Different Functions and Expression Profiles of Curcin and Curcin-L in *Jatropha curcas* L.

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To date, two types of ribosome-inactivating proteins (RIPs) have been found in *Jatropha curcas*. One is curcin, which has been isolated from the endosperm, and the other is curcin-L, which is expressed in leaves upon stress treatment. Phylogenetic analysis of the predicted amino acid sequences of the RIPs in plants revealed that these belong to a major subfamily and are close to trichosanthin (TCS). Studies on the mRNA and protein levels showed that both curcin and curcin-L have an organ-specific expression pattern. Curcin is only expressed and accumulated in the endosperm; its expression begins in the globular embryo period and peaks during the mature embryo period. In contrast, curcin-L is only expressed in the leaves, but its expression is induced by certain conditions such as treatment with phytohormones or polyethylene glycol, exposure to high and low temperatures, and fungal infection. Analysis of the 5' flanking regions of curcin and curcin-L revealed that the 5' flanking region of curcin-L has three major inserted fragments, which are not present in the corresponding region of curcin. Comparison of characteristic *cis*-elements suggests the presence of several motifs that are involved in the endosperm-specific expression in the 5' flanking region of curcin, while in curcin-L some stress- and defense-responsive motifs are found to be mainly located in the three inserted fragments. Comparison of the antifungal activity of the two RIPs showed that the one of curcin-L is higher than that of curcin. Differences in the expression and activity of curcin and curcin-L suggest that these two RIPs have different functions.

Key words: Jatropha curcas L., Ribosome-Inactivating Protein, Antifungal Activity

Introduction

Ribosome-inactivating proteins (RIPs) are a type of RNA *N*-glycosidase. They depurinate the universally conserved α-sarcin loop of ribosome large rRNAs, inactivating these and thereby inhibiting protein synthesis. Many studies have indicated that RIPs also have other biological activities such as antiviral, antifungal, and insecticidal activities, immunological effects, abortifacient activity, and transforming activity (Stirpe, 2004).

Previous studies have shown that almost all RIPs accumulate at various stages in certain plant tissues. For example, ricin and its genetic transcript begin to accumulate during the post-testa stage of seed development (Tregear and Roberts, 1992), and tricin transcripts accumulate in the endosperm during late seed development (Leah *et al.*, 1991).

In certain plant species, biotic and abiotic stresses induce the expression and accumulation of RIPs. For instance, treatment of young barley leaves with jasmonate and abscisic acid (ABA) resulted in the accumulation of jasmonate-induced protein (JIP60) at a level 5 to 10 times higher than that obtained by treatment with jasmonate alone (Chaudhry *et al.*, 1994). In another example, salt stress (0.5 M NaCl) was shown to strongly promote the expression of a type 1 RIP in the common ice plant *Mesembryanthemum crystallinum* (Rippmann *et al.*, 1997).

Two RIPs have been found in *Jatropha curcas*, a multipurpose plant with many attributes and considerable potential (Fairless, 2007). Based on the expression and accumulation characteristics of the RIPs described above, we studied curcin and curcin-L in seeds from various periods and seedlings under different artificial stress condi-

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tions. The cDNA fragments of the two RIPs were cloned and expressed in *Escherichia coli*, and the antifungal activities of the recombinant RIPs were analyzed. Our aim was to elucidate the expression profiles and determine the roles and functions of these two RIPs in *J. curcas*.

Materials and Methods

Materials

Mature seeds of *J. curcas* were collected from Panzhihua City, Sichuan Province, China. The seedlings were germinated from mature seeds and grown in a conservatory for 2 months. Methyl jasmonate (MeJA) was dissolved in *N*,*N*-dimethylformamide, while ABA and salicylic acid (SA) were dissolved in ethanol. MeJA, SA, ABA, and ethephon were diluted with 20 mM sodium phosphate buffer (pH 6.0). Their final concentrations were 1 mM, 5 mM, 15 mM, and 50 mM, respectively. The seedlings were sprayed every 2 h with one of the four solutions and left for 36 h. Polyethylene glycol 6,000 (PEG 6,000), NaCl, $MgCl_2$, and $AgNO_3$ were dissolved in water. Some seedlings were drenched with solutions of 30% PEG, 500 mM NaCl, 500 mM $MgCl_2$, or 50 mM $AgNO_3$ for 36 h. Using a sterile cotton swab, the leaves of some seedlings were inoculated with cell suspensions (cultured for 3 d) of *Curvularia lunata* (Walk.) Boed., *Gibberella zeae* (Schw.) Petch., or *Pestalotia funerea* (maintained in our laboratory). Some seedlings were also grown at 4 °C or 45 °C for 36 h. Control seedlings were continued to grow at 30 °C without being subjected to any type of treatment.

Electrophoresis and Western blot analysis of proteins

Total protein from the roots, stems, leaves, and seeds of *J. curcas* was extracted by grinding the samples in liquid nitrogen. The samples were homogenized in 5 mM phosphate buffer (pH 7.2) with 200 mM NaCl, 5 mM dithiothreitol, and 1.5% (w/v) polyvinylpolypyrrolidone and then centrifuged at 10,000 × *g* for 20 min at 4 °C to pellet the cell debris. SDS-PAGE was performed on 12% (w/v) acrylamide discontinuous gels. Western blot analysis was performed using antibodies against recombinant curcin and curcin-L. This was followed by treatment with alkaline phosphatase-labeled goat anti-rabbit IgG from Sino-American Biotechnology Company (Shanghai, China). Detection was carried out with the alkaline phosphatase system using NBT-BCIP as the substrate (Sigma, St. Louis, USA).

Total RNA extraction and RNA blot analysis

Total RNA was extracted from the plant materials using the RNeasy Plant Total RNA Kit (Qiagen, Valencia, USA). Aliquots (20 μg) were loaded on each lane of an 1.5% formaldehyde agarose gel, fractionated by electrophoresis, and transferred onto a nylon membrane (Schleicher & Schuell, Keene, USA). The membrane was briefly washed in 20 × SSC and baked at 80 °C for 2 h (Sambrook and Russell, 2001). Hybridization and immunological detection were performed according to the instructions provided with the Roche DIG high prime DNA labeling and detection starter kit II. The blotting probes (P1, P2) were synthesized using the PCR DIG probe synthesis kit. The specific paired primers of the curcin probe (P1) were as follows: Jc_curcin1 (5'-GCC AAA GTC ATA AAT GTA GCG AAT T-3') and Jc_curcin2 (5'-CAA CAA GAC TCC CAT GAC ACC TGC-3'). The specific paired primers of the curcin-L probe (P2) were as follows: Jc_curcin-L1 (5'-GGA TCC ATG GCT GGT TCC ACT CCA ACT TT-3') and Jc_curcin-L2 (5'-GAG CTC ATA CAT TGG AAA GAT GAG GA-3').

Analysis of the 5' flanking region

The sequences (curcin, GenBank accession no. AF469003; curcin-L, GenBank accession no. EU195892) were analyzed by Vector NTI. Functional sequences of the promoter region were searched in the PLACE database (plant *cis*-acting regulatory DNA elements database, http://www.dna.affrc.go.jp/PLACE/). The homology of the sequences was contrasted using the BLAST program provided by the National Center of Biotechnology Information (NCBI, http://www.ncbi.nih.gov/).

Construction of the recombinant strain

RT-PCR was used to produce the DNA sequences encoding curcin and curcin-L. The fragments were integrated into the pQE30 vector and expressed in *E. coli* strain M15 to create the recombinant strain. A single colony of recombinant *E. coli* strain M15 was subjected to double enzy-

matic digestion and DNA sequence analysis, and its recombinant vector was sequenced.

Expression and purification of the recombinant protein

The recombinant *E. coli* M15 strain was inoculated in LB medium containing ampicillin (100 $\mu g \cdot ml^{-1}$) and incubated at 28 °C with shaking at 250 rpm. IPTG was added to a final concentration of 0.5 mM to induce the expression of the recombinant vector at the time point at which the absorbance of the culture at 600 nm reached 0.5. The culture was continuously incubated for 6 h, and the cells were pelleted by centrifugation at 5,000 × *g* for 15 min at 4 °C. The harvested cell paste was resuspended in 50 ml of buffer A [50 mM Tris (hydroxymethyl aminomethane)-HCl, 0.5 mM EDTA, and 50 mM NaCl (pH 8.0)], lysed with 150 $\mu g \cdot ml^{-1}$ lysozyme and 100 mM phenylmethyl sulfonylfluoride, and ultrasonicated. The suspension was then centrifuged at 10,000 × *g* for 10 min at 4 °C. The pellet was resuspended in nine volumes of buffer B (buffer A supplemented with 0.5% Triton X-100 and 10 mM EDTA) and centrifuged at 10,000 × *g* for 10 min at 4 °C to collect the inclusion bodies. The inclusion bodies were solubilized in buffers of different pH values (pH range 4 – 12), denaturants (urea or guanidine-HCl), or in a mixture of denaturant and salt. The suspension was stored overnight at 4 °C and then centrifuged at 10,000 × *g* for 20 min. The supernatant was filtered through a 0.45-μm filter (Millipore, Bedford, USA) and was then purified by Ni-NTA agarose affinity chromatography, as described in the QIA expression manual (Qiagen). The purified protein (approx. 90% purity) was then refolded by dialysis in phosphate-buffered saline in a diminishing concentration of urea. The refolded protein was then concentrated by lyophilization at –80 °C.

Inhibition of cell-free protein synthesis and the antifungal activity assay

The enzyme activity of the purified recombinant protein was determined by a protein synthesis inhibition assay using a rabbit reticulocyte lysate system kit (Promega). The antifungal activity toward curcin and curcin-L was assayed in 90-mm diameter Petri plates containing 10 ml potato dextrose agar (PDA). After developing the fungal colony in potato dextrose, the PDA solution was autoclaved and allowed to cool to 50 °C prior to the addition of 25 ml of fungal culture. It was then shaken and returned to the water bath. The solution (10 ml) was added to each Petri dish using aseptic techniques and allowed to set at room temperature. Sterile blank paper disks (6.0 mm in diameter) containing solutions of either curcin or curcin-L were placed in the dishes. The plates were incubated at 28 °C for 72 h. Following incubation, the plates were checked for the presence of clear zones around the blank paper disks, and the distance of these zones was measured.

Results

Tissue-specific expression of curcin and curcin-L

Expression of curcin and curcin-L in *J. curcas* L. tissues was examined by Western blotting (Fig. 1). Although, expression was detected in various tissues such as ovaries, leaves, flowers, and roots, the expression levels differed sharply. Among the tissues examined, high curcin expression was found in the endosperm. PEG treatment resulted in curcin-L expression in the leaves. Curcin-L expression was not detected in preliminary experiments in which the plant was not subjected to any type of stress treatment. Undetectable or very low expression was observed in other tissues.

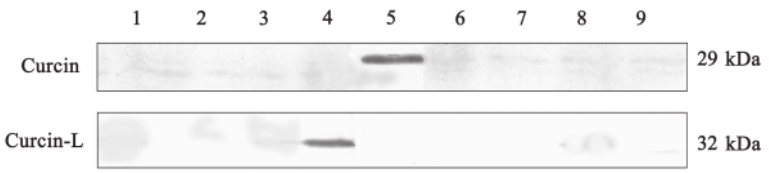

Fig. 1. Western blot demonstrating the expression of the two RIPs. The protein was isolated from the following: 1, buds; 2, roots; 3, stems; 4, leaves; 5, endosperm; 6, embryo; 7, ovaries; 8, female flowers; and 9, staminate flowers. The seedlings were either untreated or treated with PEG 6,000.

Expression of curcin at various developmental periods of the seeds

Seed formation is accompanied by the disruption and differentiation of cells, a process that involves signal regulation and signal switches of various genes related to the developmental stages. Therefore, it was necessary to study the influence of the developmental stage on curcin expression. The curcin probe could detect signals of curcin mRNA, and curcin mRNAs began to accumulate during the globular embryo or heart-shaped embryo period, reaching a steady-state peak during the mature embryo period (Fig. 2).

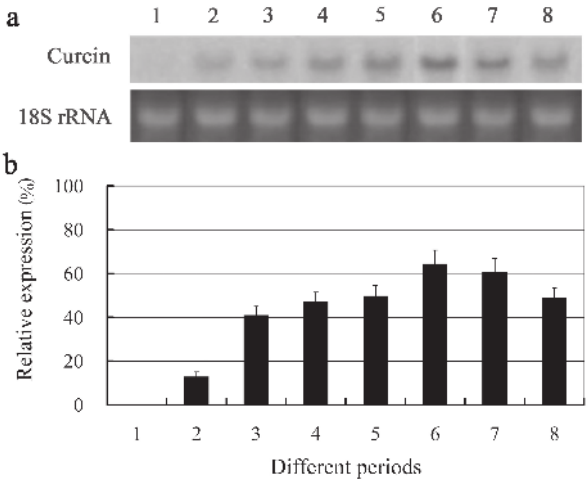

Fig. 2. RNA blot to demonstrate the expression of the two RIPs in the endosperm. RNA was isolated from the following: 1, ovaries of unpollinated female flowers; 2, ovules from the globular embryo period; 3, seeds from the heart-shaped embryo period; 4, seeds from the initial leaf embryo period; 5, endosperm from the seminal leaf embryo period; 6, endosperm from the mature embryo period; 7, endosperm from storage seeds; and 8, endosperm from germinated seeds. 18S rRNAs were used to verify equivalent mRNA loading.

Expression of curcin-L under various stress conditions

After subjecting the plants to various stress treatments, specific curcin-L probes were used to detect the mRNAs present in the leaf extracts. The results showed that the specific curcin-L probe detected a signal under most types of treatment conditions and that the mRNA yields differed under various treatment conditions. The yield obtained upon PEG stress treatment was the highest, while that obtained by $AgNO_3$ treatment was the lowest. The mRNA yields were almost undetectable upon $MgCl_2$ treatment (Fig. 3).

Sequence analysis

Comparison of the sequences of curcin and curcin-L revealed that their cDNAs had 92% identity, while their encoding amino acids had 87% identity; however, curcin-L has 16 amino acid residues more than curcin. We examined the phylogeny of various RIPs, including curcin and curcin-L, in many plants. Most RIPs were

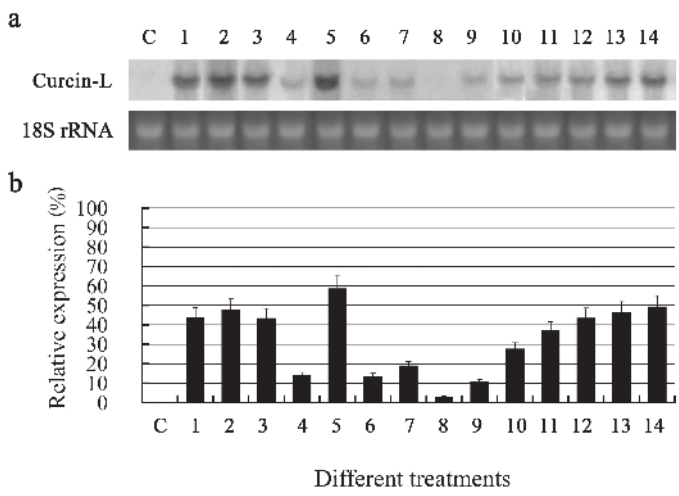

Fig. 3. RNA blot to analyze the expression of the RIPs under different types of stress conditions. RNA was isolated from leaves of the following seedlings: 1, treated with 1 mM MeJA; 2, treated with 5 mM SA; 3, treated with 15 mM ABA; 4, treated with 50 mM ethephon; 5, treated with 30% PEG 6,000; 6, treated with 500 mM NaCl; 7, having wounds; 8, treated with 500 mM $MgCl_2$; 9, treated with 50 mM $AgNO_3$; 10, treated at 4 °C; 11, treated at 45 °C; 12, infected with *C. lunata* (Walk.) Boed.; 13, infected with *G. zeae* (Schw.) Petch.; 14, infected with *P. funerea;* and C, untreated that served as the negative control. Ethidium bromide staining of 18S rRNAs was used to verify equivalent mRNA loading.

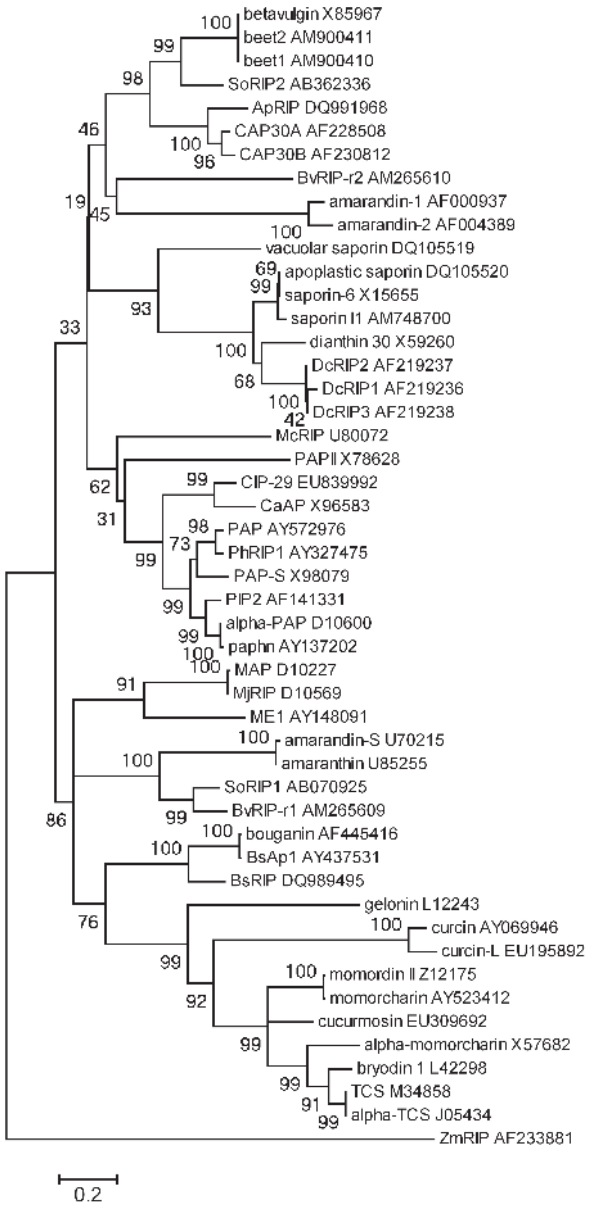

Fig. 4. The phylogenetic tree of the predicted full-length amino acid sequences of RIPs in plants. The sequence information used in this figure was obtained from the GenBank database. The GenBank accession numbers are alongside the names of the RIPs.

classified into four groups, the individual representatives of which are beetin, saporin, pokeweed antiviral protein (PAP), and trichosanthin (TCS). Curcin and curcin-L belong to the same subfamily and are phylogenetically close to TCS (Fig. 4).

Analysis of the 5' flanking region of curcin and curcin-L

After searching in the PLACE database, some putative regulatory motifs involved in seed-specific expression and activation of defense genes were found in the regions of both curcin and curcin-L. The BLASTn analysis revealed three inserted sequences (In1, In2, and In3) in the 5' flanking region of curcin-L, while there were no such sequences in the corresponding region of curcin; this was the most significant difference between curcin and curcin-L (Fig. 5). Interestingly, MYBCORE correlated with the response genes regulated by water stress inserted only in the In2 and In3 region of curcin-L.

Activity of the recombinant proteins

The two refolded recombinant proteins showed high inhibitory activity in a cell-free translation system, and when the concentration was increased to a certain extent, the inhibitory effect was also enhanced. The activity of curcin-L was observed to be higher than that of curcin (Fig. 6).

Rhizoctonia solani, *Pyricularia oryzae*, *Gibberella zeae*, *Sclerotinia sclerotiorum*, *Aspergillus oryzae*, and *Aspergillus niger* were used in an antifungal assay (Fig. 7). Recombinant curcin-L could suppress the fungal growth in these species, but recombinant curcin could not suppress the *A. niger* growth. The diameters of the circles from the fungal-static assay of the two proteins revealed that the antifungal activity of curcin-L was higher than that of curcin (Table I).

Discussion

Our results indicate that the two types of RIPs are specifically and individually expressed in different tissues and that their induced signals differ. Curcin is expressed in endosperms and accumulates in large amounts during the mature embryo period, while curcin-L is expressed in leaves and is induced by stress. The activity and function of these two RIPs might be similar to those of TCS since there is high similarity to TCS and a phylogenetically close relationship. An assay of these prokaryotic recombinant proteins showed that curcin-L has higher activity than curcin in terms of both inhibition of protein translation and antifungal effects. The results suggest that curcin and curcin-L might play different roles in *J. curcas*.

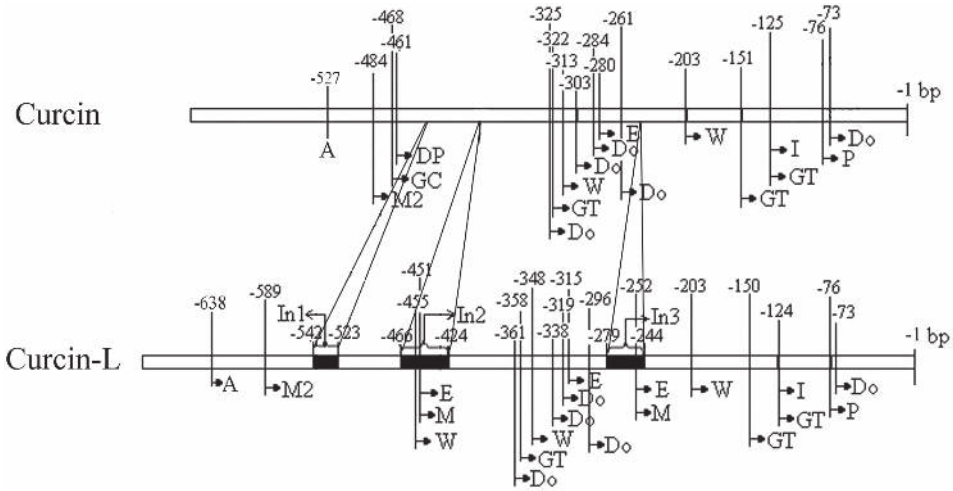

Fig. 5. Analysis of the 5' flanking region of the two RIP genes in *J. curcas.* In1, In2, and In3 represent the three inserted fragments. A represents the cores of the ABA-responsive element (ABRE: ACGTG); Do represents the core site required for the binding of Dof proteins (AAAG); DP represents DPBFCORE (ACACNNG); E represents the E box (CANNTG); GC represents the GC box; GT represents the GT-1 binding site (GRWAAW) that can stabilize the TFIIA-TBP-DNA (TATA box) complex; I represents the I box (GATAA); M represents the binding core site for MYB (CNGTTR) that responds to water stress in *Arabidopsis;* M2 represents the MYB-binding site (YAACKG) that responds to dehydration; P represents the prolamin box (TGCAAAG); W represents the W box (TTGAC) involved in the response to wounding and environmental stresses. The Dof core site, DPBFCORE, E box, and the prolamin box are involved in specific regulatory activities in the seed.

Curcin may be involved in supplying nutrition and protecting the seeds because its amino acid composition is very similar to that of other storage proteins, as determined by sequence analysis. During seedling's growth and development, curcin may function as a storage protein that provides sources of carbon, nitrogen, and sulfur. It is also a toxic protein (Stirpe, 2004) that is poisonous to animals. Therefore, it is possible that curcin may have another function, namely, preventing animals from consuming these seeds.

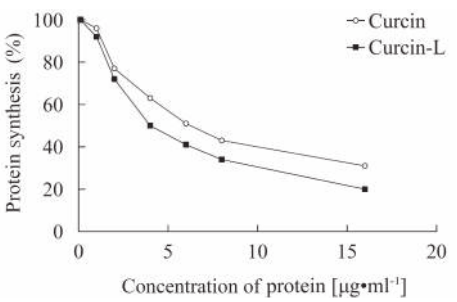

Fig. 6. Inhibitory activity of recombinant curcin and curcin-L in a cell-free protein synthesis system from the reticulocyte lysate.

Curcin-L may be a defense protein against environmental stresses. The results revealed that curcin-L is most sensitive to drought stress induced by PEG, which may be related to the growing environment of *J. curcas* (Shinozaki and Yamaguchi-Shinozaki, 1997). It is also highly sensitive to certain fungi, such as *G. zeae*, and exhibits high activity against it. Curcin-L may help *J. curcas* to survive under adverse growth conditions. Curcin-L may also be a pathogenesis-related protein in *J. curcas* since it can respond to phytohormones such as ABA and SA. Previous studies revealed that ABA and SA may help some plants to accumulate pathogenesis-related proteins in order to resist various etiological agents such as fungi, bacteria, and viruses (Malamy *et al.*, 1990). Moreover, curcin-L may play another role, namely as a regulator of signal transduction, since its synthesis could be induced by low and high temperatures, which were reported to alter the synthesis of some proteins (Murata *et al.*, 1992).

To check and explain the different functions of curcin and curcin-L, we studied their 5' flanking

regions. Sequence analysis indicated that the 5' flanking regions of the two RIPs have 88% similarity. Searches for putative elements in this region were performed using the PLACE database (Fig. 4). One ABRE (Simpson *et al.*, 2003), one DPBF, one MYB-binding site, five DOFCORE, one prolamin box, and one E box element were detected in the 5' flanking region of curcin, and one ABRE, two MYBCORE, one I box, three E box elements, and three W box elements were found in that of curcin-L. Obviously, the 5' flanking region of curcin has many motifs that are involved in endosperm-specific expression. For example, some functional elements such as DOFCORE (Yanagisawa, 2000), DPBFCORE (Finkelstein and Lynch, 2000), and E box (Hartmann *et al.*, 2005) are related to specific regulatory activities in the endosperm. The prolamin box (TGCAAAG) is also related to endosperm-specific expression. Meanwhile, the 5' flanking region of curcin-L has many motifs that respond to environmental stresses. In particular, MYBCORE (CNGTTR) (Urao *et al.*, 1993), which is located in In1 and In2 of curcin-L, is the binding site of the ATMYB1 and ATMYB2 proteins that are correlated to the response genes regulated by water stress. The W box is recognized by the WRKY transcription factor, which is involved in defense against pathogenic infection (Li *et al.*, 2004).

Acknowledgements

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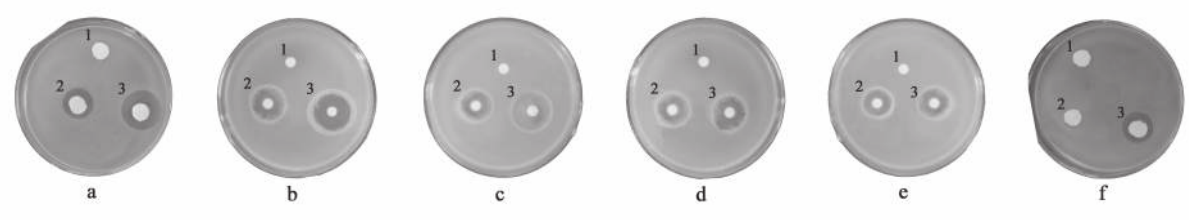

Fig. 7. The antifungal activity of the two RIPs toward fungi. 1, Control; 2, curcin; and 3, curcin-L. (a) *R. solani;* (b) *P. oryzae;* (c) *G. zeae;* (d) *S. sclerotiorum;* (e) *A. oryzae;* and (f) *A. niger.* The concentration of the RIPs used was 80 $\mu g \cdot ml^{-1}$.

Table I. Antifungal activity of curcin and curcin-L.

Protein[a]	Fungal-static circle diameter[b] [mm]					
	Rhizoctonia solani	*Pyricularia oryzae*	*Gibberella zeae*	*Sclerotinia sclerotiorum*	*Aspergillus oryzae*	*Aspergillus niger*
Curcin	10.8	12.5	11.0	11.3	10.2	7.7
Curcin-L	15.1	15.3	14.1	14.4	12.0	11.8

[a] The concentration of proteins was 80 $\mu g \cdot ml^{-1}$.
[b] Fungal-static circle diameter (± 0.3).

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