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Characterization and cDNA cloning of two glycine- and histidine-rich antimicrobial peptides from the roots of shepherd's purse, *Capsella bursa-pastoris*

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Abstract

Two novel antimicrobial peptides were isolated and characterized from the roots of shepherd's purse, *Capsella bursa-pastoris*. These antimicrobial peptides, named shepherin I and shepherin II, consist of 28 and 38 amino acids, respectively, and are glycine- and histidine-rich peptides. Shepherin I and shepherin II have 67.9% and 65.8% (mol/mol) glycine, respectively, and 28.6% and 21.1% (mol/mol) histidine, respectively. Both shepherins have a Gly-Gly-His motif. These antimicrobial peptides exhibit antimicrobial activity against Gram-negative bacteria and fungi. Circular dichroism spectra of shepherin I and shepherin II showed that shepherin I and shepherin II in 50% trifluoroethanol have 66.7% and 75% random coils, respectively, without any α-helices. cDNA sequence analysis revealed that shepherin I and shepherin II are produced from a single polypeptide, designated shep-GRP, consisting of 120 amino acids; shep-GRP has five distinct domains, an amino-terminal putative signal peptide, a shepherin I, a linker dipeptide, a shepherin II and a carboxy-terminal peptide. Southern blot analysis indicates that the gene encoding shepherins belongs to a low-complexity gene family. Northern blot analysis revealed that transcripts of shep-GRP are present in roots but not in leaves and stems.

Introduction

Antimicrobial peptides have been recognized as important molecules in the host defense of diverse species (Boman, 1995). Many different families of antimicrobial peptides, which are classified on the basis of their amino acid sequences and secondary structures, have been isolated from diverse biological sources, including plants, animals, microorganisms and insects (Cammue *et al*., 1994; Nicolas and Mor, 1995). Among these antimicrobial peptides, those from plants have attracted interest because plants can defend themselves against microbial infection even though they do not have the adaptive immune response system present in higher vertebrates (Cammue *et al*., 1994). To protect themselves from infection by pathogens, plants produce various antimicrobial compounds such as phytoalexin (Dixon *et al*., 1983), digestive enzymes (Stintzi *et al*., 1993) and antimicrobial peptides (Cammue *et al*., 1994). In recent years many plant antimicrobial peptides such as thionins (Florack and Stiekema, 1994), plant defensins (Broekaert *et al*., 1995), knottin-type antimicrobial peptides (Cammue *et al*., 1992) and hevein (Parijs *et al*., 1991) have been isolated. Most of the plant

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF180444 (shep-GRP).

antimicrobial peptides characterized so far share the property of being cationic and contain even numbers of cysteine which are all pairwise connected by disulfide bridges, but otherwise differ considerably in basic features such as their sizes and structural motifs (Broekaert *et al.*, 1997). For example, thionins (Florack and Stiekema, 1994; Broekaert *et al.*, 1997) are highly basic 5 kDa peptides with six or eight cysteines, interconnected by three or four disulfide bonds, and contain two antiparallel α-helices and an antiparallel double-stranded β-sheet. Plant defensins (Broekaert *et al.*, 1995, 1997) consist of 45–54 amino acids, including eight cysteines, and possess one α-helix and a triple-stranded antiparallel β-sheet. Knottin-type antimicrobial peptides, *Mj*-AMP1 and *Mj*-AMP2 (Cammue *et al.*, 1992), are highly basic and consist of 36 and 37 amino acids.

Although plant antimicrobial peptides were discovered from nearly all plant tissues, tissue specificity is likely to be present. Indeed, the mRNA encoding IWF4, an antimicrobial peptide isolated from sugar beet leaves, was expressed only in the aerial parts of the plant, with a constitutive expression in young and mature leaves and in young flowers but not in roots (Nielsen *et al.*, 1997). In addition, transcripts of *Mj*-AMPs, antimicrobial peptides purified from seeds of *Mirabilis jalapa*, are expressed only in near-mature and in mature seeds but not in roots and leaves (De Bolle *et al.*, 1995), and a similar expression pattern was observed for *Ac*-AMPs, which were purified from seeds of *Amaranthus caudatus* (De Bolle *et al.*, 1993).

Even though plants are usually rooted in soils that are extremely rich in microorganisms, infection remains very rare. This phenomenon suggests that antimicrobial substances may participate in the protection of plant roots against microbial infection. Antimicrobial peptides may be one of those compounds involved in the protection of plant roots. Many plant antimicrobial peptides have been isolated from seeds (Cammue *et al.*, 1992; Terras *et al.*, 1992, 1993), leaves (Molina *et al.*, 1993; Florack and Stiekema, 1994; Nielsen *et al.*, 1997), flowers (Gu *et al.*, 1992; Broekaert *et al.*, 1995), latex (Parijs *et al.*, 1991) and fruits (Vu and Huynh, 1994). However, very few antimicrobial peptides have been reported from plant roots. In this paper, we describe the characterization and cDNA cloning of two novel glycine- and histidine-rich antimicrobial peptides from the roots of shepherd's purse, *Capsella bursa-pastoris*, which has been used as a wound-healing agent in Korean traditional medicine.

Materials and methods

Microorganisms

All microorganisms used in this study were obtained from the American Type Culture Collection (ATCC). The following microorganisms were used for antimicrobial activity tests: *Erwinia herbicola* ATCC33243, *Escherichia coli* ATCC27325, *Pseudomonas putida* ATCC17426, *Pseudomonas syringae* ATCC43298, *Salmonella typhimurium* ATCC15277, *Serratia* sp. ATCC21074, *Bacillus subtilis* ATCC62037, *Staphylococcus aureus* ATCC15752, *Streptococcus mutans* ATCC25175, *Candida albicans* ATCC10231, *Cryptococcus neoformans* ATCC34881, *Saccharomyces cerevisiae* ATCC44774, *Alternaria alternata* ATCC28143, *Aspergillus flavus* ATCC46110, *Aspergillus fumigatus* ATCC9197, and *Fusarium culmorum* ATCC15620.

Peptide purification

Antimicrobial peptides were purified from the roots of shepherd's purse as described by Park *et al.* (1996) with a slight modification. The roots (300 g) were homogenized using a Waring blender (Waring, New Hartford, CT) in 1200 ml extraction medium consisting of 1% v/v trifluoroacetic acid (TFA), 1 M HCl, 5% v/v formic acid, 1% w/v NaCl and 1 μg/ml pepstatin A. The homogenate was filtered through Whatman paper No. 2 (Whatman, Maidstone, UK) and centrifuged at 20 000 $\times g$ for 30 min in a Himac SCR20BR (Hitachi, Tokyo, Japan). The peptides in the supernatant were subsequently subjected to reversed-phase concentration using a Sep-Pak C18 cartridge (Millipore, Milford, MA), which was activated with 80% acetonitrile containing 0.1% (v/v) TFA (buffer A) and flushed with 0.1% (v/v) TFA (buffer B) to remove excess acetonitrile. After being loaded with the supernatant, the Sep-Pak C18 cartridge was washed with 20 ml of buffer B and the peptides trapped in the cartridge were eluted with 6 ml of buffer A. The eluate was then lyophilized and subsequently resuspended in 10 ml of 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl. The resuspended eluate was loaded onto a 2.5 cm $\times$ 10 cm Heparin Sepharose column (Pharmacia, Uppsala, Sweden) and the bound peptides were eluted with a stepwise NaCl gradient of 100 mM, 500 mM and 1 M. All the fractions were then assayed for antimicrobial activity against *Escherichia coli*. The active fractions were applied to a C18 reversed-phase high-performance liquid chromatography (HPLC) column

(3.9 mm × 300 mm; Delta Pak, Millipore) and elution was achieved with a linear gradient of 0–80% acetonitrile in 0.1% TFA for 2 h at a flow rate of 1 ml/min. Each peak was lyophilized, resuspended in water and assayed for antimicrobial activity against *E. coli*. The peaks with antimicrobial activity were identified and pooled. The purity of the isolated peptide was assessed by reversed-phase HPLC and matrix-associated laser desorption ionization (MALDI) mass spectroscopy (Kratos Kompact MALDI, Manchester, UK). The amount of isolated peptide was determined by amino acid analysis as described by Park *et al.* (1997).

Molecular mass and amino acid sequence analysis

The molecular mass of the isolated antimicrobial peptide was determined by MALDI mass spectroscopy. About 20 nmol of the lyophilized peptide was dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a Pt probe. After removing the solvent in warm air, the peptide adsorbed to the Pt probe was analyzed after being applied to a vacuum chamber. Amino acid sequencing was performed by the automated Edman degradation method on an Applied Biosystem gas phase sequencer, Model 447 (Foster City, CA). Amino acid sequence homology searches were performed by computerized query of the GenBank/EMBL data bank. The antimicrobial peptide was synthesized at the Korea Basic Research Institute (Seoul, Korea) to further investigate antimicrobial activity.

Antimicrobial assay

Antimicrobial activity against bacteria and yeast-phase fungi was determined as described by Couto *et al.* (1992). Organisms from a single colony on an agar plate were inoculated into 5 ml nutrient broth (trypticase soy broth for bacteria; sabouraud medium for yeast-phase fungi) and cultured overnight at 37 °C. Aliquots of the overnight culture were inoculated into 20 ml of fresh nutrient broth and grown to mid-exponential phase. The resulting cultures were washed three times with cold 10 mM sodium phosphate buffer (NAPB), pH 6.5, and resuspended in 10 ml of NAPB. 1×10^4 colony-forming units (c.f.u.)/ml were incubated with serially diluted antimicrobial peptide in 10 mM NAPB for 30 min at 37 °C. After incubation, aliquots were removed, serially diluted, plated on nutrient agar plates and incubated for 24 h at 37 °C. Distilled water was used for control experiments. The concentration of the antimicrobial peptide required for 50% reduction of c.f.u. relative to that of the control was expressed as IC_{50}, against bacteria and yeast-phase fungi.

Antifungal activity against mycelial fungi was measured as previously described by Broekaert *et al.* (1992). Tests were performed in a sterilized 96-well microtiter plate (Nunc F96 microtiter plates, Denmark). 20 μl of antimicrobial peptide solution was mixed with 80 μl of fungal spore suspension (2×10^4 spores/ml) in a synthetic growth medium (2.5 mM K_2HPO_4, 50 μM $MgSO_4$, 50 μM $CaCl_2$, 5 μM $FeSO_4$, 0.1 μM $CoCl_2$, 0.1 μM $CuSO_4$, 2 μM Na_2MoO_4, 0.5 μM H_3BO_3, 0.1 μM KI, 0.5 μM $ZnSO_4$, 0.1 μM $MnSO_4$, 10 g/l glucose, 1 g/l asparagine, 20 mg/l methionine, 2 mg/l myo-inositol, 0.2 mg/l biotin, 1 mg/l thiamine-HCl, 0.2 mg/l pyridoxin-HCl). After incubation for 48 h at 25 °C, the percentage growth inhibition was determined by measuring the absorbance at 600 nm on a Model 550 Microplate Reader (BioRad, CA, USA) based on the equation $[(\Delta C - \Delta T)/\Delta C] \times 100$, where ΔC is the change of absorbance in the control well and ΔT is the change of absorbance in the test well. The concentration of antimicrobial peptide required for 50% growth inhibition was expressed as IC_{50} against mycelial fungi. The growth inhibition of *F. culmorum* by shepherin I was also examined under an Epi-F1 phase-contrast microscope (Optiphot-2 modell Nikon, Tokyo, Japan) after incubation for 48 h at 25 °C.

Circular dichroism (CD)

The CD spectra were measured in either 50 mM NAPB or 50% v/v trifluoroethanol (TFE) in 50 mM NAPB, on a Jasco model J-715 spectropolarimeter (Jasco, Tokyo, Japan) with a cell path length of 1 mm. Five scans per sample were performed and averaged over the wavelength range 200–250 nm (Chen *et al.*, 1988). Ellipticity is reported as molar ellipticity (° cm^2 $dmol^{-1}$). The contents of α-helices, β-sheet and random coils were estimated as described by Greenfield and Fasman (1969).

cDNA cloning

Total RNA was extracted from the homogenized roots of shepherd's purse by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) and poly(A)$^+$ mRNA was isolated from the total RNA using a polyAT tract mRNA isola-

tion kit (Promega, Madison, WI). To prepare a pool of cDNA, first-strand cDNA was synthesized from the poly(A)$^+$ mRNA (5 μg) using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and the poly(dT) adapter primer 1, 5′-GAGAGAGAGAGAGAGAGAGAGAACTAG TCTCGA G(T_{18})-3′ (*ol T1*, an *Xho*I site is underlined) at 37 °C. After 1 h, the reaction was stopped by diluting 10-fold with water. A cDNA library was constructed with the Uni-ZAP XR cDNA library kit (Stratagene, La Jolla, CA) according to the procedure provided by the manufacturer. A polymerase chain reaction (PCR)-based strategy was used to clone the cDNA encoding shepherin I and shepherin II in the cDNA library (Frohman *et al.*, 1988). For 3′-RACE, two primers, an adapter primer 2 (*ol T2*, 5′-CTCGAG(T_{15})-3′) and a degenerate primer (*ol D*, 5′-GGNTAYGGNGGNCAYGGNGG-3′ encoding residues 1–7 of shepherin I, where N = A, T, G, C; Y = C, T), were used as primers. 3′-RACE was done in the buffer consisting of 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 100 mM dNTPs, 5% DMSO, 25 pmol of *ol T2* primer, 100 pmol of *ol D* primer and one-sixtieth of the cDNA pool prepared as described above. To minimize the background and to increase the specificity, touchdown PCR (Hecker and Roux, 1996) was used. The touchdown PCR program was such that the annealing temperature was progressively lowered from 65 °C to 39 °C by 2 °C every cycle, followed by 20 additional cycles at 45 °C. Each cycle consisted of 1 min at 94 °C for denaturation, touchdown for annealing and 1 min at 72 °C for elongation. Based on the cDNA sequence of the 3′-RACE, a second PCR amplification was performed to obtain the 5′ end of cDNA using an antisense oligonucleotide primer (*ol P*, 5′-AACACCGGGCTGAGTCTG-3′) complementary to 360–377 of the cDNA sequence and a universal reverse primer (*URP*, 5′-GGAAACAGCTATGACCATG-3′) of the Uni-ZAP XR Vector (Stratagene). The PCR products were cloned into pT7Blue T-Vector (Novagen, Madison, WI) and then sequenced.

DNA sequencing and analysis

The nucleotide sequence was determined by an automated DNA sequencer (ABI Prism model 377, Perkin Elmer, Foster City, CA). The nucleotide and amino acid sequences were scanned and compared with databases available at National Center for Biotechnology Information (NCBI) of the National Institute of Health (NIH) through the BLAST network service.

Southern and Northern blot analysis

Genomic DNA was isolated from roots of shepherd's purse as described by Dellaporta *et al.* (1983). 50 μg of the prepared DNA was digested with either *Eco*RI, *Hin*dIII, *Bam*HI or *Xba*I. The DNA digests was fractionated on a 0.8% agarose gel and blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) after depurination. The DNA fragments were cross-linked to the membrane by UV illumination. The 377 bp cDNA fragment encoding shepherins was labeled with [^{32}P]-dCTP by the random priming method (Sambrook *et al.*, 1989) and used as a probe. Hybridization was performed at 68 °C in a solution containing 50% formaldehyde, 5× SSC, 1× Denhardt's solution, 10% dextran sulfate, 100 μg herring sperm DNA per ml and 0.1% SDS. Final washing was carried out in a solution containing 0.1× SSC and 0.1% SDS at 68 °C. For Northern blot analysis, total RNA was extracted from roots, leaves and stems of shepherd's purse by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Each 20 μg of the total RNA was fractionated on a 1% agarose gel containing 6.7% formaldehyde and 1× MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM Na_2EDTA pH 7.0) and blotted onto Hybond-N+ membrane (Amersham) by the capillary technique (Sambrook *et al.*, 1989). Hybridization and washing conditions were the same as those used for Southern blot analysis.

Results

Purification of antimicrobial peptides

Roots of shepherd's purse were homogenized in an acidic medium to maximize the solubilization of peptides. After centrifugation, the extracts were concentrated with Sep-Pak C18 cartridges and fractionated by heparin-affinity chromatography. The fractions eluted with 100 mM NaCl, which were active against *E. coli*, were further purified by C18 reversed-phase HPLC using a linear gradient of acetonitrile (Figure 1). Two peaks (Figure 1), which showed antimicrobial activity against *E. coli*, were further purified by a second reversed-phase separation, resulting in over 95% purity as determined by reversed-phase HPLC and MALDI mass spectroscopy (Figure 1, inset).

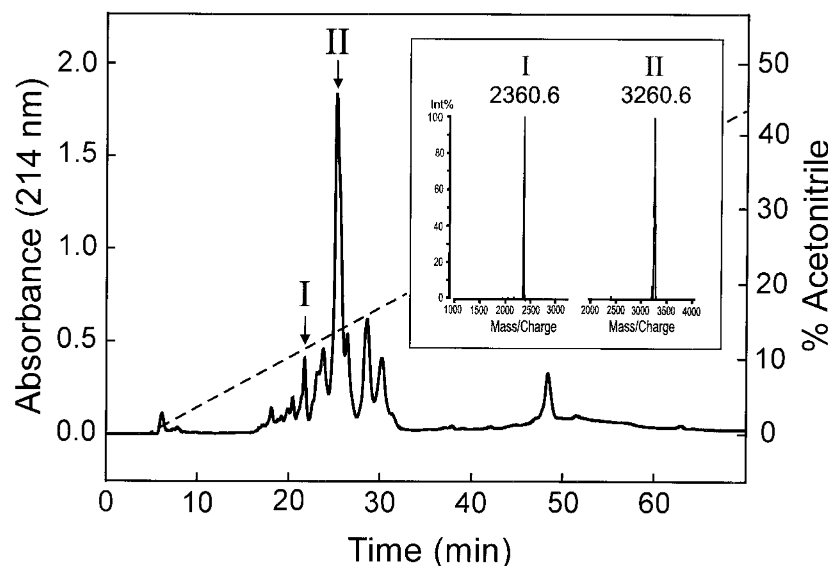


Figure 1. Purification and mass spectroscopic analysis of shepherin I and shepherin II. The active fraction after heparin chromatography was loaded on a 3.9 mm × 300 mm Delta-pak C18 column and elution was achieved with a linear gradient of acetonitrile in aqueous trifluoroacetic acid (80% acetonitrile/0.1% TFA). Absorbance was monitored at 214 nm. The peaks containing shepherin I (I) and shepherin II (II) are indicated by arrows. The inset represents the masses for shepherin I ($MH^+ = 2360.6$) (I) and shepherin II ($MH^+ = 3260.6$) (II) determined by MALDI mass spectroscopy.

The purified peptides from peak I and peak II were named shepherin I and shepherin II (derived from shepherd's purse), respectively. Approximate yields of shepherin I and shepherin II were 0.45 and 2.1 mg per 300 g of roots, respectively. The purified shepherin I and shepherin II were used for further chemical and biochemical analyses.

Primary structure determination

The molecular mass of shepherin I and shepherin II was 2360.6 and 3260.6 Da, respectively, as determined by MALDI mass spectroscopy (Figure 1, inset). After analysis of the amino acid composition of shepherin I and shepherin II, they were subjected to amino acid sequence analysis by an automated gas-phase amino acid sequencer. The complete amino acid sequence was Gly-Tyr-Gly-Gly-His-Gly-Gly-His-Gly-Gly-His-Gly-Gly- His-Gly-Gly-His-Gly-Gly-His-Gly-His-Gly-Gly-Gly-Gly-His-Gly for shepherin I and Gly-Tyr-His-Gly-Gly-His-Gly-Gly-His-Gly-Gly-Gly-Tyr- Asn-Gly-Gly-Gly-Gly-His-Gly-Gly-His-Gly-Gly-Gly-Tyr-Asn-Gly-Gly-Gly-His-His-Gly-Gly-Gly-Gly-His-Gly for shepherin II. The amino acid composition of shepherin I and shepherin II, analyzed by the acid hydrolysis method (data not shown), and their molecular mass determined by MALDI mass spectroscopy were in good agreement with those obtained from the amino acid sequence. These results indicate that post-translational modifications were not present in these peptides. Shepherin I and shepherin II were particularly rich in glycine (67.9% and 65.8% mol/mol respectively) and histidine (28.6% and 21.1% mol/mol, respectively), had a Gly-Gly-His motif and did not contain any cysteine residue, which is commonly present in most plant antimicrobial peptides known so far. The net charge per molecule for both shepherin I and shepherin II was + 8, indicating that they were highly cationic peptides.

A computer search comparing these amino acid sequences with those in the GenBank/EMBL data bank revealed that both shepherin I and shepherin II are novel antimicrobial peptides.

Antimicrobial activity

The antimicrobial activity of shepherin I and shepherin II was determined against 16 different microorganisms, including Gram-negative and Gram-positive bacteria, and fungi (Table 1). Shepherin I and shepherin II showed antibacterial activity against several Gram-negative bacteria, including *E. coli*, *P. putida*, *P. syringae* and *Serratia* sp., with IC_{50} values of 2.5–8 μg/ml, depending on the test microorganism, but did not show any antibacterial activity against Gram-positive bacteria even at a concentration up to

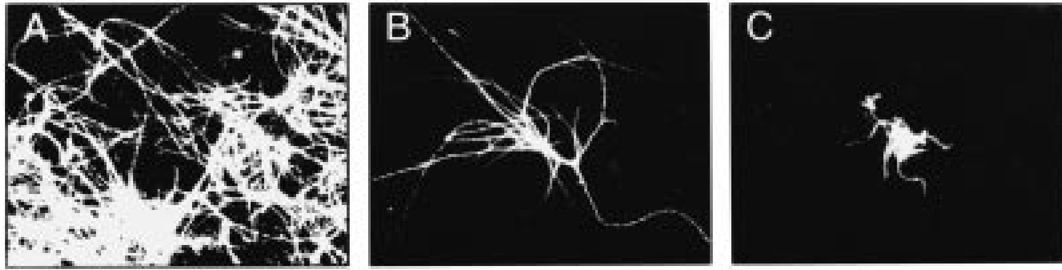
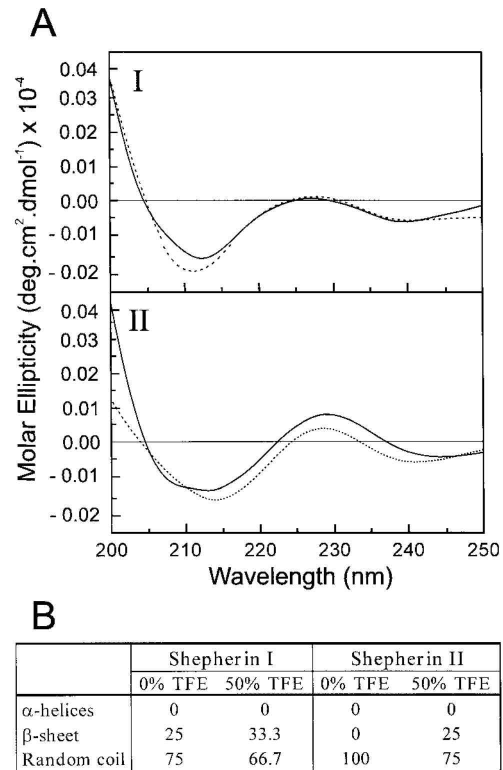


Figure 2. Growth inhibition of *F. culmorum* by shepherin I. Photographs were taken after incubation of *F. culmorum* spore suspension at 25 °C for 48 h in the absence of shepherin I (A) or in the presence of 50 μg/ml (B) and 100 μg/ml of shepherin I (C).

100 μg/ml. Shepherin I and shepherin II had marked antifungal activity against yeast-phase *C. albicans*, *C. neoformans* and *S. cerevisiae* but were moderately active against the mycelial fungi *A. alternata*, *A. niger* and *F. culmorum*. However, *A. fumigatus* was not affected by shepherin I and shepherin II even at a concentration of 100 μg/ml. In addition, shepherin GGH, which was chemically synthesized to have 6 direct repeats of the Gly-Gly-His motif, was comparable to shepherin II in antimicrobial activity. The growth inhibition of *F. culmorum* was analyzed microscopically. The morphology of fungal hyphae was altered upon treatment with shepherin I. Compared with the control culture, shorter hyphae were observed in the growth medium containing shepherin I (Figure 2).

Circular dichroism (CD)

The secondary structure of the shepherins was estimated with a spectropolarimeter in the absence and presence of trifluoroethanol (Figure 3). The CD spectra of shepherins in 50 mM NAPB were characteristic of random coils (shepherin I: 25% β-sheet, 75% random coils; shepherin II: 0% β-sheet, 100% random coils) with no α-helical content. In the presence of 50% TFE solution, the contents of β-sheet and random coils in shepherin I and shepherin II were changed to 33.3% and 66.7%, and 25% and 75%, respectively, without any increase in α-helical content.



	Shepherin I		Shepherin II	
	0% TFE	50% TFE	0% TFE	50% TFE
α-helices	0	0	0	0
β-sheet	25	33.3	0	25
Random coil	75	66.7	100	75

Figure 3. CD spectra (A) and analysis (B) of shepherin I and shepherin II in 50 mM NAPB in the absence and in the presence of TFE. The concentrations of shepherin I (A) and shepherin II (B) were 13 μM and 9 μM, respectively. Measurements were taken in 50 mM NAPB in the absence (solid line) and in the presence (dashed line) of 50% TFE.

cDNA cloning of shepherins

Using the cDNA library as a template, the 3′ end of cDNA (Figure 4, 141–575) was amplified with a degenerate primer based on the deduced nucleotide sequence for shepherin I (primer *ol D*) and an adaptor primer 2 (primer *ol T2*) described in materials and methods. The 435 bp PCR fragment was cloned into pT7Blue T-Vector (Novagen), and then sequenced. Based on the 435 bp DNA sequence of the 3′ end of cDNA, the 5′ end of cDNA (Figure 4, 1–384) was amplified from the cDNA library with a gene-specific primer (primer *ol P*, 367–384) and a universal reverse primer (*URP*). cDNA encoding shepherins was obtained by recombinant PCR using the above two PCR products as templates. The entire sequence (575 bp) for the cloned cDNA is shown in Figure 4. The

Table 1. Antimicrobial activity of shepherin I, shepherin II and shepherin GGH.

Microorganism	IC_{50}^{*}		
	shepherin I	shepherin II	shepherin GGH
Gram-negative bacteria			
Erwinia herbicola	62	25	16
Escherichia coli	<2.5	<2.5	<2.5
Pseudomonas putida	<2.5	<2.5	<2.5
Pseudomonas syringae	<2.5	<2.5	<2.5
Salmonella typhimurium	<2.5	65	75
Serratia sp.	8	<2.5	4
Gram-positive bacteria			
Bacillus subtilis	>100	>100	>100
Staphylococcus aureus	>100	>100	>100
Streptococcus mutans	>100	>100	>100
Fungi			
Candida albicans	8	5	5
Cryptococcus neoformans	<2.5	<2.5	<2.5
Saccharomyces cerevisiae	7	3	3
Alternaria alternata	7	>100	69
Aspergillus flavus	65	60	24
Aspergillus fumigatus	>100	>100	>100
Fusarium culmorum	72	68	64

*IC_{50} indicates the concentration of antimicrobial peptides required for 50% reduction in c.f.u. (bateria and yeast-phase fungi) or 50% inhibition of growth (mycelial fungi) relative to that of control.

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  1  AAGTTGAGAACAAAAAAATGGCTTCCAAGACTTTGATACTGTTGGGTCTCTTTGCAATT  59
  1                   M  A  S  K  T  L  I  L  L  G  L  F  A  I   14

 60 CTTCTCGTTGTCTCCGAAGTGTCTGCCGCAAGAGAGTCCGGCATGGTGAAGCCGGAGAGT 119
 15  L  L  V  V  S  E  V  S  A  A  R  E  S  G  M  V  K  P  E  S   34

120 GAGGAAACAGTGCAACCTGAAGGTTATGGCGGCCACGGCGGCCATGGTGGTCATGGAGGC 179
 35  E  E  T  V  Q  P  E  G  Y  G  G  H  G  G  H  G  G  H  G  G   54

180 CATGGTGGCCACGGAGGTCACGGACATGGAGGAGGAGGCCATGGACTTGACGGATACCAC 239
 55  H  G  G  H  G  G  H  G  H  G  G  G  G  H  G  L  D  G  Y  H   74

240 GGAGGACATGGTGGTCACGGAGGAGGATACAACGGAGGAGGAGGTCATGGAGGTCACGGA 299
 75  G  G  H  G  G  H  G  G  G  Y  N  G  G  G  G  H  G  G  H  G   94

300 GGAGGATACAACGGAGGAGGACACCACGGTGGAGGAGGTCACGGGCTTAACGAACCTGTT 359
 95  G  G  Y  N  G  G  G  H  H  G  G  G  G  H  G  L  N  E  P  V  114

360 CAGACTCAGCCCGGTGTTTAAAACTAATATCATGTATTCACCACCATGCATGGTTGCACT 419
115  Q  T  Q  P  G  V  *                                            120

420 TAATATATACATGTATGTGTACTCTTAATTATGCTTATGTGTGTGTATGACTATAAATAA 479

480 ACCATGGTGAGTTTGTAATGCAGTCCCTTCAGAAATGCTTTTGCAATAAAGTTGAATGAA 539

540 TATCAGCATAATTTCTCTAAAAAAAAAAAAAAAAAA                         575
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Figure 4. Nucleotide and deduced amino acid sequences of the cDNA encoding shepherin I and shepherin II. The cloned cDNA was 575 bp in length and contained an open reading frame encoding a 120 amino acid protein. The sequence of mature shepherin I (single-underlined) and shepherin II (double-underlined) is underlined. The start (ATG) and stop (TAA) codons are marked with bold letters and an asterisk, respectively. The putative signal sequence and the polyadenylation signal are marked with italic and bold letters, respectively. The nucleotide sequences encoding shepherin I and shepherin II have been submitted to the GenBank/EMBL sequence data bank under the accession number AF180444.

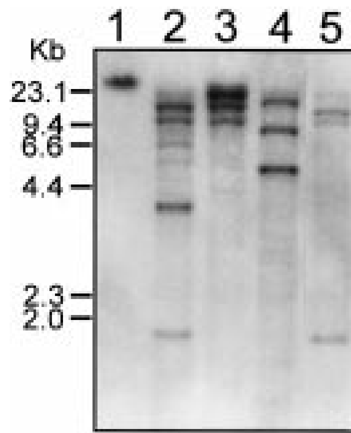

Figure 5. Southern blot analysis of shepherin I and shepherin II gene. Genomic DNA isolated from roots of *C. bursa-pastoris* was digested (50 μg) with *Eco*RI (lane 2), *Hin*dIII (lane 3), *Bam*HI (lane 4) or *Xba*I (lane 5). Lane 1 indicates undigested genomic DNA. The DNA digests were electrophoresed on a 0.8% agarose gel, blotted onto a Hybond-H+ membrane and hybridized with the ^{32}P-labeled 377 bp cDNA insert.

sequence analysis of the cDNA showed that a 363 nucleotide long open reading frame started at nucleotide 18. The sequence surrounding the putative translation initiation codon AAAAATGGC conformed well to the consensus sequence AACAATGGC known for plant genes (Lütcke *et al.*, 1987). A typical polyadenylation signal was found at nucleotide 524, 34 nucleotides upstream of the poly(A)$^+$ tail. The open reading frame may be translated into a 120 amino acid polypeptide, designated shep-GRP. The deduced amino acid sequence of the cDNA contained a putative 41 amino acid signal peptide, a shepherin I, a dipeptide linker, a shepherin II and a carboxy-terminal peptide.

Southern and Northern blot analysis

To determined the complexity of the genomic organization of the gene encoding shepherins, Southern blot analysis was carried out on genomic DNA isolated from roots of shepherd's purse. An open reading frame of the isolated cDNA was used as a probe for Southern blot hybridization of shepherd's purse DNA digested with either *Eco*RI, *Hin*dIII, *Bam*HI or *Xba*I, none of which have predicted cleaving sites within the gene encoding shepherin I and shepherin II. As shown in Figure 5, three or five discrete restriction fragments were hybridized to the probe, indicating that shepherins are a gene family of low complexity.

The expression pattern of the gene encoding shepherins, which was studied by Northern blot analysis

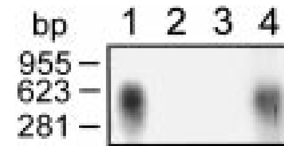

Figure 6. Northern blot analysis of the shepherin I and shepherin II transcript. Lane 1, 20 μg of total RNA of roots; lane 2, 20 μg of total RNA of leaves; lane 3, 20 μg of total RNA of stems; lane 4, 10 μg of total RNA of roots. Total RNA extracted from roots, leaves and stems from *C. bursa-pastoris* was separated on a 1.0% agarose gel containing formaldehyde, blotted onto a Hybond-H+ membrane and hybridized with the ^{32}P-labeled 377 bp cDNA insert.

(Figure 6), showed one mRNA band from roots, on a 1.0% agarose gel, whose size was estimated to be about 570 bp. This indicates that the sequence in Figure 4 represents the full-length cDNA, taking the length of the poly(A)$^+$ tail into account. The expression of the gene encoding shepherin I and shepherin II was restricted to roots. This result suggests that the shepherins may play an important role in defending roots against microbial infection.

Discussion

Antimicrobial peptides, which are now considered as one of the universal primary host defense tools of live organisms against microbial infection, have also been found in plants (Cammue *et al.*, 1994). Very few antimicrobial peptides have been found in plant roots. We describe here the purification, characterization and cDNA cloning of two novel glycine- and histidine-rich antimicrobial peptides, named shepherin I and shepherin II, from the roots of shepherd's purse, which show potent antimicrobial activity against Gram-negative bacteria and fungi. To our knowledge, this is one of the first reports on antimicrobial peptides from plant roots. Although shepherin I and shepherin II have little amino acid sequence similarity with previously described antimicrobial peptides, they have the feature common with many known plant antimicrobial peptides: shepherin I and shepherin II are highly cationic molecules (net positive charge +8) due to the presence of multiple histidine residues, which may have a role in establishing contact with negatively charged cell membranes to exert antimicrobial activity.

An interesting feature of shepherin I and shepherin II is that they do not contain cysteine which is common in most plant antimicrobial peptides known so far (Broekaert *et al.*, 1997). Plant antimicrobial pep-

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Shep-GRP    ------------MASKT--------LILLG--LFAILLVVSEVSAARESGMVKPESEETV  38
atGRP-3     ------------MASKA--------LVLLG--LFAVLLVVSEVAAAS-SATVNSESKETV  37
pUM90-1     HGGHGGGGYNGGGGHGG--------HGGGG--YNGGGHGGHGGAESVAVQTEEKTNEVN  50
zmGRP3      ------------MATNTK-------LVALG--L-AVLVSVGFSDAARVVRLGSYASAGGG  38
HvGRP3      MAETEYRCFVGGLAWATDDHNLQAAFSQYGEILDAKIINDRETGRSRGFGFVTFGSEESM  60

Shep-GRP    --------QPE--G----YGG---HGG-HGGHGGH----GGH------------GGH-GH  63
atGRP-3     --------KPDQRG----YGD---NGGNYNNGGGY----QGG------------GGN-YQ  65
pUM90-1     --------DAKYGGGS-NYNDG--RGGYNHGGGGH----GGH------------GGHGGH  83
zmGRP3      --------GGGGGGGSGSTGAAG-YGGGSGGGGGYGIGKGGGDWWNNFVSSVAGGGGGGQ  89
HvGRP3      RQAIEEMNGKELDGRQVTVNEAQSRRSAGGGGGGY----GGQR-----------GGGGGY  105

Shep-GRP    GGGG--HGLDG-YHG-GHG----------------------GHGGGYNGG--GGHGGHGG  95
atGRP-3     GGGGNYQGGGGNYQG-GGGR-YQ------------------GGGGRYQGG--GGRYQGGG  103
pUM90-1     GGGG-YNGGGG--HG-GHGG-AESVAVQTEEKTNEVNDAKYGGGSNYNDGR-GGYNHGGG  137
zmGRP3      GGGGGTNGGSGSGGGSGYGSGTSSTAASGPSSGNYANAEGKGAGGGMGGGADGAYGSGAG  149
HvGRP3      GGGGGYGGQGGGYGGQGGG----------------------GYGGQGGGG-YGGQRGGGG  142

Shep-GRP    G--------------------YNGG-----G-------HHGG---------GG-------  107
atGRP-3     G--------------------RQGG-----GGSRGSYCRHGC---------CY-------  122
pUM90-1     G--------------------YNHG-----GGGHGGHGGHGGHG-----GHGG-------  160
zmGRP3      GGVGKGQGESGVALAPSSDGYYNGGAADATGGGSAGGGHGGGAGAPSYGTGGGLAEARA  209
HvGRP3      G--------------------YGGG-----GG------GYGG---------GG-------  155

Shep-GRP    --------HGLN------------EPVQTQPGV--------------  120
atGRP-3     --------RGYNG--CSRCCSYAGEAVQTQPGH--------------  145
pUM90-1     --------HGADQ--TEDNTQNDHNDIIMHHAHFLSNICHE------  191
zmGRP3      RRQRRSWGSGYAAGIGAGTGGGGGGGFQGASGGGGGSGSGSGSGGIH  256
HvGRP3      --------GGYGG-------QRGGGDSGGQWRS--------------  173
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Figure 7. Alignment of amino acid sequence of shep-GRP and glycine-rich proteins (GRPs). The deduced amino acid sequences of shep-GRP containing shepherin I and shepherin II were aligned with atGRP-3, pUM90-1, zmGRP3 and HvGRP3 using the CLUSTALW program with default parameters. Gray shading indicates amino acid identity.

tides have been classified into distinct families such as thionins, plant defensins, lipid transfer proteins, hevein-type and knottin-type antimicrobial peptides, based on their homologies at the primary structure level (Broekaert *et al.*, 1997). All of these antimicrobial peptides contain even numbers of cysteines (4, 6, or 8) which are all pairwise connected by disulfide bridges. Therefore, shepherin I and shepherin II should be classified as a separate family since they do not contain cysteine. Another noteworthy feature of shepherins is the repetition of the Gly-Gly-His sequence motif. To examine whether this motif has any effect on antimicrobial activity, shepherin GGH containing 6 direct repeats of the motif was synthesized. Shepherin GGH showed antimicrobial activity comparable to shepherin II. This indicates that the Gly-Gly-His motif is important for antimicrobial activity in shepherin II. Motifs that may contribute to the interaction with specific membrane proteins (Duvick *et al.*, 1992) are not an uncommon characteristic in antimicrobial peptides. Several sequence motifs, including Gln-His-Gly-His-Gly-Gly-Gln in AFP from hemolymph of flesh fly, were discovered in both plant and non-plant antimicrobial peptides (Duvick *et al.*, 1992; Iijima *et al.*, 1993). However, none of these motifs are present in shepherin I and shepherin II. The high contents of glycine and histidine and the repetition of the Gly-Gly-His sequence motif undoubtedly have a major influence on the secondary structure of shepherins and hence on their mode of action. CD spectra analysis revealed that shepherin I and shepherin II have rather random coils even in 50% trifluoroethanol instead of an amphiphilic β-sheet conformation common to other plant antimicrobial peptides such as *Mj*-AMPs (Cammue *et al.*, 1992; Broekaert *et al.*, 1997) and *Ac*-AMPs (Broekaert *et al.*, 1992, 1997). Such structural characteristics might confer upon shepherin I and shepherin II a mechanism of action that is different from that of *Mj*-AMPs and *Ac*-AMPs, which are active on Gram-positive bacteria (Broekaert *et al.*, 1992; Cammue *et al.*, 1992), and may also confer upon shepherin I and shepherin II the specificity against Gram-negative bacteria and fungi.

Interestingly, shepherin I and shepherin II were produced from a single polypeptide, designated shep-GRP, which has five distinct domains, an amino-terminal putative signal peptide, a shepherin I, a linker dipeptide, a shepherin II and a carboxy-terminal peptide. A similar observation, in which two antimicrobial proteins (lectin and chitinase) were encoded by a single cDNA, was made in the stinging nettle (Lerner and Raikhel, 1992). An amino-terminal putative signal peptide and a carboxy-terminal peptide were found in plant proteins producing antimicrobial peptide such as IWF4 from sugar beet leaves (Nielsen *et al.*, 1997) and *Ac*-AMP2 from amaranth (De Bolle *et al.*, 1993). The amino-terminal putative signal sequence of shep-GRP is acidic suggesting that it is a secreted protein. The carboxy-terminal peptide of shep-GRP is also acidic. Acidic carboxy-terminal peptides are also present in the IWF4 (Nielsen *et al.*, 1997) and in the family of type I thionins, which are small, Cys-rich plant antimicrobial peptides (Florack and Stiekema, 1994). Even though the functional roles of carboxy-terminal

peptides are not known, Florack *et al*. (1994) suggested that the acidic carboxy-terminal peptide might facilitate transport through membranes. Thus carboxy-terminal peptides of shep-GRP may increase the stability of the mature protein during the transport and processing. The PSORT program, which was developed to predict subcellular localization of proteins by Nakai and Horton (1999), predicts that the shep-GRP is localized to the vacuole with a certainty of 0.90 and contains a cleavable N-terminal signal sequence. Therefore, it is likely that the putative signal sequence targets shep-GRP to the vacuole, where proteolytic enzymes convert the shep-GRP into shepherin I and shepherin II. So far, there are no reports that two antimicrobial peptides are produced from a single protein. Thus, the production of shepherin I and shepherin II from a single shep-GRP is unique. The deduced amino acid sequence of the shep-GRP showed high homology with glycine-rich proteins (GRPs) such as atGRP-3 (de Oliveira *et al*., 1990), pUM90-1 (Luo *et al*., 1992), zmGRP3 (Goddemeier *et al*., 1998) and HvGRP3 (Molina *et al*., 1997) of plants (Figure 7). GRPs are expressed in response to environmental stresses including the phytohormone abscisic acid (ABA) (de Oliveira *et al*., 1990; Luo *et al*., 1992), cold (Ferullo *et al*., 1997; Luo *et al*., 1992), drought (Laberge *et al*., 1993; Luo *et al*., 1992), wounding (Showalter *et al*., 1991), fungi (Molina *et al*., 1997) and virus infection (Linthorst *et al*., 1990). GRPs are hypothesized to either work alone or to act in concert to protect plants against adverse environments (Luo *et al*., 1992). It remains to be studied whether shep-GRP is expressed in response to environmental stress or not.

In this paper, we describe characteristics and cDNA of two novel glycine- and histidine-rich antimicrobial peptides which may play an important role in the primary host defense in the roots of shepherd's purse. The identification of antimicrobial peptides in roots of plant will help to achieve a better understanding of the defense system in roots of plants. The actual mechanism of generating shepherin I and shepherin II from shep-GRP is under study.

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