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Temporal Generation of Multiple Antifungal Proteins in Primed Seeds

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A drastic increase of antifungal activity was demonstrated during plant seed germination and in seed protein extract *in vitro.* Multiple antifungal proteins with a wide spectrum of activity were generated and identified. Chromatographic and electrophoretic analysis demonstrated that during seed germination, more fractions with potent antifungal activity were generated, and the antifungal activity shifted from small molecules to high molecular proteins. This germination-related increase of antifungal activity were observed in all three plants tested, i.e., cheeseweed, cigar tree and wheat. This rapid increase of antifungal activity was also observed with incubation of seed proteins *in vitro,* suggesting that at least part of the antifungal protein generation is independent of gene expression. Seven antifungal proteins with activities against five different plant pathogens were isolated from the active fractions. However, random digestion of purified seed protein with multiple proteinases failed to generate any antifungal proteins. It is suggested that during plant seed germination, a regulated biochemical process takes place that results in the generation of multiple peptides or proteins with antifungal activities. This onset of antifungal proteins is transitional in nature, but could play an important role in the protection of plants in early stage of development when the more sophisticated defense system has yet to develop. © 2002 Elsevier Science (USA)

In animals, antimicrobial proteins constitute part of the innate immunity system; peptides and small proteins with potent antimicrobial activity are used by the host to fight off dangerous microbes (1–4). Because of their distinct mechanism of action, there is increasing interest for using antimicrobial peptides as antibiotics for pathogen control (5–7). Plants, and especially their seeds, are good source of antimicrobial proteins (8–12). Recently, there have been reports that some antifungal proteins show high homology to seed storage proteins or the seed storage protein itself has antifungal activity. For example, it has been shown that some basic antifungal proteins are related to vicilin, a major seed storage protein (13). Another report indicated that vicilin isolated from cowpea showed antifungal activity (14, 15). In our previous studies, five potent antifungal proteins were isolated from a single source, and more interestingly two heterologous AFPs with fungicidal activity were first reported (16, 17). These two antifungal proteins each composed of a 5 and a 3 kDa peptide respectively, also showed high homology to the abundant seed storage protein, vicilin (18). It is believed that antimicrobial peptides play an important role in the protection of plant seeds and vulnerable young plants (19–22). During the effort to isolate more potent AFPs, we noticed that there was significant variation of antifungal activity among different batches of seed proteins even though they were extracted from the same seeds. A close examination of the variation suggested that the difference was possibly associated with the storage time of the extracted seed proteins. With this observation, a hypothesis was proposed that some seed proteins could be processed by endogenous proteinases to release fragments with antifungal activities, those antifungal proteins would function together with other pre-existing antifungal proteins to form the early plant defense system. This could be very important in the early stages of plant development especially during germination, because at this stage the more complex plant defense system has not been fully developed. Therefore, the antifungal proteins or peptides generated may be the major defense system that plants rely on during the germination period. It is proposed that plants use this proteolysis mechanism to generate a transitional onset of various antimicrobial proteins with different avidities to fight off pathogen attack during their early development.

MATERIALS AND METHODS

Chemicals, seeds and antifungal assay. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Precasted gels for

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electrophoresis were purchased from NOVEX (San Diego, CA). Cigar tree (*Catalpa speciosa*) seeds were collected from Missouri Botanical Garden (St. Louis, MO). Two collections were made. In the first collection, seeds were collected shortly after they became matured but still on the tree, the second collection was made in the winter, the seeds were collected from the ground. Cheeseweed (*Malva parviflora*) seeds were purchased from Valley Seed Services (Fresno, CA). Wheat (*Triticum durum*) NING 7840 seeds were obtained from within Monsanto Company (St. Louis, MO). Reversed phase C-18 columns were purchased from Vydac Co. (Hesperia, CA) and C-16 amide columns were purchased from SUPELCO (Bellefonte, PA). Ion exchange and gel filtration columns were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

The bioassay for antifungal activity was done in half area 96 well plate. In a total of 20 μl volume, 18 μl of spore suspension containing about 100 fungal spores was first distributed into the wells, followed by 2 μl of protein sample. The plate was incubated at room temperature overnight, and antifungal activities were rated according to the degree of growth inhibition. An arbitrary rating system of 0 to 2 was used to evaluate the fungal inhibition, 2 indicated complete inhibition, and 1 indicated good but not complete inhibition and 0 indicating no inhibition. In the case of analyzing proteins released from polyacrylamide gels, an activity rating of 0 to 3 was used where 3 indicated complete inhibition, 2 indicated good but not complete inhibition, 1 indicated weak inhibition and 0 indicated no inhibition. 25 μl of protein sample was used in the assay, and 5 μl of spore suspension (250 spores) was added to the protein sample. Seed Treatment and Seed Protein Extraction. Two treatments were used for seed priming. In the first treatment, seeds were treated with 10% sulfuric acid for 24 hr, and washed extensively to remove the acid. The primed seeds were dried down for later use. In the second treatment, the seeds were soaked in water for 24 hr and used directly in the experiments described in the text.

For seed protein extraction, 20 grams of cigar tree seeds and 200 grams each of cheeseweed and wheat NING 7840 seeds were homogenized to powder separately in liquid nitrogen and the proteins were extracted in extraction buffer (100 mM Tris, pH 7.4; 1.5% polyvinylpolypyrrolidone; 10 mM Chaps; 5 mM DTT; 1 μg/ml Leupeptin; 1 mM Benzamadine) at 4°C overnight at a ratio of 1:5 (seed: buffer). The extracted proteins were collected by centrifugation at 21,000*g* for 20 min, and precipitated with 80% ammonium sulfate in an ice-bath. The protein precipitate was collected by centrifugation and dialyzed for 48 hr with one change of buffer (4 liters, 50 mM Tris–HCl, pH 8.0, 1 mM PMSF). After dialysis, the protein preparations were centrifuged at 21,000xg for 20 min and the supernatant was used for the experiments described in the text.

Seed protein self-digestion and proteinase digestion. 100 mg of protein prepared after ammonium sulfate precipitation and dialysis was incubated at room temperature for self-digestion by endogenous proteinases for different periods of time. After self-digestion, the protein mixture was passed through a 0.4 μm filter and separated on a C-4 preparative reversed phase column (15–20 μM, 22 $\times$ 250 mm). The fractions were dried down in a SpeedVac lyophilizer (Savant Inc., Farmingdale, NY) and dissolved in 500 μl of Tris buffer, 2 μl aliquots were used to test for antifungal activity.

To test the possibility that some fragments from the seed storage protein vicilin or vicilin itself could have antifungal activity, vicilin type protein from cheeseweed was prepared according to Sammours *et al.* (23) with some modification. The protein precipitate between 70% and 90% ammonium sulfate was collected and dialyzed against water and purified by preparative MonoQ column (HR, 16/10) followed by Superdex 200 (HR 10/30) gel filtration. The identity of the protein was confirmed by Mass Spectrometry of the trypsin digest (data not shown) and the purified protein was estimated to have molecular weight of 40 kDa on SDS–PAGE with at least 90% purity.

Eight proteinases (Sigma-Aldrich, St. Louis, MO) were used to test if the digestion could release vicilin fragments with antifungal activity. These proteinases were: Pepsin; Bromelain; Proteinase XX-S (Endoproteinase Arg-C, mouse submaxillary gland); *Streptomyces griseus* proteinase; Papain; Proteinase XXVII (Nagarase, E.C.3.4.21.62); α-chymotrypsin and protease V8 (*Staphlococcus aureus* strain V8). The amount of proteinase was adjusted relative to vicilin (0.5 mg per reaction) so that the digested products remained as major bands on SDS–PAGE. After each digestion, the protein fragments were separated on a C-18 reversed phase column (5 μM, 4.5 $\times$ 50 mm), and tested separately for antifungal activity.

Electrophoretic analysis. A modified SDS–PAGE was used to demonstrate the shift of antifungal activity from small molecules to high molecular proteins. The following gel running conditions were used in the electrophoresis so that bioassay could be used to test antifungal activity directly. (1) No reducing reagent was added to the sample. (2) The sample was incubated at 42°C for 20 min before loading to the gel. (3) SDS concentration was reduced to 0.04% in the running buffer.

After gel electrophoresis, the gel was incubated in sterile PBS for 30 min, and protein lanes were cut along the gel running direction. Each protein lane was further cut into 1 mm wide gel pieces perpendicular to the gel running direction and put into 96-well plate. 80 μl of sterile water was added to the well. The plate was sonicated for 5 min to release proteins from the gel; 25 μl of solution from the sonicated sample was used for the antifungal activity assay. It was found that under these conditions, the majority of the antifungal proteins tested retained their antifungal activity (data not shown).

RESULTS

Antifungal activity shifted to high molecular weight region in primed seeds. Cheeseweed (*Malva parviflora*) seed proteins were primed with a sulfuric acid solution, and total seed proteins were extracted from the primed and untreated dry seeds. 1 mg of total protein from each preparation was separated by gel electrophoresis, and the distribution of antifungal activity was analyzed after proteins being released from the gel by sonication. Figure 1 showed that antifungal activity in primed cheeseweed seeds was shifted from small molecule region ($<$5 kDa) to higher molecular weight proteins (5–200 kDa). In contrast, proteins from the untreated seeds showed strong antifungal activity only in the low molecular weight range (1–5 kDa).

More active fractions against different pathogens were generated after seed protein self-digestion. To test the possibility that during germination, antifungal proteins were generated from seed proteins by endogenous proteinase digestion, a protein self-digestion was carried out. Protein extracts from untreated cheeseweed seeds were incubated at room temperature for digestion by endogenous proteinases, and the digested proteins were separated by chromatography. All the separated fractions were tested for antifungal activity against two fungal pathogens, *Fusarium graminearum* (*Fg*) and *Phytophthora infestans* (*Pi*). In Fig. 2, it is shown that without digestion, only 4 protein fractions exhibited low antifungal activities, whereas after digestion, 10 fractions exhibited strong antifungal activity. Moreover, 9 of the active protein fractions demonstrated complete inhibition against *Fg*, indicating drastic increase in antifungal activity. Figure 3 indicated that after protein digestion, there were also more

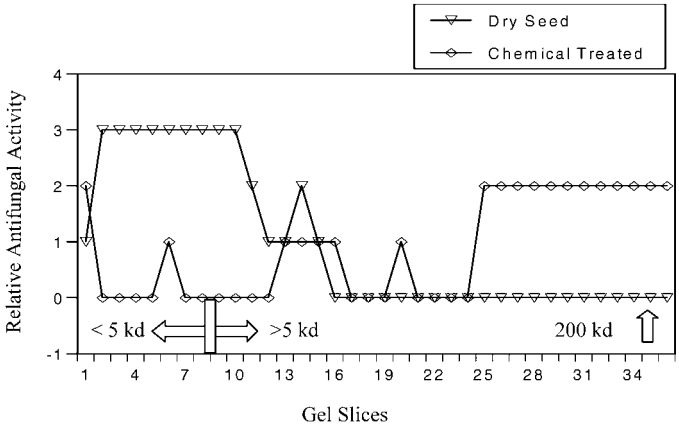

FIG. 1. Gel electrophoresis analysis of the distribution of antifungal activities from cheeseweed seeds with different treatment. Detailed assay procedures are under Materials and Methods. By using a 4–20% Tris-glycine gel, 36 gel pieces were analyzed from small molecular weight to high molecular weight region (1 through 36) with molecular size from less than 1 to 200 kDa, according to protein standards. In the relative antifungal activity rating, 3 indicated complete inhibition, 2 indicated good but not complete inhibition, 1 indicated weak inhibition, and 0 indicated no inhibition. The inhibition was rated after 24 h growth of *Fusarium graminearum* (*F.g*). In the blank treatment (b), no proteins were loaded on the lane. The minor inhibition in the first two gel slices was caused by the SDS detergent front.

fractions showing antifungal activity against *Pi,* suggesting a general increase in antifungal activity against different pathogens. A time course study demonstrated that the highest antifungal activities against *Fg* and *Pi* were peaked after 24 and 48 h incubation respectively, and decreased afterward (Fig. 4A), indicating the temporal nature of antifungal protein generation in this *in vitro* system.

To determine whether this phenomenon was present in other plant species, seed proteins from cigar tree (*Catalpa speciosa*) and wheat (*Triticum durum*) were extracted and tested after temporal incubation. It was shown that in both cigar tree and wheat seeds, the antifungal activity profile was similar to those found in cheeseweed, (data not shown). A time course study using seed proteins from cigar tree showed that anti-*Fg* and anti-*Pi* activities peaked at 24 and 48 h respectively (Fig. 4B). The observation that this drastic increase in antifungal activity occurs in all three-plant seeds suggested that this could be a general biochemical phenomenon in plants.

Since it has been reported that some potent antifungal proteins show high homology to vicillin, a major seed storage protein (18), the possibility that active antifungal proteins could be generated from vicillin through proteolytic cleavage was tested. Vicillin from cheeseweed was purified and subject to partial digestion with 8 different proteinases, and the fragments were separated to test for antifungal activity. Antifungal assay indicated that neither the purified vicilin nor the digested fragments exhibited any antifungal activity (data not shown). This result suggested that either the self-digestion process was mediated by specific proteinases, or the antifungal proteins purified earlier with homology to vicilin were actually derived from other proteins instead of vicillin (13, 16).

Seven antifungal proteins were purified after self-digestion. To further characterize the temporal increase of antifungal activity observed in the primed seeds and during seed protein incubation, antifungal proteins were purified from the active fractions by a combination of ion exchange, gel filtration and reverse phase chromatography. After the proteins were purified, the N-terminal amino acid sequences were determined by Edman Degradation, and the IC_{50} of all the purified proteins were

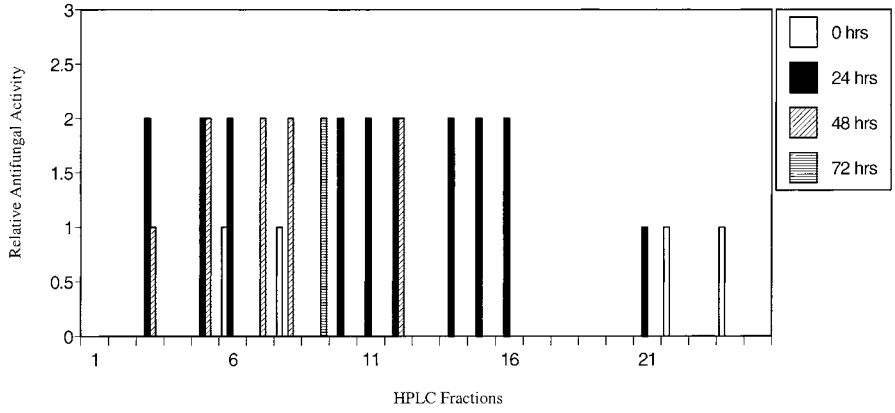

FIG. 2. Antifungal protein profile against *Fusarium graminearum* by proteins separated on a C-4 reverse phase column after self-digestion of cheeseweed seed proteins. Proteins extracted from cheeseweed seeds were digested by endogenous proteinase(s) for 0, 24, 48, and 72 h, and separated by C-4 chromatography. Antifungal activity was rated after 24 h with minor modification from the standard rating (Materials and Methods). 0 indicated no inhibition; 1 indicated minor to good inhibition; 2 indicated complete inhibition. The fractions (1 to 24) were eluted from the C-4 reverse-phase column with an acetonitrile gradient of 0 to 100% in 300 ml.

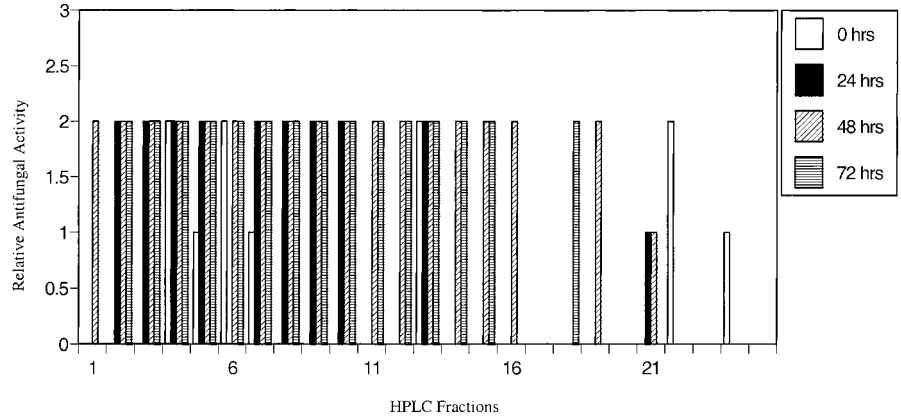

FIG. 3. Antifungal protein profile against *Phytophthora infestans* by proteins separated on a C-4 reverse phase column after self-digestion of cheeseweed seed proteins. All the assay conditions were the same as in Fig. 2.

measured against five plant pathogens (Table 1). In this study, only a few active fractions were selected for further protein purification, yielding a total of 7 antifungal proteins. A protein database search indicated that the purified antifungal proteins share sequence homology to variety of proteins, such as γ-purothionin, β-amylase inhibitor, and protein with no known functions (Table 2). Even though some of the antifungal proteins showed low potency toward the pathogens tested in this study, their relative high abundance could still provide sufficient antifungal activity to be a strong barrier for pathogen infestation. Furthermore, it is reasonable to believe that additional antifungal proteins are present in other fractions that were not characterized in this study.

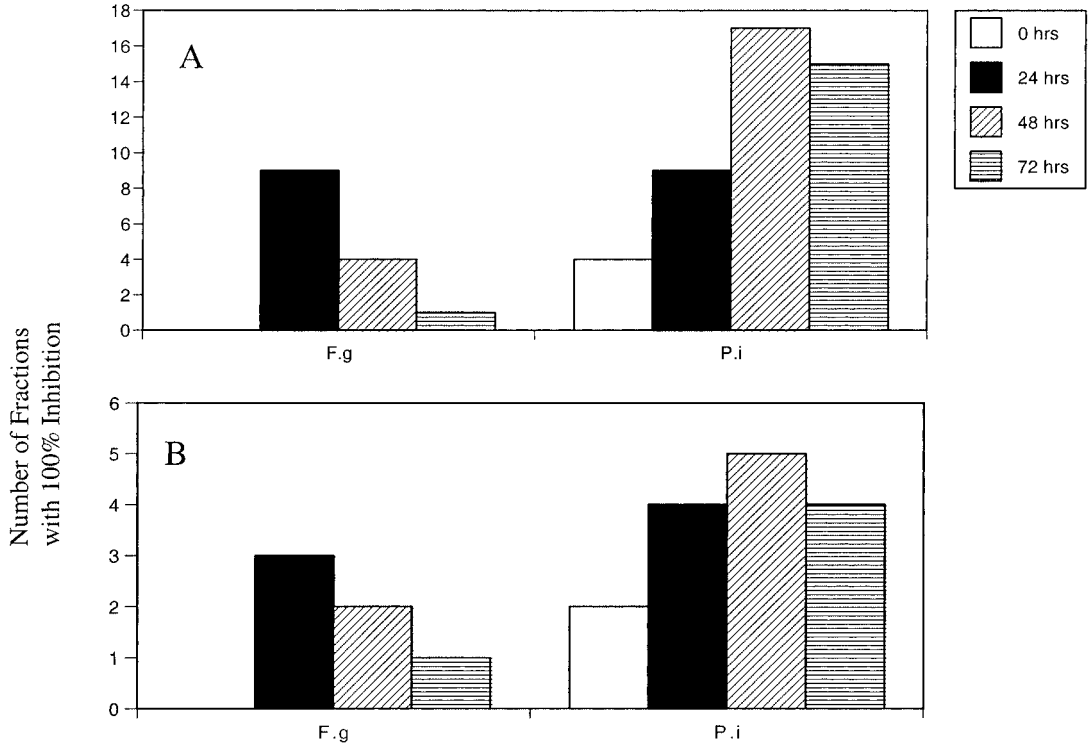

FIG. 4. Time course of antifungal activity generation from cheeseweed seed proteins after self-digestion. Only the number of fractions with complete fungal inhibition was compared. All the assay conditions were the same as in Fig. 2. F.g, *Fusarium graminearum;* P.i, Phytophthora *infestans.* (A) Active fractions with complete fungal inhibition from cigar tree. (B) Active fractions with complete fungal inhibition from wheat.

TABLE 1

Antifungal Proteins Purified from Self-Digested Seed Proteins

AFP	Source	Size (kDa)	Amino acid sequence
AFP-1	Wheat	6	KVCRQKSAGFKGPCVSDKNC
AFP-2	Wheat	4	KIXTGKSQHFKFPXVSDKN
AFP-3	Wheat	14	SGPWMCYPGQAFQVP
AFP-4	Wheat	35	ALAGESQHQXP
AFP-5	Wheat	12	IDXGHVDSLVRP
AFP-6	Cigar tree	14	EGQYE
AFP-7	Cigar tree	6	GETKGEQARKNY

DISCUSSION

Previous studies showed that in germinating seeds, upon pathogen infection, pathogenesis-related proteins (PR) were induced. Some of the induced proteins demonstrated antifungal activity (24–27). But it is not clear what biochemical mechanism is involved in the process. During the effort to screening for antifungal proteins from plant seeds, it was noticed that cigar tree seeds collected from high moisture ground showed more potent antifungal activity than those collected shortly after seeds were formed, suggesting that additional antifungal activities were generated in the former condition. Another observation was that if protein extract from seeds were kept at 4°C for an extended period of time, its antifungal activity would increase significantly. These observations led to the hypothesis that there is a biochemical process in plant seeds that is responsible for antifungal protein generation during seed germination, those antifungal proteins will form part of the initial plant innate defense system. To test this hypothesis, cheeseweed seeds were primed for germination, and antifungal activity was compared between primed and untreated seeds. Figure 1 indicated that primed seeds possess much potent antifungal activity in the higher molecular weight region (5–200 kDa) whereas untreated seeds showed strong antifungal activity in the small molecule region (<5 kDa). Similar results were observed in cigar tree and wheat, suggesting that this germination-related increase of antifungal protein might be a general phenomenon. The decrease of antifungal activity at the small molecule region from the primed seeds is attributed to the diffusion of the small antifungal molecules during seed treatment. In fact, a similar decrease of small antifungal molecules and increase of antifungal proteins were observed in both cigar tree and wheat seeds after they were incubated in water for 24 h (data not shown). Further testing in a large number of plant seeds indicated that the majority of seeds have strong antifungal activity in the small molecule region, whereas only a low percentage of seeds tested possess potent antifungal proteins without priming (data not shown).

Antifungal activity was significantly increased with *in vitro* incubation of seed proteins. To further study the germination-related antifungal activity increase, proteins from unprimed seeds were extracted and incubated at room temperature for different time periods. It is hypothesized that if the increase of antifungal activity observed in the primed seeds is a biochemical process, it is possible to reproduce the antifungal activity generation *in vitro* without gene expression. Figures 2 and 3 indicated that upon incubation, the number of protein fractions with strong antifungal activity increases drastically. In the case of proteins active against *F.g*, the most dramatic change is after 24 h incubation, active fractions with complete inhibition of *F.g* went from 0 to 9. At 72 h, only one fraction remained with complete inhibition of *F.g.* This result indicated that the antifungal protein generation is a rather transitional process *in vitro.* Further test in *P.i.* indicated that fractions with anti-*P.i* activity does not decrease significantly, suggesting that antifungal proteins with different specificity might be processed differently.

This *in vitro* generation of antifungal protein was also observed in other seeds tested. As indicated in Fig.

TABLE 2

IC_{50} Determination and Homology Analysis of Antifungal Proteins Isolated from Self-Digested Seed Proteins

AFP	Fg.	Pi.	Sn.	Mg.	Vt	Homology
AFP-1	270	70	140	0*	70	70% identical to γ-purothionin from wheat
AFP-2	46	0	0	0	0	63% identical to Ω-hordothionin from barley
AFP-3	240	0	240	0	120	100% identical to α-amylase inhibitor from wheat
AFP-4	3.3	0	0	0	2	71% identical to rice 1-3, 1-4-β-glucanase
AFP-5	84	0	84	ND**	ND	100% identical to LTP from wheat
AFP-6	0	0	4	ND	ND	100% identical to a hypothetical protein from Thiobacillus. F.
AFP-7	30	15	30	ND	ND	64% identical to an embryonic abundant protein from common sunflower

Note. Fg, *Fusarium graminearium;* Pi, *Phytophthora infestans;* Sn, *Stagonospora nodorum;* Mg, *Magnaporthe grisea;* Vt, *Verticillium daliae.* *IC_{50} > 500 ppm; ND**, Not determined.

4, seeds from both cigar tree and wheat showed transitional generation of antifungal protein against *F.g* and *P.i* respectively. Since several antifungal proteins with high homology to seed storage proteins have been isolated recently (13, 16), it is hypothesized that some of the antifungal activity observed above might be derived from limited seed protein digestion. To test this hypothesis, vicillin, a major seed storage protein from cheeseweed was purified, and test on the protein itself does not show any antifungal activity. The purified protein was partially digested with 9 different proteinases, and fragments were isolated by chromatography. No antifungal activity was tested from any of the fragments isolated (data not shown). This result suggested that both the *in vivo* and *in vitro* generation of antifungal activity might be a highly regulated biochemical process, requiring specific protein cleavage and processing. It will be interesting to know what kind of proteinases were involved in this process in the primed seeds.

Multiple antifungal proteins were isolated from self-digested seed protein. To demonstrate that the observed increase in antifungal activity is the results of *de novo* generation of antifungal proteins, a few active fractions were selected for further protein purification. Tables 1 and 2 showed that seven antifungal proteins were isolated. It is important to note that only a few fractions of the many showing complete pathogen inhibition were selected for antifungal protein isolation. The total number of antifungal proteins generated during the self-digested process could be much higher. From Table 2, it is obvious that the antifungal proteins isolated have a wide range of antifungal spectra and potency. It has been demonstrated that antifungal proteins could have synergistic enhancement against pathogens (28). Since some of the antifungal peptides could be derived from abundant seed proteins, those antifungal proteins together could form a strong defense system for the germinating plants as indicated previously.

In conclusion, this study presented preliminary evidence that there may be a regulated biochemical process in plant seeds that, during germination, could generate a wide range of novel antifungal proteins. Plant defense system is a complicated and highly regulated system (29–32), the process of multiple antifungal protein generation described above might play an important role during the early stage of plant development. Further elucidation of this process and identification of the proteinases involved in the specific cleavage should help us to understand this physiological process, and provides us a rich source of antifungal proteins with potential applications in plant disease control and development of novel antibiotics.

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