

# A Potent Antimicrobial Protein from Onion Seeds Showing Sequence Homology to Plant Lipid Transfer Proteins<sup>1</sup>

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An antimicrobial protein of about 10 kD, called *Ace*-AMP1, was isolated from onion (*Allium cepa* L.) seeds. Based on the near-complete amino acid sequence of this protein, oligonucleotides were designed for polymerase chain reaction-based cloning of the corresponding cDNA. The mature protein is homologous to plant nonspecific lipid transfer proteins (nsLTPs), but it shares only 76% of the residues that are conserved among all known plant nsLTPs and is unusually rich in arginine. *Ace*-AMP1 inhibits all 12 tested plant pathogenic fungi at concentrations below 10  $\mu\text{g mL}^{-1}$ . Its antifungal activity is either not at all or is weakly affected by the presence of different cations at concentrations approximating physiological ionic strength conditions. *Ace*-AMP1 is also active on two Gram-positive bacteria but is apparently not toxic for Gram-negative bacteria and cultured human cells. In contrast to nsLTPs such as those isolated from radish or maize seeds, *Ace*-AMP1 was unable to transfer phospholipids from liposomes to mitochondria. On the other hand, lipid transfer proteins from wheat and maize seeds showed little or no antimicrobial activity, whereas the radish lipid transfer protein displayed antifungal activity only in media with low cation concentrations. The relevance of these findings with regard to the function of nsLTPs is discussed.

Although plant seeds are usually sown on a substrate that is extremely rich in microorganisms, infection of seeds or seedling tissues normally occurs at relatively low frequency. It is believed that seed proteins that exhibit antimicrobial activity may participate in the protection of seeds against potential microbial invaders. Different types of antimicrobial proteins have been purified from plant seeds

including chitinases (Roberts and Selitrennikoff, 1986; Huynh et al., 1992),  $\beta$ -1,3-glucanases (Manners and Marshall, 1973; Leah et al., 1991), permatins (Vigers et al., 1991), thionins (Fernandez de Caleyá et al., 1972), ribosome-inactivating proteins (Roberts and Selitrennikoff, 1986; Leah et al., 1991), Cys-rich antimicrobial peptides (Broekaert et al., 1992; Cammue et al., 1992; Duvick et al., 1992), plant defensins (Terras et al., 1992b, 1993, 1995), 2S albumins (Terras et al., 1992b, 1993), and nsLTPs (Terras et al., 1992a). The antimicrobial activity of some of these proteins has been reported to be drastically reduced in the presence of physiological concentrations of inorganic cations, and it may be questioned whether they can actually exert their antimicrobial activity in vivo (Roberts and Selitrennikoff, 1990; Broekaert et al., 1992; Cammue et al., 1992; Terras et al., 1992b, 1993).

In this paper we describe the purification and cDNA cloning of an antimicrobial protein from onion (*Allium cepa* L.) seeds whose antimicrobial activity is not or is only very weakly affected by physiological concentrations of inorganic cations. This protein shows sequence homology to nsLTPs. nsLTPs are a family of proteins of unknown function that are classified as lipid transfer proteins based on their ability to shuttle phospholipids between membrane vesicles or organelles in vitro (Arondel and Kader, 1990). Unlike many nsLTPs, however, *Ace*-AMP1 was not able to transfer either phosphatidylcholine or phosphatidylinositol from liposomes to mitochondria. On the other hand, *Ace*-AMP1 showed a much higher antimicrobial activity compared to nsLTPs purified from radish, wheat, and maize seeds. These findings suggest that different types of nsLTP-like proteins may fulfill different functions.

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Abbreviations: *Ac*-AMP1, *Amaranthus caudatus* antimicrobial peptide 1; *Ace*-AMP1, *Allium cepa* antimicrobial protein 1; IC<sub>50</sub>, concentration required for 50% growth inhibition; NH<sub>4</sub>Ac, ammonium acetate; nsLTP, nonspecific lipid transfer protein; RP-HPLC, reversed-phase HPLC; ssDNA, single-stranded DNA.

## MATERIALS AND METHODS

### Materials

Onion (*Allium cepa* L. cv Vaugirard) seeds were obtained from a local seed supplier. The nsLTPs from wheat and maize seeds were kindly provided by Dr. Didier Marion (Institut National de la Recherche Agronomique, Nantes, France), and the nsLTP from radish was purified as described by Terras et al. (1992a). Ac-AMP1 was isolated from amaranth seeds as described by Broekaert et al. (1992), and  $\beta$ -purothionin was purified from wheat flour by the method of Redman and Fisher (1969).

### Microorganisms

Filamentous fungi were grown on five-cereal agar (15 g L<sup>-1</sup> five-cereal baby food instant flakes, Nestle, Vevey, Switzerland; 15 g L<sup>-1</sup> agar) and spores were harvested and stored as previously described (Broekaert et al., 1990).

The following fungal strains were used for determining antifungal activity: *Alternaria brassicola* MUCL 20297, *Ascochyta pisi* MUCL 30164, *Botrytis cinerea* MUCL 30158, *Colletotrichum lindemuthianum* MUCL 9577, *Fusarium culmorum* IMI 180420, *Fusarium oxysporum* f.sp. *pisii* IMI 236441, *Fusarium oxysporum* f.sp. *lycopersici* MUCL 909, *Nectria haematococca* Collection Van Etten 160-2-2, *Phoma betae* MUCL 9916, *Pyrenophora tritici-repentis* MUCL 30217, *Pyricularia oryzae* MUCL 30166, and *Verticillium dahliae* MUCL 6963. The following bacteria were used for determining the antibacterial activity: *Bacillus megaterium* ATCC 13632, *Sarcina lutea* ATCC 9342, *Agrobacterium tumefaciens* LMG 188, *Alcaligenes eutrophus* LMG 1195, *Azospirillum brasilense* ATCC 29145, *Erwinia carotovora* subsp. *carotovora* LMG 2458, *Escherichia coli* strain HB101, *Pseudomonas solanacearum* LMG 2293, and *Pseudomonas syringae* pv *tabaci* LMG 5192.

### Antifungal and Antibacterial Activity Assays

Antifungal activity was measured by microspectrophotometry as previously described (Broekaert et al., 1990). Routinely, tests were performed with 20  $\mu$ L of a filter-sterilized test solution and 80  $\mu$ L of a suspension of fungal spores ( $2 \times 10^4$  spores mL<sup>-1</sup>) in either half-strength potato dextrose broth (medium A) or half-strength potato dextrose broth supplemented with CaCl<sub>2</sub> and KCl to final concentrations of 1 mM and 50 mM, respectively (medium B). Alternatively, a synthetic growth medium with low ionic strength (SMF-) was used. The synthetic growth medium consisted of K<sub>2</sub>HPO<sub>4</sub> (2.5 mM), MgSO<sub>4</sub> (50  $\mu$ M), CaCl<sub>2</sub> (50  $\mu$ M), FeSO<sub>4</sub> (5  $\mu$ M), CoCl<sub>2</sub> (0.1  $\mu$ M), CuSO<sub>4</sub> (0.1  $\mu$ M), Na<sub>2</sub>MoO<sub>4</sub> (2  $\mu$ M), H<sub>3</sub>BO<sub>3</sub> (0.5  $\mu$ M), KI (0.1  $\mu$ M), ZnSO<sub>4</sub> (0.5  $\mu$ M), MnSO<sub>4</sub> (0.1  $\mu$ M), Glc (10 g L<sup>-1</sup>), Asn (1 g L<sup>-1</sup>), Met (20 mg L<sup>-1</sup>), *myo*-inositol (2 mg L<sup>-1</sup>), biotin (0.2 mg L<sup>-1</sup>), thiamine-HCl (1 mg L<sup>-1</sup>), and pyridoxine-HCl (0.2 mg L<sup>-1</sup>).

Unless otherwise stated the fungal test organism was *F. culmorum* (strain IMI 180420) and incubation was at 25°C for 48 h. The antifungal activity of a sample (units per mL) is defined as the total volume of the assay mixture divided by the volume of the sample in the assay mixture that gives

50% growth inhibition (= dilution factor for 50% growth inhibition). Percent growth inhibition is defined as  $100 \times$  the ratio of the corrected *A* of the control microculture minus the corrected *A* of the test microculture over the corrected *A*<sub>595</sub> of the control microculture. The corrected *A* values equal the *A*<sub>595</sub> of the culture measured after 48 h minus the *A*<sub>595</sub> measured after 30 min.

Antibacterial activity was measured microspectrophotometrically as follows. Bacteria were precultured overnight in 2% (w/v) tryptone at 30°C in a rotary shaker. A soft agarose medium (2% [w/v] tryptone; 0.5% [w/v] low-melting-point agarose) was inoculated with the bacteria to a cell density of 10<sup>5</sup> colony-forming units mL<sup>-1</sup>. Aliquots (80  $\mu$ L) of the bacterial suspension were added to filter-sterilized samples (20  $\mu$ L) in flat-bottom 96-well microplates and allowed to solidify. The *A*<sub>595</sub> of the culture was measured with the aid of a microplate reader after 30 min and 24 h of incubation at 28°C. Percent growth inhibition was calculated as described above for the antifungal activity assay.

### Human Cell Membrane Integrity Assay and Hemolytic Activity Assay

Disruption of membrane integrity of cultured human cells was tested on skin muscle diploid fibroblasts using the neutral red uptake method as described previously (Terras et al., 1992b). Hemolytic activity was assayed using human blood-group A erythrocytes as described previously (Terras et al., 1992b), except that the erythrocytes were suspended in PBS instead of 150 mM *myo*-inositol.

### Lipid Transfer Activity Assay

The lipid transfer activity was measured by following the transfer of either phosphatidylcholine or phosphatidylinositol between liposomes and mitochondria as previously described (Grosbois et al., 1989). Liposomes containing either [<sup>3</sup>H]phosphatidylcholine or [<sup>3</sup>H]phosphatidylinositol (260 nmol, 740 Bq) as a lipid to be transferred and [1-<sup>14</sup>C]cholesteryl oleate (1 nmol, 740 Bq) as a nontransferable tracer were incubated at 30°C for 30 min with maize mitochondria (2 mg of protein) in the presence of protein. The <sup>3</sup>H label recovered in mitochondria and collected by centrifugation indicated the extent of transfer of either phosphatidylcholine or phosphatidylinositol (as percent of the initial radioactivity of lipids), whereas the <sup>14</sup>C label (usually low, around 1%) allowed the determination of the contamination of mitochondria by intact liposomes. The lipid transfer activity is expressed as nmol of phospholipid transferred per min per mg of protein.

### Protein Analysis Methods

All protein concentrations were determined by the bicinchoninic acid method (Smith et al., 1985) using BSA as a standard. SDS-PAGE was performed on precast commercial gels (PhastGel High Density from Pharmacia) using a PhastSystem (Pharmacia) electrophoresis apparatus. The sample buffer contained 200 mM Tris-HCl (pH 8.3), 1% (w/v) SDS, 1 mM EDTA, 0.005% (w/v) bromphenol blue,

and 1% (w/v) DTE. Proteins were fixed after electrophoresis in 6% (v/v) glutaraldehyde and stained with Coomassie blue.

Determination of free thiol groups was done by the dithionitrobenzoic acid method as described previously (Terras et al., 1992b). Amino acid sequence analysis was performed on a 477A Protein Sequencer (Applied Biosystems) with on-line detection of phenylthiohydantoin amino acid derivatives in a 120A Analyser (Applied Biosystems).

Cys residues were modified by *S*-carboxyamidomethylation as described (Cammue et al., 1992). Reagents were removed by RP-HPLC on a Pep-S (porous silica C<sub>2</sub>/C<sub>18</sub>) (Pharmacia) column (25 × 0.4 cm). The *S*-carboxyamidomethylated protein was recovered by eluting the column with a linear gradient from 0.1% (v/v) TFA to acetonitrile containing 0.1% (v/v) TFA. Digestion of the *S*-carboxyamidomethylated protein with the endoproteinases Arg-C and Asp-N (both of sequencing grade from Boehringer Mannheim) was carried out according to the supplier's instructions applying minimal advised enzyme to protein ratios (w/w) and maximal advised incubation times. Digested peptides were subsequently separated by RP-HPLC on a Pep-S (porous silica C<sub>2</sub>/C<sub>18</sub>) (Pharmacia) column (25 × 0.4 cm) using a linear elution gradient from 0.1% (v/v) TFA to acetonitrile containing 0.1% (v/v) TFA in 100 min at 1 mL min<sup>-1</sup>.

#### Extraction of Basic Heat-Stable Proteins from Onion Seeds

One hundred grams of onion seeds were ground in a coffee mill and the resulting meal was extracted for 2 h at 4°C with 200 mL of an ice-cold extraction buffer containing 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 15 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM KCl, 2 mM EDTA, and 2 mM thiourea. After extraction the slurry was mixed in a Waring blender and subsequently squeezed through a jam mincer to separate the extract from the solid residue. The resulting extract was clarified by centrifugation (10 min at 5000g). Solid ammonium sulfate was added to the supernatant to obtain 85% relative saturation and the precipitate was allowed to form by standing overnight at 4°C. Following centrifugation at 7000g for 30 min, the precipitate was redissolved in 100 mL of distilled water and dialyzed extensively against distilled water. After di-

alysis the solution was adjusted to 50 mM NH<sub>4</sub>Ac (pH 9) by addition of the 10-fold-concentrated buffer and passed over a Q-Sepharose Fast Flow (Pharmacia) column (12 × 5 cm) equilibrated in 50 mM NH<sub>4</sub>Ac (pH 9.0). The protein fraction that passed through the column was lyophilized and redissolved in 200 mL of 50 mM NH<sub>4</sub>Ac (pH 5.5). This material represents the basic (pI > 9) protein fraction of the seeds and was further purified as described in "Results."

#### PCR-Based Cloning of the 5' and 3' Parts of *Ace*-AMP1 cDNA

Total RNA was extracted by the method of Logemann et al. (1987) from a mixture of immature seeds collected 15, 21, and 30 d postanthesis. The 3' part of *Ace*-AMP1 cDNA was cloned as follows. Total RNA (1 μg) was reverse transcribed in a 30-μL reaction mixture containing 12 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), appropriate buffer constituents (Sambrook et al., 1989), and 10 pmol of a modified oligo(dT) primer (primer OWB114, see Table I) and incubated for 30 min at 52°C. A fraction of the reverse-transcription reaction (0.5 μL) was transferred to a 25-μL PCR reaction mixture containing 5 pmol of the antisense primer OWB114, 5 pmol of the sense primer OWB111 (a degenerate primer corresponding to an internal amino acid sequence of *Ace*-AMP1, namely PRFQNIP), 5 nmol dNTPs, 0.5 unit of *Taq* polymerase, and *Taq* polymerase buffer constituents (Sambrook et al., 1989). Temperature cycling for PCR was done according to standard conditions (Sambrook et al., 1989) using a primer annealing temperature of 55°C. PCR reaction products were analyzed by agarose gel electrophoresis and a band of approximately 400 bp (which was absent from control PCR reactions containing the same template but only one of both primers) was isolated using a Prep-a-Gene kit (Bio-Rad) according to the manufacturer's instructions. The PCR product was digested with *Xba*I and subcloned into the plasmid pEMBL18+ (Boehringer Mannheim). Both strands of the insert were sequenced on an ALF automated sequencer (Pharmacia) using the Autoread sequencing kit (Pharmacia) with fluorescein-labeled M13 forward and reverse primers.

The 5' part of the *Ace*-AMP1 cDNA was cloned as follows. Total RNA was reverse transcribed as described

**Table I.** Characteristics of the oligonucleotides used for *Ace*-AMP1 cDNA cloning

Name	Sense (S) <sup>a</sup> or Antisense (AS)	Sequence <sup>b</sup>	Position Relative to <i>Ace</i> -AMP1 cDNA Nucleotide Sequence
OWB114	AS	5'-CCACTCTAGAGAATTCACCTTTTTTTTTTTTTTTTTTT-3'	Poly(A <sup>+</sup> ) tail
OWB116	AS	5'- <sup>c</sup> AGAATTCGCATTGCATCGGATCCATGATCGAT-3' <sup>d</sup>	5' end
OWB117	S	5'-ATCGATCATGGATCCGATGCAATGC-3' <sup>e</sup>	5' end
OWB111	S	5'-AATTCTAGACCNMGNTTYCARAAYATHCC-3' <sup>f</sup>	307 to 326
OWB132	AS	5'-ATCGGATCCGAATTCGTTGCCGACAATCACGAGG-3'	325 to 344
OWB133	AS	5'-ATCGGATCCGAATTCAGGACGAACAAAGGTGTTGC-3'	338 to 354
OWB158	S	5'-TAAGGTACCATGGTTCGGCTTGATC-3'	35 to 53
OWB160	AS	5'-TAAGGATCCCTTCATTCCTCAGCGTCCAAG-3'	417 to 437

<sup>a</sup> Relative to *Ace*-AMP1 mRNA. <sup>b</sup> Restriction sites are underlined. <sup>c</sup> 5' end is phosphorylated. <sup>d</sup> 3'OH at 3' end is aminated. <sup>e</sup> Sequence is complementary to nucleotides 8 to 32 of OWB116. <sup>f</sup> N = G, A, T, C; H = A, C, T; M = A, C; Y = C, T; R = A, G.

above using either OWB114 or OWB133 (an *Ace*-AMP1-specific primer derived from the nucleotide sequence of the 3' part of *Ace*-AMP1 cDNA) as a primer. Excess primer was removed by gel filtration over a Chromaspin + TE-100 column (Clontech, Palo Alto, CA) equilibrated in 10 mM Tris, 1 mM EDTA, 300 mM NaCl, 0.05% (w/v) SDS (pH 8.0). RNA was subsequently removed by alkaline hydrolysis, and the ssDNA was ethanol precipitated as described by Delort et al. (1989) and finally redissolved in 10  $\mu$ L of distilled water. The 3' ends of these ssDNAs (corresponding to the 5' ends of the mRNAs) were ligated to the oligonucleotide OWB116, which was synthesized with a phosphate group at its 5' end (to allow for ligation to the ssDNA) and an amino group at its 3' end (to avoid primer self-ligation). The ssDNA ligation reaction mixture (30  $\mu$ L) contained 5 pmol of primer OWB116, 2.5  $\mu$ L of ssDNA (see above), 10 units of T4 RNA ligase (New England Biolabs), and T4 RNA ligase buffer constituents (Tessier et al., 1986), and incubation was done at 22°C for 16 h. A fraction (0.1  $\mu$ L) of the ssDNA ligation mixture was transferred to a 25- $\mu$ L PCR reaction mixture containing 5 pmol of primer OWB117 (which is partially complementary to OWB116), 5 nmol of dNTPs, and 1.25 units of *Taq* polymerase and *Taq* polymerase buffer constituents. After five PCR cycles with an annealing temperature of 60°C, 25 pmol of an *Ace*-AMP1-specific primer (OWB132, corresponding to a position on *Ace*-AMP1 cDNA immediately upstream of that of OWB133) was added to the reaction mixture and 30 additional PCR cycles with an annealing temperature of 55°C were carried out. A PCR product of approximately 400 bp, which was not present in single-primer PCR controls, was gel purified as described above. The same 400-bp PCR band was obtained irrespective of whether OWB133 or OWB114 were used in the first-strand synthesis. This PCR product was *Bam*HI-digested and subcloned into pEMBL18+, and the nucleotide sequence of the insert was determined as described above.

To verify the nucleotide sequences obtained for the 5' and 3' parts of *Ace*-AMP1 and to facilitate the construction of chimeric genes for future heterologous expression experiments, two novel primers were designed, OWB158 and OWB160, which are situated around the start codon at the 5' end of *Ace*-AMP1 cDNA and around the stop codon at the 3' end of *Ace*-AMP1 cDNA, respectively. These primers were used for PCR amplification using OWB114-primed reverse-transcribed mRNA as a template. The resulting 400-bp PCR product was digested with *Kpn*I and *Bam*HI and subcloned into pEMBL18+. Nucleotide sequencing of this insert confirmed the nucleotide sequence of the *Ace*-AMP1 coding region shown in Figure 4.

## RESULTS

### Purification of an Antimicrobial Protein from Onion Seeds

The starting material for the isolation of the *A. cepa* antimicrobial protein was the basic protein fraction extracted from the mature seeds as described in "Materials and Methods." This extract was further purified by cation-exchange chromatography using a linear gradient of am-

monium acetate at pH 6.0. The fractions were lyophilized and assayed for antifungal activity against *F. culmorum* both in half-strength potato dextrose (medium A) and in the same medium supplemented with 1 mM CaCl<sub>2</sub> and 50 mM KCl (medium B). The chromatogram of this separation (Fig. 1A) shows a first group of peaks eluting between 0.3 and 0.6 M ammonium acetate that are devoid of antifungal activity, a second group of peaks eluting between 0.8 and 1.2 M ammonium acetate that have markedly stronger antifungal activity in medium A relative to medium B, and finally, a well-resolved peak eluting at approximately 1.5 M ammonium acetate whose antifungal activity in medium A is virtually as high as that in medium B. As illustrated in Figure 1B, the latter peak could further be separated by RP-HPLC into two well-resolved symmetric peaks. The first of these peaks co-eluted with the cation-insensitive antifungal activity. The antifungal factor contained in this peak is further referred to as *Ace*-AMP1. The approximate yield of *Ace*-AMP1 using this procedure is about 10 mg/kg of seeds.

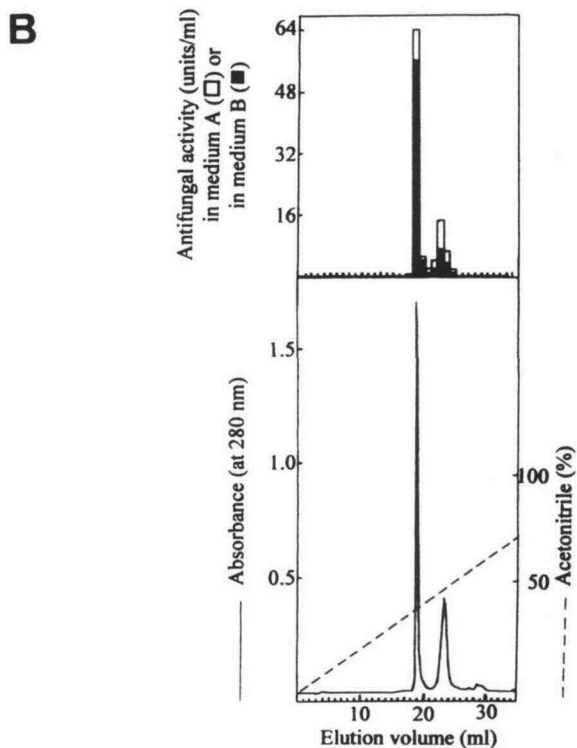
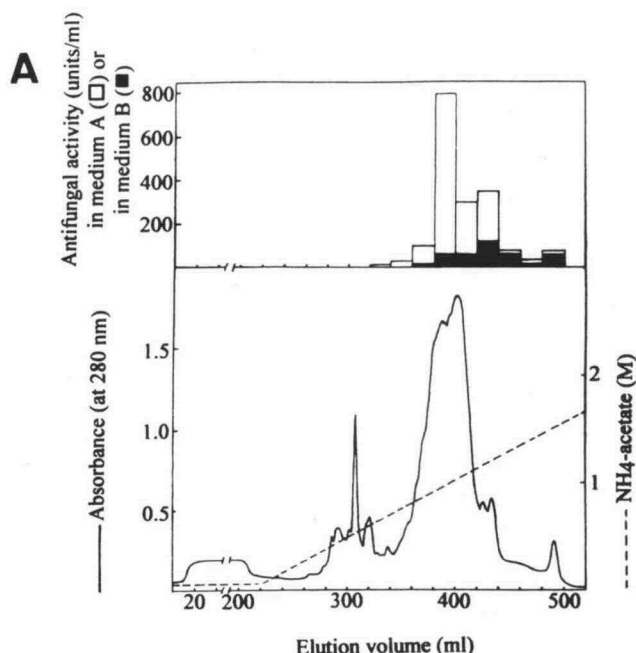
### Characterization of *Ace*-AMP1

SDS-PAGE analysis of purified *Ace*-AMP1 revealed that it migrates as a single band of approximately 10 kD (Fig. 2). Molecular mass estimation of native *Ace*-AMP1 by gel filtration on Superose-12 (Pharmacia) yielded a value of 7.5 kD (results not shown). Taken together, these results suggest that *Ace*-AMP1 is a monomeric protein with a molecular mass of around 7.5 to 10 kD. Determination of covalently bound sugars using the phenol-sulfuric acid method (Dubois et al., 1956) indicated that *Ace*-AMP1 contains less than 0.1% sugar, indicating that *Ace*-AMP1 is not glycosylated. Moreover, treatment of *Ace*-AMP1 with endoglucosidase H did not result in a shift of the mobility of *Ace*-AMP1 upon SDS-PAGE analysis (results not shown).

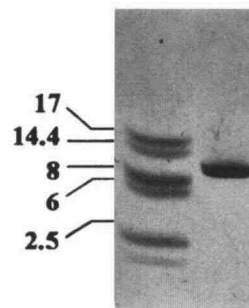
Initial attempts to sequence *Ace*-AMP1 showed that the protein was N-terminally blocked. Since deblocking with pyroglutamate aminopeptidase also proved unsuccessful, *Ace*-AMP1 was digested with the endoproteases Arg-C and Asp-N. Prior to digestion, the protein was treated with DTT and iodoacetamide to derivatize the Cys residues. Upon digestion with Arg-C and separation of the proteolytic peptides by RP-HPLC, 10 peptides could be resolved, 8 of which yielded unambiguous sequence signals. Treatment with Asp-N generated three protein fragments, two of which could be sequenced. Based on the sequence information of these peptides, the primary structure of *Ace*-AMP1 could be reconstructed with the exception of the blocked N-terminal part (Fig. 3).

Since the amino acid sequence of *Ace*-AMP1 pointed to the presence of at least seven Cys's, we questioned whether free thiol groups occur in this protein. Using the Ellman reagent, however, no free thiol groups could be detected in native *Ace*-AMP1 unless the protein was first reduced with DTT. This indicates that all Cys residues participate in disulfide bonds.

The stability of *Ace*-AMP1 was next assessed using its antifungal activity as a measure for its integrity. As shown in Table II, *Ace*-AMP1 is remarkably heat stable, since it



**Figure 1.** Purification of Ace-AMP1 from onion seeds. A, Approximately 200 mL of the basic protein fraction (see "Materials and Methods") was applied to a S-Sepharose High Performance (Pharmacia) column (10 × 1.6 cm) equilibrated in 50 mM ammonium acetate, pH 5.5. The column was eluted at 2.0 mL min<sup>-1</sup> with a linear gradient from 50 mM to 2 M ammonium acetate, pH 5.5, over 180 min. The eluate was monitored for protein by on-line measurement of the A<sub>280</sub> (lower panel) and collected in 20-mL fractions. One-milliliter samples from each fraction were lyophilized and redis-



**Figure 2.** SDS-PAGE analysis of Ace-AMP1. Two micrograms of purified Ace-AMP1 were subjected to SDS-PAGE (right lane). The molecular mass was estimated by comparison with myoglobin fragments (left lane). The molecular mass of the myoglobin fragments is indicated in kD.

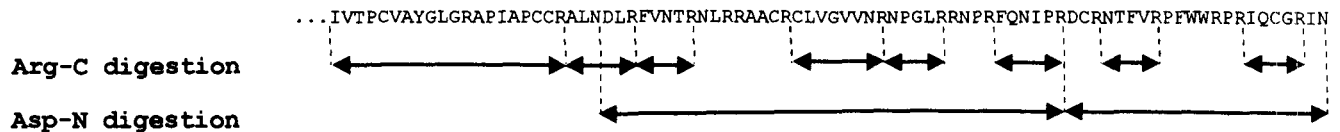
withstands boiling for 10 min. Moreover, Ace-AMP1 was relatively resistant to treatment with the proteases chymotrypsin, trypsin, and proteinase K, whereas digestion with pronase E reduced the activity almost completely.

#### cDNA Cloning of Ace-AMP1

Based on the partial amino acid sequence of Ace-AMP1, a degenerate oligonucleotide mixture corresponding to the amino acid sequence PRFQNIP was synthesized. This primer was used in combination with an olig(dT) primer to amplify by PCR a 400-bp fragment using reverse-transcribed mRNA from developing onion seeds as a template. Nucleotide sequencing revealed that this fragment corresponded to the 3' part of Ace-AMP1 cDNA. Based on this nucleotide sequence information, new oligonucleotides were designed in order to amplify the 5' part of Ace-AMP1 cDNA following a modified rapid amplification of cDNA ends PCR procedure. This second PCR reaction yielded another 400-bp fragment that was confirmed by nucleotide sequencing to correspond to the 5' part of Ace-AMP1 cDNA and that overlapped by 38 nucleotides with the sequence of the 3' part of Ace-AMP1.

Figure 4 shows the 686-bp nucleotide sequence of the full-length Ace-AMP1 cDNA, which was reconstructed from the nucleotide sequences of the 5' and 3' parts. Ace-AMP1 cDNA contains a 396-bp open reading frame coding

solved in 1 mL of distilled water, of which 20  $\mu$ L was assayed for antifungal activity as described in "Materials and Methods" (upper panel) in both medium A and medium B. The peak eluting at approximately 1.5 M ammonium acetate was further purified by RP-HPLC. B, Amounts equivalent to 2 mg of protein were loaded on a Pep-S (porous silica C<sub>2</sub>/C<sub>18</sub>) (Pharmacia) column (25 × 0.4 cm) equilibrated with 0.1% (v/v) TFA. The column was developed at 1 mL min<sup>-1</sup> with a linear gradient of 0.1% (v/v) TFA to 99.9% (v/v) acetonitrile:0.1% (v/v) TFA over 50 min. The eluate was monitored for protein by on-line measurement of the A<sub>280</sub> (lower panel). One-milliliter fractions were collected, vacuum dried, and redissolved in 1 mL of distilled water, of which 20  $\mu$ L was used in an antifungal activity assay using both medium A and B (upper panel). Chromatography was performed on a Waters 600 HPLC station.



**Figure 3.** Amino acid sequence of *Ace*-AMP1. The amino acid sequence (with the exception of the blocked N-terminal part) was determined by sequencing proteolytic peptides obtained by endoproteinase Arg-C and Asp-N digestion.

for 132 amino acids, a 36-bp 5' leader sequence, and a 3' untranslated region of 232 bp up to the poly(A<sup>+</sup>) tail. Analysis of the coding region reveals the presence of a putative signal peptide of 27 amino acids. The predicted signal peptide cleavage site is in agreement with the rules of von Heijne (1986). The amino acid sequence comprised the area between amino acids 37 and 120 of the coding region and was identical to the amino acid sequence determined experimentally for mature *Ace*-AMP1. The cDNA-derived coding region predicts that mature *Ace*-AMP1 has nine additional amino acids at the N terminus relative to the sequence shown in Figure 4. This sequence could not be determined experimentally due to the presence of a blocked N-terminal amino acid in mature *Ace*-AMP1. Thus, mature *Ace*-AMP1 contains 93 amino acids in total. Furthermore, the primary translation product of *Ace*-AMP1 mRNA is predicted to have an additional 12-residue C-terminal propeptide, which is absent from mature *Ace*-AMP1.

#### *Ace*-AMP1 Is Homologous to Lipid Transfer Proteins

The amino acid sequence of mature *Ace*-AMP1 was found to be partially homologous with that of a number of plant-derived nsLTPs. Figure 5 shows the alignment of the amino acid sequences of *Ace*-AMP1 and 13 different proteins classified as nsLTPs or nsLTP-like proteins. From a comparison of all nsLTP sequences shown in Figure 5 (excluding *Ace*-AMP1), the following consensus motif can be derived. All eight Cys's are at conserved positions 4, 14,

30, 31, 51, 53, 77, and 93 (numbering as in Fig. 5); hydrophobic residues (L, I, A, V, M) or aromatic residues (F, W, Y) appear at positions 2, 7, 15, 17, 18, 34, 37, 41, 54, 61, 64, 69, 73, 80, 82, 85, 87, and 96; Pro's are present at positions 25 and 74; basic residues (H, R, K) are conserved at positions 47 and 55; hydroxy residues (S, T) appear at positions 43 and 88; and a conserved Asp occupies position 46. *Ace*-AMP1 corresponds largely to this consensus motif, but deviates at the following positions: it does not have hydrophobic/aromatic residues at positions 2, 18, 61, 69, and 80; and it does not have the conserved Asp, Lys, and Ser at positions 46, 55, and 88, respectively. Hence, about 24% of the conserved residues in plant nsLTPs are altered in *Ace*-AMP1. Moreover, *Ace*-AMP1 distinguishes itself from all known nsLTPs by a much higher Arg content. Indeed, *Ace*-AMP1 contains 19 Arg's, whereas the number of Arg's in other nsLTPs varies from 1 for the spinach leaf nsLTP to 6 for the nsLTP from maize seedlings.

#### Antibiotic Activities of *Ace*-AMP1

*Ace*-AMP1 was purified by monitoring chromatographic separations using an assay for antifungal activity against

**Table II.** Stability of the antifungal activity of *Ace*-AMP1

Tests for antifungal activity were performed with 20- $\mu$ L samples diluted 5-fold with growth medium containing *F. culmorum* spores, according to the assay method described in "Materials and Methods." Untreated control samples consisted of the test proteins at 500  $\mu$ g mL<sup>-1</sup> in 10 mM sodium phosphate buffer (pH 7.0). Heat-stability tests were performed by heating aliquots of the test proteins for 10 min at different temperatures up to 100°C. For digestions, proteases were added at 400  $\mu$ g mL<sup>-1</sup> and incubated at 37°C for 16 h. Under the same conditions, BSA was completely digested by any of the proteases, as could be visualized by SDS-PAGE analysis (not shown).

Treatment	Relative Antifungal Activity
	% of control activity
Control	100
Heating at 80°C, 10 min	100
Heating at 90°C, 10 min	100
Heating at 100°C, 10 min	100
Chymotrypsin digestion	80
Pronase E digestion	5
Proteinase K digestion	60
Trypsin digestion	90

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1 AACGAAAATTACGAAATACATCAATATCTCGAGCCaTgTTCGCCTTGATCTTTACTT
                                     M V R V V S L L -20
61 GCAGCAGTCGACCTTCATCCTGTTGATTGATGATAATCAGCAGTCGGTATGCAAAATAGTCAG
   A A S T F I L L I M I I S S P Y A N S Q +1
121 AACATATGCCCAAGGGTTAATCGAATTTGTGACACCCCTGTGGCCACGGACTCGGAAGG
   N I C P R V N R I V T P C V A Y G L G R +21
181 GCACCAATCGCCCATGCTGCAGAGCCCTGACAGATCTACGGTTTGTGAATACTAGA AAC
   A P I A P C C R A L N D L R F V N T R N +41
241 CTACGACGTGCTGCATGCCCTGCTGCTGCTAGGGGTAGTGAACCGGAACCCGGTCTGAGA
   L R R A A C R C L V G V V N R N P G L R +61
301 CGAAACCCTAGATTTGAGAACATTCCTCGTGATGTCGCAACACCTTTGTTGCTCCCTTC
   R N P R F Q N I P R D C R N T F V R P F +81
361 TGGTGGCGTCCAAGAAATCAATGCCGAGGATTAACCTTACGGATAAGCTTATATACTTG
   W W R P R I Q C G R I N L T D K L I Y L +101
421 GACGCTGAGGAATgaAGACTAGGCTCTACTGTTATGCACTATAGTTTATAGTATATATAC
   D A E E -
481 TAAATAAAACAGTATGTGCTGATAAATTTGCAATATGGACTTATTATAGCAAGTCCTAA
541 TGGTGTCTGCTACTTGGGTCAGCATTGAGCACTATATAGGCACTATATAGGGTACTATG
601 GGCTGATTATGATGTCAACGGCGTACTTTTACTTCCATATAaataaaTAATGGGTTTACTT
661 TGCTTGAAAAAAAAAAAAAAAAAAAAA

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**Figure 4.** Nucleotide sequence and deduced amino acid sequence of *Ace*-AMP1 cDNA. The start codon, stop codon, and polyadenylation signal are indicated in lowercase letters. The putative signal peptide cleavage site is marked by the arrow. The amino acid sequence corresponding to the experimentally determined sequence of *Ace*-AMP1 (see Fig. 3) is underlined.

	10	20	30	40	50	60	70	80	90
<i>Ace</i> -AMP1	QNICPRVNRIVTPCVAYGLGRA	--PIA--PCCRALNDLR--FVNTRNLRRAACRCLVGVVNRNPGRLRRNPRFONIPRDCRNTEFVRPFWRPPIQCGRIN							
Consensus	.L.C.V.....CV.YL.....P....CC..L.L..V.T..DR...C.CLK.....I.L...A...IP..C.V.I..F.IS....C..I.								
	I	I FA	V	A S K	I	V I L L	I V Y V	V	
	V	L IV	I		V	F A	L V		
		A	Y			Y	I		
						L			
<i>Rs</i> -nsLTP	ALSCGTVNSLNAACIGYLTQNA	--PLARGCCTGVTNLNNMA?TTP??							
<i>So</i> -nsLTP	GITCGMVSSKLAPCIGILKGG	---PLGGGCGGKIKALNAAAATTPDRKTACNCLKSAANAIRGINYGKAAG-LPGMC-GVHI-PYAIISPSTNCNAHV							
EP2	VLTCGQVTGALAPCLGYLRSQVNVVPLTCCNVVRGLNNAARTTLDRKTACGCLKQTANAVTGLNLNAAAG-LPARC-GVNI-PYKISPTTDCNRV								
TobLTP	ALSCGQVQSGLAPCLPYLQGRG	--PLG-SCCGGVKGLLGAAKSLDRKTACICLKSAANAIRGIDMGKAAG-LPGAC-GVNI-PYKISPSTDCSKVQ							
<i>Le</i> -nsLTP	ALTTCGQVTAGLAPCLPYLQGRG	--PLG-GCCGGVKNLLGSAKTTADRKTACTCLKSAANAIRGIDLNKAAG-IPVSC-KVNI-PYKISPSTDCSTVQ							
CB-A	-VDCGQVNSLASCIPFLTGGVASPSA	-SCCAGVQNLKTLAPTSADRRRAACECIKAAARFPTIKQDAASS-LPKKC-GVDI-NIPISKTTNCQAIN							
CB-B	-VNCGQVNSLASCIPFLTGGVASPSA	-SCCAGVQNLKTLAPTSADRRRAACECIKAAARFPTIKQDAASS-LPKKC-GVDI-NIPISKTTNCQAIN							
CB-C	AVPCSTVDMKAAACVGFATGKDSKPSQ	-ACCTGLQQLAQTVKTVDDKKAICRCLKASSKSL-GIKDQFLSK-IPAAAC-NIKV-GFPVSTNTNCETIH							
PAPI	ALNCGQVDSKMKPCLTYVQGGPGPSG	-LCCNGVRDLHNQAQSSGDRQTVNCNCKLGIARGIHNLNLNNAAS-IPSKC-NVNV-PYTIISPDIKDCSRIY							
CW18	AITCGQVSSALGPAAAYAKGSSTSPSA	-GCCSGVKRLAGLARSTADKQATCRCLKSVAGAY-NA--GRAAG-IPSRC-GVSV-PYTIISASVDCSKIH							
CW21	AISCGQVSSALSPCISYARGNGAKPPA	-ACCSGKRLAGAAQSTADKQATCRCLKSAAGGL-NA--GKAAG-IPSMC-GVSV-PYAIISASVDCSKIR							
<i>Ta</i> -nsLTP	-IDCGHVDLSLVRPCLSYVQGGPG	-PSG-QCCDGVKNLHNQAQSSGDRQTVNCNCKLGIARGIHNLNEDNARS-IPPKC-GVNL-PYTIISLINDCSR							
<i>Zm</i> -nsLTP	AISCGQVASAIAPCISYARGQSGPSA	-GCCSGVRSLNNAARTTADRRRAACNCLKNAAGVSGLNAGNAAS-IPSKC-GVSI-PYTIISTSTDCSRVN							

**Figure 5.** Amino acid sequence alignment between mature *Ace*-AMP1 and nsLTPs or nsLTP-like proteins. The following sequences of nsLTPs or nsLTP-like proteins are included in the comparison: *Rs*-nsLTP from *Raphanus sativus* seeds (Terras et al., 1992a); *So*-nsLTP from *Spinacia oleracea* leaves (Bernhard et al., 1991); EP2 from *Daucus carota* zygotic embryos (Sterk et al., 1991); TobLTP from *Nicotiana tabacum* flowers (Masuta et al., 1992); *Le*-nsLTP from *Lycopersicon esculentum* (Torres-Schumann et al., 1992); CB-A, CB-B, and CB-C from *Ricinus communis* seedlings (Takishima et al., 1988); PAPI from *Hordeum vulgare* seeds (Mundy and Rogers, 1986); CW18 and CW21 from *Hordeum vulgare* leaves (Molina et al., 1993); *Ta*-nsLTP from *Triticum aestivum* seeds (Simorre et al., 1991); and *Zm*-nsLTP from *Zea mays* seedlings (Tchang et al., 1988). Gaps introduced for optimal alignment are indicated by dashes and question marks represent unknown residues. The consensus motif represents conserved residues in all nsLTP sequences shown (excluding *Ace*-AMP1). Residues of *Ace*-AMP1 belonging to the same homology group as the consensus residues are indicated with vertical bars, and nonconserved amino acids are indicated with asterisks.

*F. culmorum*. To assess the antimicrobial activity spectrum of *Ace*-AMP1, its inhibitory activity was tested on various fungi and bacteria. Table III shows the  $IC_{50}$  values of *Ace*-AMP1 on 12 different plant pathogenic fungi, where they are compared with those determined under the same conditions for three other nsLTPs of seed origin, namely *Rs*-nsLTP (from radish, *Raphanus sativus*), *Ta*-nsLTP (from wheat, *Triticum aestivum*), and *Zm*-nsLTP (from maize, *Zea mays*). In each case the antifungal activity was tested in a defined synthetic growth medium with low ionic strength SMF<sup>-</sup> (see "Materials and Methods" for composition) and the same medium supplemented with 1 mM CaCl<sub>2</sub> and 50 mM KCl (SMF<sup>+</sup>). Both in media SMF<sup>-</sup> and SMF<sup>+</sup>, *Ace*-AMP1 inhibited all 12 tested fungi at concentrations equal to or below 10  $\mu\text{g mL}^{-1}$  (corresponding to about 1  $\mu\text{M}$ ). In contrast, at the same concentrations ( $\leq 10 \mu\text{g mL}^{-1}$ ) *Rs*-nsLTP inhibited only two, and *Ta*-nsLTP and *Zm*-nsLTP inhibited none of the tested fungi in SMF<sup>-</sup>. No antifungal activity at all could be observed in SMF<sup>+</sup> for these nsLTPs at concentrations up to 100  $\mu\text{g mL}^{-1}$ .

The sensitivity of the antifungal activity of *Ace*-AMP1 to the presence of cations in the growth medium was assessed

by measuring the  $IC_{50}$  value of *Ace*-AMP1 in various media differing in ionic constitution (Table IV). For comparative purposes, *Ac*-AMP1, an antimicrobial peptide from *Amaranthus caudatus* seeds (Broekaert et al., 1992), and  $\beta$ -purothionin, an antifungal protein from wheat seeds (Fernandez de Caleyra et al., 1972; Cammue et al., 1992), were also included in these tests. The antifungal activity of *Ace*-AMP1 was slightly stimulated in the presence of 50 mM K<sup>+</sup>, 50 mM Na<sup>+</sup>, 50 mM NH<sub>4</sub><sup>+</sup>, 5 mM Mg<sup>2+</sup>, or 5 mM Ba<sup>2+</sup>, and the activity decreased 2-fold in the presence of 5 mM Ca<sup>2+</sup>.  $\beta$ -Purothionin responded in a similar way, except that its activity was reduced by almost 10-fold in the presence of 5 mM Ca<sup>2+</sup>. In contrast, the activity of the amaranth protein *Ac*-AMP1 was strongly decreased in the presence of all tested cations.

The antibacterial activities of *Ace*-AMP1, *Rs*-nsLTP, *Ta*-nsLTP, and *Zm*-nsLTP are summarized in Table V. *Ace*-AMP1 inhibited growth of both Gram-positive bacteria tested (*B. megaterium* and *S. lutea*) but had no effect on any of the seven different Gram-negative bacteria tested. *Rs*-nsLTP and *Zm*-nsLTP were inhibitory only to *B. megaterium*, but their  $IC_{50}$  values were 25- and 75-fold higher than

**Table III.** Antifungal activity of *Ace-AMP1*, *Rs-nsLTP*, *Ta-nsLTP*, and *Zm-nsLTP*

Fungus	IC <sub>50</sub> (μg mL <sup>-1</sup> )							
	<i>Ace-AMP1</i>		<i>Rs-nsLTP</i> <sup>a</sup>		<i>Ta-nsLTP</i>		<i>Zm-nsLTP</i>	
	SMF-	SMF+	SMF-	SMF+	SMF-	SMF+	SMF-	SMF+
<i>A. brassicicola</i>	2.5	1.5	48	500	>200	>200	>200	>200
<i>A. pisi</i>	1	10	41	700	>200	>200	>200	>200
<i>B. cinerea</i>	3	7	45	680	N.D. <sup>b</sup>	N.D.	N.D.	N.D.
<i>C. lindemuthianum</i>	1.5	1.5	25	>1000	>200	>200	>200	>200
<i>F. culmorum</i>	6	10	20	520	>200	>200	200	>200
<i>F. oxysporum</i> f.sp. <i>pisi</i>	3.5	4	58	900	>200	>200	200	>200
<i>F. oxysporum</i> f.sp. <i>lycopersici</i>	3	10	54	>1000	>200	>200	200	>200
<i>N. haematococca</i>	3.5	7	100	>1000	>200	>200	60	>200
<i>P. betae</i>	1.5	7	18	750	>200	>200	150	>200
<i>P. tritici-repentis</i>	3	3.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
<i>P. oryzae</i>	3	7	10	>1000	N.D.	N.D.	N.D.	N.D.
<i>V. dahliae</i>	0.25	0.5	7	135	>200	>200	200	>200

<sup>a</sup> Data from Terras et al. (1992a).<sup>b</sup> N.D., Not determined.

that of *Ace-AMP1*, respectively. No antibacterial activity was detected for *Ta-nsLTP* at the highest concentration tested (200 μg mL<sup>-1</sup>).

In addition, the specificity of the antibiotic activity of *Ace-AMP1* was addressed by testing its effect on human erythrocytes and on cell cultures of human skin muscle fibroblasts. When added at concentrations up to 200 μg mL<sup>-1</sup>, *Ace-AMP1* did not cause lysis of erythrocytes, nor did it affect the viability of the cultured fibroblasts. Similarly, *Rs-nsLTP*, *Ta-nsLTP*, and *Zm-nsLTP* did not show any adverse effects in these tests when included at up to 200 μg mL<sup>-1</sup>.

#### Lipid Transfer Activity of *Ace-AMP1*

Because of the homology at the amino acid sequence level between *Ace-AMP1* and plant nsLTPs, it was worthwhile to test whether *Ace-AMP1* is capable of transferring lipids from liposomes to mitochondria in vitro, an activity associated with nsLTPs. To our surprise, however, we could not detect any lipid transfer activity for either phosphatidylcholine or phosphatidylinositol associated with *Ace-AMP1*, even when included at a concentration of 100 μg mL<sup>-1</sup> in the assay mixture. Under the same assay conditions, *Rs-nsLTP* and *Zm-nsLTP* transferred phosphatidylcholine at 3.2 and 6.8 nmol min<sup>-1</sup> mg<sup>-1</sup> protein, respectively, and transferred phosphatidylinositol at 5.4 and 3.1 nmol min<sup>-1</sup> mg<sup>-1</sup> protein, respectively.

#### DISCUSSION

Based on amino acid sequence homology, *Ace-AMP1* can be classified as a member of the nsLTP protein family. On the other hand, this protein distinguishes itself from regular nsLTPs by several features. *Ace-AMP1* was found to be unable to transfer either phosphatidylcholine or phosphatidylinositol from liposomal to mitochondrial membranes, the canonical activity of true nsLTPs. However, it cannot be excluded that *Ace-AMP1* can transfer lipids other than the phospholipids tested, thereby exhibiting a binding specificity that differs from that of true nsLTPs. The inability of *Ace-AMP1* to transfer phosphatidylcholine may be the result of deviations from the nsLTP consensus at the amino acid sequence level. For instance, the Asp at position 48, which is conserved in all known nsLTPs, is substituted by a Leu in *Ace-AMP1*. According to current models of the nsLTP-lipid interaction (Tchang et al., 1988; Madrid and von Wettstein, 1991; Désormaux et al., 1992), the conserved Asp could well be involved in the stabilization of the positively charged amino function in the polar head of phosphatidylcholine. This assumption, however, awaits further detailed studies of the nsLTP-phospholipid complex by NMR or crystallography. On the other hand, *Ace-AMP1* was found to be unable to transfer the acidic phospholipid phosphatidylinositol, which indicates that other residues involved in phospholipid binding by nsLTPs may be altered in *Ace-AMP1*.

In contrast to all nsLTPs or nsLTP-like proteins characterized to date, which are all translated as preproteins

**Table IV.** Antifungal activity of *Ace-AMP1* in the presence of different cations

Protein	IC <sub>50</sub> (μg mL <sup>-1</sup> ) <sup>a</sup>						
	SMF-	+50 mM K <sup>+</sup>	+50 mM Na <sup>+</sup>	+50 mM NH <sub>4</sub> <sup>+</sup>	+5 mM Mg <sup>2+</sup>	+5 mM Ba <sup>2+</sup>	+5 mM Ca <sup>2+</sup>
<i>Ace-AMP1</i>	3	2	2	1.5	2	2	6
<i>Ac-AMP1</i>	4	100	100	50	>200	>200	>200
β-Purothionin	4	2	3	2	2	2.5	35

<sup>a</sup> The IC<sub>50</sub> values of *Ace-AMP1*, *Ac-AMP1*, and β-purothionin on *F. culmorum* were determined in a synthetic growth medium (SMF-) and in SMF- supplemented with the chloride salts of the indicated cations at the indicated concentrations.



**Table V.** Antibacterial activity of *Ace*-AMP1, *Rs*-nsLTP, *Ta*-nsLTP, and *Zm*-nsLTP

Bacterium	IC <sub>50</sub> (μg mL <sup>-1</sup> )			
	<i>Ace</i> -AMP1	<i>Rs</i> -nsLTP	<i>Ta</i> -nsLTP	<i>Zm</i> -nsLTP
<i>B. megaterium</i>	0.8	20	>200	60
<i>S. lutea</i>	8	>200	>200	>200
<i>A. tumefaciens</i>	>200	N.D.	N.D. <sup>a</sup>	N.D.
<i>A. eutrophus</i>	>200	N.D.	N.D.	N.D.
<i>A. brasilense</i>	>200	N.D.	N.D.	N.D.
<i>E. carotovora</i>	>200	>200	>200	>200
<i>E. coli</i>	>200	N.D.	N.D.	N.D.
<i>P. solanacearum</i>	>200	N.D.	N.D.	N.D.
<i>P. syringae</i>	>100	>200	>200	>200

<sup>a</sup> N.D., Not determined.

(Madrid and von Wettstein, 1991; Kader, 1993), *Ace*-AMP1 appears to be synthesized as a preproprotein. Indeed, the amino acid sequence deduced from the *Ace*-AMP1 cDNA predicts the presence of 12 amino acids at the C terminus that are absent from the mature protein. This C-terminal propeptide is rich in hydrophobic and acidic residues, a characteristic feature of C-terminal propeptides present in the precursors of many vacuolar plant proteins (Nakamura and Matsuoka, 1993). Such C-terminal propeptides have, in a number of cases, been demonstrated to be determinants for sorting of the protein to the vacuole (Bednarek and Raikhel, 1991; Neuhaus et al., 1991). The subcellular location of *Ace*-AMP1, however, remains to be determined. The cDNA-deduced amino acid sequence of *Ace*-AMP1 also shows a putative *N*-glycosylation site at the C-terminal Gln residue of the mature domain. However, it is highly unlikely that mature *Ace*-AMP1 is actually glycosylated, since the C-terminal Gln of the mature protein was clearly revealed by amino acid sequence analysis (which would not be the case if it were glycosylated) and since we have failed to detect covalently linked carbohydrate in purified *Ace*-AMP1.

The other striking finding of our study is the fact that *Ace*-AMP1 displays very strong antimicrobial activity, whereas *Zm*-nsLTP and *Ta*-nsLTP are virtually devoid of antimicrobial activity. *Ace*-AMP1 is also more potent than *Rs*-nsLTP, a recently described nsLTP-like antimicrobial protein from radish seeds (Terras et al., 1992a). Indeed, *Ace*-AMP1 inhibits all 12 tested fungi at concentrations equal to or below 10 μg mL<sup>-1</sup> both in the low ionic strength medium SMF<sup>-</sup> and in the medium ionic strength medium SMF<sup>+</sup>. In contrast, *Rs*-nsLTP inhibits only two of the same 12 fungi in SMF<sup>-</sup> at concentrations equal to or below 10 μg mL<sup>-1</sup>, whereas none of these fungi are inhibited in SMF<sup>+</sup> at concentrations below 100 μg mL<sup>-1</sup> (Terras et al., 1992a). The IC<sub>50</sub> value of nsLTP-like proteins isolated from barley leaves (including CW18 and CW21, see Fig. 5) on the fungus *N. haematococca* (syn. *Fusarium solani*) varied from approximately 25 to 180 μg mL<sup>-1</sup>, depending on the isoform, when assessed in potato dextrose broth medium (Molina et al., 1993). However, the activity of these proteins on other fungi and their sensitivity to cations remains to be determined. On the other hand, the barley leaf nsLTPs

were active against at least one Gram-negative bacterium (*P. solanacearum*), and we were unable to detect any activity against a number of Gram-negative bacteria for *Ace*-AMP1, *Rs*-nsLTP, *Ta*-nsLTP, or *Zm*-nsLTP (see Table V).

The function of nsLTPs is still a matter of controversy. The original hypothesis that nsLTPs play a role in the intracellular trafficking of phospholipids from the ER to other cellular components (Arondel and Kader, 1990; Kader, 1993) has been contradicted by recent findings that at least some nsLTPs occur predominantly in specific cell types, most often the peripheral cell layer of an organ (Koltunow et al., 1990; Sossountzov et al., 1991; Sterk et al., 1991; Fleming et al., 1992; Molina and Garcia-Olmedo, 1993; Thoma et al., 1994) and, moreover, appear to be located extracellularly (Sterk et al., 1991; Molina and Garcia-Olmedo, 1993; Thoma et al., 1993; Pyee et al., 1994). These findings have led to a reformulation of the original hypothesis, i.e. that nsLTPs play a role in the transport of fatty acid-type cutin precursors to the extracellular space (Sterk et al., 1991), which is corroborated by the observation that the carrot nsLTP binds fatty acids and fatty acid-CoA esters to some extent (Meijer et al., 1993) and that the major protein in the surface wax of broccoli leaves is an nsLTP-like protein (Pyee et al., 1994). Alternatively, it has been suggested that nsLTPs may play a role in host defense (Terras et al., 1992a; Molina et al., 1993; Segura et al., 1993), based on the antimicrobial properties they exert in vitro. The observation that barley leaf nsLTP-like proteins with antimicrobial properties are up-regulated in response to challenge by a fungal pathogen provides indirect support for the latter hypothesis (Molina and Garcia-Olmedo, 1993). We have now demonstrated that not all nsLTPs possess antimicrobial properties, which implies that a defensive function does not apply for all nsLTP types. It is possible, however, that some nsLTP types or isoforms take part in defense reactions, whereas others fulfill a role in the deposition of extracellular lipids such as cutin monomers. Moreover, as originally suggested by Terras et al. (1992a), these functions are not mutually exclusive, since some nsLTPs may exert their defensive role after being deposited in the cell wall together with the transported cutin precursors. Hence, the antimicrobial properties of some nsLTPs may be regarded as an acquired secondary function that confers increased fitness on the plant.

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