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Cloning and characterization of a differentially expressed cDNA encoding myo-inositol-1-phosphate synthase involved in response to abiotic stress in *Jatropha curcas*

Yong Wang · Jing Huang · Chun Bao Gou · Xiao Dai · Fang Chen · Wei Wei

Abstract D-myo-inositol-3-phosphate synthase (MIPS) catalyzes the reaction from D-glucose 6-phosphate to D-myo-inositol 3-phosphate (MIP), which is the first and rate-limiting step in myo-inositol biosynthesis. In this study, *Jatropha curcas* MIPS cDNA (JcMIPS) (GenBank accession no. EF 185781) has been isolated using mRNA differential display technology (DDRT) and the rapid amplification of cDNA ends (RACE). The cDNA clone of JcMIPS is comprised of 1,957 bp, encoding 509 amino acids, with a predicted molecular weight of 56.4 kDa. The JcMIPS protein is highly homologous to those from other plant species, ranging from 88.4 to 91.18% homology at the amino acid levels. Real-time quantification polymerase chain reaction (PCR) analysis has revealed that JcMIPS transcripts are highly present in seed and leaf tissues, but are at low levels in stem and flower tissues. Furthermore, the transcription of JcMIPS in leaves is up-regulated by abscisic acid (ABA) (100 μM), drought (30% PEG-6000), NaCl (200 mM), and low-temperature (4°C) treatments. The observed increase of JcMIPS enzyme activity is also detected following treatments with ABA, drought, and NaCl. Interestingly, JcMIPS enzyme activity is only slightly changed following low-temperature treatment.

Keywords DDRT-PCR · MIPS · ABA · *Jatropha curcas*

Y. Wang · J. Huang · C. B. Gou · X. Dai · F. Chen ·
W. Wei (✉)
Key Laboratory of Bio-resources and Eco-environment, Ministry of Education, College of Life Sciences, Sichuan University, 24 South Section 1, Yihuan Road, Chengdu, Sichuan 610064, People's Republic of China
e-mail: wwxfbxw@gmail.com

Introduction

Abiotic stress, including drought, high salinity, extreme temperatures, and heavy metal toxicity, limits plant development. Plants resist abiotic stress by changing the morphological, biochemical, and metabolic processes (Ingram and Bartels 1996; Lokhande et al. 2010). All of these changes result from the changes of genes expression. Although a large number of genes have been recently identified, such as *GmDREB1* (Jin et al. 2010) and *PgCDPK1a* (Kiselev et al. 2010), their physiological roles in relation to stress are largely unknown in plants (Ingram and Bartels 1996; Seki et al. 2001, 2002).

Jatropha curcas belongs to Euphorbiaceae and it is distributed in many tropical and subtropical areas (He et al. 2009). It can be used as a biological pesticide and in various medicinal purposes. Recently, *J. curcas* has drawn much attention for its toxicity and high content of seed oil (Singh et al. 1984; Muanza et al. 1995; Misra et al. 2010) and could confer drought and heavy metal tolerance (Zhang et al. 2007; Juwarkar et al. 2008). However, the mechanism of its tolerance to stress remains poorly understood.

Myo-inositol 1-phosphate synthase (MIPS) catalyzes the conversion of D-glucose 6-phosphate to 1-myo-inositol-1-phosphate, the first and rate-limiting step in the biosynthesis of all inositol-containing compounds (Abreu and Aragão 2007). MIPS is a highly conserved enzyme. The binding motif site, which is required for catalytic reaction, is similarly found in amino acid sequences from diverse sources such as bacteria, green algae, fungi, higher plants, and animals (Majumder et al. 2003). MIPS genes have been isolated and characterized from a number of plant species and found to be representing multigene families in some plant species (Ju et al. 2004; Park and Kim 2004).

The regulation of MIPS has also been studied in some higher plants. For example, transcripts encoding MIPS have been shown to be up-regulated during osmosis stress in *Mesembryanthemum crystallinum* (Ishitani et al. 1996). The activity of MIPS enzyme in *Oryza sativa* L. could be enhanced by salinity (Raychaudhuri and Majumder 1996).

There has been limited molecular information about the *MIPS* gene in *J. curcas* so far. In this study, we have cloned and characterized MIPS cDNA from seedlings under drought stress and studied its differential expression in different tissues and under diverse stresses. Furthermore, JcMIPS enzyme activity under abiotic stress and exogenous abscisic acid (ABA) treatment is also discussed.

Materials and methods

Plant materials and stress treatments

The seeds of *J. curcas* were collected from Panzhihua, Sichuan Province, China. Seeds were sterilized by 0.1% HgCl$_2$ and germinated in seedbeds containing a 1:1:1 (v/v/v) mixture of perlite, peat moss, and sandy loam soil, and irrigated daily with a half-strength Hoagland solution, as described by Saneoka et al. (2001). After the seeds germinated, they were transferred to flower pots and grown at 25°C with a photoperiod of 18 h light/6 h dark. Two weeks later, plants were selected to be used for the stress treatment.

Drought and salinity stress treatments were carried out by incubating the seedlings in 100 ml of Murashige–Skoog (MS) liquid medium, containing 30% PEG-6000 and 200 mM NaCl at 25°C for 24, 48, and 72 h, respectively. The degree of water stress was determined by the relative water content (RWC) of the plants. The RWC was measured as described by Ma et al. (2006). The seedlings were incubated at 4°C for 72 h for the low-temperature treatment. For exogenous ABA treatment (ABA dissolved in methanol and 2.5% methanol used as a control), the seedlings were carried out by incubating plants in MS medium containing 100 μM ABA at 25°C for 24 h. These plant tissues were immediately frozen in liquid nitrogen and kept at −80°C until use.

RNA isolation and mRNA differential display

To search for the differentially expressed cDNAs, mRNA differential display analysis was performed as described by Liang and Pardee (1992), with slight modifications. Total RNA was extracted from 2-week-old light-grown *J. curcas* leaves subjected to 72-h drought stress using plant RNA isolation reagent (QIAGEN RNeasy Plant Mini Kit, QIAGEN, Holland) following the manufacturer's instructions. Total RNA from untreated leaves was used as a control. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase, and then used for reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR was followed by PCR amplification using three anchored primers (APs) and each of the ten arbitrary primers (ARPs). The primers are shown in Table 1. Twenty-five cycles of PCR were carried out, each consisting of 3 min at 95°C, 30 s at 94°C, 2 min at 42°C, and 1 min at 72°C. Then, ten cycles of PCR were carried out, each consisting of 30 s at 94°C, 1 min at 50°C, and 1 min

Table 1 List of primer sequences used in the mRNA differential display

Primers	Sequences
Oligo (dT) anchored	5′-ACGACTCACTATAGGGCTTTTTTTTTTTTMN-3′ (N = A,C,G, or T; M = A,G, or C)
Arbitrary primers (ARPs)	M13r ARP1: 5′-ACAATTTCACACAGGAGTTGCGATCC -3′
	M13r ARP2: 5′-ACAATTTCACACAGGAACGGACGTCA -3′
	M13r ARP3: 5′-ACAATTTCACACAGGAGATCGCATTG -3′
	M13r ARP4: 5′-ACAATTTCACACAGGATACAACGAGG -3′
	M13r ARP5: 5′-ACAATTTCACACAGGAGGTACTAAGG -3′
	M13r ARP6: 5′-ACAATTTCACACAGGATCGGTCATAG -3′
	M13r ARP7: 5′-ACAATTTCACACAGGAGATGCCAGAC -3′
	M13r ARP8: 5′-ACAATTTCACACAGGAGGATGCCACT -3′
	M13r ARP9: 5′-ACAATTTCACACAGGAAGCCAGCGAA -3′
	M13r ARP10: 5′-ACAATTTCACACAGGACTTTGGCTCC -3′
Anchored primers (APs)	AP3: 5′-ACGACTCACTATAGGGCTTTTTTTTTTTTG-3′
	AP6: 5′-ACGACTCACTATAGGGCTTTTTTTTTTTTC-3′
	AP8: 5′-ACGACTCACTATAGGGCTTTTTTTTTTTTA-3′
	M13 Reverse: 5′-AGCGGATAACAATTTCACACAGGA
	T7 promoter: 5′-GTAATACGACTCACTATAGGGC-3′

Table 2 Primers for 5′-RACE and RT-PCR

Primer	Sequence
YH1	5′-CTTCTTGCTGAGCTGAGCACCA-3′
AP1	5′-GCTGTCAACGATACGCTACGTAACGGCATG ACAGTG(T)$_{18}$-3′
AP2	5′- GTCAACGAT ACGCTACGTAACG-3′
GSP1	5′-AAACACAGGCCAAAGCATACAG-3′
GSP2	5′-AACAGAC TCTCCACAGTGTC-3′
YH3	5′-GCGGTCGACTCATTTGTACTCCAAGATCATGT-3′
YH5	5′ -GCGGAATTCATGTTTATTGAGA AGTTTAAGGT-3′

Table 3 Primers for real-time quantitative RT-PCR

Primer	Sequence	Product
18SrRNA-F	5′-ATAAACGATGCCGACCAG-3′	460 bp
18SrRNA-R	5′-ACAAATCGCTCCACCAAC-3′	
JcMIPS-F	5′-AAGTACACGGATGATGAGA-3′	280 bp
JcMIPS-R	5′-TGAGTAAGTGAACCGAAGT-3′	

at 72°C, with an additional 10 min at 72°C in an automatic thermal cycler (Bio-Rad, USA). PCR products were separated on 6% polyacrylamide sequencing gels and visualized by silver staining. Bands of interest were recycled from the gels and reamplified with M13 primer and T7 primer.

Reverse Northern blot, 5′-RACE, and RT-PCR amplification

Reverse Northern blot was carried out to reduce false-positives arising from the differential display. Each reamplification mixture was dotted on Hybond N+ membrane filter (Schleicher & Schuell, Dassel, Germany). Total cDNA probes were synthesized by reverse transcription of 2 μg total RNA for drought-treated and untreated *J. curcas* leaves. Hybridization was performed following the manufacturer's instructions (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche, Switzerland). The cDNA fragments were cloned into the pMD18-T Easy vectors and were sequenced (Invitrogen, China). One of these differential display segments shared high similarity to other plant MIPS. The full length of cDNA was cloned by RT-PCR and 5′-rapid amplification of cDNA ends (5′-RACE). The sequences of all primers used are listed in Table 2.

Real-time quantification PCR analysis

SuperScript^{TM} II Reverse Transcriptase (Invitrogen, USA) was used to transcribe 2.5 μg total plant RNA into cDNA. *J. curcas* 18S ribosomal RNA (GenBank accession no. AY823528) was used as an internal standard in the real-time PCR reaction. All primers are as listed in Table 3. Real-time PCR was carried out in a BioRad iCycler using SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, USA). The program was as following: 45 cycles of 2 min at 50°C, 5 min at 95°C, 15 s at 55°C, 20 s at 72°C, and the data collection during the extension step (3 min at 72°C). Melting curves were run immediately after the last cycle to exclude any influence of primer–

dimer pairs. Cycle numbers at which the fluorescence passed the cycle threshold (Ct) were further analyzed using the DDRT method and the Relative Expression Software Tool (REST) (Pfaffl et al. 2002).

For the amplification of rare mRNA species, 1 μg of total RNA was reverse transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad) with an oligo dT primer and enhancer according to the manufacturer's protocol. PCR products from templates of leaves of unstressed and stressed plants were compared by standard agarose gel electrophoresis.

Preparation of crude enzyme and enzyme assay

Root, stem, leaf, flower, and seed (5 g of each) tissues were homogenized in 50 ml of ice-cold buffer A (20 mM Tris–HCl pH 7.5, 10 mM NH$_4$Cl, 10 mM β-mercaptoethanol, 2 mM PMSF). The homogenate was centrifuged at 8,000g at 4°C for 45 min. The supernatant was stored at 4°C for protein concentration and the JcMIPS enzyme activity assay. The enzyme was assayed colorimetrically by the periodate oxidation method (Barnett et al. 1970). One unit of enzyme activity is defined as the μmol amount of 1 mg protein converting the substance D-glucose-6-phosphate to inositol-1-phosphate during 1 h (μmol h^{-1} mg^{-1}). Protein was determined according to the method of Bradford (1976) with BSA as a standard.

Results

Screening the differential expression genes under drought stress by DDRT-PCR

Some partial cDNA fragments were identified by mRNA differential display technology (DDRT-PCR) induced by water deficit from leaves of 2-week-old light-grown *J. curcas* plants (Fig. 1). A 527-bp fragment that showed significant positive signal in reverse Northern blot was selected for further investigation. Nucleotide BLAST search suggested that the isolated cDNA fragment shared high similarity with other known MIPS genes, indicating that the partial MIPS gene of *J. curcas (JcMIPS)* was obtained (data not shown).

Fig. 1a–c The partial results of mRNA differential display PCR. **a** Electrophoresis of mRNA differential display PCR between control and treatment (1, 3, 5, 7: control; 2, 4, 6, 8: treatment). **b** Electrophoresis of denaturing polyacrylamide gel of mRNA differential display PCR between control and treatment. The *arrows* indicate different bands of PCR-amplified cDNA fragments between control and treatment. **c** Reverse Northern blot analysis between control and treatment. The *arrows* indicate the positive dots
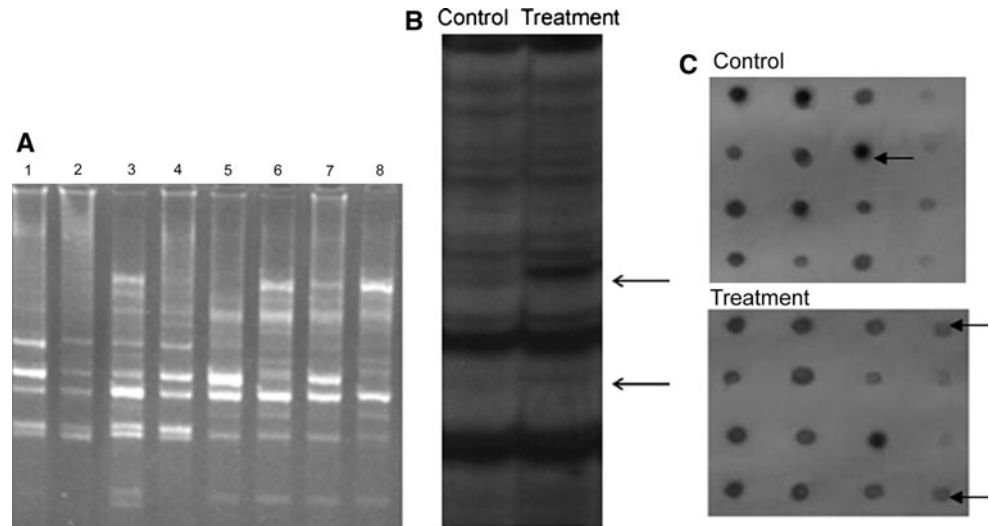
Cloning and characterization of the full-length cDNA encoding *JcMIPS*

A *J. curcas* MIPS cDNA (*JcMIPS*) was isolated from *J. curcas* seedlings using the DDRT-PCR and RACE systems. The 1,957-bp cDNA sequence contained a 1,530-bp coding sequence that could encode a protein of 509 amino acids, which correspond to a polypeptide with a predicted molecular weight (MW) of 56.4 kD (Fig. 2). The nucleotide sequence of the *JcMIPS* gene was deposited in the GenBank database under accession number EF 185781.

Sequence alignment showed that *JcMIPS* shared a high similarity to the other plant MIPS from *Glycine max* (89.22%, AY038802), *Citrus paradisi* (88.63%, Z32632), *Phaseolus vulgar* (88.63%, AJ853494), *Brassica napus* (88.43%, FJ429169), and *Arabidopsis thaliana* (87.08%, U04876). The JcMIPS protein had the highest identity with *Nicotiana paniculata* MIPS (91.18%, AB032073) (Fig. 3).

In addition, four highly conserved motifs similar to those of other plant MIPS were also found in the JcMIPS polypeptide. The four conserved domains are GWGGNNG (domain I), LWTANTERY (domain II), NGSPQNTFVPGL (domain III), and SYNHLGNNDG (domain IV) (Fig. 3), which are essential for MIPS functions such as the binding of cofactor NAD^+ and catalysis of the reaction (Majumder et al. 1997; Bachhawat and Mande 1999).

Tissue-specific expression of the *JcMIPS* mRNA in *J. curcas*

The expression pattern of *JcMIPS* in various tissues of *J. curcas*, the *JcMIPS* gene, was transcriptionally active in all of the five detected tissues at different levels (Fig. 4).

The mRNA levels of *JcMIPS* were high in leaf and seed, but lower in root and flower and the lowest in the stem. The mRNA level of *JcMIPS* in leaf was nearly eight times higher than that in stem. The expression pattern of *JcMIPS* demonstrated that this gene was differentially expressed in various tissues.

Expression analysis of the *JcMIPS* gene and JcMIPS enzyme activity under abiotic stress and exogenous ABA treatment

Expression patterns of the *JcMIPS* gene were studied in leaves under abiotic stress and exogenous ABA treatment. When exposed to 30% PEG 6000, 200 mM NaCl, or 4°C cold stress, *JcMIPS* transcripts in *J. curcas* seedlings were all up-regulated clearly (Fig. 5a). The mRNA levels of *JcMIPS* under drought and salt treatments were higher (24 and 36%, respectively) than that of the control. However, the expression of *JcMIPS* in response to cold treatment was lower and increased only 15% after the seedlings were exposed to a 4°C environment for 72 h. In order to study the transcription level of *JcMIPS* after exogenous hormone treatment, *J. curcas* seedlings were treated by 100 μM exogenous ABA at 25°C for 24 h. Figure 5a shows that the transcription level reached 1.56 times that of the control. The data indicates that the expression of *JcMIPS* was related to drought, salt and cold stress, and exogenous ABA treatment.

The JcMIPS enzyme activity in *J. curcas* leaf under different stress treatments and ABA induction had been detected. The results showed that JcMIPS enzyme activity was also induced by drought, salt, and exogenous ABA treatment (Fig. 5b). The activity of JcMIPS increased 1.15 and 1.52 times when exposed to 30% PEG and 200 mM NaCl, respectively, for 24 h. However, it seemed that the

Fig. 2 Full-length cDNA sequence and amino acid sequence encoding JcMIPS. *Gray background*: initiation codon; *boxed section*: termination codon; *underlined*: poly (A)
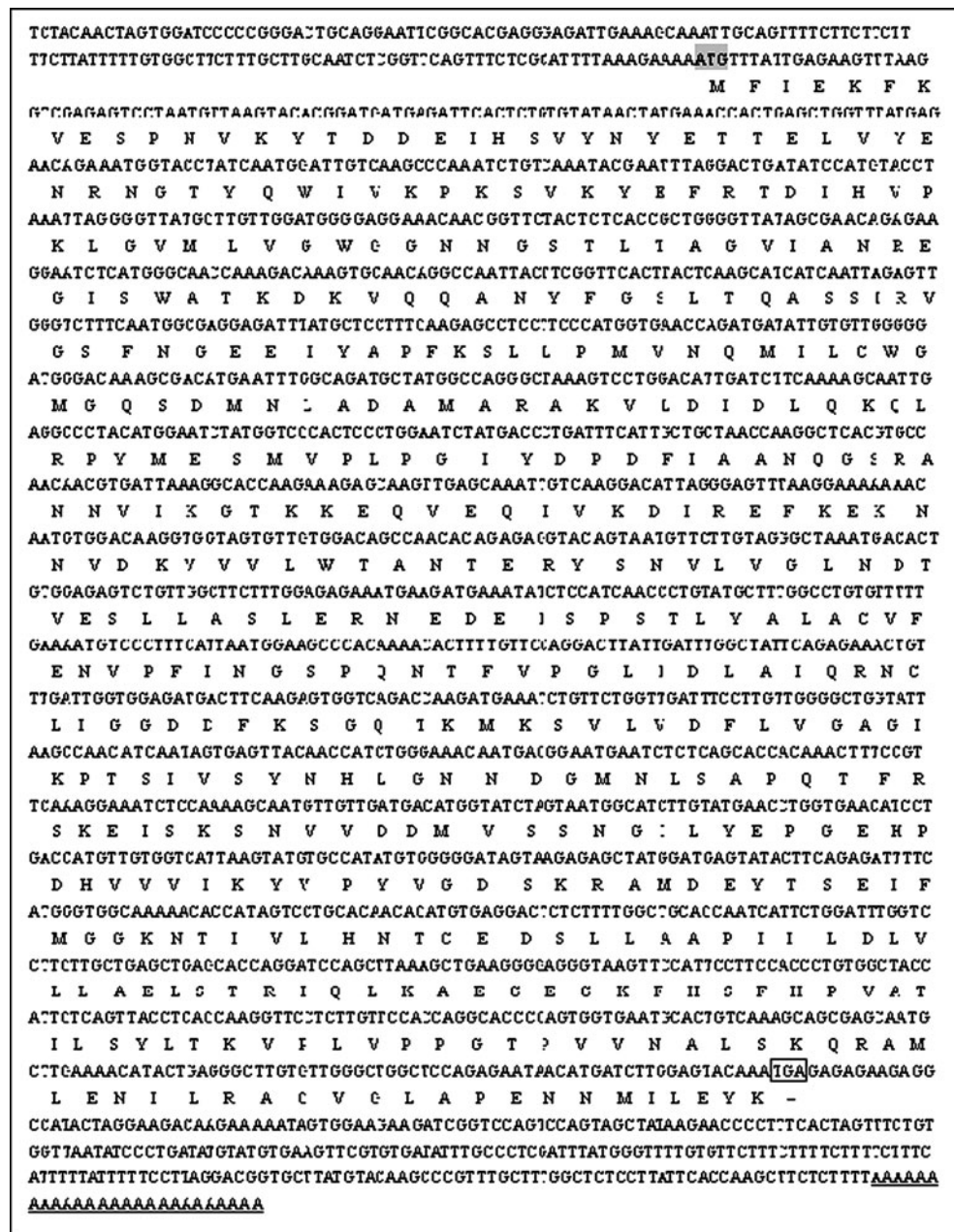

JcMIPS enzyme activity was not sensitive to cold treatment. There was only a 10% increase when exposed to 4°C for 72 h. Interestingly, the activity of the JcMIPS enzyme suffered sharply from the induction of ABA and increased 2.16 times more than control plants after 100 μM ABA treatment on *J. curcas* seedlings for 24 h.

Time courses of mRNA expression and enzymatic activity changes of JcMIPS in response to drought stress

Figure 6a shows that the average value of RWC of the control group leaves is 77.6%. This index decreased to 64, 57, and 52% after 24, 48, and 72 h of drought stress treatment, but recovered to 62% quickly after one day of re-watering. Figure 6b shows that the mRNA level of *JcMIPS* in *J. curcas* leaf increased gradually with the intensification of drought stress degree and reached 1.24 and 1.45 times that of the control in the first two days, but it decreased on the third day, and nearly decreased to the control level after re-watering treatment. The changes of JcMIPS enzyme activity in *J. curcas* leaves along with the stress degree were detected (Fig. 6c). The results suggested that JcMIPS activities in *J. curcas* leaves were further enhanced as plants were exposed to more severe water loss. JcMIPS enzyme activity began to rise slowly (114.7%) at

Fig. 3 Alignment of the amino acid sequences of myo-inositol-1L-phosphate synthase from *Jatropha curcas* (EF185781), *Glycine max* (AY038802), *Citrus paradisi* (Z32632), *Phaseolus vulgar* (AJ853494), *Brassica napus* (FJ429169), *Arabidopsis thaliana* (U04876), and *Nicotiana paniculata* (AB032073). The four highly conserved motifs (I, II, III, and IV) are indicated by the *gray box outline*
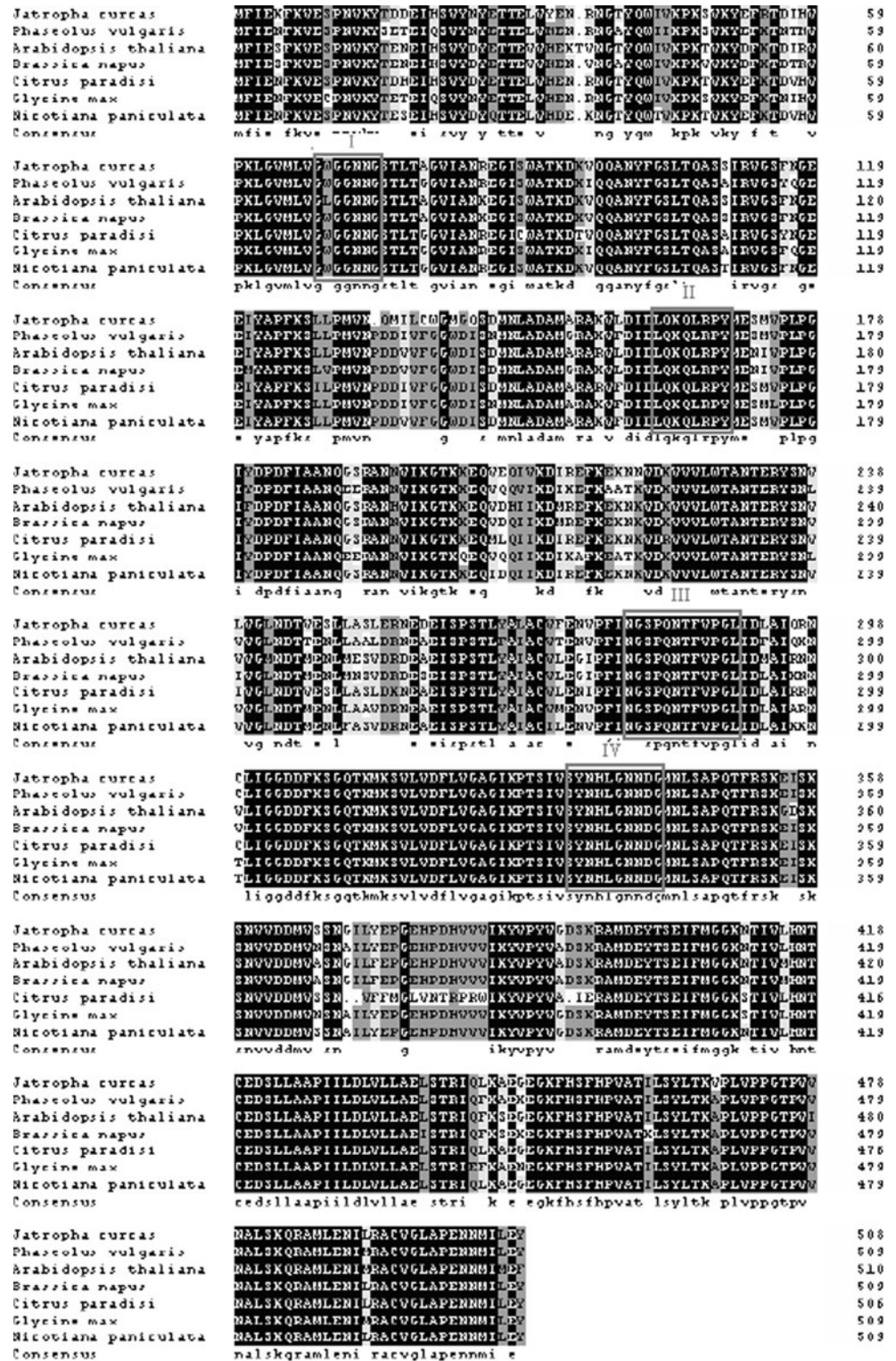
24 h and continued to rise after 48 h of treatment (124.2%), reaching the peak at 72 h, which was nearly one times higher than the control. After one day of re-watering, it decreased quickly and was similar to the level after 24 h of treatment.

Discussion

In this study, the cloning of JcMIPS and its expression in response to stress tolerance in *J. curcas* provided opportunities for developing *J. curcas* lines capable of growing

on poor and marginal soils and yielding acceptable levels of oil. Furthermore, JcMIPS can also be used for the genetic improvement of other agriculturally important crops for tolerance to salt and drought stresses (Eswaran et al. 2010).

The expression pattern was shown to be organ-specific in different plant species, such as sesame (Chun et al. 2003). In soybean, MIPS1 was highly expressed in developing seeds, but it was not expressed or minimally expressed in flowers, leaves, and roots (Chappell et al. 2006). Our data suggested that JcMIPS transcription was the highest in seed and the lowest in stem. The MIPS gene in different organs belonged to the multigene family. They may play different functions in plants but would coordinate inositol metabolism with cellular growth (Ishitani et al. 1996; Majumder et al. 1997).
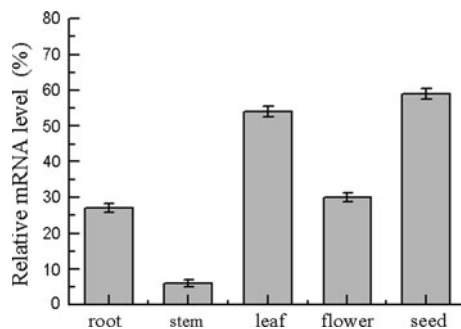

Fig. 4 Expression of the *JcMIPS* gene in different tissues of *J. curcas* (given as relative % yield of mRNA level normalized to 18S rRNA). Total RNAs were isolated from different tissues of *J. curcas* and 1 μg of total RNA was used for the real-time PCR assay. The cDNA fragments were amplified by PCR reaction with primers for the *JcMIPS* gene and 18S rRNA
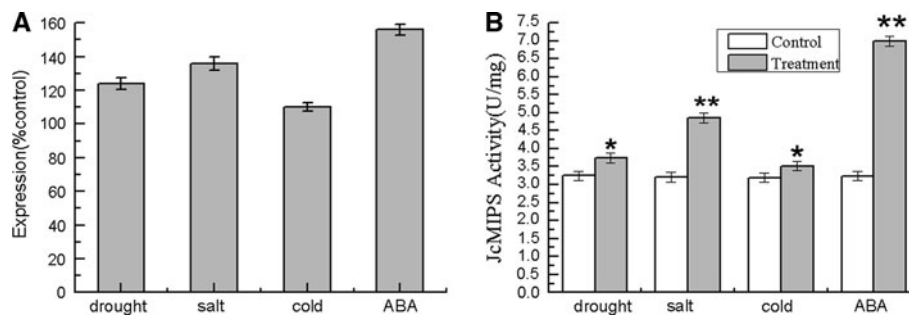
The studies revealed that the transcription of MIPS could be induced by different abiotic stresses (Ishitani et al. 1996; Abreu and Aragão 2007). Our results showed that *JcMIPS* transcription would be up-regulated by drought, salt, and low-temperature stress, and it is the most sensitive to salt stress. However, *JcMIPS* transcription, but not the enzyme activity, is slightly induced by low-temperature treatment. Luo et al. (2005) showed that the seedlings of *J. curcas* had a lower tolerance to low temperature. The results indicated that JcMIPS may participate in the response of drought stress but not cold stress.

JcMIPS transcripts would increase after drought stress, but decreased dramatically after 24 h of re-watering. The changes of gene expression at the enzymatic level also are consistent with the transcriptional level. The RWC in *J. curcas* leaf continued decreasing with the prolongation of stress treatment. When the drought stress degree was aggravated, the *JcMIPS* gene transcription and protein translation level would gradually increase. The RWC increased in leaves, indicating that the stress degree was mitigating. At the same time, the *JcMIPS* transcription level and enzymatic activity would decrease. So, *JcMIPS* was closely related to *J. curcas* in response to water stress.

Most of the stress-responsive genes, including the *MIPS* gene, have been found to be induced by exogenous application of ABA, which plays an important role in signal transduction pathways responding to abiotic stress. The endogenous ABA content would increase when plants perceived drought and salt stress, which mediate rapid physiological response through gene transcription (Bray 1997; Leung and Giraudat 1998). Our research is similar to the previous results. *JcMIPS* is induced fiercely by exogenous ABA. The up-regulation degree of *JcMIPS* induced by ABA treatment is higher than the other stresses.

Fig. 5a, b Expression analysis of *JcMIPS* mRNA and changes of enzyme activity under abiotic stress and exogenous ABA treatment. **a** Expression of the *JcMIPS* gene in leaves of *J. curcas* treated by drought (30% PEG-6000, 24 h), salt (200 mM NaCl, 24 h), cold (4°C, 72 h), and ABA (100 μM ABA, 24 h). Total RNAs were isolated from different leaves of *J. curcas* exposed to different stress treatment, and 1 μg of total RNA was used for real-time PCR. The cDNA fragments were amplified by PCR reaction with primers for the *JcMIPS* gene and 18S rRNA. The transcript levels of the JcMIPS at each treatment were plotted as percentages of the control. **b** The changes of activity of JcMIPS enzyme in leaves of *J. curcas* in control and those treated by different stress treatments (drought, salt, cold, and exogenous ABA). All of the presented data are averages and standard errors (*bars*) of three independent experiments. The *double asterisks* indicate that the treatment was significantly different from the control at $P < 0.01$. The *single asterisk* indicates that the treatment was different from the control at $P < 0.05$
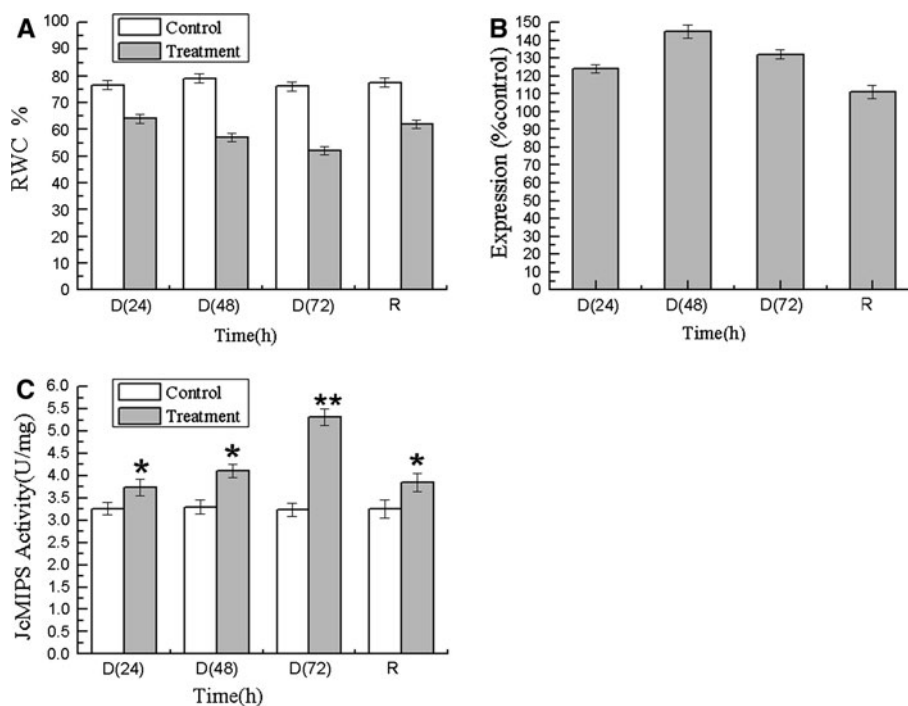
Fig. 6 Time courses of *JcMIPS* mRNA expression and activity changes of JcMIPS enzyme in response to drought stress. D24, D48, and D72 represent drought treatment for 24, 48, and 72 h, respectively; R represents re-watering after drought treatment for 72 h. **a** Changes of the relative water content (RWC) of *J. curcas* leaves at different stress treatments. The presented data are averages and standard errors (*bars*) of three independent experiments. **b** Expression of the *JcMIPS* gene upon different drought level treatments. The transcript levels of the *JcMIPS* at each treatment were plotted as percentages of the control. **c** The enzymatic activities of JcMIPS in the leaves of *J. curcas* as control and treated by drought. All of the presented data are averages and standard errors (*bars*) of three independent experiments. The *double asterisks* indicate that the treatment was significantly different from the control at $P < 0.01$. The *single asterisk* indicates that the treatment was different from the control at $P < 0.05$

In summary, we have identified the *MIPS* gene in *J. curcas* and studied its expression pattern. The data showed that the *JcMIPS* gene was highly conserved at the amino acid levels compared to other plants, and the differential expressions of *JcMIPS* and enzyme activity were induced by abiotic stress and ABA treatment. Furthermore, the copy number of the *JcMIPS* gene would be investigated, which may be of fundamental importance in order to understand this gene across phylogenetic groups.

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