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Characterization of antimicrobial peptides against a US strain of the rice pathogen *Rhizoctonia solani*

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ABSTRACT

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Aim: To identify antimicrobial peptides with high lytic activity against *Rhizoctonia solani* strain LR172, causal agent of rice sheath blight and aerial blight of soyabeans in the US.

Methods and Results: Among 12 natural and synthetic antimicrobial peptides tested *in vitro*, the wheat-seed peptide, purothionin, showed the strongest inhibitory activity that was similar to the antifungal antibiotics, nystatin and nikkomycin Z. Cecropin B, a natural peptide from cecropia moth, and synthetic peptide D4E1 produced the highest inhibitory activity against *R. solani* among linear peptides. Membrane permeabilization levels strongly correlated with antifungal activity of the peptides. Noticeable changes in membrane integrity were observed at concentrations of ≥0·5 μmol l^{-1} for purothionin, 2 μmol l^{-1} for cecropin B, D4E1, D2A21, melittin, and phor21, and 8 μmol l^{-1} for magainin II and phor14. An increase of nuclear membrane permeabilization was observed in fungal cells treated with cecropin B, but not with purothionin. Diffusion of nuclear content was observed by fluorescent microscopy 10 min after adding a lethal concentration of cecropin B. Evaluation by electron microscopy confirmed severe cytoplasmic degradation and plasma membrane vesiculation. Purothionin and cecropin B were the most stable against proteolytic degradation when added to liquid cultures of *R. solani*.

Conclusions: Purothionin, cecropin B, D4E1 and phor21 were shown to exhibit high *in vitro* lytic activity against *R. solani* strain LR172 for rice and soyabean. These peptides are greater than 16 amino acids long and rapidly increase fungal membrane permeabilization. Resistance to proteolysis is important for sufficient antifungal activity of antimicrobial peptides.

Significance and Impact of the Study: Selected antimicrobial peptides offer an attractive alternative to traditional chemicals that could be utilized in molecular breeding to develop crops resistant to rice sheath blight and aerial blight of soyabean.

Keywords: antimicrobial peptides, membrane permeabilization, plant pathogen, *Rhizoctonia solani*.

INTRODUCTION

Each year fungal diseases cause millions of dollars of crop damage despite extensive use of pesticides. The fungus *Rhizoctonia solani* Kühn causes sheath blight on rice in all rice-growing countries. Moreover, *R. solani* has a wide host range infecting more than 27 families in both monocots and dicots (Roy 1993). Genes conferring complete or high levels of resistance to this fungus have not been discovered, although extensive evaluation of rice germplasm has been conducted. Only partial resistance to the disease has been reported to date (Krishnamurthy *et al.* 2001; Shanmugam *et al.* 2002). Therefore, identification and subsequent introduction of novel genes into plant hosts encoding effective antifungal compounds against *R. solani* causing sheath blight on rice is an area of great interest.

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Antimicrobial peptides are now recognized as an important component of nonspecific host defence systems, and innate immunity in insects, amphibians, plants and mammals (Hancock and Lehrer 1998; Dathe and Wieprecht 1999). They are diverse in structure and characterized by broad range of activity against bacteria, fungi, viruses and protozoa with disruption of membrane integrity (Niidome and Anzai 1999). Emergence of resistance against antimicrobial peptides is shown to be less probable than observed for conventional antibiotics (Zasloff 2002). Antimicrobial peptides interact with each other in an additive, synergistic or antagonistic manner (Westerhoff *et al.* 1995; Hong *et al.* 1999; Vorland *et al.* 1999). Utilizing combinations of peptides with synergistic interactions could lower effective inhibitory concentrations. These characteristics make antimicrobial peptides attractive targets for developing durable disease resistance against *Rhizoctonia*. Several research groups have tested the expression of antimicrobial peptides in plants as antimicrobial agents (Francois *et al.* 2002; Morassutti *et al.* 2002). Expression of sarcotoxin IA, a peptide from *Sarcophaga peregrina* in transgenic tobacco, resulted in enhanced resistance to different pathogens including *R. solani* (Mitsuhara *et al.* 2000). Overexpression of thionins in *Arabidopsis*, tobacco and rice increased resistance against fungal and bacterial pathogens, *Fusarium oxysporum* and *Pseudomonas syringae* (Epple *et al.* 1997; Iwai *et al.* 2002).

The majority of natural antimicrobial peptides including cecropin B, magainin II and melittin are cationic amphipathic molecules composed of 20–40 amino acid residues. They contain no cysteine residues, and form linear α-helical structures when bound to membranes. Cecropin B and magainin II display strong activity against both Gram-positive and Gram-negative bacteria, but exhibit low toxicity against mammalian cells (Bevins and Zasloff 1990; Boman 1995), whereas melittin produces strong lytic effects against both bacteria and mammals (Boman *et al.* 1989). Plant antimicrobial peptides such as thionins are characterized as cysteine-rich, highly basic, 40–60 amino acids long peptides, arranged in a stem and arm configuration. Results from two independent studies suggest that thionins bind to membranes for permeabilization, but do not create bona fide ion channels such as those associated with various linear antimicrobial peptides (Thevissen *et al.* 1996; Vila-Perello *et al.* 2003). Thionins display a broad spectrum of antibacterial and antifungal activity (Thevissen *et al.* 1996; Garcia-Olmedo *et al.* 1998). Automated peptide synthesis and computer-assisted combinatorial peptide chemistry have led to the design of synthetic peptides, thus extending the range of activity against important bacterial and fungal pathogens, in addition to natural peptides (Javadpour *et al.* 1996; Mayo 2000; Tossi *et al.* 2000).

The majority of antimicrobial peptides exhibit antibacterial rather than antifungal activity, although some natural and synthetic antimicrobial peptides were shown to be active against various pathogenic fungi (Zasloff 1987; Powell *et al.* 1995; Hancock and Lehrer 1998). Four synthetic linear peptides, pep6, pep7, pep11 and pep20, were reported to be effective, both *in vitro* and *in vivo*, against fungal pathogens *Phytophthora infestans* and *Alternaria solani* (Ali and Reddy 2000). Plant thionins were reported to produce both antifungal and antibacterial activity (Hancock and Lehrer 1998; Thevissen *et al.* 1999). A few lytic peptides, including cecropin B and D4E1, but not thionins, were previously evaluated for activity against *R. solani* (Owens and Heutte 1997; Reed *et al.* 1997; Rajasekaran *et al.* 2001). As fungi excrete a variety of proteolytic enzymes (De Lucca *et al.* 1998) selection of peptides resistant to fungal proteolysis could result higher antifungal activity.

The objective of our research was to evaluate and characterize antimicrobial peptides for activity against strain LR172 of *R. solani*, a highly virulent pathogen of rice (Marshall and Rush 1980; Pan *et al.* 1999; Shahjahan *et al.* 2001) and soyabeans (O'Neill *et al.* 1977) in the US. To identify the most effective peptides, we compared natural and synthetic linear peptides and wheat purothionin, previously reported to have a high antimicrobial activity, with effective antifungal antibiotics, nystatin and nikkomycin Z (Georgopapadakou 1997; Ghannoum and Rice 1999). In addition we evaluated permeabilization of *R. solani* exposed to antimicrobial peptides, degradation properties of peptides in presence of *R. solani*, and effects of peptide–peptide interactions on growth of *R. solani*.

MATERIALS AND METHODS

Peptides and fluorescent dyes

Pep6 (Reed *et al.* 1997), pep7 (Zasloff 1987) and pep11 (Cavallarin *et al.* 1998) were kind gifts from Dr ASN Reddy (Department of Biology, Colorado State University, Pittsburg, PA, USA). D4E1 and D2A21 were obtained from Demegen, Inc. Phor21, phor14 (Javadpour *et al.* 1996) and MP (Hong *et al.* 1999) were synthesized by fmoc solid-phase peptide synthesis. The wheat purothionin was kindly provided by Dr Ronald Skadsen (USDA, ARS, Madison, WI, USA). SYTOX Green and FUN-1 were obtained from Molecular Probes (Eugene, OR, USA).

Fungal isolate

Strain LR172 of *R. solani* (AG-1) (Marshall and Rush 1980) was maintained as described by Pan *et al.* (1999). It is important to note that strain LR172 is representative of *R. solani* for infection level and host range of US rice (Rush and Lee 1992), has never lost pathogenicity and that distinct races or strains of *R. solani* have never been detected in commercial US rice or soyabean fields.

Antifungal bioassay

Fungal cultures of LR172 were grown on potato dextrose agar (Difco Co., Detroit, MI, USA) and inoculum was prepared as described by Reed *et al.* (1997). Ninety-microlitre aliquots of hyphal suspensions, adjusted to 3×10^4 mycelial fragments per millilitre, were mixed with 10 μl of peptide or antibiotic at final concentrations of 0, 0·25, 0·5, 1, 2, 4, 8, 16 and 32 μmol l^{-1} in 96-well microtitre plates (model 3596; Corning, Inc., Corning, NY, USA). Plates were incubated for 48 h at 28°C without shaking in the dark. Absorbance was measured at 595 nm using the photometric Microplate Reader (SLT Rainbow; TECKAN, Research Triangle Park, NC, USA). Fungal inhibition data were expressed as percentage mortality compared with the control treatment in each experiment. IC_{50} was defined as the concentration of a compound that inhibits 50% growth of fungal cultures relative to control. MIC was defined as the minimum concentration of compound that completely inhibited growth of fungal cells. IC_{50} and MIC values were generated for each peptide using dose–response curve-fitting procedures described in SigmaPlot 5·0 (SPSS Inc., Chicago, IL, USA).

Membrane permeabilization assay

Membrane permeabilization was measured by uptake of Sytox Green, a high affinity nuclear stain that penetrates cells with compromised membranes and fluoresces upon binding to nucleic acids (Roth *et al.* 1997). Fungal inoculum was prepared as described for the Antifungal bioassay in SMF1 medium (Thevissen *et al.* 1996) and 80-μl aliquots were incubated as described above. Plates were placed on ice after 48 h and supplemented with 0·5 μmol l^{-1} Sytox Green diluted in the same medium. Antifungal compounds were added to final concentrations 0, 0·25, 0·5, 1, 2, 4, 8 and 16 μmol l^{-1}. Plates were transferred to 28°C and fluorescence was measured with a Cytofluor 4000 Plate Reader (Perceptive Biosystems, Framington, MA, USA) at an excitation wavelength of 485 nm (slit 20 nm) and an emission wavelength of 530 nm (slit 25 nm) at 1, 10, 20, 30, 40, 50, 60, 120 and 180 min after exposure to peptide. Fluorescence was normalized for each well by subtracting the fluorescence of the wells containing dye and fungal culture without antifungal compounds (average of five wells) at corresponding time point.

Fluorescent and electron microscopy

Fungi were grown as for membrane permeabilization assay. Metabolic activity was visualized with the viability stain FUN-1 for fungal cells (Millard *et al.* 1997). Hyphae were treated with 16 μmol l^{-1} cecropin B for 10 min at 28°C in the dark, and FUN-1 was added at a final concentration of 5 μmol l^{-1}. Following a 10-min incubation period, hyphae were examined with a Zeiss AxioPlan fluorescence microscope (Carl Zeiss, Göttingen, Germany) using a Rodamine filter set.

To visualize membrane permeabilization, fungal cultures were supplemented with 0·5 μmol l^{-1} Sytox Green and peptide diluted in SMF1. Treated and untreated samples were immediately monitored under a fluorescent microscope with an FITC filter set.

For examination by transmission electron microscopy, cultures were collected from 10-microtitre wells and exposed to 16 μmol l^{-1} cecropin B for 10 min at 28°C in the dark. Fungal pellets were fixed in 1·25% glutaraldehyde with 2% paraformaldehyde in 0·1 mol l^{-1} sodium cacodilate buffer, pH 7·3 for 1 h at room temperature. Postfixation was performed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. Samples were then dehydrated in an ethanol series and embedded in Epon-Araldyte resin (Polysciences, Inc., Warrington, PA, USA), after which thin sections were prepared with MTXL ultratome (RMC Products, Boeckeler Instruments, Inc., Tucson, AZ, USA), stained with uranyl acetate and lead citrate, and observed with a Zeiss-10 electron microscope.

Peptide degradation assay

Fungal cultures were prepared as for antifungal bioassay and peptides were added in potato dextrose broth after 24 h of growth at a final concentration of 16 μmol l^{-1}. Cultures were then incubated for 1, 10 and 60 min. Samples were spun down for 30 s, sodium dodecyle sulphate dye (Bio-Rad, Inc., Hercules, CA, USA) was added immediately to the supernatant, and tubes were placed in a 95°C water bath for 5 min. Samples were subjected to polyacrylamide gel electrophoresis using 10–20% linear gradient Tris-tricine/peptide gels (Bio-Rad, Inc.) and silver-stained according to the manufacturer.

Analysis of peptide interactions

After determination of IC_{50} values, peptides active against *R. solani* were tested in different combinations with cecropin B or nikkomycin Z. Eighty-microlitre aliquots of fungal suspension prepared as described above for antifungal assay were mixed with 10 μl of cecropin B or nikkomycin Z and 10 μl of a second peptide or water. Samples were incubated and measured as described above to give the observed percentage inhibition (I_{obs}) (Ali and Reddy 2000). Expected efficacy of a mixture was determined by the Abbott formula: $I_{exp} = X + Y - (XY/100)$, where I_{exp} is the expected percentage inhibition for an additive interaction, X is the percentage growth inhibition by first compound, Y is the percentage growth inhibition by second compound, XY is the percentage growth inhibition by mixture of both com-

pounds. Interaction ratios (IR = I_{obs}/I_{exp}) of 0·5–1·5, more than 1·5, and <0·5 were interpreted as additive, synergistic and antagonistic interactions, respectively (Gisi 1996).

Statistical analysis

Bioassays were performed using a completely random design. Data were analysed by the one-way ANOVA with the least significant difference test. A 95% level of significance was employed.

RESULTS

Activity of natural and synthetic peptides against *R. solani*

Using the microtitre 96-well dilution assay, we determined 50% inhibitory concentrations (IC_{50}) for antifungal peptides and wheat purothionin against *R. solani* strain LR172 and compared results with antifungal antibiotics, nystatin and nikkomycin Z (Table 1). Cecropin B produced the lowest IC_{50} and MIC values among linear natural peptides, with inhibitory effects only twofold less than the strong antifungal antibiotics nikkomycin Z and nystatin. Melittin and magainin II displayed 1·5- and threefold less inhibitory effect than cecropin B, respectively. Purothionin was the most active with inhibition levels similar to those of nystatin and nikkomycin Z. D4E1 produced the highest inhibitory levels among the synthetic peptides tested with activity identical to that of cecropin B (Table 1). The synthetic D2A21 and phor21 peptides also displayed relatively high inhibitory effects. Phor21 and phor14, evaluated for the first time for activity against any fungal pathogen, were designed as a trimer and dimer from the sequence KFAKFAK (Javadpour *et al.* 1996). The dimer phor14 displayed nearly twofold less activity than phor21. The short antimicrobial peptides MP, pep6 and pep7 showed no effect on growth of *R. solani* even at 32 μmol l^{-1} concentrations.

Permeabilization of *R. solani* exposed to antimicrobial peptides

The most active peptides and nikkomycin Z were tested for the ability to permeabilize hyphae by measuring fluorescence using Sytox Green. Nikkomycin Z had no effect on fungal membrane permeabilization at 0·25–2 μmol l^{-1} in 1 h after addition to fungal cultures (Fig. 1). In contrast, increase of membrane permeabilization was observed in 1 h for purothionin and cecropin B from 0·5 and 2 μmol l^{-1} concentrations, respectively (Fig. 1). Significant correlations ($r = 0{\cdot}86$–$0{\cdot}99$, $P = 0{\cdot}05$) between induced membrane permeabilization as measured by fluorescence using Sytox Green (Fig. 2) and growth inhibition (Table 1) was observed for all peptides, except phor14. A dramatic increase in permeabilization by cecropin B and melittin treatments was observed at 4 μmol l^{-1} (Fig. 2a). However, nikkomycin Z produced only low levels of fluorescence above control values at ≥4 μmol l^{-1} concentrations. Magainin II showed no

Table 1 Origin, sequence and inhibitory concentrations of antimicrobial peptides and antibiotics tested against *Rhizoctonia solani*

Compound	Source	Amino acid (aa) sequence	Length (aa)	IC_{50} (μmol)	IC_{50} 95% confidence interval	MIC (μmol)
Cecropin B	Cecropia moth	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	35	4·6	(4·96, 4·24)	9·8
Magainin II	African frog	GIGKFLHSAGKFGKAFVGEIMKS	23	15·7	(14·76,16·64)	30·2
Melittin	Honey bee	GIGAVLKVLTTGLPALISWIKRKRQQ	26	7·1	(6·48, 7·72)	13·1
Purothionin	Wheat	MVCLLILGLVLEQVQVEGKSCCRSTLGRNCYNL CRVRGAQKLCAGVCRCKLTSSGKCPTGFPK	63	1·14	(0·79, 1·49)	5·02
D4E1	Synthetic	FKLRAKIKVRLRAKIKL	17	4·5	(4·18, 4·82)	8·75
D2A21	Synthetic	FAKKFAKKFKKFAKKFAKFAFAF	23	6·5	(4·56, 8·46)	18·4
MP	Synthetic	KKVVFKVKFK	10	>20		ND
pep6	Synthetic	FRLKFH	6	>20		ND
pep7	Synthetic	RLARLAR	7	>20		ND
pep11	Synthetic	WKLFKKILKVL	11	11·2	(9·03, 13·27)	24·1
phor14	Synthetic	KFAKFAKKFAKFAK	14	19·2	(16·07, 22·09)	37·9
phor21	Synthetic	KFAKFAKKFAKFAKKFAKFAK	21	8·4	(7·27, 9·42)	18·6
Nikkomycin Z	*Streptomycetes*	Antifungal antibiotic		0·86	(0·71, 1·01)	4·5
Nystatin	*Streptomycetes*	Antifungal antibiotic		1·1	(0·76, 1,44)	5·2

IC_{50} was defined as the concentration of a compound that inhibits 50% growth of fungal cultures relative to control. Data from one representative experiments of two, with five replications, were used to generate dose–response curves and determine IC_{50} and MIC values.
MIC was defined as the minimum concentration of compound that completely inhibited growth of fungal cells.
ND, not determined.

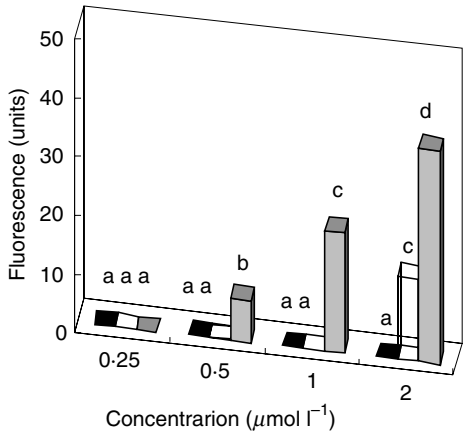

Fig. 1 Membrane compromising effects of (■) antibiotic, nikkomycin Z, (□) linear antimicrobial peptide, cecropin B and (▩) plant antimicrobial peptide, purothionin on *Rhizoctonia solani* at low concentration range 0·25–2 μmol l^{-1} as measured by Sytox Green fluorescence in SMF1 medium 1 h after exposure to antifungal agents. Data obtained from five replications were averaged across one representative experiment of two. Columns for each series marked with different letters are significantly different ($P = 0{\cdot}05$)

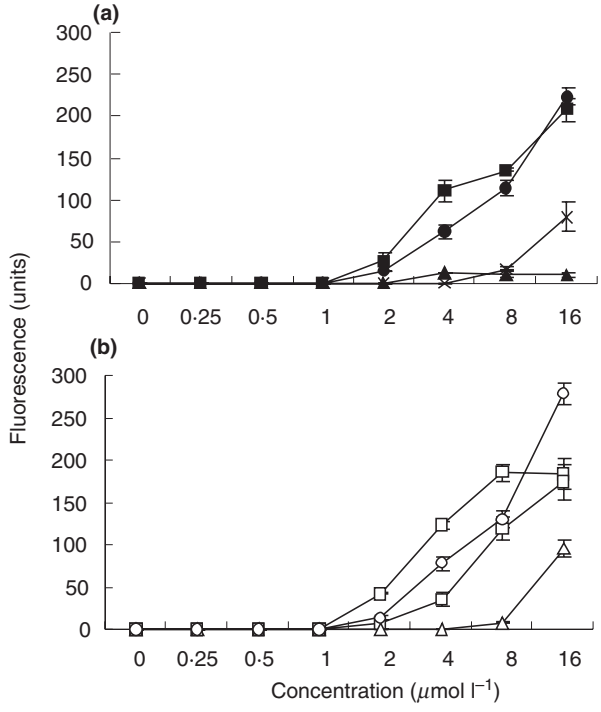

Fig. 2 Comparison of dose–response curves of membrane permeabilization of *Rhizoctonia solani* exposed to antibiotic nikkomycin Z and linear antimicrobial peptides as measured by Sytox Green fluorescence in SMF1 medium after 1 h exposure. Fungal hyphae were treated with (a), natural agents: (●) cecropin B, (×) magainin II, (■) melittin and (▲) nikkomycin Z, and (b), synthetic agents: (◆) D4E1, (□) D2A21, (▲) phor14, and (○) phor21. Data obtained from three replications were averaged across one representative experiment of two. Vertical bars represent standard errors of the mean

difference in fluorescence compared with nikkomycin Z except at 16 μmol l^{-1}. Therefore, natural peptides produced permeabilization profiles that were consistent in ranking with corresponding IC_{50} and MIC results.

Synthetic peptides, in contrast, produced permeabilization profiles (Fig. 2b) that differed in ranking from IC_{50} results. Phor21 exhibited stronger permeabilization effects at 16 μmol l^{-1} than D4E1, although the former displayed stronger inhibitory activity. D4E1, in contrast to natural peptides, produced the highest permeabilization levels at 8 μmol l^{-1}. Phor21 produced a permeabilization pattern, similar to that of cecropin B, that resulted in the highest fluorescence among all tested peptides at 16 μmol l^{-1}. A significant increase in permeabilization was detected with phor14 starting from 8 μmol l^{-1} that was similar to magainin II. Interestingly, the level of permeabilization of phor21 at 16 μmol l^{-1} was nearly threefold greater than that of phor14.

Monitoring changes in membrane permeabilization caused by lytic peptides over time revealed similar responses among peptides (Fig. 3). Rapid increase of fluorescence was observed after addition of all peptides at corresponding IC_{50} concentrations except D4E1. Fluorescence could be detected after only 1 min of incubation for the majority of peptides active against *R. solani*. The highest permeabilization level after 1 min was detected with cecropin B and phor21 at 16 μmol l^{-1}. In general, plateau values were reached within *ca* 10 min after adding linear peptides. Addition of purothionin at 0·5–2 μmol l^{-1} produced detectable membrane permeabilization to Sytox Green after only 1 min. Fluorescence slowly increased, reaching a plateau in 40 min.

Fluorescent microscopy

Alteration of viability of *R. solani* hyphae, caused by a lethal concentration of cecropin B (16 μmol l^{-1}), was monitored using the fungal-specific fluorescent dye FUN-1. Fungal hyphae, treated with the peptide, displayed significant differences in label uptake from untreated fungi. Numerous red-fluorescing points along the cell walls were observed in untreated hyphae, indicating sites of metabolically active transport of FUN-1 into cells (Fig. 4a). *Rhizoctonia solani* treated with cecropin B for 10 min showed no fluorescent label, indicating a dramatic decrease in metabolic activity (Fig. 4b).

To visualize changes in fungal membrane permeabilization, fungal hyphae were stained with Sytox Green, treated with cecropin B or purothionin, and immediately observed under a fluorescent microscope. Two minutes after addition of cecropin B at concentrations 2 and 16 μmol l^{-1}, fungal nuclei could be observed with fluorescence that increased over time. Figure 4d shows that the impermeant dye entered

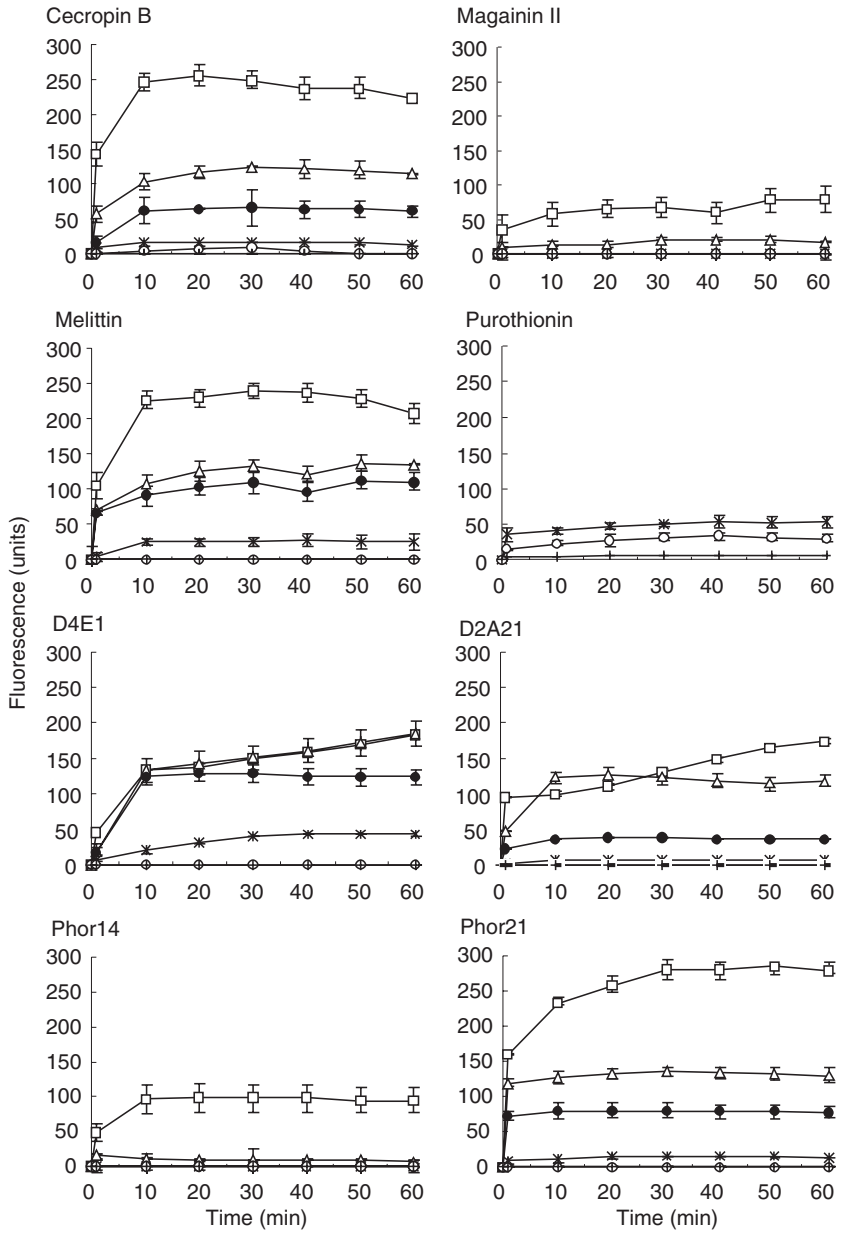

Fig. 3 Time course of *Rhizoctonia solani* membrane permeabilization, induced by antimicrobial peptides in SMF1 medium. Peptides were added at concentrations of (+) 0·5 μmol l^{-1}, (○) 1, (✳) 2 μmol l^{-1}, (●) 4 μmol l^{-1}, (▲) 8 μmol l^{-1} and (□) 16 μmol l^{-1}, and membrane permeabilization was measured by Sytox Green fluorescence at 1, 10, 20, 30, 40, 50 and 60 min. Time 0 min corresponds to fluorescence before the addition of compounds. Values are averages with standard deviations of triplicate measurements combined across one representative experiment of two. Note: permeabilization produced by purothionin was tested at range of concentrations 0·5–2 μmol l^{-1}

the hyphae treated with 16 μmol l^{-1} cecropin B and accumulated in nuclei. Within 10 min after exposure to 2 μmol l^{-1} cecropin B, intensity of fluorescence did not change and nuclei were brightly fluorescent similar to results shown in Fig. 4d. In contrast, after 10 min from addition of 16 μmol l^{-1} cecropin B, the majority of nuclei could not be distinguished. Diffuse fluorescence was observed throughout the cell cytoplasm indicating the absence of defined nuclei (Fig. 4e). When 2 μmol l^{-1} purothionin was added, low fluorescence of hyphae was observed in 2 min, however, no nuclei were visible. Fluorescence of fungal cells increased after 10 min of incubation with purothionin and was noticeably brighter than fluorescence of the culture treated with 2 μmol l^{-1} cecropin B, although nuclei were not detectable (data not shown). No fluorescence was observed with untreated hyphae within 10 min interval (Fig. 4c).

Transmission electron microscopy

Mycelia exposed to 16 μmol l^{-1} cecropin B showed structural alterations that were detected 10 min after adding the peptide (Fig. 5). Fungal cells were severely damaged as evidenced by cytoplasmic disorganization and aggregation accompanied by vacuolization (Fig. 5c,d), as opposed to control untreated cells (Fig. 5a,b). The peptide treatment resulted in retraction of the cell wall and alteration of

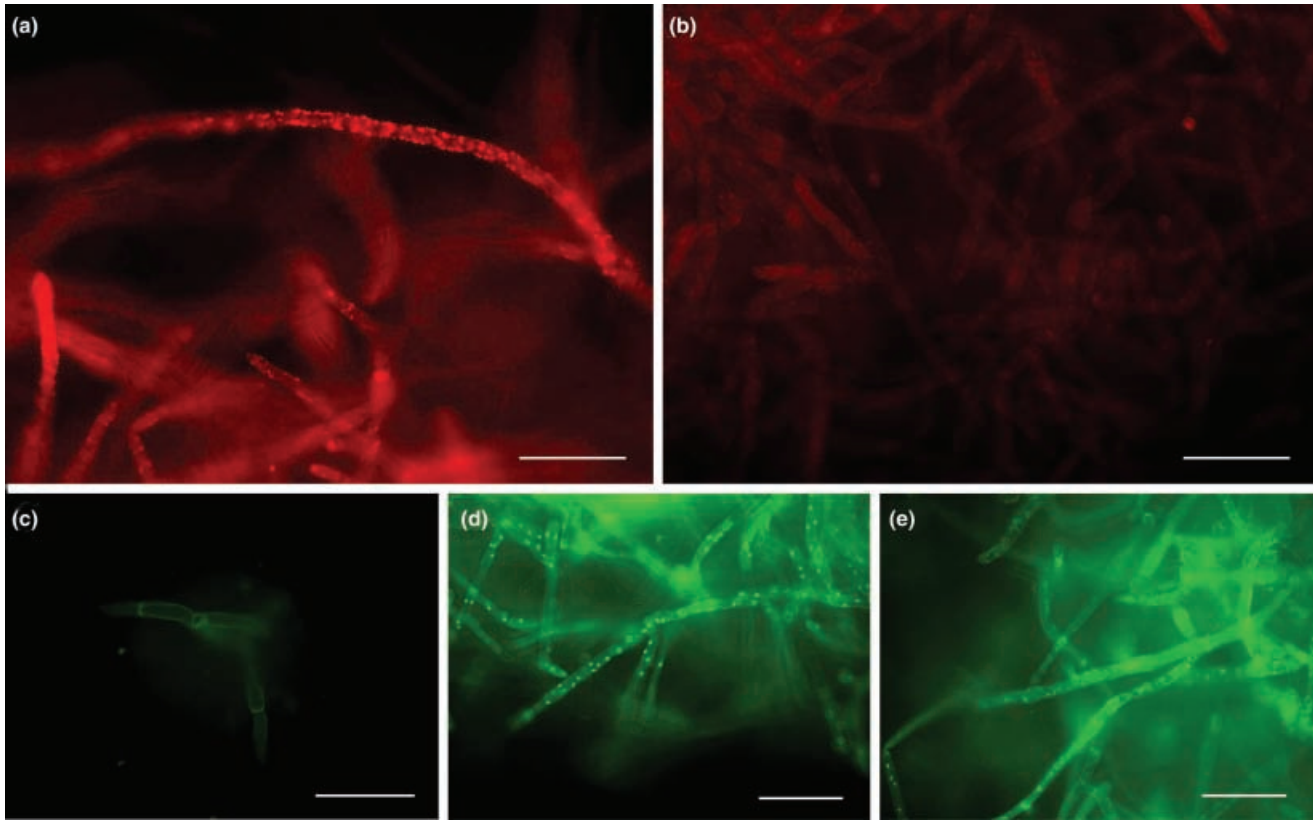

Fig. 4 Fluorescence microscopy of *Rhizoctonia solani* cells in the presence of FUN-1 and Sytox Green in SMF1 medium. Cells were supplied with H_2O (a) or 16 μmol l^{-1} cecropin B (b), and incubated for 10 min then 5 μmol l^{-1} FUN-1 was added. Fungal cultures were supplied with 0·5 μmol l^{-1} Sytox Green, then with H_2O (c) or 16 μmol l^{-1} cecropin B (d, e) and incubated for 2 min (d) and 10 min (c, e). The bar is 20 μm

integrity of the nuclear membrane (Fig. 5d). Comparison with untreated fungal cells at higher magnification (Fig. 5b) revealed changes in plasma membrane of cells treated with cecropin B (Fig. 5e). The integrity of plasma membrane of treated cells was disrupted accompanied by frequent invaginations. Formation of membrane vesicles was apparent at some locations.

Degradation of peptides in presence of *R. solani*

Persistence of the most active linear peptides and wheat purothionin in fungal culture was analysed using PAGE as all peptides examined could be detected at 16 μmol l^{-1} with silver staining except D4E1 and melittin (Fig. 6). No changes in amounts of any peptide were detected from 1 to 60 min of incubation in culture medium alone. Purothionin exhibited degradation or dimer formation within 1 min in culture medium, but remained relatively stable up to 60 min. When peptides were added to *R. solani*, all displayed reduced amounts in culture after 1 min with no apparent degradation products observed. Purothionin displayed the least decrease in peptide amount after 1 min (Fig. 6c) of incubation with fungal culture. No further changes in quantity or stability were observed for any peptide after 10 min of incubation with fungi. Purothionin was the most stable after 1 h of incubation in fungal culture (Fig. 6c). Cecropin B showed moderate degradation in culture after 1 h of incubation (Fig. 6a). D2A1 exhibited a higher rate of degradation compared with cecropin B, however its degradation produced a shorter, stable fragment (Fig. 6d). All other peptides suffered apparent degradation after 1 h in fungal culture. Magainin II and phor21 were the most susceptible as almost no peptides or their fragments were detected after 1 h (Fig. 6b,f). Interestingly, phor14 showed better stability than phor21 (Fig. 6e).

Effect of peptide interactions on growth of *R. solani*

Linear peptides that showed high activity against *R. solani* were subsequently tested in combination with nikkomycin Z or cecropin B to evaluate potential interactions (Table 2). All antifungal agents were used in concentrations less than IC_{50} values (Table 1). When each peptide was combined

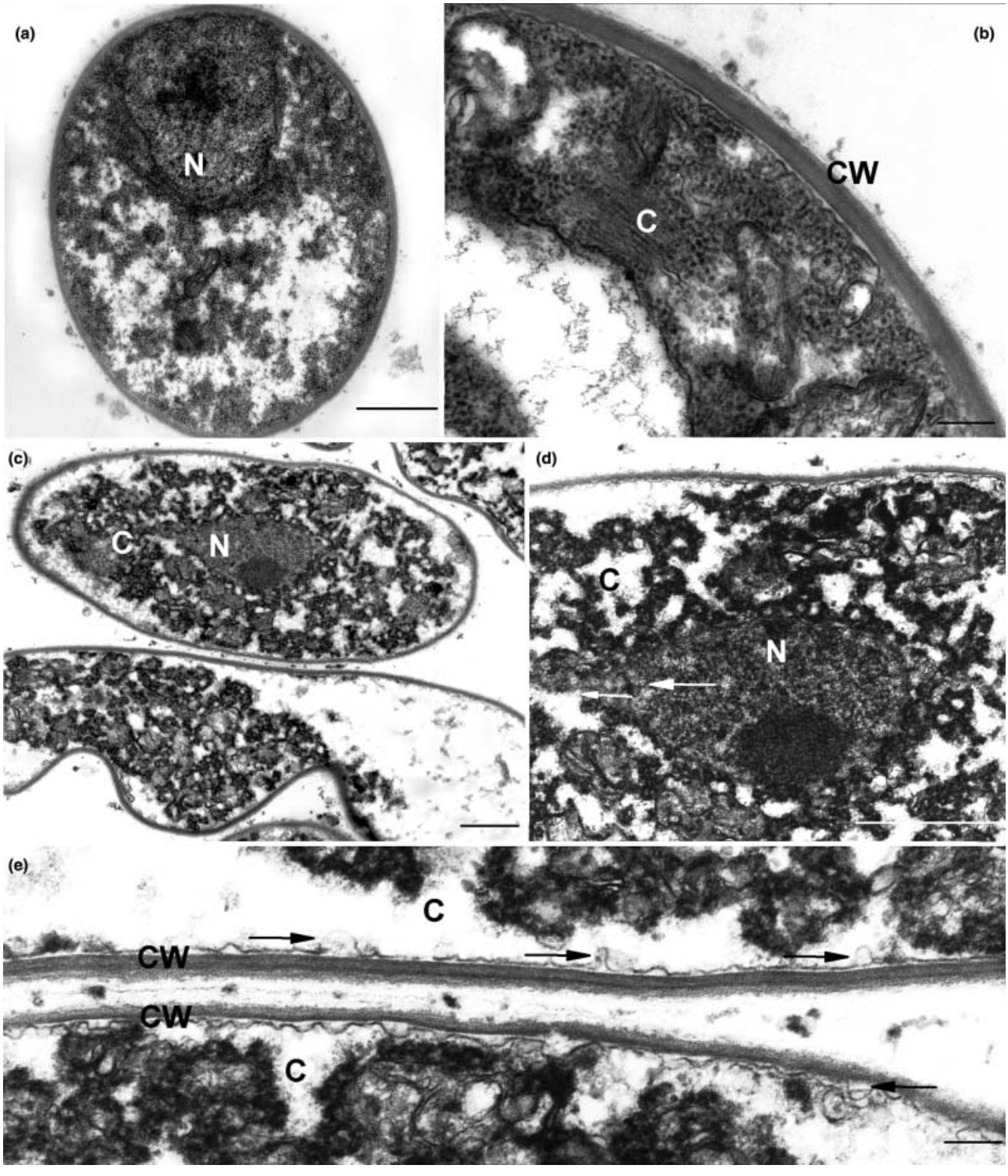

Fig. 5 Transmission electron micrographs of *Rhizoctonia solani* cells exposed to 16 μmol l^{-1} cecropin B in SMF1 medium. Hyphae in absence of cecropin B showed a dense cytoplasm adhering to a plasma membrane (a) and cell wall (b). Fungal cells treated with cecropin B after incubation for 10 min exhibited significant disorganization and aggregation of cytoplasm (c), loss of nuclear membrane integrity (arrows) (d) and invaginations of plasma membrane with forming vesicles (arrows) (e). C, cytoplasm; CW, cell wall; N, nucleus. The bar in (a, c, d) is 1 μm and in (b,e) is 0·20 μm

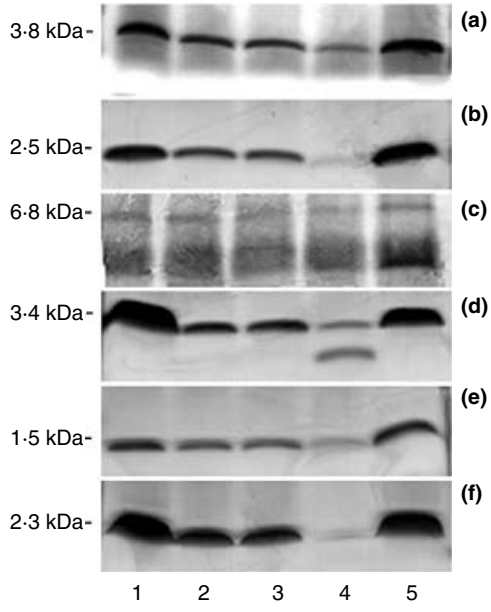

Fig. 6 Proteolytic degradation of antimicrobial peptides by *Rhizoctonia solani*. The 24-h-old fungal culture grown in PDB was treated with cecropin B (a), magainin II (b), purothionin (c), D2A21 (d), phor14 (e) and phor21 (f), at concentration of 16 μmol l^{-1}. Lane 1: no fungi, 1 min after addition of peptide; lane 2: fungi, 1 min; lane 3: fungi, 10 min; lane 4: fungi, 60 min; and lane 5: no fungi, 60 min. Supernatants were subjected to polyacrilamide gel electrophoresis and silver-stained

with nikkomycin Z, an inhibitor of fungal cell-wall chitin production, the observed percentage fungal growth inhibition indicated a lack of interaction in all combinations. Specifically, calculated interaction ratios of all mixtures of antimicrobial peptides with nikkomycin Z fell within a range between 0·8 and 1·1 which corresponded to an additive response. In contrast, when each peptide was combined with cecropin B, the observed inhibition of *R. solani* was higher than the expected value. The interaction ratios were between 1·7 and 4·4, indicating a synergistic interaction between cecropin B and other peptides. The highest synergistic effect in inhibiting *R. solani* growth was observed between cecropin B and phor14, with an interaction ratio of 4·4, despite the relatively weak inhibition shown by phor14 when used alone. Phor21, a longer peptide with the same amino acid sequence, followed phor14 in increase of inhibitory activity in combination with cecropin B (Table 2).

Table 2 Growth inhibition of *Rhizoctonia solani* and interaction of different peptides with cecropin B and the antibiotic nikkomycin Z

Nikkomycin Z and peptide combinations (μmol)	Percentage growth inhibition of *R. solani*		
	Observed*	Expected†	IR‡
Nikkomycin Z (0·5)	25	–	–
Cecropin B (2)	9	–	–
Magainin II (10)	29	–	–
Melittin (2)	17	–	–
D4E1 (2)	18	–	–
D2A21 (4)	26	–	–
phor14 (10)	1	–	–
phor21 (2)	5	–	–
Nikkomycin Z (0·5) + nikkomycin Z (0·5)	36	44	0·8
Nikkomycin Z (0·5) + cecropin B (2)	33	32	1·0
Nikkomycin Z (0·5) + magainin II (10)	52	47	1·1
Nikkomycin Z (0·5) + melittin (4)	42	38	1·1
Nikkomycin Z (0·5) + D4E1 (2)	41	39	1·0
Nikkomycin Z (0·5) + D2A21 (4)	44	45	0·9
Nikkomycin Z (0·5) + phor14 (10)	27	26	1·0
Nikkomycin Z (0·5) + phor21 (2)	31	29	1·1
Cecropin B (2) + cecropin B (2)	56	17	3·2
cecropin B (2) + magainin II (10)	62	35	1·7
Cecropin B (2) + melittin (4)	62	25	2·4
Cecropin B (2) + D4E1 (2)	69	25	2·7
Cecropin B (2) + D2A21 (4)	65	33	1·9
Cecropin B (2) + phor14 (10)	44	10	4·4
Cecropin B (2) + phor21 (2)	54	14	3·8

*Values are averages of five replications and correspond to one representative experiment of two.
†Expected inhibition of growth determined by the Abbot formula (Ali and Reddy 2000).
‡IR, interaction ratios (IR = I_{obs}/I_{exp}) between 0·5 and 1·5, >1·5, and <0·5 represent additive, synergetic and antagonistic interactions, respectively.

DISCUSSION

In this work, we have identified biologically active agents against a representative strain of *R. solani* that has been highly virulent against all commercial varieties of rice and soyabeans for the past 20 years in the US. Results from our study have important implications for application in molecular breeding of rice sheath blight and aerial blight-resistant crops. The wheat purothionin was the most effective among the tested peptides with activity similar to the known antifungal compounds nystatin and nikkomycin Z. Cecropin B was the most effective among 11 natural and synthetic linear peptides tested. Both purothionin and cecropin B meet the following criteria to be potential antifungal compounds for molecular breeding: low minimal inhibitory concentration, broad spectrum of action, nontoxicity for higher eucaryotes, rapid membrane permeabilization and stability against degradation.

Data for purothionin against R. *solani* was similar to effectiveness of purothionin against *Neurospora crassa* and *Plectosphaerella cucumerina* (Thevissen *et al.* 1999; Vila-Perello *et al.* 2003). Cecropin B displayed the highest antifungal activity among linear peptides with MIC values

only twice less than that of purothionin. In our study, magainin II was found to be substantially less active than cecropin B. This contrasts with results by Powell *et al.* (1995) who reported higher antifungal activity of magainin II than for cecropin B when tested against *F. oxysporum*. Dependence of inhibitory activity of cecropin B and magainin II on specific microbial species was previously reported. Thus, identical inhibition levels were observed for cecropin B and magainin II against *Penicillum* and *A. solani*, but twice higher activity was detected for cecropin B than for magainin II against *P. infestans* (Alan and Earle 2002). The peptide D4E1 displayed the highest antifungal activity of all synthetic peptides in our study with IC_{50} and MIC values similar to those of cecropin B. Rajasekaran *et al.* (2001) observed different levels of inhibition for D4E1 against several bacteria and fungi with IC_{50} values ranging from 0·19 to 13 μmol l^{-1}. We observed that *R. solani* was moderately susceptible to D4E1 at IC_{50} of 4·5 μmol l^{-1}. Comparison of activity of phor14 and phor21, a dimer and trimer of the same amino acid sequence, respectively (Javadpour *et al.* 1996), suggested that peptide length was associated with growth inhibition of *R. solani*. Greater inhibition of phor21 over the shorter phor14 counterpart was consistent with the postulate that optimum peptide lengths to span and bind to membranes should be greater than 16 amino acids (Lear and Wasserman 1988; Dathe and Wieprecht 1999). Short-chain peptides can span membrane by forming oligomeric bundles of head-to-tail dimmers (Anzai *et al.* 1991; Bessalle *et al.* 1993) and require higher concentrations to permeabilize membranes. None of the peptides with ≤10 amino acids in our study were effective against *R. solani* in the range of tested concentrations despite their antimicrobial activity previously reported against a wide variety of bacteria and fungi (Hong *et al.* 1999). Pep6 rapidly compromised the cell membrane of *R. solani* (AG-1), *F. oxysporum*, *Ceratocystis fagacearum* and *Pythium ultimum* with inhibitory activity three to 10 times lower than nystatin (Zasloff 1987; Reed *et al.* 1997; Ali and Reddy 2000). These results suggest that peptides with ≤10 amino acids have a narrower spectrum of antifungal activity than longer peptides. Dependence of antimicrobial activity on specific microbial species underscores the necessity to test various antimicrobial peptides against organisms of interest to identify compounds producing the greatest inhibitory effects.

For the majority of peptides tested in our study, levels of growth inhibition correlated with membrane permeabilization activity, suggesting a link of mechanism of action with membrane compromising ability. Purothionin significantly permeabilized fungal membranes at the lowest concentration, which was fourfold lower than for cecropin B. Moreover, a fourfold higher level of membrane permeabilization was observed for purothionin than for cecropin B at 2 μmol l^{-1}. Similarly, a fourfold difference in IC_{50} values was detected between purothionin and cecropin B. Membrane permeabilization effects of purothionin on *R. solani* were very similar to those for *N. crassa* where permeabilization was detected at initial concentrations of 0·5 μmol l^{-1} for purothionin (Thevissen *et al.* 1999). We observed a noticeable increase in membrane permeabilization for linear peptides only at concentrations ≥2 μmol l^{-1}. These results are similar to previous research with mammalian cells that showed cell toxicity of membrane-compromising peptides was negligible at concentrations <2 μmol l^{-1} (Felder *et al.* 1993; Martin *et al.* 1996). An association between the ability to permeabilize fungal membranes and inhibitory activity of linear peptides was evident as phor21 exhibited nearly threefold stronger permeabilization and 2·5-fold higher inhibitory activity against *R. solani* than phor14.

Our fluorescent studies showed that uptake of peptides occurred rapidly within 1 min reaching plateau values in *ca* 10 min. Rapid uptake of cecropin B was confirmed by observing accumulation of Sytox Green in fungal nuclei under fluorescent microscope and reduced amount of peptide detected after 1 min in liquid culture with *R. solani*. Similarly, a purothionin from barley was shown to significantly permeabilize membranes of *N. crassa* within 10 min while three structurally diverse plant defensins only gradually increased permeabilization during 2 h of treatment (Thevissen *et al.* 1999). The mode of linear peptide uptake involves both temperature-sensitive and temperature-insensitive processes, indicating endocytic and nonendocytic uptake (Scheller *et al.* 1999). Because peptides were added on ice in our study, the activity detected 1 min after peptide addition corresponded to a temperature-insensitive mode of uptake, which is preferable for growth inhibitors. A rapid mode of action of peptides could be valuable in defense against *R. solani* to minimize exposure to proteolytic enzymes and to quickly disrupt pathogenic cells preventing further production and release of toxins.

Previous evidence has suggested that permeabilization of target cells is nonlethal unless followed by peptide entry into the cell to cause intracellular damage (Lichenstein 1991). We showed by fluorescent microscopy the rapid incorporation of Sytox Green into the nuclei within 2 min by both, 2 μmol l^{-1} which is less than IC_{50} and lethal concentrations of cecropin B. However, lethal concentration of peptide caused disruption of nuclei and diffusion of nuclear contents after 10 min of exposure. Moreover, our electron microscopy studies revealed severe cellular degradation with damage of plasma and nuclear membranes after the same treatment, suggesting that disruption of plasma and nuclear membrane were the primary cause of fungal cell death. In contrast, purothionin did not increase nuclear membrane permeabilization as we could not observe accumulation of Sytox Green in nuclei. This evidence, together with the fact that the level of membrane permeabilization at IC_{50}

concentration was significantly lower for purothionin than for any linear peptide, suggests differences in mechanisms of growth inhibition between the linear peptides and purothionin.

Supporting the importance of proteolytic stability for antifungal activity of peptides, wheat purothionin was the most stable peptide in the presence of *R. solani*. Cecropin B was second to purothionin in stability when exposed to fungal cultures. The link between proteolytic resistance and inhibitory activity of linear peptides was reported previously for D4E1 and cecropin A (De Lucca *et al.* 1998). High proteolytic susceptibility of magainin II and phor21 was consistent with lower inhibitory activity of these peptides. Interestingly, degradation of D2A1 resulted in a shorter, proteolytically resistant fragment which could contribute to its high antifungal activity. Selecting peptides that resist degradation by fungal proteases could increase the accumulation of active peptides that reach effective inhibitory concentrations in the immediate proximity of fungal cells.

We hypothesize that different combinations of antifungal agents with different mechanisms of action could produce desired synergistic effects. However, we found that nikkomycin Z, an inhibitor of fungal chitin synthase, produced only additive effects when combined with any of the tested peptides. The lack of interaction may be due to very rapid activity of the lytic peptides that cause cell damage before the enzyme inhibitor could exert any appreciable effects. Synergistic interactions between cecropin B and six other linear peptides strongly suggested a similar mode of action for these compounds (Westerhoff *et al.* 1995; Hong *et al.* 1999). Combinations of cecropin B and phor21 or phor14 could significantly enhance levels of resistance against *R. solani* in transgenic rice and soyabeans. In conclusion, the results from our study suggest that use of genes that encode the selected active peptides, wheat purothionin, cecropin B and phor21, either individually or in synergistic combinations, should boost defense levels in molecular breeding efforts against *R. solani* causing rice sheath blight and aerial blight in the US.

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