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A Novel Family of Small Cysteine-rich Antimicrobial Peptides from Seed of *Impatiens balsamina* Is Derived from a Single Precursor Protein*

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Four closely related peptides were isolated from seed of *Impatiens balsamina* and were shown to be inhibitory to the growth of a range of fungi and bacteria, while not being cytotoxic to cultured human cells. The peptides, designated Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, are 20 amino acids long and are the smallest plant-derived antimicrobial peptides isolated to date. The Ib-AMPs (*I. balsamina* antimicrobial peptides) are highly basic and contain four cysteine residues which form two intramolecular disulfide bonds. Searches of protein data bases have failed to identify any proteins with significant homology to the peptides described here. Characterization of isolated cDNAs reveals that all four peptides are encoded within a single transcript. The predicted Ib-AMP precursor protein consists of a prepeptide followed by 6 mature peptide domains, each flanked by propeptide domains ranging from 16 to 35 amino acids in length. Such a primary structure with repeated alternating basic mature peptide domains and acidic propeptide domains has, to date, not been reported in plants.

An increasing number of cysteine-rich antifungal and antimicrobial peptides have been isolated from plants and in particular from plant seed. These peptides may have an important role to play in the protection of plants from microbial infection, and they could prove to be useful tools for the genetic engineering of fungal resistance in transgenic plants (1). Based on amino acid sequence homology, these peptides fall into at least six different classes. They include peptides isolated from seed of *Mirabilis jalapa* (1), *Amaranthus caudatus* (3), and *Zea mays* (4), members of the thionin family of peptides (5), members of the lipid transfer proteins (6–8), and members of the plant defensins (9–14).

From extracts of seed of *Impatiens balsamina*, we have isolated four small peptides the amino acid sequences of which are very closely related to each other but that do not resemble any peptides previously characterized from plants or other organisms. This paper describes the purification of the peptides and reports on their antimicrobial properties, in particular with respect to the inhibition of the growth of plant pathogenic fungi. Furthermore, a single class of cDNA has been identified that encodes all four members of this family of peptides as part of a preproprotein. Details of the characterization of the unusual structure of this cDNA and its products, as well as of their expression patterns, are presented.

EXPERIMENTAL PROCEDURES

Biological Materials—Seeds of *I. balsamina* were purchased from Sandeman Seeds (Pulborough, United Kingdom). Fungi and bacteria were grown and maintained as described previously (14, 15). The following fungal strains were used: *Alternaria longipes* (CBS62083); *Botrytis cinerea* (K1147); *Cladosporium sphaerospermum* (K0791); *Colletotrichum gloeosporioides* (SR24BTA); *Fusarium culmorum* (K0311); *Gloeodes pomigena* (field isolate; T. Sutton); *Gloeosporium solani* (CBS19432); *Nectria galligena* (MUCL6128); *Penicillium digitatum* (K0879); *Phialophora malorum* (field isolate; D. Sugar); *Sclerotinia sclerotiorum* (SES A); *Trichoderma viride* (K1127); and *Verticillium alboatrum* (K0937). The following Gram-positive bacterial strains were used: *Bacillus subtilis* (JHCC 55331); *Micrococcus luteus* (ATCC 9341); *Staphylococcus aureus* (ATCC 25923); *Streptococcus faecalis* (ATCC 29212); and the following Gram-negative bacterial strains: *Erwinia amylovora* (CFBP1430); *Escherichia coli* (HB101); *Proteus vulgaris* (JHCC 558711); *Pseudomonas solanacearum* (R48/a); *Xanthomonas campestris* pathovar *pelargonii* (INRA 10342); and *Xanthomonas oryzae* (ETH 698).

Extraction of Peptides—The purification of antimicrobial peptides from the basic protein fraction of *I. balsamina* seed was essentially as described previously (10). One-kilogram amounts of seed were ground in a coffee mill, and protein was extracted by stirring overnight at 4 °C in extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, pH 7). Ammonium sulfate was added to 80% relative saturation, and precipitated proteins were collected by centrifugation, resuspended in distilled water, and extensively dialyzed against distilled water using 2000-Da cutoff dialysis tubing (Sigma). The extract was adjusted to 50 mM NH_4Ac (pH 9) and passed over a Q-Sepharose Fast Flow column (12 × 5 cm, Pharmacia) equilibrated in 50 mM NH_4Ac (pH 9). The unbound fraction represents the basic protein fraction, and this was adjusted to pH 6 with acetic acid and passed over a S-Sepharose Fast Flow column (10 × 2.6 cm, Pharmacia) equilibrated in 50 mM NH_4Ac (pH 6). Bound proteins were eluted with a linear gradient of 50 mM–1.5 M NH_4Ac (pH 6) over 325 min at a flow rate of 3 ml/min. Proteins were monitored by the on-line measurement of the absorbance at 280 nm. Fractions with the highest antifungal activity were pooled for each peak and further purified by RP-HPLC[1] on a Pep-S column (C_2/C_{18} silica, 25 × 0.93 cm, Pharmacia). Peptides were eluted with linear gradients of 0.1% (v/v) trifluoroacetic acid to 99.9% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid over 100 min at a flow rate of 3 ml/min. Elution of peptides was monitored by absorbance at 210 nm.

Electrophoresis and Amino Acid Sequencing—The purified peptides were analyzed by SDS-PAGE on precast high density gels (PhastSys-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y14369.

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[1] The abbreviations used are: RP-HPLC, reversed phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AMP, antimicrobial peptide; Ib, *Impatiens balsamina*; SSPE, saline/sodium/phosphate/EDTA.

tem, Pharmacia), the sample buffer containing 200 mM Tris-HCl (pH 8.3), 1% (w/v) SDS, 1 mM EDTA, 0.005% (w/v) bromphenol blue, and 1% (w/v) dithiothreitol. Peptides were diffusion blotted onto 0.2-μm pore size nitrocellulose (Schleicher & Schuell) and visualized by silver staining (16). For N-terminal amino acid sequence analysis, peptides were reduced and cysteine residues were alkylated with 4-vinylpyridine prior to digestion with either trypsin (EC 3.4.21.4) or chymotrypsin (EC 3.4.21.1), both from Promega. Following protease digestion, peptide fragments were separated by RP-HPLC (Pep-S column, C_2/C_{18} silica, 25 × 0.93 cm, Pharmacia) and eluted with linear gradients of 0.1% (v/v) trifluoroacetic acid to 99.9% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid over 100 min at a flow rate of 3 ml/min. Peptide fragments were subjected to N-terminal sequencing by automated Edman degradation using a 477A Protein Sequencer (Applied Biosystems).

Mass Spectrometry—Matrix-assisted laser desorption/ionization-time of flight mass spectrometry was performed by M-Scan Ltd. (Ascot, UK) using a PerSeptive Biosystems Voyager™ Elite Biospectrometry ™ Research Station laser-desorption mass spectrometer coupled with Delayed Extraction™.

Antifungal and Antibacterial Assays—Antifungal and antibacterial assays were conducted as described previously (11, 14). The growth medium for the antifungal assays was either potato dextrose broth (Difco) at 12 g/liter (medium A), or medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl (medium B).

Antibacterial assays were carried out in 1% (w/v) tryptone (Sigma), 0.5% (w/v) low melting point agarose for *B. subtilis, Escherichia coli*, *M. luteus, Proteus vulgaris, Staphylococcus aureus,* and *Streptococcus faecalis* and in 1% (w/v) Bacto-Peptone (Difco), 0.5% (w/v) low melting point agarose for *Erwinia amylovora, Pseudomonas solanacearum, X. campestris*, and *X. oryzae*. Magainin I (Sigma) was used as a positive control in the antibacterial assays. Growth in each assay was assessed after the appropriate incubation period (48 h at 24 °C for fungi and 24 h at 28 °C for bacteria).

Human Cell Membrane Integrity Assay and Hemolytic Activity Assay—Membrane integrity of cultured human cells was tested on skin muscle diploid fibroblasts using the neutral red uptake method as described previously (10). Hemolytic activity was assayed using human blood group A erythrocytes as described previously (10) except that the erythrocytes were suspended in phosphate-buffered saline instead of 150 mM myo-inositol.

Construction and Screening of cDNA Library—Total RNA was purified from dry seed of *I. balsamina* by grinding in liquid nitrogen, extraction with phenol/cresol and phenol/chloroform, followed by lithium chloride precipitations (17). Poly(A)$^+$ RNA was isolated by a Poly(A)Ttract mRNA Isolation System utilizing magnetic beads (Promega). cDNA was synthesized using a ZAP-cDNA synthesis kit (Stratagene) and, following the ligation of *Eco*RI linkers and size fractionation on a Sephacryl S-400 column (Pharmacia), a library constructed in λ-ZAP (Stratagene). A DNA probe for screening the cDNA library was generated by performing PCR on the above seed cDNA fractions using a pair of degenerate oligonucleotide PCR primers based on reverse translation of the available amino acid sequence of Ib-AMP1 peptide. The degenerate primers utilized were IbAMP1-C (5′-GITG^T/$_C$TG^T/$_C$GGITGGGGICC-3′) and IbAMP1-B (5′-CACCAIC^T/$_G$IAC^G/$_A$CA^G/$_A$TA-3′), where I represents an inosine residue. The resulting 46-base pair PCR product was eluted from an acrylamide gel, purified using a Mermaid Kit (Bio101), and labeled with [α-^{32}P]dCTP using a Random Prime DNA Labeling Kit (Boehringer). The labeled PCR product was used to screen the cDNA library by plaque hybridization, following the transfer of near-confluent plaques to nylon membrane (Hybond-N, Amersham) and the UV cross-linking of DNA. Nonstringent hybridization was performed in 0.25% (w/v) Marvel (Premier Beverages, UK), 5× SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, pH 7.4), 0.1% (w/v) SDS at 35 °C and washing in 6 × SSC followed by 3 × SSC at 42 °C (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). A total of 12 hybridizing plaques were purified and subsequently *in vivo* excised to generate subclones in pBluescript SK (−) phagemid vectors (Stratagene). Sequencing was performed using a Sequenase kit (United States Biochemical). Nucleotide sequence assembly and analysis was performed using PC/GENE and IG-SUITE Intelligenetics software.

Northern and Western Blot Analysis—Seed representing a range of developmental stages was harvested from glasshouse-grown plants. Developing seed was arbitrarily classified into 5 stages based on morphology and size: stage 1 = white seed, ~1 mm in diameter; stage 2 = pale green/white, 2 mm; stage 3 = pale green/white dry, 3 mm; stage 4 = gray, dry, 4 mm; and stage 5 = brown seed coat, 3–5 mm. For germination studies, seed were germinated on damp filter paper at 28 °C in the dark, and material was harvested at 24-h intervals.

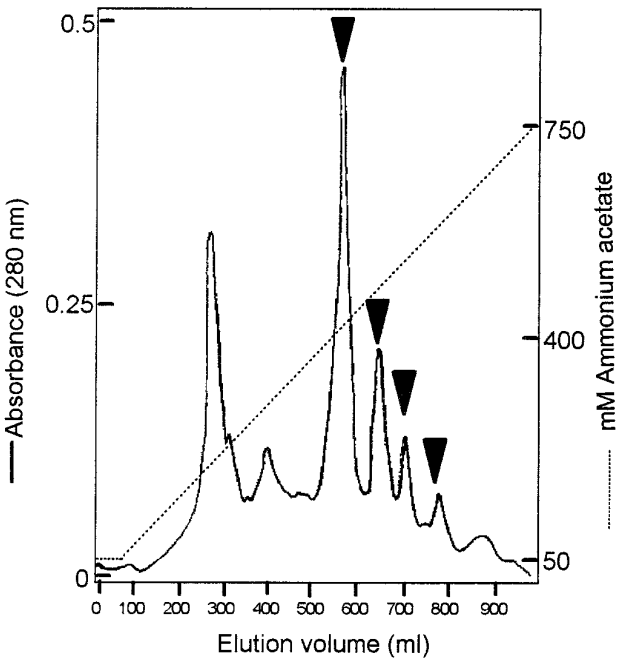

FIG. 1. **Separation of proteins in the basic protein fraction from seed of *I. balsamina*.** The basic protein fraction was loaded on a S-Sepharose high performance cation-exchange column in equilibrium with 50 mM NH_4Ac at pH 6. The column was washed with 50 mM NH_4Ac until the absorbance at 280 nm dropped below 0.01 absorbance unit, and the bound fraction eluted at 3 ml/min with a linear gradient of 50–750 mM NH_4Ac at pH 6 over 325 min. The eluate was monitored for proteins at 280 nm and collected in 10-ml fractions. Aliquots from each fraction were freeze-dried, resuspended in sterile water, and assayed for antifungal activity using a *F. culmorum* spore germination assay. Peaks of absorbance at 280 nm that correspond to fractions with antifungal activity when tested at 100 μg of total protein/ml or below in the assays are *arrowheaded*.

For Northern analysis, total RNA was isolated from seed as described by Jepson *et al.* (17) and blotted from a 1.5% (w/v) agarose gel onto Hybond-N membrane (Amersham). Hybridization was performed in 0.25% (w/v) Marvel (Premier Beverages, UK), 5 × SSPE, 0.01% (w/v) SDS at 65 °C and washing in 0.2 × SSC, 0.1% SDS at 65 °C. The [α-^{32}P]dCTP-labeled probe used for these studies was a purified DNA fragment representing the full length of the cDNA insert. The signal was detected by autoradiography.

For immunoblot analysis, total protein extracts were prepared from seed as described by Terras *et al.* (1). Two μg of total protein were electroblotted from a 15% nonreducing SDS-PAGE gel onto 0.2-μm pore size nitrocellulose membrane (Schleicher & Schuell) as described (1). The antiserum used had been raised in rabbits against bovine serum albumin-conjugated Ib-AMP1 peptide. Detection was performed using an enhanced luminescence method (Amersham) as described (1).

RESULTS

Purification and Primary Structure Determination—The basic proteins from *I. balsamina* seed were fractionated by cation exchange chromatography, and fractions assayed for antifungal activity against *F. culmorum* spores. Following chromatography, the extract yielded four peaks of activity eluting between 400 mM and 700 mM NH_4Ac (Fig. 1, *arrowheaded*). Fractions from each peak that showed the highest levels of activity were pooled and further purified by preparative RP-HPLC. Each pooled fraction yielded a single peak of absorbance at 210 nm which exactly matched the antifungal activity eluting from the column (data not shown). These active peaks were designated Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, respectively, according to the order of their elution from the cation exchange column.

The purified active fractions were further analyzed by SDS-

PAGE, and each was shown to contain a peptide of ~2–3 kDa (Fig. 2). The peptides ran identically whether reduced or unreduced prior to loading on the gels (data not shown), indicating that they are each composed of a single polypeptide chain.

Initial attempts to sequence the Ib-AMPs by automated Edman degradation indicated that all four peptides were N-terminally blocked. To obtain their sequences, each peptide was digested with either trypsin or chymotrypsin following modification of cysteine residues with 4-vinylpyridine, and the resulting fragments were purified by RP-HPLC prior to sequencing. The following partial amino acid sequences were generated for peptides Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, respectively: GRRCCGWGPGRRYCVRW*X*, GRRCCN*x*GPGRRYCKRWC, RHRCCAWGPGRKYCKRWC, and GRRCCGWGPGRRYCRRWC. Residue 7 of Ib-AMP2 could not be unambiguously identified as one of the common amino acids. Clearly, all four peptides are very close homologues of each other, with as little as one, and no more than five, amino acid differences between any pairwise alignment of the above sequences.

Electrospray Mass Spectrometry—The molecular mass of each of the purified Ib-AMP peptides was experimentally determined by electrospray mass spectrometry as 2464.6, 2527.4, 2536.6, and 2522.6 Da for Ib-AMP1, -2, -3, and -4, respectively (data not shown). These molecular mass determinations are consistent with the estimation based on SDS-PAGE.

Antifungal Activity of the Ib-AMPs—The antifungal activity of the purified peptides was assessed on 13 fungal strains, many of which are plant pathogens of significant importance to agriculture, using a standard antifungal activity assay (14). In medium A, all four peptides showed similar levels of broad spectrum activity (Table I, medium A). For the majority of the assays in this medium, the IC_{50} values were <10 μg/ml. The antifungal activity of the peptides is, however, sensitive to the ionic strength of the assay medium and in the same medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl, the activity of Ib-AMP1, Ib-AMP2, and Ib-AMP3 is severely reduced (Table I, medium B). Only Ib-AMP4 maintains any significant inhibitory activity even though its activity is also markedly reduced. On some fungi, notably *F. culmorum*, the Ib-AMPs cause a very distinct swelling and hyperbranching in the spore germination assay at subinhibitory rates (Fig. 3*A*). The Ib-AMPs also inhibit the growth of germlings, and in the case of Ib-AMP4 this is also apparent in medium B (Table I). On germlings, the Ib-AMPs cause swelling and branching along the length of hyphae and at the hyphal tip (Fig. 3*B*).

Antibacterial Assays—In addition to their broad spectrum antifungal activity, the Ib-AMPs are also inhibitory to the growth of a range of bacteria, especially Gram-positive bacteria (Table II). On the Gram-positive bacteria tested, the IC_{50} values of Ib-AMP4 are lower than those obtained with the antibiotic peptide magainin I (18).

Human Cell Integrity Assays—The effect of Ib-AMP2 and Ib-AMP4 on human erythrocytes and cell cultures of human

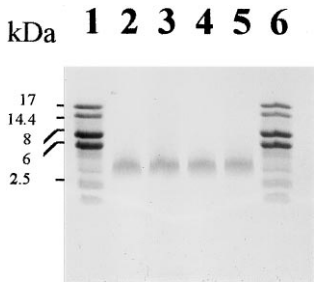

FIG. 2. **SDS-PAGE analysis of the purified Ib-AMPs.** 200 ng of each peptide were dissolved in reducing sample buffer and separated on a High Density Phastgel (Pharmacia). Peptides were transferred to nitrocellulose by capillary blotting and visualized by silver staining. *Lanes 1* and *6*, myoglobin fragments with molecular masses in kDa as indicated; *lane 2,* Ib-AMP1; *lane 3,* Ib-AMP2; *lane 4,* Ib-AMP3; *lane 5,* Ib-AMP4.

TABLE I
Antifungal activity of the Ib-AMP peptides

Protein concentrations required for 50% growth inhibition 48 h after addition were determined from dose-response curves. Proteins were added either directly to spores or to 24-h pregerminated mycelium (germlings). Assays were carried out either in medium A or in medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl (medium B).

Fungus	IC_{50}			
	Ib-AMP1	Ib-AMP2	Ib-AMP3	Ib-AMP4
			$\mu g/ml$	
Medium A, spores				
Alternaria longipes	3	12	6	3
Botrytis cinerea	12	25	6	6
Cladosporium sphaerospermum	1	6	3	1
F. culmorum	1	6	6	1
Penicillium digitatum	3	6	3	3
T. viride	6	12	12	6
V. alboatrum	3	12	6	6
Medium B, spores				
Alternaria longipes	50	>200	>200	12
Botrytis cinerea	>200	>200	>200	200
Cladosporium sphaerospermum	50	>200	100	6
F. culmorum	50	>200	100	6
Penicillium digitatum	200	>200	100	25
T. viride	>200	>200	>200	150
V. alboatrum	>200	>200	>200	50
Medium B, germlings				
Colletotrichum gloeosporioides	ND[a]	ND	ND	25
Gloeodes pomigena	ND	ND	ND	>100
Gloeosporium solani	ND	ND	ND	>100
Nectria galligena	ND	ND	ND	6
Phialophora malorum	ND	ND	ND	6
Sclerotinia sclerotiorum	ND	ND	ND	25

[a] ND, not determined.

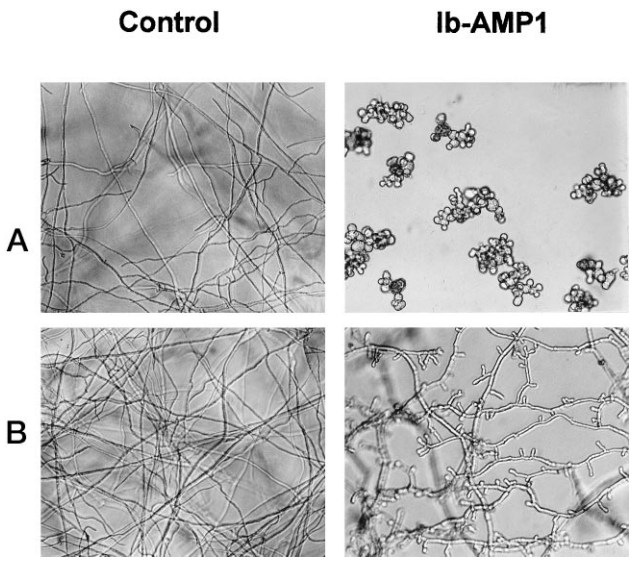

FIG. 3. **Morphological changes induced by Ib-AMP1 on partially inhibited *F. culmorum*.** Fungal spores were either germinated and grown in medium A in the presence of the peptides and observed after 24 h of incubation (*A*) or pregerminated for 24 h prior to the addition of the peptide and observed 8 h later (*B*). The concentration of Ib-AMP1 was 0.5 μg/ml in *A* and 5 μg/ml in *B*. Control incubations were carried out with the addition of water.

skin fibroblasts was investigated. At a concentration of 200 μg/ml, these peptides did not cause lysis of erythrocytes nor did they perturb membrane integrity of the cultured fibroblasts (data not shown).

cDNA Isolation and Characterization—A total of 12 cDNA clones were isolated from the dry seed cDNA library by hybrid-

TABLE II
Antibacterial activity of the Ib-AMP peptides

Protein concentrations required for 50% growth inhibition were determined after 24 h of incubation.

Bacteria	IC_{50}		
	Ib-AMP1	Ib-AMP4	Magainin I
		$\mu g/ml$	
Gram-positive bacteria			
Bacillus subtilis	10	5	20
Micrococcus luteus	10	5	20
Staphylococcus aureus	30	20	30
Streptococcus faecalis	6	5	20
Gram-negative bacteria			
Erwinia amylovora	ND[a]	>100	ND
Escherichia coli	>500	>500	ND
Proteus vulgaris	>500	>500	ND
Pseudomonas solanacearum	>500	>100	ND
X. campestris	ND	6	ND
X. oryzae	ND	15	ND

[a] ND, not determined.

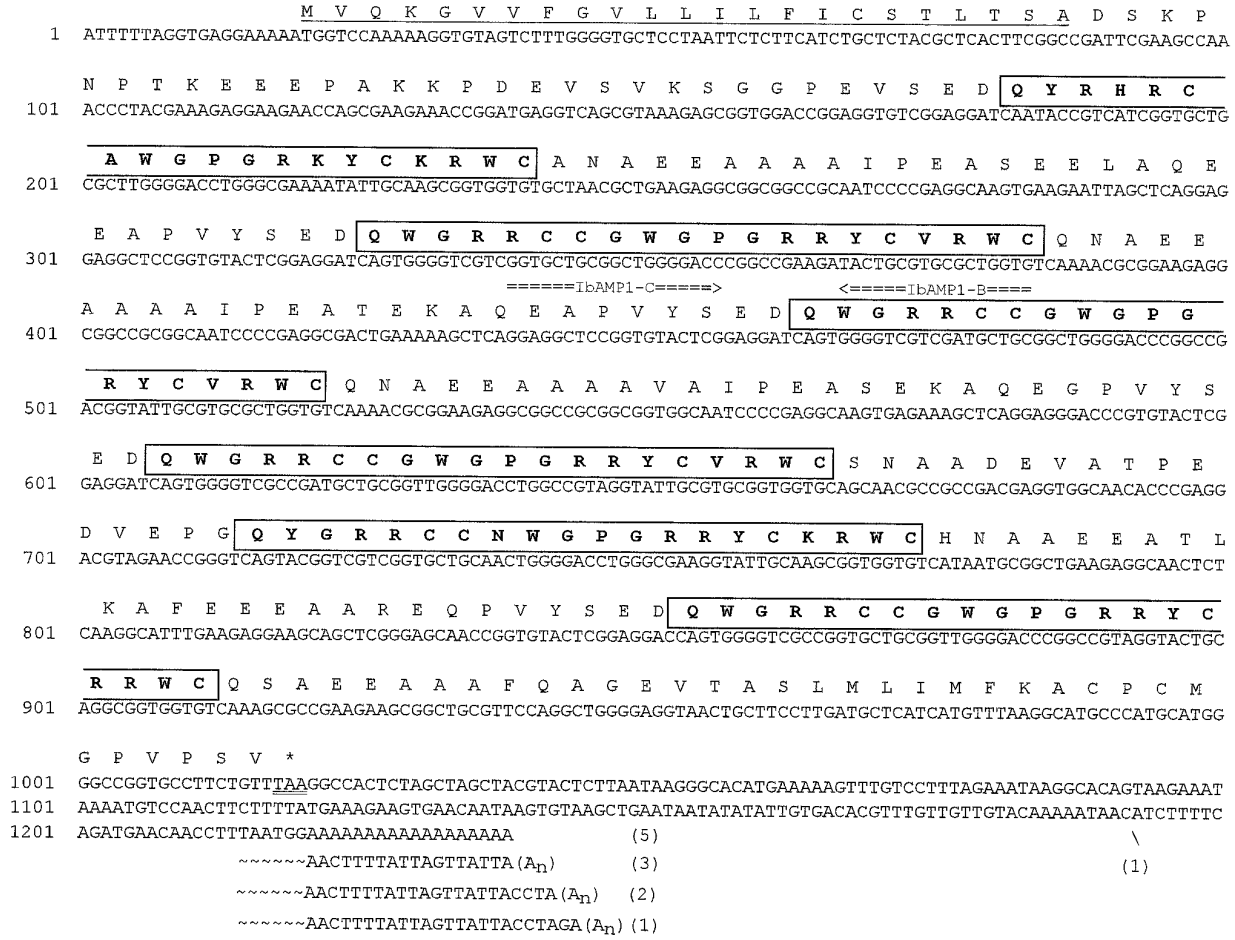

FIG. 4. **Complete nucleotide sequence of cDNA clone Ib22 insert and the predicted translation product.** Amino acid residues comprising the domains representing the mature Ib-AMP peptides are in *bold* and *boxed*; those comprising the predicted signal sequence are *underlined*. The predicted termination codon is *double underlined*. The DNA sequences immediately upstream of poly(A) tails in otherwise identical cDNA clones are shown. The number of independently isolated cDNA clones exhibiting these different sites of polyadenylation is indicated in *parentheses*. The annealing positions of the named degenerate oligonucleotide PCR primers used to generate the initial hybridization probe are indicated by *double arrows* underneath the first of the Ib-AMP1 repeat regions.

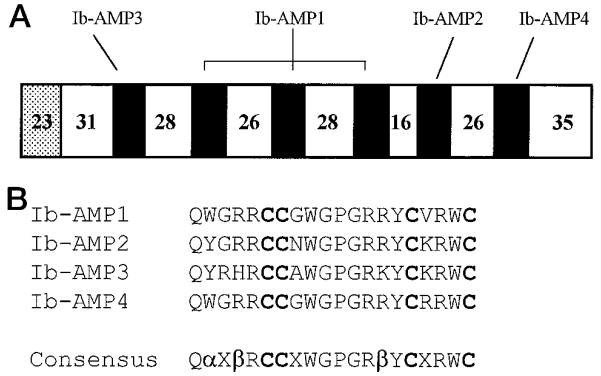

FIG. 5. **General gene structure and alignment of peptide sequences.** *A*, the generalized structure of the predicted 333 amino acid primary translation product in which the individual 20-amino acid domains representing the mature Ib-AMP peptides are *shaded*, with the Ib-AMP member indicated immediately above. The propeptide regions are *unshaded*, the predicted prepeptide (signal sequence) region is *hatched*, and the number of amino acids comprising each of these regions is indicated. *B*, amino acid sequence alignment of the predicted translation products of each mature domain. A consensus sequence for the Ib-AMPs appears below in which *upper case letters* represent invariant residues, α an aromatic residue, β a basic residue, and *X* a nonconserved residue. Cysteine residues are in *bold text.*

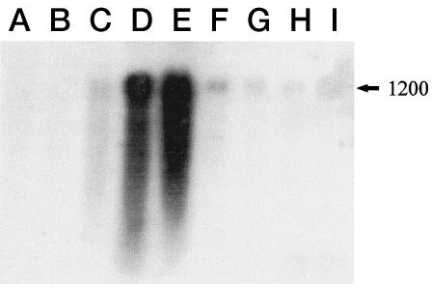

FIG. 6. **A Northern blot of total RNA from developing, mature, and germinating *I. balsamina* seed.** Total RNA isolated from developing seed was from the developmental stages described under "Experimental Procedures." Each lane on the 1.5% (w/v) agarose gel was loaded with 5 μg of total RNA. Northern blots were probed with radiolabeled fragment representing the insert of cDNA clone Ib22. The *arrow* indicates the length, in nucleotides, of the hybridizing band as determined by comparison with the migration of RNAs of known lengths (not shown). Sources of extracts on the gel were as follows: *lanes A-D*, seed at developmental stages 2–5, respectively; *lane E*, dry mature seed; *lanes F–I,* seed at 24, 48, 72, and 96 h postgermination, respectively.

ization, and the insert of the largest clone (Ib22) fully sequenced following *in vivo* excision of the phagemid. The 1238-base pair DNA sequence of the insert from this clone is presented in Fig. 4 and discussed further below. Approximately 500 base pairs of DNA sequence were obtained for each of the other cDNA clones. Although some clones appeared to be truncated at the 5′ end, the DNA sequence of each was identical to that of clone Ib22 in regions of overlaps, the only differences being apparently due to the exact position of polyadenylation. As indicated in Fig. 4, there appeared to be 5 different polyadenylation sites utilized among the 12 cDNA clones within a region spanning 52 base pairs of the 3′-untranslated region, the majority being at the same position as that of clone Ib22.

Clone Ib22 is considerably larger than would be anticipated for a cDNA encoding a single IbAMP peptide. In fact it contains an open reading frame encoding a predicted protein comprising 333 amino acids, with a molecular mass of 37,262 Da. Analysis of this predicted translation product reveals within it the presence of domains that correspond exactly in amino acid sequence to the four antimicrobial peptides described above. Ib-AMP1 is represented three times in consecutive repeats, the other Ib-AMPs once each. The six *boxed* regions in Fig. 4 represent, consecutively, Ib-AMP3, Ib-AMP1, Ib-AMP1, Ib-AMP1, Ib-AMP2, and Ib-AMP4. Hydropathy plots and sequence analysis (not shown) of the predicted translation product deriving from clone Ib22 predict a 23-amino acid N-terminal signal sequence, consistent with the fact that many plant antifungal peptides are extracellularly located (1). The general structure of the predicted translation product and an amino acid alignment of those regions assumed to encode the individual mature peptides are presented in Fig. 5.

The proprotein domains flanking each Ib-AMP peptide domain vary in length between 16 and 35 amino acids but display some degree of homology with each other, each containing at least five negatively charged amino acids, generally organized as doublets in the vicinity of the presumed cleavage sites (see Fig. 4). This composition is in contrast to the highly basic Ib-AMP domains, which are separated by these proprotein regions. Although there is no clear homology to other peptide sequences or processing sites in the data bases, it is assumed that these regions contain information required for the correct processing of the preproprotein into constituent mature peptides.

Analysis of Expression—To investigate the accumulation of Ib-AMP-related transcripts and protein during seed development, both Northern and Western blot analysis was performed on material isolated from developing, dry, and germinating seed. The hybridization pattern resulting from Northern blots probed with the entire insert of clone Ib22 is shown in Fig. 6. There appears to be hybridization to a single class of transcript of ~1200 nucleotides, which is in accordance with the size predicted from the cDNA sequence analysis of clone Ib22. The pattern of hybridization indicates that the highest accumulation of related transcript is found in dry seed (Fig. 6, *lane E*) and in the stage of development immediately prior to this (Fig. 6, *lane D*).

Western blots using Ib-AMP1 antibody indicate that immunoreactive material is most abundant in dry seed, from which the Ib-AMPs were originally isolated, and in seed undergoing the early stages of germination (Fig. 7, *lanes E, F,* and *G*). There appear to be significant quantities of such material also present in the developmental stage immediately preceding seed dry-down (Fig. 7, *lane D*). Immunoreactive material migrating at a higher position on the gel (Fig. 7, *lane C*) may represent unprocessed or incompletely processed precursor protein. An investigation into the processes involved in maturation of the precursor protein is currently under way.

DISCUSSION

Four closely related, small, basic, cysteine-rich peptides have been purified from seed of *I. balsamina* and shown to be active *in vitro* against a range of fungal and bacterial species. The majority of the amino acid sequence of the four peptides could be determined experimentally, although complete assignment was prevented because the N terminus of each was blocked. The molecular mass of Ib-AMP1 as determined by electrospray mass spectrometry indicated that the full-length Ib-AMP1 sequence was only 2 amino acids longer at its N terminus than the 18 amino acids assigned by direct amino acid sequencing. It can be predicted that the amino acid residue N-terminally adjacent to the sequenced region must be either a tryptophan or a tyrosine for chymotrypsin to have cleaved Ib-AMP1 in that position. Furthermore, based on the molecular mass estimation and the fact that the peptide is N-terminally blocked, it can be considered likely that the unassigned N-terminal residue is a cyclized glutamine. Both these speculations were indeed confirmed for all four Ib-AMP peptides by the subsequent analysis

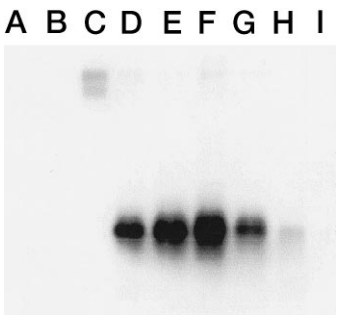

FIG. 7. **An immunoblot of protein extracts from developing, mature, and germinating *I. balsamina* seed.** Total protein was isolated from seed at the developmental stages described under "Experimental Procedures." Each lane on the 15% (w/v) nonreducing SDS-PAGE gel was loaded with 2 μg of total protein extract. Western blots were immunostained with an antiserum raised against Ib-AMP1. Sources of extracts on the gel were as follows: *lanes A–D*, seed at developmental stages 2–5, respectively; *lane E*, dry mature seed; *lanes F–I,* seed at 24, 48, 72, and 96 h postgermination, respectively.

of the cDNA clone reported here.

The molecular mass of each peptide determined experimentally by matrix-assisted laser desorption/ionization-time of flight electrospray analysis is consistently ~21 Da lower than that theoretically predicted for each by direct translation of the gene sequence. This difference is precisely accounted for if it is assumed that the N-terminal glutamine residue of each Ib-AMP peptide is cyclized and that the four internal cysteine residues form two disulfide bridges. The former supposition is supported by the fact that all classes of the Ib-AMP peptides were found to be N-terminally blocked when amino acid sequencing was attempted. The latter is supported by the fact that the native Ib-AMP peptides do not react with Ellman's free thiol reagent.

When the theoretical molecular masses for Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4 are adjusted to take into account the N-terminal cyclization and disulfide bridge formation, the respective values of 2464.3, 2527.4, 2536.5, and 2521.4 match the experimentally determined values of 2464.6, 2527.4, 2536.6, and 2522.6 Da almost exactly. This provides further evidence that the gene represented by the Ib22 cDNA does indeed encode all four classes of isolated peptides. Furthermore, these data confirm that the unassigned residue of Ib-AMP2 is a tryptophan, as predicted from the cDNA sequence. Interestingly, a residue of the plant defensin peptide *Br*-AFP2 could not be identified by direct sequencing (11) but might reasonably be assumed to be a tryptophan residue because of close homology to other Brassicaceae antifungal proteins.

The Ib-AMP family is clearly distinct from all other families of plant antimicrobial peptides reported previously (Ref. 19; Table III). Searches of protein data bases did not identify any peptides or proteins with significant homology to either the mature Ib-AMP peptides or the intervening propeptide regions. Limited homology was noted between the Ib-AMPs and the N-terminal region of the mature peptide domain of the eight-cysteine type plant thionins, particularly with respect to the cysteine residue pattern (Table III). This might indicate an evolutionary relationship between the Ib-AMP and thionin genes involving processes of truncation and duplication. In addition, the sequence Gly-Pro-Gly-Arg-Arg-Tyr, corresponding to the conserved residues 10–15 in Ib-AMP1, Ib-AMP2, and Ib-AMP4, was identified in a number of other proteins, notably viral proteins (22). In a number of these proteins, this sequence has been shown to form a β-turn, and it is possible that a similar turn is occurring at this sequence in Ib-AMP1.

All four Ib-AMPs are inhibitory to the growth of a wide range of filamentous fungi when assayed in medium A. When this medium is supplemented with $CaCl_2$ and KCl, only Ib-AMP4 retains any significant inhibitory activity, even though it differs from the more sensitive Ib-AMP1 by only a single amino acid residue substitution. A reduction in antifungal activity in media of increased ionic strength is a common feature of most of the small cationic peptides isolated to date and probably reflects the weakening of electrostatic interactions with the target rather than any alteration of the structure of the peptide by the binding of ions from the medium (11). This is supported by the fact that the degree of reduction in antifungal activity is dependent on the test fungus (Table I). Moreover, the finding that the only one of the four Ib-AMP peptides that remains active in medium B is the most basic homologue would also support this. On the whole, the antifungal activity of the Ib-AMPs compares favorably with the more active antifungal and antimicrobial peptides purified to date from plants (2–4, 8, 10, 11).

In addition to their antifungal activity, the Ib-AMPs were also inhibitory to the growth of the four Gram-positive bacteria that were tested and to the growth of two Gram-negative *Xanthomonas* species. In these assays, their activity was compared with the antibiotic peptide magainin I (18), and on all four of the Gram-positive bacteria tested the Ib-AMPs were equally active as or more active than magainin I. Few of the other antifungal peptides isolated to date from plants show significant levels of activity on bacteria (2, 14).

From the literature, it is apparent that peptides with very different functions can share a common structure. The α-conotoxins, which have been purified from marine snails, have a similar cysteine arrangement to the Ib-AMPs, although the spacing between the two C-terminal cysteines is different (23). The solution structure for α-conotoxin G1 has been determined using two-dimensional NMR and shows that the two disulfide bonds stabilize a nonrandom coil structure with two β-turns (24). Preliminary work involving the analysis of purified products released from unreduced Ib-AMP1 following digestion by trypsin showed that Cys_{16} could not be connected to Cys_{20} but rather that the C-terminal cysteine was connected to either Cys_6 or Cys_7 (data not shown). The two remaining possible combinations of pairwise connectivities (*i.e.* Cys_6-Cys_{16} and Cys_7-Cys_{20} or Cys_6-Cys_{20} and Cys_7-Cys_{16},) could not be resolved by protease digestion methods. However, NMR has since been used to determine a solution structure for Ib-AMP1, enabling a comparison with that of the α-conotoxins, and will be reported elsewhere.[2]

The amino acid sequence of each of the four Ib-AMP peptides isolated from dry seed can be identified within the predicted translation product of a single class of cDNA obtained from RNA also isolated from dry seed. One of the peptides is represented three times, the other three once. The fact that 12 individual cDNAs have essentially identical DNA sequences suggests that, at least in seed, only a single gene encoding such peptides is expressed. Southern blotting of genomic DNA suggests that there is only one gene (data not shown).

cDNAs encoding antibacterial peptides processed from a multipeptide precursor have been described previously, albeit not in plants. Inducible, proline-rich, 18-amino acid apidaecin peptides from bees are encoded by a family of cDNAs which contain up to 12 peptide repeats separated by well conserved "processing" regions (25). The cDNA sequence coding for *Xenopus* prepromagainins also has a multipeptide structure (26). In plants, there are two published examples of cDNAs encoding multipeptide precursors. The first well known example is that

[2] S. Patel and J. Thornton, personal communication.

TABLE III
Comparison of plant antifungal peptides

The size of the mature peptide (number of amino acids) and spacing of cysteine residues within its sequence is presented for Ib-AMP1 and representative members of other reported plant antifungal peptide families. Figures in the final column represent the number of amino acid residues flanking the cysteine residues indicated.

Peptide family	Representative member	Ref.	Size	Spacing of cysteine residues
Plant defensins	Rs-AFP2	1, 10	51	3-C-10-C-5-C-3-C-9-C-8-C-1-C-3-C
Knottin-type	Mj-AMP1	2	36	1-C-6-C-8-CC-3-C-10-C-3
Lipid transfer protein	Ace-AMP1	8	93	3-C-9-C-12-CC-18-C-1-C-23-C-15-C-4
Hevein-type	Ac-AMP2	3	30	3-C-4-C-4-CC-5-C-6-C-2
Macadamia	Mi-AMP1	20	76	10-C-9-C-1-C-25-C-14-C-11-C
Maize basic protein	MBP-1	4	33	6-C-3-C-13-C-3-C-4
Thionin (8-Cys type)	α-Purothionin	21	45	2-CC-7-C-3-C-8-C-3-C-1-C-8-C-6
Impatiens	Ib-AMP1	This paper	20	5-CC-8-C-3-C

of polyubiquitin cDNA, which encodes head to tail repeats of ubiquitin peptides, apparently without linker domains (27). The other example is that of a cDNA from *Nicotiana alata* encoding stigma proteinase inhibitor peptides separated from each other by dibasic dipeptide linkers (28). No structure similar to that of the Ib-AMP precursor, with acidic linker domains separating basic mature peptide domains, has yet been reported in plants. The mechanism of processing of the Ib-AMP preproprotein into its constituent mature peptides is not apparent from the inferred amino acid sequence of the translation product of the cDNA since no previously well characterized processing sites are apparent within the preproprotein on the basis of sequence homology alone. However, the presence of negatively charged amino acid doublets (EE or DE) at equivalent positions within each propeptide (including immediately upstream of five of the six mature peptide regions) might suggest the presence of specific processing sites.

The above examples of multiple mature peptides being generated from a single precursor differ in many respects from each other and from the Ib-AMP gene structure described here, possibly reflecting differences in the mechanism of processing. Nevertheless, it is reasonable to assume that concatenation of such small peptide domains is a general mechanism for enhanced simultaneous production of related peptides. Should these related peptides individually exhibit different activity profiles, such a mechanism would enable a broader activity spectrum to be achieved from a single gene transcript.

The mode of action of the Ib-AMPs is presently unknown. Of particular interest is whether the peptides are interacting directly with microbial membranes or whether they have a protein/receptor target. Even at very high rates (500 μg/ml), the Ib-AMPs do not cause any visible cell lysis or membrane collapse on fungi. The peptides were shown not to affect human cells, and they are noncytotoxic to cultured insect and plant cells (data not shown). Taken together, these preliminary data suggest that the Ib-AMPs are not acting as ionophores but rather that they are inhibiting a distinct cellular process.

As a rough estimate from the yields obtained, the Ib-AMPs account for ~0.5% of the total protein in mature *I. balsamina* seed, where they may play a role in the defense of the germinating seed. Assuming that genes in dry seed are generally transcriptionally inactive, the patterns of hybridizing mRNA revealed by Northern blot hybridization analysis might indicate that the gene is highly transcribed immediately prior to seed maturation and that the RNA remains undegraded in dry seed. Within 24 h of imbibition, the RNA level is markedly decreased, whereas protein levels are at a peak, perhaps suggesting that RNA stored in dry seed is translated and rapidly degraded at the onset of germination. It is not known whether the expression of the Ib-AMP peptides is limited to the seed, but as in the case of other plant antimicrobial peptides (1, 29) the Ib-AMPs may also be present, perhaps inducibly, in other tissues of the plant and may play a more general role in protecting the plant from microbial infections.

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