the antifungal activity of Brassicaceae AFPs and 2S albumins does not result from complexation of cations but rather from a direct interaction of the proteins with a target structure on the fungi. Our preliminary results indicate that in the case of 2S albumins this target structure may be the fungal plasma membrane as these proteins cause efflux of K^+ ions out of treated hyphae (H.M.E. Schoofs et al., unpublished results).

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REFERENCES

[1] Bowles, D.J. (1990) Annu. Rev. Biochem. 59, 873–907.
[2] Raikhel, N.V. and Broekaert, W.F. (1992) in: Control of Plant Gene Expression (D.P. Verma Ed.) Telford Press (in press).
[3] Roberts, W.K. and Selitrennikoff, C.P. (1986) Biochim. Biophys. Acta 880, 161–170.
[4] Vigers, A.J., Roberts, W.K. and Selitrennikoff, C.P. (1991) Mol. Plant–Microbe Interact. 4, 315–323.
[5] Huynh, Q.K., Borgmeyer, J.R. and Zobel, J.F. (1992) Biochem. Biophys. Res. Commun. 182, 1–5.
[6] Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., van den Elzen, P.J.M. and Cornelissen, B.J.C. (1991) Plant Cell 3, 619–628.
[7] Hejgaard, J., Jacobsen, S. and Svendsen, I. (1991) FEBS Lett. 291, 127–131.
[8] Cammue, B.P.A., De Bolle, M.F.C., Terras, F.R.G., Proost, P., Van Damme, J., Rees, S.B., Vanderleyden, J. and Broekaert, W.F. (1992) J. Biol. Chem. 267, 2228–2233.
[9] Terras, F.R.G., Schoofs, H.M.E, De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A. and Broekaert, W.F. (1992) J. Biol. Chem. 267, 15301–15309.
[10] Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A. and Vanderleyden, J. (1990) FEMS Microbiol. Lett. 69, 55–60.
[11] Mikola, J. and Suolinna, E.-M. (1969) Eur. J. Biochem. 9, 555–560.
[12] Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, C., Royo, J. and Carbonero, P. (1987) Oxford Surv. Plant Mol. Cell Biol. 4, 275–334.
[13] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mollia, A.K., Gartner, F.H., Provenzano, M.D., Fujitomo, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[14] Heukeshoven, J. and Dernick, R. (1985) Electrophoresis 6, 103–112.
[15] Kovarik, A., Hlubinova, K., Vrbenska, A. and Prachar, J. (1987) Folia Biologica 33, 253–257.
[16] Terras, F.R.G., Goderis, I.J., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A. and Broekaert, W.F. (1992) Plant Physiol. (in press).
[17] Roberts, W.K. and Selitrennikoff, C.P. (1990) J. Gen. Microbiol. 136, 1771–1778.
[18] Broekaert, W.F., Mariën, W., Terras, F.R.G., De Bolle, M.F.C., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S.B., Vanderleyden, J. and Cammue, B.P.A. (1992) J. Biochem. 31, 4308–4314.
[19] Krebbers, E., Herdies, L., De Clercq, A., Seurinck, J., Leemans, J., Van Damme, J., Segura, M., Gheysen, G., Van Montagu, M. and Vandekerckhove, J. (1988) Plant Physiol. 87, 859–866.
[20] Lefebvre, D.D. (1990) Plant Physiol. 93, 522–524.