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Enzymatic specificity of three ribosome-inactivating proteins against fungal ribosomes, and correlation with antifungal activity

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Abstract Ribosome-inactivating proteins (RIPs) are enzymes that cleave a specific adenine base from the highly conserved sarcin/ricin (S/R) loop of the large ribosomal RNA, thus arresting protein synthesis at the translocation step. In the present study, we employed three RIPs to dissect the antifungal activity of RIPs as plant defense proteins. We measured the catalytic activity of RAT (the catalytic A-chain of ricin from *Ricinus communis* L.), saporin-S6 (from *Saponaria officinalis* L.), and ME (RIP from *Mirabilis expansa* R&P) against intact ribosomal substrates isolated from various pathogenic fungi. We further determined the enzymatic specificity of these three RIPs against fungal ribosomes, from *Rhizoctonia solani* Kuhn, *Alternaria solani* Sorauer, *Trichoderma reesei* Simmons and *Candida albicans* Berkhout, and correlated the data with antifungal activity. RAT showed the strongest toxicity against all tested fungal ribosomes, except for the ribosomes isolated from *C. albicans*, which were most susceptible to saporin. RAT and saporin showed higher enzymatic activity than ME against ribosomes from all of the fungal species assayed, but did not show detectable antifungal activity. In contrast, ME showed substantial inhibitory activity against fungal growth. Using *N*-hydroxysuccinimide–fluorescein labeling of RIPs and fluorescence microscopy, we determined that ME was targeted to the surface of fungal cells and transferred into the cells. Thus, ME caused ribosome depurination and subsequent fungal mortality. In contrast, saporin did not interact with fungal cells, correlating with its lack of antifungal activity.

Keywords Antifungal activity · Cytotoxicity · *N*-Glycosidase · Pathogenesis-related protein · Ribosome-inactivating protein · RIP–fungus interaction

Abbreviations ME: *Mirabilis expansa* protein · NHS–fluorescein: *N*-hydroxysuccinimide–fluorescein · RAT: a catalytic A-chain from the heterodimeric toxic lectin ricin · RIP: ribosome-inactivating protein · S/R loop: sarcin/ricin loop

Introduction

Ribosome-inactivating proteins (RIPs) are widely distributed cytotoxic enzymes that are found in over 100 different plant species (for review, see Stripe and Barbieri 1986; Mehta and Boston 1998), and inhibit protein synthesis by virtue of their *N*-glycosidic activity. RIPs selectively cleave a specific adenine residue from a highly conserved and surface-exposed stem-loop structure termed the sarcin/ricin (S/R) loop in the large rRNA (Endo and Tsurugi 1987). RIP site-specific de-adenylation interrupts the interaction of elongation factors EF1 and EF2 with the S/R loop and blocks protein synthesis at the translocation step (for review, see Neilson and Boston 2001). Several studies have demonstrated the specificity of RIPs towards different ribosomal substrates from sources including animal, plant and bacterial species. Ricin (RIP from *Ricinus communis*), for example, is highly active toward mammalian ribosomes, but shows less activity against wheat ribosomes (Cawley et al. 1977; Lord and Roberts 1996). Maize RIP also shows higher activity against rabbit ribosomes than plant ribosomes (Bass et al. 1992; Hey et al. 1995). This enzymatic specificity suggests that RIP–ribosome interaction depends on the ribosomal conformation of the affected organism (Krawetz and Boston 2000).

Apart from enzymatic activity and substrate specificity, very little is known about the biological function and role of RIPs. A potential role for RIP as a plant defense

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protein has been deduced from its enzymatic activity and extracellular localization (Ready et al. 1986). It has been proposed that RIPs are synthesized as inactive proteins sequestered in the cell wall matrix, and re-enter the cytoplasm along with the pathogen at infection sites. Thus RIPs are thought to inhibit pathogen multiplication by inactivating the host ribosomes (Ready et al. 1986). However, recent studies suggest that RIPs can directly inhibit pathogens by inactivating their ribosomes and causing cell death (Vivanco et al. 1999; Lam and Ng 2001a, b). ME (RIP from *Mirabilis expansa*) showed direct inhibitory activity against an array of fungal and bacterial plant pathogens (Vivanco et al. 1999), and hypsin and lyophyllin (RIPs from *Hypsizigus marmoreus* and *Lyophyllum shimeji*) demonstrated the inhibition of mycelial growth in various fungal species (Lam and Ng 2001a, b). Furthermore, a recent study indicates that RIPs can inhibit fungal growth directly by entering fungal cells through synergism with other defense proteins such as chitinases, β -1,3-glucanases and proteases (Park et al. 2002). However, the actual defense mechanism of RIPs has not been clearly defined. In this communication, we further the understanding of the enzymatic specificity of three RIPs, RAT, saporin-S6 and ME, by showing that RIPs have substrate specificity and that some RIPs, such as ME, exert fungicidal activity after internalization into fungal cells via an as-yet-unknown mechanism.

Materials and methods

Protein materials

ME was purified by ion-exchange chromatography according to Vivanco et al. (1999), and saporin-S6 (SO-6) and RAT were purchased from Sigma. The proteins were dissolved in 25 mM phosphate buffer (pH 7.0), and the purity of the RIPs was confirmed by Coomassie and silver staining of SDS-polyacrylamide gels, the concentration being determined by the Bradford (1976) method using a protein assay kit purchased from Bio-Rad.

Isolation of ribosomes from fungi

Plant pathogenic fungi, *Rhizoctonia solani*, *Alternaria solani* and *Trichoderma reesei*, and human pathogenic yeast, *Candida albicans*, were grown in potato dextrose media. Mycelial tissues of fungi and yeast were collected by vacuum-filtration. The yeast and fungal hyphae were ground in a mortar with liquid N₂, and dissolved in 100 ml of extraction buffer (200 mM KCl, 25 mM MgCl₂, 25 mM EGTA, 200 mM sucrose and 25 mM β -mercaptoethanol in 200 mM Tris-HCl, pH 9.0). The supernatant was collected by centrifugation at 10,000 g for 20 min at 4 °C, carefully pipetted onto a sucrose cushion (1 M sucrose, 20 mM KCl and 5 mM MgCl₂ in 25 mM Tris-HCl, pH 7.6) in 70 Ti tubes (Beckman), and centrifuged at 55,000 rpm for 4 h at 4 °C (L-70 Ultracentrifuge; Beckman). The pellets were resuspended in 25 mM Tris-HCl buffer (pH 7.6) with 25 mM KCl and 5 mM MgCl₂, and stored at -80 °C until use.

Analysis of enzymatic activity of RIPs

The depurination assay was conducted according to Tumer et al. (1997). Briefly, fungal ribosomes were resuspended in RIP buffer (100 mM Tris-HCl, 167 mM KCl, 100 mM MgCl₂, pH 7.2) and

incubated with each RIP at 30 °C for 30 min in a total volume of 100 μ l. RNA incubated in the absence of RIPs served as a negative control. Following incubation, the RIPs were removed from the mixture by phenol:chloroform extraction and the RNA was divided in half. Half of the extracted RNA was incubated on ice for 30 min with 1 M aniline acetate (pH 4.5) and precipitated with ethanol. Both aniline-treated and untreated RNAs were subjected to electrophoresis in a 7 M urea/6% polyacrylamide gel and stained with ethidium bromide.

Antifungal assay

Antifungal activity of each RIP was determined by a radial growth-inhibition assay adapted from the method of Schlumbaum et al. (1986). Fungal plugs were placed in the center of potato-dextrose-agar (PDA) plates, and sterilized paper discs were placed around the fungal plugs. Subsequently, various amounts of sterilized RIPs ranging from 25 to 100 μ g ml⁻¹ were pipetted onto the discs. Proteins were sterilized using Ultrafree-MC Sterile (0.22 μ m GV Durapore; Millipore). The plates were incubated in the dark at room temperature. Antifungal activity was observed as a crescent-shaped zone of inhibition at the mycelial front. The effect on fungal growth was expressed qualitatively, according to the procedure of Schlumbaum et al. (1986).

Preparation of NHS-fluorescein-labeled proteins

ME and saporin were fluorescence-labeled using *N*-hydroxysuccinimide (NHS)-fluorescein (Pierce, Rockford, Ill., USA) according to the manufacturer's instructions. Briefly, 1 mg of each sample was dissolved in 100 μ l of conjugation buffer (0.1 M phosphate, 0.15 M NaCl, pH 7.2). Subsequently, 50 μ l of 2 M NHS-fluorescein dissolved in dimethyl sulfoxide (DMSO; Sigma) was added dropwise to each protein sample, and mixed by stirring. Samples were placed on ice and incubated for 2 h. The unreacted NHS-fluorescein was then removed by dialysis, and the protein concentration was determined by the Bradford (1976) method using a protein assay kit purchased from Bio-Rad. Fluorescein-labeled proteins were stored at 4 °C in 0.1% NaN₃ until use.

Fluorescence microscopy

Fluorescence-labeled ME and saporin were applied to *R. solani* using the modified plate assay method (Schlumbaum et al. 1986). Briefly, a fungal plug was placed in the center of a PDA plate, in all treatments, and sterilized paper discs were placed around the fungal plug. Subsequently, each labeled RIP was sterilized using Ultrafree-MC Sterile (0.22 μ m GV Durapore; Millipore), and pipetted onto the discs. The plates were covered with aluminum foil to exclude light, and incubated at room temperature. After 96 h incubation, fungal hyphae were carefully collected from the halo where fungal growth inhibition occurred. Collected hyphae were briefly washed with 10% glycerol, and placed on a glass plate. As a negative control, 100 μ l of 2 M NHS-fluorescein solution was applied in the antifungal assay using the same procedure as for the labeled proteins. Fluorescent images and phase images were taken using a fluorescence microscope (JEOL 2000 EXII).

Results

Substrate specificity of RAT, saporin and ME on the ribosomes isolated from *R. solani*, *A. solani*, *T. reesei* and *C. albicans*

The substrate specificities of three type-I RIPs, RAT, saporin and ME, were examined against the fungal

ribosomes isolated from *R. solani*, *A. solani*, *T. reesei* and *C. albicans*. When RIP-depurinated rRNA is treated with aniline, cleavage occurs at the depurinated site and a small nucleotide fragment is released (Fig. 1). Ribosomes from the four fungi were individually incubated with five different concentrations of each RIP (RAT, saporin and ME). rRNAs were then extracted, treated with aniline, and analyzed by urea-polyacrylamide gel electrophoresis (PAGE). Figure 1 shows the RNA depurination of *R. solani* ribosomes incubated with RIPs. RAT showed the strongest activity against *R. solani* ribosomes. Efficient depurination was observed with as low as 1 ng ml⁻¹ of RAT. Saporin and ME showed less

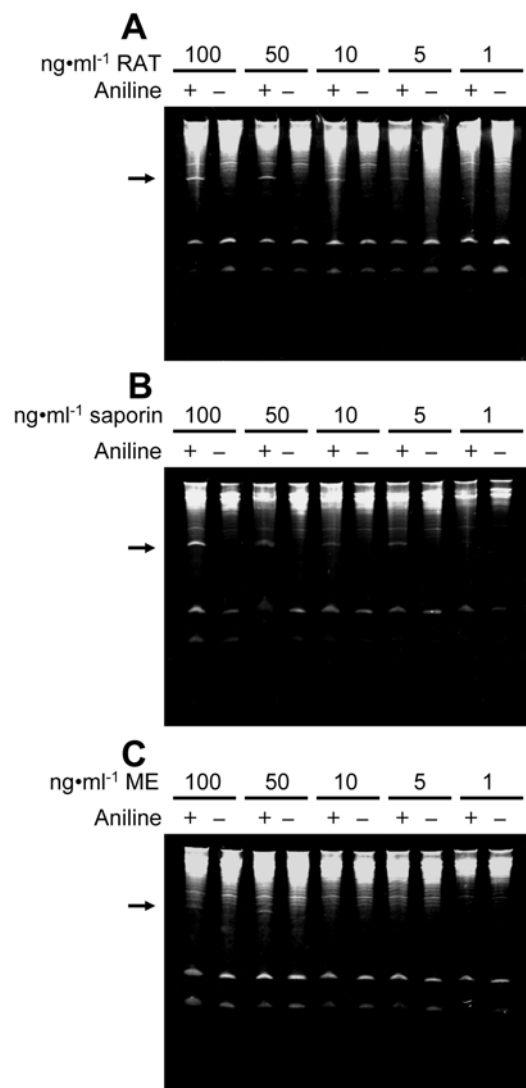


Fig. 1A–C Enzymatic activity of RAT, Saporin and ME on *R. solani* ribosomes. The ribosomes were isolated from *Rhizoctonia solani* and incubated with RAT (A), saporin (B) and ME (C) as described in *Materials and methods*. rRNAs were extracted, treated with aniline, separated on a 4.5% urea-polyacrylamide gel, and stained with ethidium bromide. RIP concentrations (ng ml⁻¹) are indicated above the lane, and the presence (+) or absence (-) of aniline is denoted. The arrow shows the presence of the diagnostic nucleotide cleavage product of rRNA

depurination activity against *R. solani* ribosomes than RAT. The enzymatic activity of saporin was approximately 5-fold less against *R. solani* ribosomes and a dose of 5 ng ml⁻¹ showed the minimum enzymatic activity. ME was approximately 50-fold less active than RAT, and a dose of 50 ng ml⁻¹ showed the minimum depurination activity.

A similar pattern of RIP activity profiles was observed against *A. solani* ribosomes as was seen against *R. solani* ribosomes (Table 1). However, the density and thickness of diagnostic fragments indicated that RAT was more active against *A. solani* than *R. solani* ribosomes (Table 1). In contrast, saporin showed enhanced depurination activity against *R. solani* ribosomes compared with *A. solani* ribosomes. Saporin showed depurination activity at concentrations as low as 5 ng ml⁻¹ against both *R. solani* (Fig. 1B) and *A. solani* ribosomes (Table 1). However, comparison of the diagnostic fragments indicated that saporin was more active against *R. solani* ribosomes than against *A. solani* ribosomes (Table 1). The enzymatic activity of ME against *A. solani* ribosomes was lower than the activities of the other two RIPs, a finding similar to the results obtained with *R. solani* ribosomes. However, ME exhibited enhanced enzymatic activity against *A. solani* ribosomes as determined by gel analysis. A sparse band was detected in *A. solani* ribosomes incubated with 10 ng ml⁻¹ of ME (data not shown); the same amount of ME did not produce a detectable fragment against *R. solani* ribosomes (Fig. 1C).

Saporin showed enzymatic activity against *T. reesei* ribosomes with 10 ng ml⁻¹ as its lowest concentration, and RAT demonstrated the strongest enzymatic activity with 1 ng ml⁻¹ as its lowest concentration (Table 1). Nevertheless, diagnostic fragments observed by gel analysis indicated that *T. reesei* ribosomes were less susceptible to RAT than *A. solani* and *R. solani* ribosomes. Interestingly, *C. albicans* ribosomes were the most sensitive to saporin with doses as low as 5 ng ml⁻¹ causing depurination (Table 1). RAT was relatively less active against *C. albicans* ribosomes than against other fungal ribosomes, creating only faint aniline fragments at a concentration of 50 ng ml⁻¹ (Table 1). Unexpectedly, ME showed no depurination activity against either *T. reesei* or *C. albicans* ribosomes upto 100 ng ml⁻¹ (Table 1).

Antifungal activity of RAT, saporin and ME

To explore the biological significance of the enzymatic activity of RIPs on fungal ribosomes, we tested the antifungal activity of RAT, saporin and ME against *R. solani* and *T. reesei* (see *Materials and methods*). Various concentrations of each enzyme were tested against the fungi, and the antifungal activity was observed as a crescent-shaped zone of inhibition at the mycelial front. RAT and saporin did not inhibit the growth of *R. solani* (Fig. 2) or that of *T. reesei* (data not shown) in plate

assays. A number of different concentrations as high as $100 \mu\text{g ml}^{-1}$ of RAT and saporin were tested against both *R. solani* and *T. reesei*, but no inhibitory activity was observed (data not shown). As summarized in Table 2, RAT and saporin had no antifungal activity even though these enzymes showed stronger enzymatic activity than that of ME against intact ribosomes isolated from *R. solani* and *T. reesei*. As shown in Fig. 2A–C, $50 \mu\text{g ml}^{-1}$ of ME substantially inhibited the growth of *R. solani* during a time course. *R. solani* was treated with various doses of ME. As little as $10 \mu\text{g ml}^{-1}$ of ME showed inhibitory activity after 96 h of incubation, but ME did not show growth inhibition against *T. reesei* (data not shown). These results indicate that ME has selective inhibitory activity against certain fungi, and correlate with the gel depurination analysis result that ME depurinated ribosomes from *R. solani* but not those isolated from *T. reesei* (Table 1).

ME interactions with the fungal hyphae of *R. solani*

To dissect the antifungal activity of RIPs at the cellular level, ME and saporin were labeled with a fluorescent dye using NHS–fluorescein. Subsequently, the enzymatic activities of labeled RIPs were tested against *R. solani* ribosomes using depurination gel analysis. As shown in Fig. 3A, 100 ng ml^{-1} of labeled ME and saporin were active against the ribosomes and released diagnostic fragments. Labeled RIPs were then applied to *R. solani* as described in *Materials and methods*. *R. solani* was incubated with the labeled RIPs in an absolutely dark chamber to prohibit the light's effect on NHS–fluorescein. As shown in Fig. 3B, NHS–fluorescein-labeled ME created an inhibitory zone against *R. solani* after 96 h of incubation, and this inhibition lasted up to 142 h (data not shown). However, labeled saporin up to $100 \mu\text{g ml}^{-1}$ did not show any inhibitory

Table 1 Comparison of enzymatic activities of RAT, saporin and ME against ribosomes isolated from *Alternaria solani*, *Rhizoctonia solani*, *Trichoderma reesei*, and *Candida albicans*. Three RIPs at five different concentrations (100 , 50 , 10 , 5 , and 1 ng ml^{-1}) dissolved in $10 \mu\text{l}$ of 25 mM phosphate buffer ($\text{pH } 7.0$) were used for the determination of enzymatic activity. The RIP effect on fungal ribosomes is expressed in qualitative terms, in which + + + + +

+ stands for the strongest activity, + for the weakest activity, and – for no activity. The activity was based on the relative intensity of the RIP-depurinated nucleotide fragments as observed in 4.5% urea–PAGE gels, and was analyzed using Gel Doc 2000 gel documentation system and Quantity One Quantitation Software (Bio-Rad)

Tested fungal pathogens	RAT (ng ml^{-1})		Saporin (ng ml^{-1})		ME (ng ml^{-1})	
	100, 50, 10	5, 1	100, 50, 10	5, 1	100, 50, 10	5, 1
<i>R. solani</i>	+ + + + +	+ + +	+ + + +	+ +	+	–
<i>A. solani</i>	+ + + + + + +	+ + + +	+ + +	+	+ +	–
<i>T. reesei</i>	+ + + + +	+ +	+ + +	–	–	–
<i>C. albicans</i>	+ +	–	+ + +	+	–	–

Fig. 2A–C Assay of the inhibition by RIPs of *R. solani* radial growth during a time course. Filter-sterilized aliquots ($50 \mu\text{g}$) of saporin (1), RAT (2) and ME (3) were applied to the discs, and tested for antifungal activities as described in *Materials and methods*. The control (4) consisted of 25 mM NaPO_4 buffer, $\text{pH } 7.5$

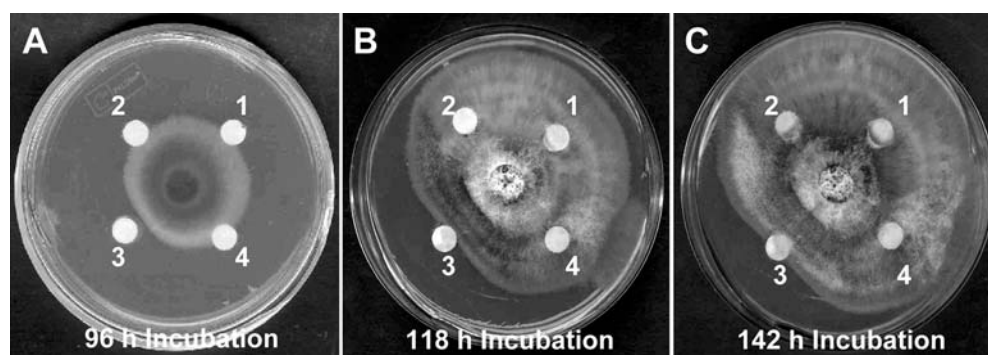


Table 2 Comparison of enzymatic and antifungal activities of RAT, saporin and ME against *R. solani* and *T. reesei*. Ten ng ml^{-1} of each RIP dissolved in $10 \mu\text{l}$ of 25 mM phosphate buffer ($\text{pH } 7.0$) was used for the determination of enzymatic activity, and $50 \mu\text{g ml}^{-1}$ of each RIP dissolved in $50 \mu\text{l}$ of 25 mM phosphate buffer ($\text{pH } 7.0$)

was used for determination of antifungal activity. The effects on fungal ribosomes and growth are expressed in qualitative terms in which + + + + + represents the strongest activity, + the weakest activity, and – no activity

Tested fungi	Enzymatic activity on ribosomes			Inhibitory activity on fungal growth		
	RAT	Saporin	ME	RAT	Saporin	ME
<i>R. solani</i>	+ + + + +	+ + +	+	–	–	+ + + + +
<i>T. reesei</i>	+ + + + +	+ +	–	–	–	–

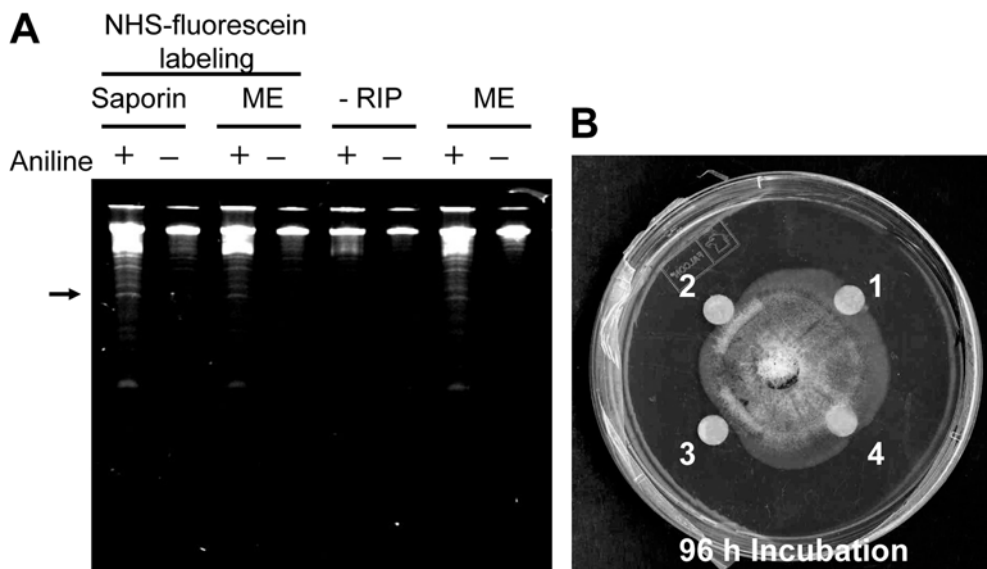


Fig. 3A, B Enzymatic and antifungal activity of NHS-fluorescein-labeled saporin and ME. **A** Ribosomes were isolated from *R. solani* and incubated with 100 ng ml^{-1} each of fluorescence-labeled saporin and ME for 30 min. rRNAs were extracted with phenol/chloroform, treated with 1 M aniline, separated by 4.5% urea-polyacrylamide gel electrophoresis, and stained with ethidium bromide. The presence (+) or absence (-) of aniline is denoted, and the arrow shows the presence of the diagnostic nucleotide cleavage product of rRNA. As a control, 100 ng ml^{-1} of non-labeled ME was used. **B** Antifungal activity of NHS-fluorescein-labeled saporin and ME against *R. solani*. Filter-sterilized $100 \mu\text{g ml}^{-1}$ of saporin (1), $50 \mu\text{g ml}^{-1}$ (2), and $100 \mu\text{g ml}^{-1}$ (3) of ME were applied to the discs, and tested for antifungal activities as described in *Materials and methods*. The control (4) consisted of $100 \mu\text{l}$ of 2 M NHS-fluorescein dissolved in 25 mM NaPO_4 buffer, pH 7.5. After 96 h incubation, fungal hyphae were carefully collected from very near the discs to monitor RIP-fungus interaction

activity, and neither did the buffer solution containing 2 M NHS-fluorescein (Fig. 3 B). Taken together, these results demonstrate that NHS-fluorescein did not interfere with the enzymatic activity of the proteins, and that antifungal activity was most likely the result of the biological function of the RIPs tested, and not a side-effect of NHS-fluorescein.

To observe the RIP-fungus interaction, carefully collected hyphae growing in the proximity of applied labeled enzymes were used to detect the localization of fluorescence by fluorescence microscopy. As shown in Fig. 4A, B, a fluorescent signal indicated that ME interacted with the fungal mycelium of *R. solani*. ME showed strong interaction with the surface of hyphae in all collected samples. Interestingly, fluorescent signal was also detected in the region between hyphal cells, demonstrating that ME could penetrate into the septa, or that ME, having penetrated the surface of cells, may move to the septa. Further, a dispersed staining pattern of fluorescent signal was observed in the cytoplasmic space of *R. solani* hyphae (Fig. 4C-F). These images indicate that internalization of ME occurs in the outer envelope after its interaction with the cell. As shown in Fig. 4F, ME then spreads out of hyphal

cells, appearing as lysed fungal cells, suggesting that ME depurinates ribosomes, and thus causes cell mortality. In contrast, hyphae collected after incubation with labeled saporin showed no fluorescent signal, indicating that saporin did not interact with *R. solani* (Fig. 4G, H), which correlates with its lack of antifungal activity.

Discussion

Although several studies have examined RIP antimicrobial properties, such as antiviral and antifungal activities (Lodge et al. 1993; Tumer et al. 1997; Wang et al. 1998; Vivanco et al. 1999; Zoubenko et al. 2000), the role of RIPs in plant defense is not yet clear. To explore the potential efficacy of RIPs as a plant defense agent, we isolated ribosomes from various plant pathogenic fungi and characterized their sensitivity to three type-I RIPs (RAT, saporin and ME). As summarized in Table 1, RAT, saporin, and ME exhibited depurination activity against fungal ribosomes in a substrate-specific manner. RAT showed overall the strongest activity toward fungal ribosomes.

In our studies comparing the catalytic activities of RAT, saporin, and ME, each RIP clearly demonstrated enzymatic specificity (Fig. 1, Table 1), indicating that the depurination activity of RIPs depends on specific RIP-substrate interaction. The RIP-mediated substrate-specificity may be dependent on the flexibility of ribosomes and/or RIP structure (Monzingo et al. 1993; Chaddock et al. 1996; Maguire and Zimmermann 2001). The RIP-ribosome interaction may contain a double-step mechanism comprised of an initial interaction with ribosomal proteins and then the depurination of the rRNA (Hudak et al. 1999; Savino et al. 2000). Saporin showed covalent linkage to yeast ribosomal proteins (Ippoliti et al. 1992), and ricin was similarly linked to L9 and L10e proteins in mammalian systems (Valter et al.



Fig. 4A–H Fluorescence images monitoring interaction between RIPs and *R. solani*. The hyphae of *R. solani* were briefly washed with 10% glycerol, and placed on a glass plate. Fluorescence images and phase images were taken using a fluorescence microscope (JEOL 2000 EXII). **A–F** Interaction between ME and *R. solani*. **G** Phase image taken from the hyphae incubated with saporin. **H** No fluorescence signaling was detected as a result of interaction between saporin and *R. solani*. Control fungal hyphae images incubated with 50 μl of 2 M NHS–fluorescein solution did not show a fluorescent signal, indicating that the signal is only detected during the RIP–fungus interaction (data not shown)

1995). PAP (RIP from *Phytolacca americana*) was also shown to interact with *Escherichia coli* L3 ribosomal protein (Hudak et al. 1999). These findings indicate that RIPs possess a structural domain involved in the recognition of specific ribosomal proteins. Recently, Savino et al. (2000) suggested the C-terminal region of saporin SO6 might be involved in this interaction. In addition, the conformational changes of the rRNA, which have been proposed to occur in the S/R loop during the elongation step, may enhance or interrupt RIP interaction with the target site (Marchant and Hartley 1995). Therefore, substrate-specificity of RIPs may be caused by (i) the C-terminal region in RIPs that recognize and interact with ribosomal proteins, (ii) unique ribosomal proteins from various ribosome sources that enhance or diminish interaction with RIPs, and (iii) differential exposure of the S/R loop during the elongation step depending on different RIP-ribosomal conformations.

We found that the *N*-glycosidic activity of RIPs on fungal ribosomes did not completely account for their antifungal activity. RAT and saporin showed approximately 10- to 50-fold higher activity against isolated fungal ribosomes than did ME (Fig. 1, Table 1). Nevertheless, RAT and saporin did not show antifungal

activity in plate assays (Fig. 2). In contrast, ME showed inhibitory activity against *R. solani* growth at a dose as low as $10 \mu\text{g}\cdot\text{ml}^{-1}$, although ME was less active toward intact ribosomes of *R. solani* than RAT and saporin (Table 2). The potential function of RIPs as defense proteins has been supported by their enzymatic activity on ribosomal substrates isolated from several pathogenic microbes (for reviews, see Tumer et al. 1999; Neilson and Boston 2001). However, in this communication, we show experimental evidence that certain type-I RIPs have biological activity as defense proteins by directly inhibiting fungal growth, and that this antifungal activity is independent of the level of their ribosome-depurinating activity.

To further our understanding of the antifungal mechanism of RIPs, we labeled ME and saporin with NHS–fluorescein and monitored the interaction between labeled RIPs and *R. solani*. As shown in Fig. 4, labeled ME showed strong interaction with *R. solani* hyphae, in contrast to no interaction between labeled saporin and *R. solani*. Furthermore, fluorescent signals were observed in the septa between two hyphal cells, and the density and thickness of signals shown within the septa and other cell barriers were similar, indicating that ME not only interacted with the surface of cell walls but also was embedded in them. These results suggest that RIPs such as ME interact with functional domains located in the fungal cell membranes and/or cell wall. Interestingly, we have also shown that ME was internalized into fungal cytoplasmic space (Fig. 4C–F). Type-I RIPs lack the lectin chain contained in type-II RIPs, and thus are believed to be incapable of penetrating undamaged cells. Here we report that ME, a type-I RIP, is capable of penetrating undamaged fungal cells. Our results also showed that ME selectively recognizes fungi and inhibits

their growth, which leads us to hypothesize that a fungal membrane receptor is required for the action mechanism of ME, and that the internalization of ME is necessary to exert its fungicidal activity. However, the mechanism of ME internalization is not clear at this point. As a possible explanation, posttranslational modifications may exist in ME similar to those of some type-I RIPs such as gelonin and dianthin (Stirpe et al. 1980, 1981), which may be utilized for binding to carbohydrate receptors on the fungal cell membrane (Lord and Roberts 1996; Sandvig and van Deurs 1996).

Internalization of ME into the fungal cytoplasm accounts for the actual antifungal action mechanism of ME by which it depurinates fungal ribosomes and causes protein synthesis to cease, thus causing cell death. Accordingly, fluorescence imaging reveals that ME bursts out of the mycelium cells through broken cell walls, causing fungal cell death (Fig. 4). In contrast, fluorescence images taken after the incubation of labeled saporin with *R. solani* show no indication of interaction between saporin and the fungus (Fig. 4G, H). This result correlates with the lack of antifungal activity of saporin, although saporin did show higher depurination activity against *R. solani* ribosomes compared with ME.

In summary, we employed the ribosome-depuration assay, the plate antifungal activity assay, and NHS-fluorescent labeling techniques to elucidate the antifungal role and function of several RIPs. Our findings provide experimental evidence that the enzymatic activity of RIPs on certain ribosomes does not fully explain their inhibitory function against those pathogens. However, the RIP ME did show direct inhibitory action against fungi. We hypothesize here that certain RIPs recognize specific fungi, are internalized into the cytoplasmic space, and kill the targeted cell by interrupting protein synthesis.

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