Expression of a ribosome inactivating protein (curcin 2) in *Jatropha curcas* is induced by stress

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The open reading frame (ORF) encoding curcin 2 was cloned from total genomic and cDNA of *Jatropha curcas* leaves, which were treated by drought, temperature stress and fungal infection, by polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR amplification. The ORF has 927 bp that encodes a precursor protein of 309 amino acid residues. There are high similarities with curcin and the conserved domain of ribosome inactivating proteins (RIPs). Antiserum to curcin recognized one band of 32 kDa on Western blot of the leaves treated by temperature stresses at 4°C and 50°C and by fungal infections of *Pestalotia funerea*, *Curvularia lunata* (Walk) Boed, *Gibberelle zeae* (Schw.) Petch. Two bands of 32 kDa and 65 kDa were recognized on Western blot of the leaves treated by 10%–40% polyethylene glycol (PEG). In addition, the 32 kDa band is nearly the molecular weight of curcin 2. This finding suggests that the protein of 32 kDa should be related to curcin 2. The presence of this protein molecular marker under stresses may provide an experimental foundation to study the stress proteins in *J. curcas*.

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1. Introduction

Plants are always exposed to various abiotic stresses and biotic stresses, which cause considerable reduction in plant growth and yield. Plants respond to conditions of drought or cold stress through a number of physiological and developmental changes (Kazuo and Kazuko 1996), and plants have developed complex defense mechanisms against pathogen attacks during evolution (Bowles 1990). The defense mechanisms employed by a number of higher-plant species involve defensive chemicals, which including ribosome inactivating proteins (RIPs). RIPs may be induced by mechanical wounding, jasmonic acid, abscisic acid, salt stress, virus and fungi (Leah *et al* 1991; Logemann *et al* 1992; Desvoyes *et al* 1997; Joerg *et al* 1997; Song *et al* 2000).

The previous investigations (Leah *et al* 1991; Desvoyes *et al* 1997; Lin *et al* 2003a) indicated there are a number of RIPs in the same plant species. It was reported that RIP gene *curcin* was cloned from *Jatropha curcas* seeds (Lin *et al* 2003b), and the anti-tumour activity of curcin was demonstrated (Lin *et al* 2003c). Our original intention was to examine whether *curcin* gene is expressed in roots, stems and leaves of seedlings growing under stress conditions, but the finding was a curcin-related RIP (designated as curcin 2) is induced in leaves of seedlings growing under stress conditions. The present paper reports the inducible expression of *curcin 2* gene under drought, temperature stress and fungal infection conditions. This work may provide useful information on defense mechanisms of *J. curcas*.

Keywords. Curcin 2; *Jatropha curcas*; protein induction; stress

Abbreviations used: ORF, open reading frame; PEG, polyethylene glycol; RIPs, ribosome inactivating proteins; RT-PCR, reverse transcriptase-polymerase chain reaction.

2. Materials and methods

2.1 *Plant materials*

The mature seeds of *J. curcas* were obtained from Panzhihua City, Sichuan Province, China.

2.2 *Treatment on seedlings of* J. curcas *with polyethylene glycol, temperature and fungi*

2.2a *Polyethylene glycol treatment*: After normally irrigated with water, one-month-old seedlings of *J. curcas* were treated with 10%–40% PEG-6000 solution. The seedlings treated with distilled water served as control.

2.2b *Temperature treatment*: After normally growing in phytotron at 25°C, one-month-old seedlings were transferred to the temperature conditions at 4°C or 50°C, respectively. The control seedlings were allowed to continue growth at 25°C.

2.2c *Fungal infection*: Using a sterile cotton swab, the leaves of one-month-old seedlings were inoculated with cell suspension (cultured for 3 days) of *Gibberelle zeae* (Schw.) Petch., *Rhizoctonia solani* Kuha, *Curvularia lunata* (Walk) Boed, *Sclerotinia sclerotiorum* Lib de Bary, *Colletotrichum gloeosporioides* (Perz.) sacc. and *Pestalotia funerea* (maintained by our laboratory), respectively. The uninfected seedlings served as control.

The seedlings were grown in greenhouse conditions. The various root, stem and leaf samples were collected at 12 h and 24 h after treatment, respectively (only at 24 h after treatment in PEG treatment), and stored at – 70°C until needed. The experiment was carried out three times independently in the same conditions.

2.3 *Reverse transcriptase-polymerase chain reaction analysis*

Total RNAs were extracted by the method described by Malnoy *et al* (2001). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was conducted with the One Step RNA PCR Kit (TaKaRa) by using forward primer P1′ : 5′-[5′-GGATCC (*Bam*HI) TGAAATCAATATGAAAGGTGGCA-3′] and reverse primer P2 : 3′-[5′-GAGCTC (*Sac*I) ATACATTGGAAAGATGAGGA-3′], which were designed according to the cDNA sequence of *curcin* (Lin *et al* 2003b). RT-PCR parameters: after reverse transcription at 50°C for 30 min, 94°C denaturing for 2 min; 20 or 30 cycles with 94°C for 20 s, 60°C for 30 s, 72°C for 1 min; 72°C extension for 5 min. The RT-PCR product was recovered and cloned into the pMD-T vector (TaKaRa). It was sequenced by Shanghai Biotech Co. Ltd., China.

Specific-primer RT-PCR was run by using *curcin 2* specific-primer [forward P3: (5′-TAGCCAAAGTCATAAATTCTGGGGACA-3′) and reverse P4: (5′-CATTCAACAAGACTCCCATGAGACCTTT-3′)]. The *curcin* specific-primer RT-PCR served as control by using its specific-primer [forward P5: (5′-TTGTAGCCAAAGTCATAAATGTAGCGAATT-3′) and reverse P6: (5′-TCAACAAGACTCCCATGACACCTGC-3′)]. Based on the signal intensity of the amplified products, the relative amount of cDNA in different stress conditions was examined by simultaneity using *J. curcas* 18S rRNA specific primers (forward P7: 5′-CCTACCATGGTGGTGACGGGT-3′ and reverse P8: 5′-GTCTGTCAATCCTTACTATGT-3′) in PCR amplification. Primers P7–P8 were designed according to 18 S rRNA sequence (GenBank code: AY823528) of *J. curcas*. The masses (M_{570} and M_{923}) of PCR products of 570 bp (amplified by P3–P4) and 923 bp bands (amplified by P7–P8) were detected with gene tools software. The PCR parameters were the same as the above. *Curcin* cDNA gene used as templet was provided by our laboratory.

2.4 *PCR amplification from genome*

DNA was extracted by the method described by Roger and Bendich (1988). PCR amplification was conducted by using two pairs of primers: P1: 5′-[5′-GGATCC (*Bam*HI) ATGGCTGGTTCCACTCCAACTTT-3′]-P2 and P1′–P2. PCR parameters: 94°C denaturing for 5 min; 30 cycles with 94°C for 30 s, 55°C for 1 min, 72°C for 1·5 min; 72°C extension for 5 min. The PCR product was recovered and cloned into the pMD-T vector. Four single colonies harbouring target fragments were randomly selected to determine the sequence.

2.5 *The sequence analysis*

The encoding amino acid sequence of *curcin 2* was deduced with DNA tools 5·0. The analysis and comparison of the deduced amino acid sequence with curcin and conserved domain of RIPs was searched in GenBank.

2.6 *Protein analysis of seedlings by SDS-PAGE and Western blotting*

Total soluble protein extracts from leaves were prepared by grinding 100–200 mg of leaves in liquid nitrogen. The samples were homogenized in 5 mmol/l phosphate buffer (pH 7·2), centrifuged at 12000 r/min for 10 min at 4°C to pellet out the cell debris. Western blot analysis was performed using antiserum against curcin, which has 92% homology (searched in GenBank) with putative amino acid sequence of curcin 2, followed by treatment with an

alkaline phosphatase labelled goat anti-rabbit IgG from Sino-American (Towbin *et al* 1979).

3. Results

3.1 *cDNA isolation of the open reading frame encoding curcin 2 precursor protein*

No band was amplified with P1′–P2 from cDNA of roots and stems whether non-stress or stress (data not shown).

No band was also amplified from cDNA of control leaves (figure 2A), but sequencing results of cDNA amplified with P1′–P2 showed that the stresses tested, except *R. solani* Kuha, *C. gloeosporioides* (Perz.) sacc. and *S. sclerotiorum* Lib de Bary infection, caused expression of *curcin 2* which is related to but distinct from curcin, at transcript level (figure 1). This finding was also validated by the specific-primer RT-PCR. The specific-primer RT-PCR products of the expected size (570 bp) were detected in cDNA clones of leaves stressed with 20%–40% PEG (in 20 cycles) and 10%–40% PEG (in 30 cycles) (figure

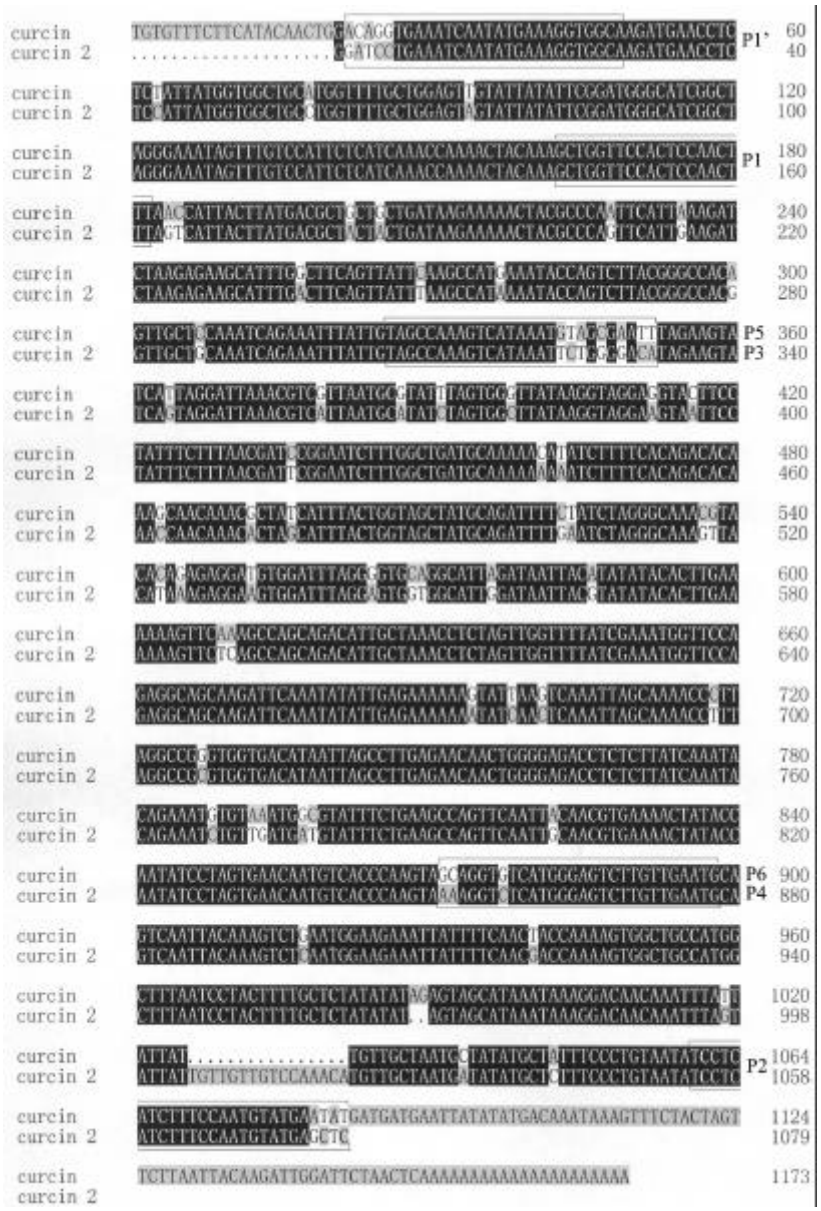

Figure 1. The cDNA sequence of ORF encoding curcin 2 and comparison with curcin cDNA. Primers are in box. Identical nucleotides are shown with black background; positive nucleotides are shown in gray background.

2A), temperature at 4°C and 50°C (in both 20 cycles and 30 cycles) or infected with *P. funerea*, *C. lunata* (Walk) Boed and *G. zeae* (Schw.) Petch (in both 20 cycles and 30 cycles) (figure 2B). But no band of 570 bp was detected in cDNA clones of all mRNA using *curcin* sequence-specific primers (P5–P6) (data not shown). We examined the cDNA mass using trace mRNA. The 18 S rRNA fragment of 923 bp was amplified and served as controls for cDNA original quantities. The intensity was expressed with $mass_{570}/mass_{923}$ (M_{570}/M_{923}). The values of M_{570}/M_{923} showed increased intensity with stress as a function of time (PEG concentrations, temperature and fungal infection) (figure 2).

3.2 *Cloning of open reading frame encoding curcin 2 from genome*

By PCR amplification using primers P1–P2 and P1′–P2, the target bands with approximately 1000 bp and 930 bp were seen on 0·8% agrose, respectively. After the purified PCR products were cloned into the pMD18-T vector, four single colonies on LB agar media with ampicillin were randomly selected to determine the sequence. Of 4 positive sequenced clones, two sequences from P1′–P2 was in agreement with cDNA sequence of *curcin 2*, in addition to another 42 amino acids at N-terminus. One sequence from P1–P2 was also in agreement with cDNA sequence of *curcin 2*; the other from P1–P2 was the same with curcin cDNA (AY069946).

3.3 *Characterization of the open reading frame encoding curcin 2*

The open reading frames (ORFs) encoding precursor protein and mature protein have 927 and 801 nucleotides,

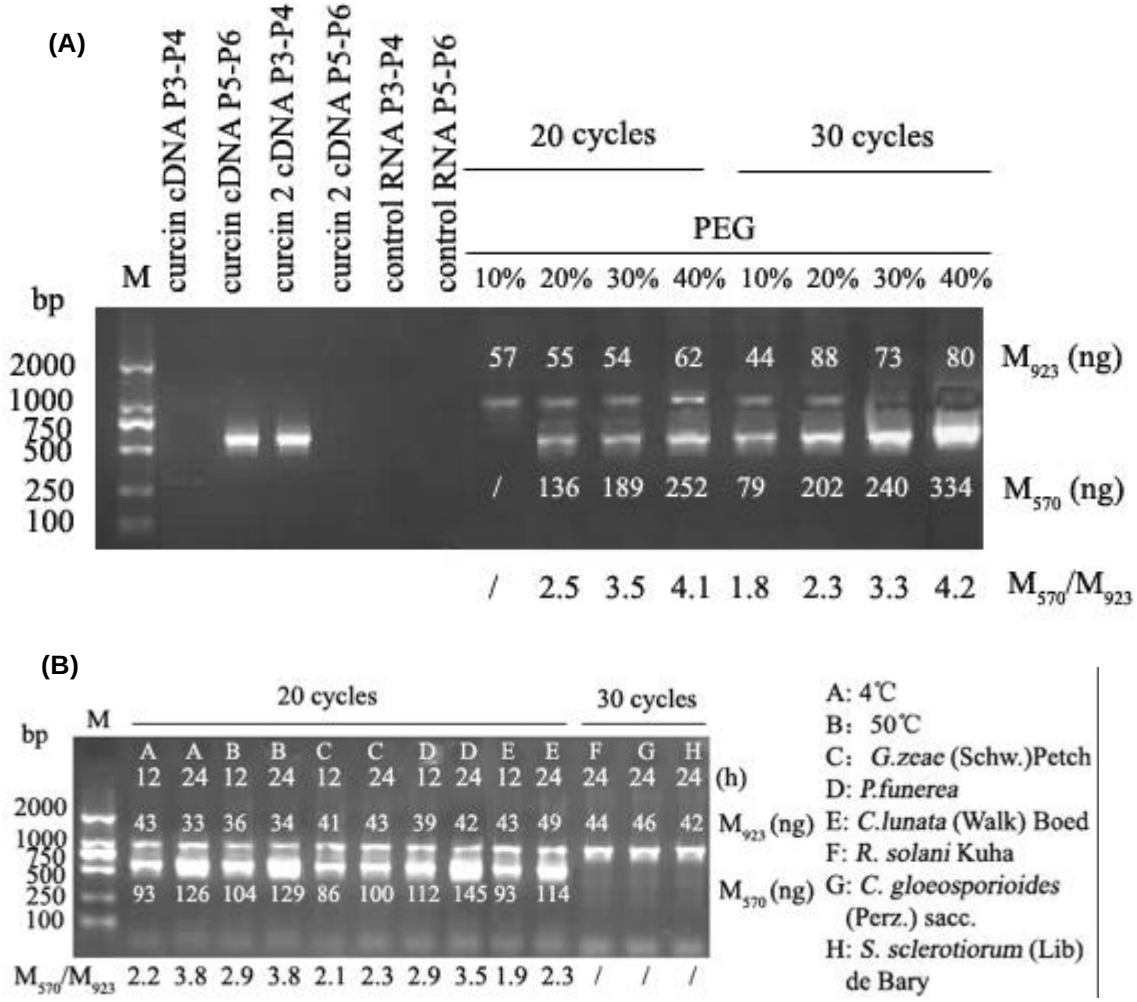

Figure 2. The electrophoregram of RT-PCRs of *J. curcas* leaves of seedlings growing under stress condition. (**A**) Treatment with PEG. (**B**) Treatment with temperature and fungal infections. M, DNA size markers; M_{570}/M_{923}, $mass_{570}/mass_{923}$.

respectively. They encode 309 and 267 amino acids with the calculated relative mass of 34·9 kDa and 30·1 kDa and pI of 6·56 and 5·25, respectively. The deduced curcin 2 precursor protein contains a segment of 42 amino acid residues at the N-terminus for a signal peptide base, which has 98% similarity with signal peptide of curcin (Lin *et al* 2003b). *Curcin 2* has 83% identity with nucleotides of *curcin*. The conserved domain of RIPs in GenBank contains 249 amino acids. Curcin 2 has 32% identity and 67% similarity with the conserved domain of RIPs. Two octapeptides have been found in the regions, one is EAARFKYI in residues 209–216, and the second is ISLENNWG in residues 235–242 in curcin 2. The highly conserved and consensus amino acid residues are involved in the active site of RIPs (figure 3).

3.4 *Immunoblot analysis of curcin 2 on stress response*

After protein samples were electrophoretically separated on 15% SDS-PAGE, the proteins were transferred to nitrocellulose membrane. The immuneserum recognized a band with molecular weight of 32 kDa and a band of 65 kDa in the extracts of leaves treated by PEG. A single band of 32 kDa was recognized in the extracts of leaves that were treated by temperature at 4°C and 50°C and by three fungal infections of *G. zeae* (Schw.) Petch., *P. funerea* and *C. lunata* (Walk) Boed. No band was recognized in the extracts of leaves infected by *R. solani* Kuha, *S. sclerotiorum* (Lib) de Bary and *C. gloeosporioides* (Perz.) sacc (figure 4).

4. Discussion

No introns were typical of other RIPs gene that has been sequenced (Juan *et al* 2003). Based on the result that curcin 2 genomic DNA sequence matched with its cDNA sequence, we suggest that there should be no intron in *curcin 2*.

The expression profiles of *curcin 2* revealed that the accumulation of *curcin 2* is expressed specifically in *J. curcas* leaves of seedlings growing under drought, temperature stress and partial fungal infection conditions, and it is not expressed at transcript level in non-stressed seedlings (figure 2). We suggest that differential expression of *curcin 2* in roots, stems and leaves might be due to the sensitivity or specificity of each organ towards various treatments, and the leaf different response to different fungi species might be due to different resistance to different fungi species. The cDNA mass increased with stress time and PEG concentration (figure 2). It is showed that transcript is related to stress intensity.

RIPs are generally viewed as pathogen-related proteins because of their toxicity towards animals, bacteria, fungi or viruses (Barbieri *et al* 1993; Lam *et al* 1992). So far,

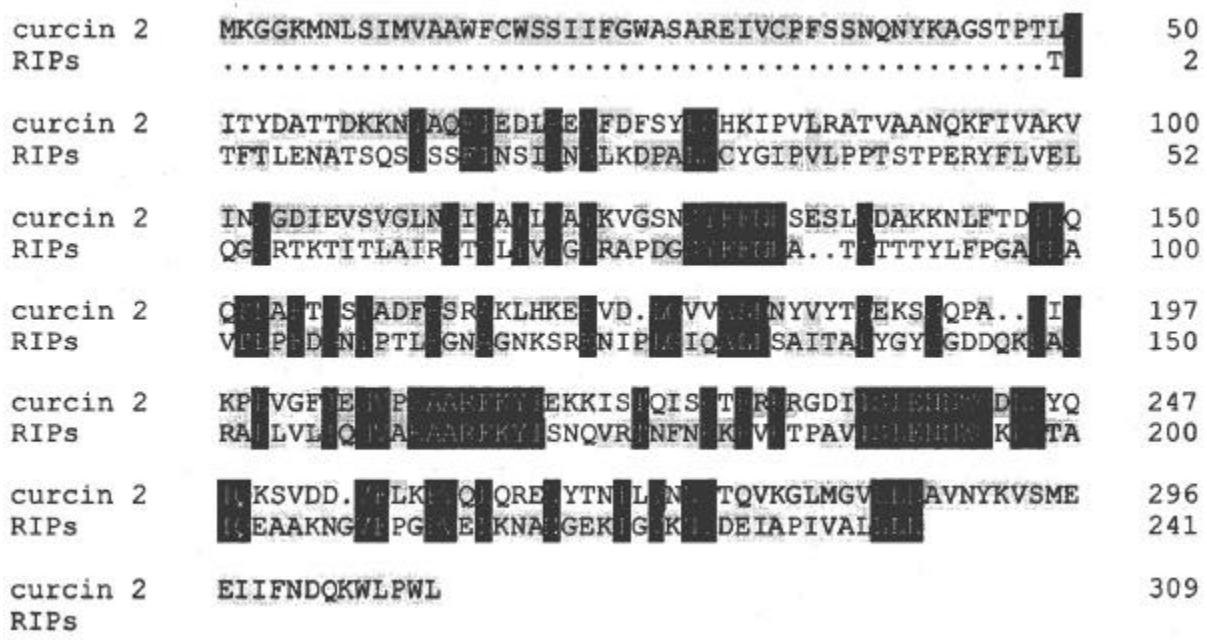

Figure 3. Alignment between *curcin 2* cDNA sequence with RIPs conserved domain searched in GenBank. Identical amino acids are shown with black background; positive amino acids are shown in gray background.

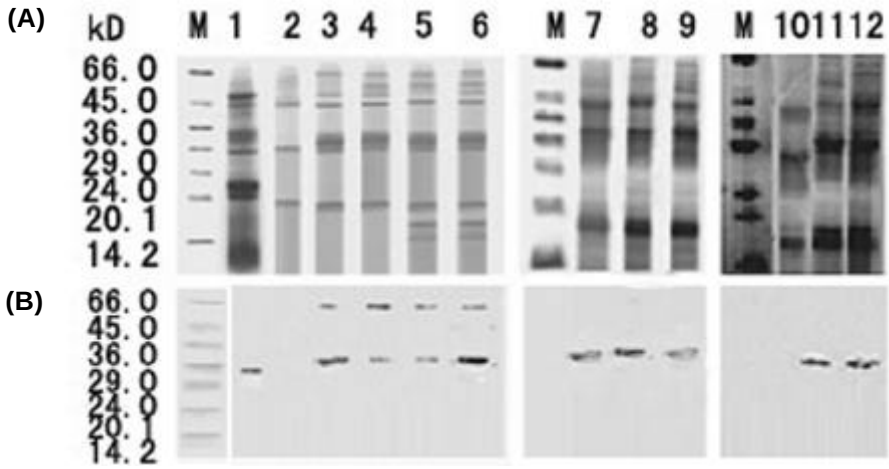

Figure 4. The induced proteins and immunoblot of *J. curcas* leaves of seedlings growings under 10%–40% PEG-water stress conditions. (**A**) SDS-PAGE. (**B**) Western blot. Lane M, Protein markers; lanes 1, crude proteins extracted from *J. curcas* seeds; 2, control (no PEG treatment); 3, stressed by 10% PEG ; 4, stressed by 20% PEG; 5, stressed by 30% PEG; 6, stressed by 40% PEG; 7, infected by *P. funerea*; 8, infected by *C. lunata* (Walk) Boed; 9, infected by *G. zeae* (Schw.) Petch.; 10, control (at 25°C); 11, stressed at 50°C; 12, stressed at 50°C.

only a few environmental stress-inducible RIP have been described (Reinbothe *et al* 1994; Stirpe *et al* 1996). Our finding revealed that curcin 2 expression should be induced by biotic and abiotic stresses. It may be explained by the use of common, or partly overlapping, signalling pathways (Joerg *et al* 1997). *Curcin* gene cannot express in leaves whether in stress or non-stress condition. However, *curcin 2* gene expresses in leaves of seedlings growing under stress conditions (figure 2).

Curcin is extracted from seeds other than leaves, and its molecular weight is 28·2 kDa (Lin *et al* 2002). The antiserum against curcin can recongnize a band of 32 kDa band (figure 4), which is near the deduced molecular weight of curcin 2 (30·1 kDa). It indicated that the 32 kDa band has high homology with curcin (a RIP) in protein structure. This finding was in agreement with curcin 2 property. So we suggest that the 32 kDa band might just be curcin 2. Desvoyes *et al* (1997) have described inactive dimer formation PAPi of PAP, a antiviral protein as well as RIP isolated from pokeweed. A dimeric form of *α*-sarcin, a RIP with a ribonuclease activity, has been identified in a purified toxin preparation. Recently, these dimers have been shown to be inactive and it has been suggested that a dimer formation could play a biological role in preventing the cytotoxicity of *α*-sarcin. In our study, we suggest that the band of 65 kDa maybe is the dimer of curcin 2 in order to decrease the cell toxicity according to Desvoyes *et al* (1997). This is a kind of cell-self defence reaction. Nevertheless, the existence of an unknown protein associated to curcin 2 in a heterodimer cannot be excluded. However, further determination is needed.

To our knowledge, this is the first report on RIP gene expressed by stress induction in *J. curcas*. Curcin 2 may play a role in plant defence and hence can be exploited in plant protection. The presence of this protein molecular marker under drought, temperature and fungal infection conditions may provide experiment foundation to study the stress proteins.

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