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Research Note

Synthetic Antimicrobial Peptide Design

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To guide the design of potential plant pathogen-resistance genes, synthetic variants of naturally occurring antimicrobial gene products were evaluated. Five 20-amino acid (ESF1, ESF4, ESF5, ESF6, ESF13), one 18-amino acid (ESF12), and one 17-amino acid (ESF17) amphipathic peptide sequences were designed, synthesized, and tested with *in vitro* bioassays. Positive charges on the hydrophilic side of the peptide were shown to be essential for antifungal activity, yet the number of positive charges could be varied with little or no change in activity. The size could be reduced to 18 amino acids, but at 17 amino acids a significant reduction in activity was observed. ESF1, 5, 6, and 12 peptides were inhibitory to the germination of conidia from *Cryphonectria parasitica*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Septoria musiva* but did not inhibit the germination of pollen from *Castanea mollissima* and *Salix lucida*. ESF12 also had no effect on the germination of *Malus sylvestris* and *Lycopersicon esculentum* pollen, but inhibited the growth of the bacteria *Agrobacterium tumefaciens*, *Erwinia amylovora*, and *Pseudomonas syringae*. The minimal inhibitory concentrations of the active ESF peptides were similar to those of the naturally occurring control peptides, magainin II and cecropin B. The significant differential in sensitivity between the microbes and plant cells indicated that the active ESF peptides are potentially useful models for designing plant pathogen-resistance genes.

Additional keywords: antibiotic, amphipathic helix.

The purpose of designing synthetic peptides is to optimize their desired activity as gene products prior to cloning them into a plant. A steadily increasing interest is being focused on small defensive peptides produced by a variety of organisms and their prospects for genetically engineering disease resistance in plants (Rao 1995; current review). Most of these small (<50 amino acids), lytic, antimicrobial peptides have been grouped into four chemically distinct groups; the magainins, the cecropins, the defensins, and the proline-rich peptides (Agerberth et al. 1991). Our research was initiated to evaluate several synthetic antimicrobial peptides which mimic the amphipathic α-helix found in magainins (Zasloff 1987; Soravia et al. 1988; Bevins and Zasloff 1990) and cecropins (Hultmark et al., 1982, Christensen et al., 1988, Durell et al., 1992) and to select one design for use in a putative plant disease-resistance gene.

Our first peptide design, ESF1, mimicked the charge distribution and amphipathic α-helical structure found in the magainin, PGLa (Soravia et al., 1988). However, ESF1 contained a different amino acid sequence and was one amino acid shorter. The α-helical structure was predicted using Garnier-Robson and Chou-Fasman models within a Lasergene program (DNASTAR, Inc., Madison, WI). Synthetic analogs of ESF1 were designed which removed the positive charge of the molecule, ESF4 and ESF13; increased or reduced the positive charge, ESF5 and ESF6; or reduced the size of the molecule, ESF12 and ESF17. The resulting amino acid sequences of the ESF peptides are shown in Table 1. The hydrophobic and charge density relative to the α-helical distribution for ESF5 and 6 are shown in Figure 1. The peptides were synthesized and purified to >80% by Genosys Biotechnologies (The Woodlands, TX).

The minimal inhibitory concentrations (MIC) of ESF1, two naturally occurring antimicrobial peptides, magainin II and cecropin B, and one magainin II analog, ($Ala^{8,13,18}$) magainin II (Chen et al. 1988), were determined for conidial germination of three plant-pathogenic fungi, *Cryphonectria para-*

Table 1. Sequences of ESF1 peptide and analogs

AA#	ESF1[a]	ESF4	ESF5	ESF6	ESF12	ESF13	ESF17
1	Met	Met	Met	Met	Met	Met	–[b]
2	Ala	Ala	Ala	Ala	Ala	Ala	Ala
3	Ser	Ser	Ser	Ala[b]	Ser	Ser	Ser
4	Arg	Gln[b]	Arg	Arg	Arg	Asp[b]	Arg
5	Ala	Ala	Ala	Ala	Ala	Ala	Ala
6	Ala	Ala	Ala	Ala	Ala	Ala	Ala
7	Gly	Gly	Gly	Gly	Gly	Gly	Gly
8	Leu	Leu	Leu	Leu	Leu	Leu	Leu
9	Ala	Ala	Ala	Ala	Ala	Ala	Ala
10	Ala	Ala	Arg[c]	Ala	Ala	Ala	Ala
11	Arg	Gln[c]	Arg	Arg	Arg	Asp[c]	Arg
12	Leu	Leu	Leu	Leu	Leu	Leu	Leu
13	Ala	Ala	Ala	Ala	Ala	Ala	Ala
14	Arg	Gln[c]	Arg	Ala[c]	Arg	Asp[c]	Arg
15	Leu	Leu	Leu	Leu	Leu	Leu	Leu
16	Ala	Ala	Ala	Ala	Ala	Ala	Ala
17	Leu	Leu	Arg[c]	Leu	Leu	Leu	Leu
18	Arg	Gln[c]	Arg	Arg	Arg	Asp[c]	Arg
19	Ala	Ala	Ala	Ala	–[b]	Ala	–[b]
20	Leu	Leu	Leu	Leu	–[b]	Leu	–[b]

[a] First peptide design from which anologs are based.
[b] Amino acid deletions.
[c] Amino acid substitutions.

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sitica (Murrill) Barr, *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder, and H.N. Hans., and *Septoria musiva* Peck. Three or more repeats of each bioassay were performed in 96-well microtiter plates containing PDAmb medium (Anagnostakis 1982). The results are shown in Table 2. The MIC is the lowest peptide concentration tested which prevents any detectable growth. The MICs for ESF1 in these tests were as low as or lower than those for Magainin II, ($Ala^{8,13,18}$) magainin II, and cecropin B, indicating that this peptide design was successful at mimicking the natural peptides' activity. There were small differential activity spectra among these peptides with respect to the MICs for the three fungi.

Eliminating positively charged amino acids destroys bioactivity. ESF4 is an analog of ESF1 in which all four of the positively charged arginines were replaced with the uncharged polar amino acid, glutamine (Table 1). This maintained the amphipathic nature of the peptide but removed all positive charges, producing an uncharged polar side to the peptide. Similarly, all the positively charged arginines were replaced with the negatively charged amino acid, aspartic acid, to produce the analog ESF13 (Table 1). Again, this maintained the amphipathic nature of the peptide but removed all positive charges, producing a negatively charged side to the peptide. The loss of the positively charged amino acids in both of these peptide analogs increased the MICs to a level above the highest concentration tested, 250 μM (Table 2). Therefore the inhibitory activities of ESF4 and ESF13 were less than one fiftieth as potent as ESF1.

Change in positive-charge density has only a modest effect on bioactivity. ESF5 contained an increased positive charge density on the hydrophilic side of the α-helix as compared to ESF1. This was accomplished by replacing two nonpolar amino acids, alanine and leucine, with positively charged arginine at amino acid positions 10 and 17 (Table 1), increasing the number of positively charged amino acids from four to six. ESF6 contained a decreased positive charge density on the hydrophilic side of the α-helix by replacing one positively charged arginine, position 14, and uncharged polar serine, position 3, with the nonpolar amino acid alanine (Table 1). This decreased the number of positively charged amino acids from four to three. Though the charge density was significantly changed in both ESF5 and ESF6 (Fig. 1), their MICs were similar and varied only 0- to 4-fold from the MICs for ESF1, depending on the fungal species tested (Table 2).

Decreasing the size from 20 to 18 amino acids lowers bioactivity. ESF12 was decreased in length to 18 amino acids by eliminating the last two amino acids in the 20-amino acid ESF1 sequence (Table 1). This decreased the peptide's size below the estimated 19 to 20 amino acids needed to form an α-helix that could span the width of a biological membrane (Wolfe 1993). The MIC of ESF12 for the germinating conidia was 2- to 8-fold higher than that of ESF1 (Table 2), indicating a small decrease in activity. Decreasing the size of the peptide to 17 amino acids, ESF17, by eliminating the methionine on the amino end of the peptide produced a 40- to 100-fold higher MIC than ESF1 (Table 2). This indicated that the smaller size of ESF17 or the loss of the amino-terminal methionine caused a significant reduction in activity. ESF12 was

Table 2. Minimal inhibitory concentrations (MIC) of antimicrobial peptides in μM for selected fungi

Peptides	Fungal species[a]		
	C. parasitica	*F. oxysporum*	*S. musiva*
ESF1	2.5	2.5	5
ESF4	>250	>250	>250
ESF5	10	5	5
ESF6	10	10	5
ESF12	10	20	10
ESF13	>250	>250	>250
ESF17	100	250	250
Magainin II ($Ala^{8,13,18}$)	10	15	5
Magainin II	30	15	10
Cecropin B	15	20	2.5

[a] Approximately 100 conidia were inoculated on the surface of 100 μl of PDAmb medium in 96 well microtiter plates containing 0, 1.25, 2.5, 5, 10, 15, 20, 25, 50, 100, 150, 200, and 250 μM concentrations of the peptide tested. Each bioassay was replicated 3 or more times.

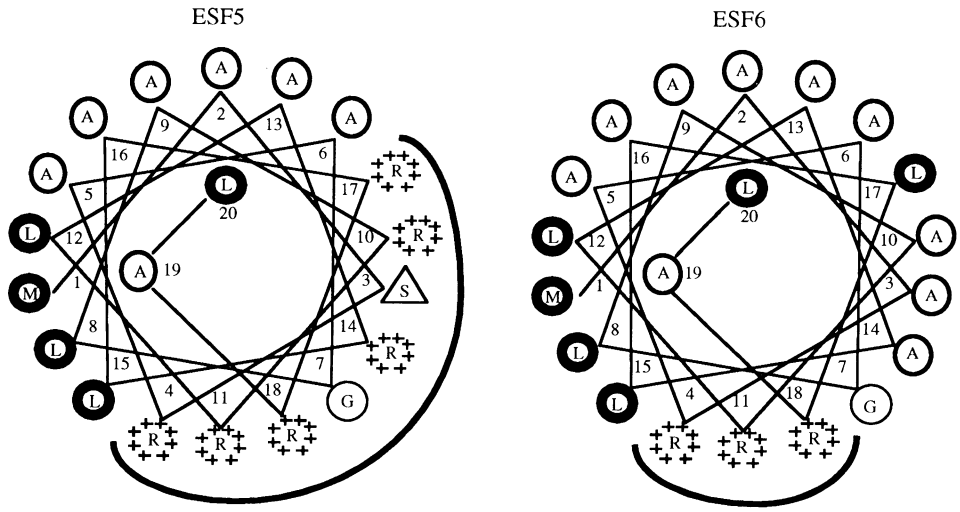

Fig. 1. Helical wheel diagrams of ESF5 and ESF6 peptides indicating differences in positive charge distributions. The numbers at the angles between the lines indicate the amino acid position, starting at the amino terminal end. Bold circles = strongly hydrophobic; lighter circles = weakly hydrophobic; triangle = polar, uncharged; and + = positively charged amino acids. Straight lines indicate bonds between amino acids.

Table 3. Minimal inhibitory concentrations (MIC) of antimicrobial peptides in μM for selected bacteria

Plant-pathogenic bacteria[a]	Peptides	
	($Ala^{8,13,18}$) magainin II	ESF12
Agrobacterium tumefaciens	100	50
Erwinia amylovora	250	250
Pseudomonas syringae	250	250

[a] Ten microliters of log phase bacteria cell suspensions (OD_{600} of 0.7–0.85) was inoculated on the surface of 100 μl of PDAmb medium in 96-well microtiter plates containing 0, 0.625, 1.25, 2.5, 5, 10, 15, 20, 25, 50, 100, and 250 μM concentrations of the peptide tested. Each bioassay was replicated 3 or more times.

chosen for further testing before using it as a putative pathogen-resistance gene product.

Proteolysis destroys peptide activity. Pretreating ESF12 with trypsin or proteinase K increased the MIC above the highest level tested (250 μM) for *F. oxysporum* f. sp. *lycopersici* conidia. The positive control, ESF12 without either enzyme, inhibited fungal growth at 20 μM. This indicated the ESF12 peptide was sensitive to inactivation by both of these two proteolytic enzymes and confirmed that the activity was due to the peptide and not to some contaminating nonprotein compound. Controls containing the proteolytic enzymes without ESF12 did not inhibit conidial germination. Pretreating ESF12 with 10 mM HCl did not change the MIC as compared to the controls, indicating insensitivity to low pH treatments.

The activity of the ESF12 peptide is comparable to a magainin II analog in bacterial bioassays. The MICs of ESF12 were compared to the MICs of ($Ala^{8,13,18}$) magainin II from bioassays utilizing the plant-pathogenic bacteria *Agrobacterium tumefaciens*, *Erwinia amylovora*, and *Pseudomonas syringae* (Table 3). ESF12 was as inhibitory to bacterial growth as the magainin analog. Both ESF12 and ($Ala^{8,13,18}$) magainin II were more inhibitory to fungal conidial germination than to bacterial growth.

Pollen germination is not affected by the ESF peptides. Plant pollens were collected (de Niella and Maynard 1993) and bioassayed in 96-well microtiter plates containing 50-μl sucrose-boric acid medium, pH 6.2 (8 pollen grains/μl). In the bioassays, germination of *Castanea mollissima* Bl. and *Salix lucida* Muhl. pollen varied from 5 to 15% among controls receiving no peptides. Pollen samples receiving ESF1, 4, 5, 6, 12, or 13, showed no inhibition of germination at concentrations up to 250 μM. Consequently, ESF1, 5, 6, and 12 demonstrated a 50-fold greater inhibition against fungal conidia germination than pollen germination. When ESF12 was tested against pollen from *Malus domestica* Borkh. and *Lycopersicon esculentum* Mill., no inhibition of germination was observed at concentrations up to 250 μM.

The goal of this study was to design and evaluate small antimicrobial peptides (≤20 amino acids) for potential use in developing plant-pathogen resistance genes. Of 7 designs tested, 4 demonstrated antifungal activity at concentrations similar to or lower than the magainin, the magainin analog, and the cecropin tested. The synthetic peptides did not demonstrate inhibitory effects on pollen germination at concentrations 50- to 100-fold the MICs for the fungi. This differential may be greater, but due to the small quantities of peptides available, the highest tested concentration was 250 μM. The MIC of ESF12 was equal to or lower than that of ($Ala^{8,13,18}$) magainin II, a strong antibacterial magainin analog (Chen et al. 1988), when tested against *E. amylovora*, *P. syringae*, and *A. tumefaciens*. Therefore, ESF12 also has potential for use in genes designed to resist some bacterial pathogens. Although the MICs reported measured total inhibition of growth, it was observed that partial inhibition had occurred at lower concentrations. However, these bioassays were not designed to quantify the percent inhibition, therefore only total inhibition is described. Further investigations will be necessary to determine the peptide's LD_{50}.

In vitro bioassays do not prove the utility of these antimicrobial peptides for constructing plant pathogen-resistance genes, but they do give insights into peptide design, such as the necessity of positive charges. To elucidate the effects of these peptides in vivo, a gene encoding ESF12 has been constructed and is being used to transform several plant species to determine if the antimicrobial activity of ESF12 can be retained and thereby increase disease resistance.

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