Novel defensin subfamily from spinach (*Spinacia oleracea*)

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Received 9 July 1998; revised version received 13 August 1998

Abstract Antimicrobial peptides (So-D1-7) were isolated from a crude cell wall preparation from spinach leaves (*Spinacia oleracea* cv. Matador) and, judged from their amino acid sequences, six of them (So-D2-7) represented a novel structural subfamily of plant defensins (group IV). Group-IV defensins were also functionally distinct from those of groups I–III. They were active at concentrations <20 μM against Gram-positive (*Clavibacter michiganensis*) and Gram-negative (*Ralstonia solanacearum*) bacterial pathogens, as well as against fungi, such as *Fusarium culmorum*, *F. solani*, *Bipolaris maydis*, and *Colletotrichum lagenarium*. Fungal inhibition occurred without hyphal branching. Group-IV defensins were preferentially distributed in the epidermal cell layer of leaves and in the subepidermal region of stems.

Key words: Antimicrobial peptide; Innate immunity; Plant defense; Plant defensin; Plant pathogen; Spinach

1. Introduction

Defensins are among the best characterized cysteine-rich antimicrobial peptides in plants (see [1,2] for a review). All known members of this family have 4 disulfide bridges and are folded in a globular structure that includes three β-strands and an α-helix [3,4]. This structure resembles that of antimicrobial defensins from insects [5,6]. Inhibition of fungal growth by plant defensins seems to occur by permeabilization of the plasma membrane through binding to a putative receptor [7,8].

Genes encoding plant defensins are developmentally regulated, with a predominant expression in outer cell layers [9–11], and can be induced above basal levels in response to pathogen infection and other stresses [10–14]. Additionally, certain defensin genes are down-regulated by some pathogens [10]. Gene expression patterns of defensins are thus consistent with a hypothetical role in plant defense [1,2]. The observation of enhanced tolerance (reduced lesion area) to the fungus *Alternaria longipes* in transgenic tobacco overexpressing a radish plant defensin (Rs-AFP2) further supports this hypothesis [11].

Known defensins have been classified into three groups or subfamilies [1,2,8,13,15], based on structural and functional considerations: group I includes defensins that inhibit growth of *Fusarium culmorum* and cause increased hyphal branching; group II includes those that inhibit the fungus but do not cause hyphal branching; and group III, those that are inactive against the tested fungi. Apart from highly conserved amino acid residues that are common to all three subfamilies, there are residues that are conserved only in one or two of them, which allows to discern a closer relationship between groups I and II than between any of these and group III.

We report here six new defensins isolated from crude cell wall preparations from spinach leaves which represent a novel defensin subfamily (group IV), both in structural terms and in its pathogen specificity. Defensins of this new type coexist with those of group III in the same tissue.

2. Materials and methods

2.1. Purification and analysis of proteins

Spinach, *Spinacia oleracea* cv. Matador, was used in this study. Frozen leaves (20 g) were ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 ml buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml of H_2O. The resulting pellet was then extracted with 50 ml 1.5 M LiCl at 4°C for 1 h, and the extract dialyzed against 5 l H_2O, using a Spectra/Por 6 (MWCO: 3000) membrane, and freeze-dried [16]. The extract was fractionated by reverse-phase HPLC (RP-HPLC) as previously described [16]. The proteins were subjected to SDS-PAGE in preformed gradient gels (4–20%; Bio-Rad) according to the manufacturer's instructions. MALDI mass spectrometry of proteins was done in a Voyager Biospectrometry Workstation (PerSeptive Biosystems) using α-cyano-4-hydroxycinnamic acid (Aldrich) as matrix. Amino acid sequencing of intact proteins or of chymotryptic peptides was done by automated Edman degradation. Detection of proteins by Western-blot and by the tissue-print technique was done as previously described using an 1:500 dilution of the So-D2-7 antiserum [17].

2.2. Pathogen inhibition tests

Inhibition tests were carried out as previously described [16] and hyphal branching of *Fusarium culmorum* was investigated as indicated by Broekaert et al. [18]. The following microbial strains were used: bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus* strain C5, *Ralstonia solanacearum* strain P2; and fungal pathogens *Fusarium solani* strain 1, and *Trichoderma viridae* from the ETSIA collection (Madrid, Spain), and *Septoria nodorum*, *Bipolaris maydis*, *Colletotrichum lagenarium* and *Fusarium culmorum* from the collection of Novartis (NC, USA).

3. Results

3.1. Purification of spinach defensins

A crude cell wall preparation from spinach leaves was obtained as previously described [16,19,20] and bound proteins were extracted with 1.5 M LiCl. The extract was fractionated by RP-HPLC as shown in Fig. 1A and the fractions screened for antibacterial activity at 100 μg/ml. Homogeneity of active fractions was tested by SDS-PAGE and by RP-HPLC, using a less steep gradient. All active fractions were homogeneous according to these two criteria, except for the fraction designated (2–5) in Fig. 1A that yielded 4 homogeneous components upon rechromatography (Fig. 1B,C). The purified pro-

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PII: S0014-5793(98)01060-6

teins, So-D1-7, also appeared homogeneous when subjected to MALDI mass spectrometry analysis and to N-terminal amino acid sequencing. A gene-bank search indicated that all of these proteins were homologous to previously reported plant defensins and a comparison of all known sequences showed that six of the new proteins, So-D2-7, appeared to represent a new defensin subfamily, whereas protein So-D1 belonged to the previously proposed group III (Fig. 2A,B). The complete amino acid sequence of So-D2 was determined after chymotryptic digestion and the MW calculated from this sequence (5804 Da) was within 1 Da of that directly determined by MALDI mass spectrometry. The new group is structurally closer to group III than to groups I and II, but shows divergence from group III at the N-terminal half, including a 5-residue extension. Some common amino acid residues are shared by defensins of groups I and II with drosomycin, a defensin from the insect *Drosophila melanogaster* [6], and by those of groups III and IV with tenecin, a defensin from the insect *Tenebrio molitor* [22].

3.2. Distribution of group-IV defensins in the plant

Rabbit antiserum raised against protein So-D2 recognized proteins So-D2-7 and did not significantly bind protein So-D1 (Fig. 3). Using this antiserum, group-IV defensins were detected in spinach leaves and stems (not in roots) at concentrations that were in the range of 1–3 μmol/kg of fresh weight in the homogenized proteins (Fig. 3). As shown by tissue-print analysis, the distribution of the proteins in these tissues was peripheral, as they were at higher concentrations in the epidermal cell layer of leaves and occupied a wide subepidermal band in stems (Fig. 4). The actual concentrations in the deposition sites are probably up to 10-fold higher, well above the concentrations required for inhibition in vitro. As judged from the tissue prints of young and mature leaves (Fig. 4B,C), the protective defensin shield seems to be present throughout the life of this organ. This type of distribution is common to other defensins and antimicrobial peptides from plants [1].

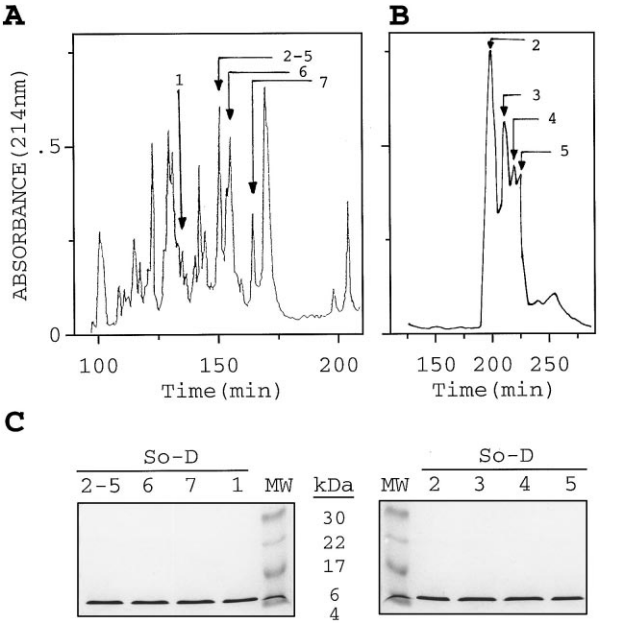

Fig. 1. Purification of spinach defensins. A: RP-HPLC fractionation of the 1.5-M LiCl extract from a crude cell-wall preparation from spinach leaves. The gradient used was H_2O (0.1% trifluoroacetic acid)-2-propanol, linear 0–30% for 180 min, 30–50% for 15 min. B: RP-HPLC separation of fractions from A. Same conditions, except that gradient was linear 0–30% for 360 min. C: Separation by SDS-PAGE of the indicated purified proteins. Molecular mass markers (MW) were the multi-colored standard mix from Novex.

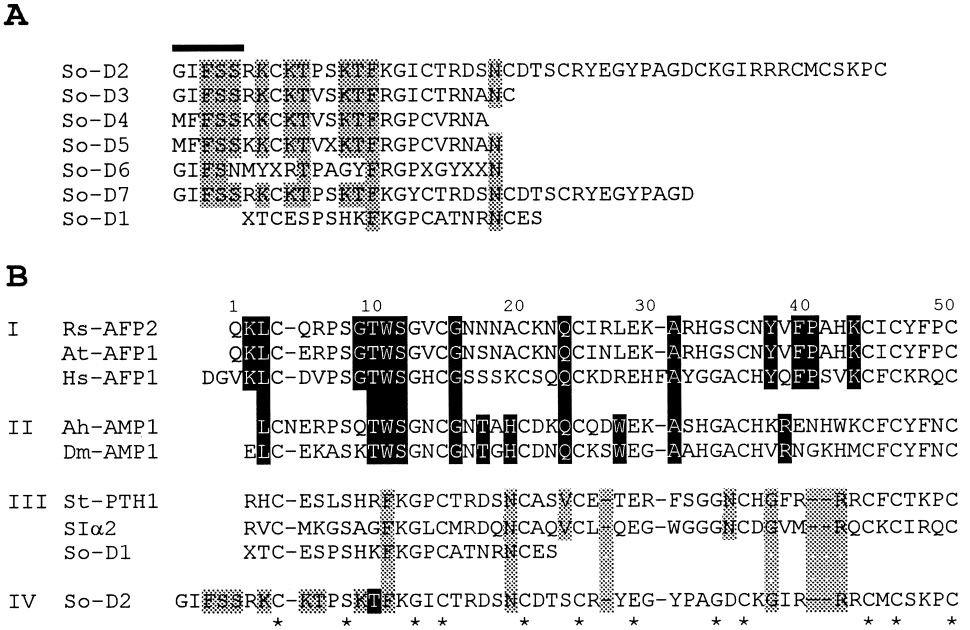

Fig. 2. Alignment of defensin amino acid sequences. A: So-D1-7 defensins. Black horizontal bar indicates the N-terminal extension of group-IV defensins. Highly conserved residues that are relevant for the classification are shaded. B: Comparison of amino acid sequences of defensin groups I–IV. Highly conserved residues that are relevant for the classification (conserved in non-represented, known members of each type) are shaded (black or grey). Residues conserved across all groups are indicated by stars (*). Representative defensin sequences for each group have been taken from the indicated references: Rs-AFP2 [11]; At-AFP1 [14]; Hs-AFP1, Ah-AMP1 and Dm-AMP1 [15]; St-PTH1 [10]; and SIα2 [21].

Fig. 3. Western-blot analysis of spinach defensins. So-D2 (1 μg), SO-D1 (1 μg) and total protein extracts from 50 mg of fresh tissue from roots (R), leaves (L) and stems (S). Quantitation by densitometry of Western-blot bands indicates concentrations of defensins of 3 μmol/kg fresh leaves and 1 μmol/kg fresh stems.

3.3. Antimicrobial properties of group-IV defensins

Inhibitory properties of group-IV defensins (So-D2,6,7) were compared with those of two type-III defensins (So-D1; St-PTH1) and of Ta-THα thionin (Table 1). Spinach defensins of groups III and IV were similarly active against the bacteria tested, whereas only those of type IV were active against *Fusarium* spp. Inhibition of *F. culmorum* occurred without hyphal branching and was abolished when salt (1 mM $CaCl_2$ + 50 mM KCl) was added to the medium. Other fungi, such as *Colletotrichum lagenarium* (EC_{50} = 11 μM) and *Bipolaris maydis* (EC_{50} = 6 μM) were also found to be sensitive to So-D2, whereas growth of *Trichodema viridae* and *Septoria nodorum* was not affected at So-D2 concentrations of up to 20 μM.

4. Discussion

We have isolated six new peptides from spinach (So-D2-7) that represent a novel defensin subfamily, as well as one (So-D1) which belongs to the previously described group III of defensins [15]. The new group is structurally closer to group III than to the other two groups, but shows significant divergence with respect to group III at the N-terminal half of the molecule. Of particular interest is a 5-residue N-terminal extension (GIFSS in So-D2) that is present in group-IV and absent in group-III defensins. An unrelated amphibian defensin, esculentin from *Rana esculentum*, has the sequence GIFS at the N terminus [23] and a similar difference is shown by two groups of brevinins, the defensins from *Rana brevipoda porsa* [24]; i.e. brevinins 1 and 2 lack an extension that is present in brevinins 1E and 2E (GLLDSLKG and GIMDTLKN, respectively).

Inhibitory properties of group-IV defensins are summarized and compared with those of the other groups in Table 2. Members of this subfamily are characterized by their ability to inhibit the test fungus *F. culmorum*, without causing multiple budding and swelling of germ tubes and hyphae, as well as both Gram-positive (*Clavibacter michiganensis*) and Gram-negative (*Ralstonia solanacearum*) bacterial pathogens. This means that group-IV defensins resemble those of group II in their antifungal activity and those of group III in their antibacterial activity. The evolution of this peptide family seems to be congruent with its defense role, as the observed structural and functional divergence could have been driven, at least in part, by different challenges represented by the main pathogens of the different plant species. The coexistence of defensins belonging to different subfamilies in the same tissue,

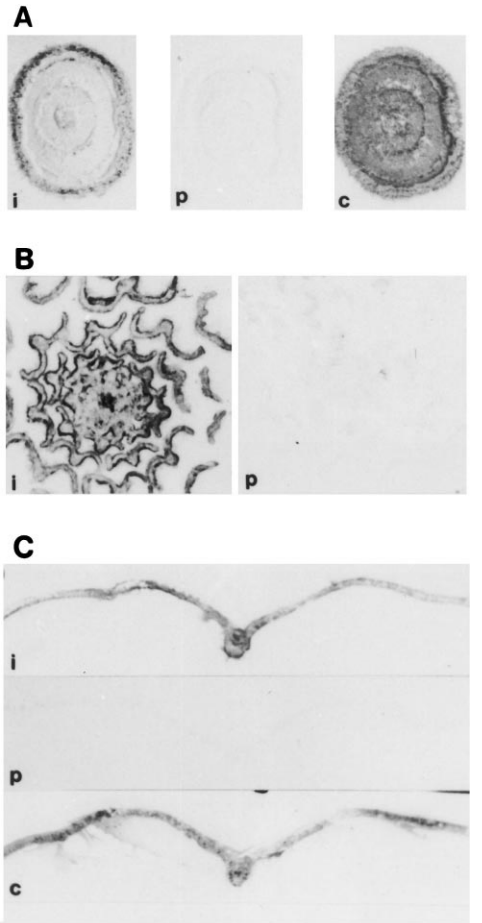

Fig. 4. Tissue-print localization of group-IV defensins from spinach. A: Stem; B: young leaves; C: older leaves. Equivalent sections were stained with immune serum (i), preimmune serum (p) and amido black (c).

Table 1
Inhibition of bacterial and fungal plant pathogens by spinach defensins (So-D1,2,6,7), potato defensin (St-PTH1) and wheat thionin (Ta-THα)

Pathogen	Protein (EC_{50}, μM)[a]					
	So-D				St-PTH	Ta-TH
	1	2	6	7	1	α
Bacteria						
C. michiganensis	1	1	1	0.1	0.2	1
R. solanacearum	15	2	6	1	3	1
Fungi						
F. culmorum	NA	0.2	–	–	NA	0.3
F. solani	NA	11	11	9	7	10
Trichodema viridae	NA	NA	–	–	NA	5

[a]EC_{50} = effective concentration for 50% inhibition; NA, not active at concentrations < 20 μM.

Table 2
Inhibitory properties of defensin subfamilies

Pathogen type	Subfamily			
	I[a]	II[a]	III[a]	IV
Bacteria				
Gram+ ($EC_{50} < 20$ μM)	+	−	+	+
Gram− ($EC_{50} < 20$ μM)	−	−	+	+
Fungus				
F.culmorum ($EC_{50} < 20$ μM)	+	+	−	+
Hyphal branching	+	−	−	−

[a]See [8] and Table 1.

as is reported here, represents a way to achieve a broader antimicrobial barrier in that tissue.

Structure-activity relationships have been investigated in the type-I defensin Rs-AFP2 by site-directed mutagenesis [8]. It is to be noted that, out of 11 positions at which a mutational change produced a significant decrease in the activity of this peptide, only two are conserved in So-D2, namely a T at position 10 and a P at position 50. Furthermore, the change Y→G at position 38 inactivated Rs-AFP2, whereas a G is at that position in So-D2. All these differences would be consistent with the hypothesis of Broekaert and coworkers [1,8], which postulates the existence of more than one mechanism of action among the different defensin groups. Thus, residues that are essential for the activity of group-I defensins would not be necessarily required for activity in groups II and IV.

Acknowledgements: We are indebted to G. Nye (Novartis) and Dr. F. Madueño for their help with MALDI mass spectrometry and amino acid sequencing and to G. Lopez, D. Lamoneda and J. García for techinical assistance. This work was supported by Grant no. PB92-0325 (Dirección General de Investigación Científica y Técnica).

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