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ORIGINAL PAPER

Molecular and functional characterization of the *JcMYB1*, encoding a putative R2R3-MYB transcription factor in *Jatropha curcas*

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Abstract The cDNA encoding the R2R3MYB transcription factor, designated as *JcMYB1*, was isolated from *Jatropha curcas* using rapid amplification of cDNA ends. *JcMYB1* contains a 942 bp open reading frame that encodes 313 amino acids. The deduced JcMYB1 protein was predicted to possess the conserved R2R3 domain and the signature motif specific for the interaction between MYB and bHLH proteins in the R3 domain. JcMYB1 is a member of the R2R3-MYB transcription factor subfamily. *JcMYB1* expressed at different levels with the highest transcription in the roots, followed by the leaves and stems. *JcMYB1* transcription was up-regulated by drought, polyethylene glycol, NaCl and cold treatments. *JcMYB1* transcription was up-regulated by abscisic acid and jasmonic acid treatment, whereas *JcMYB1* expression was not induced by ethylene. *JcMYB1* over-expression improved the drought and salt stress tolerance of transgenic tobacco. *JcMYB1* has an important function in modulating responses to abiotic stresses.

Keywords Abiotic stress · Gene expression · *Jatropha curcas* · R2R3 MYB transcription factor · Transgenic tobacco

Abbreviations

ABA	Abscisic acid
bHLH	Basic helix-loop-helix
GFP	Green fluorescent protein
JA	Jasmonic acid
ORF	Open reading frame
PEG	Polyethylene glycol
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription polymerase chain reaction
TF	Transcription factor
UTR	Untranslated region

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Introduction

The MYB transcription factor (TF) family is one of the largest TF families in plants (Allan et al. 2008). More than 100 MYB proteins are encoded in the *Arabidopsis* and soybean genomes (Dubos et al. 2010; Du et al. 2012). MYB proteins are characterized by a highly conserved MYB domain, and each consists of about 50 amino acids and a helix-turn-helix structure (Jin and Martin 1999). MYB TFs are classified into four subfamilies, R2R3-MYB, 1R-MYB, 3R-MYB, and 4R-MYB factors, depending on the number of adjacent repeats (R1, R2, and R3) in the DNA-binding domain (Dubos et al. 2010). MYB TFs have diverse functions in plant growth, developmental processes, and stress responses. In plants, the MYB family has selectively expanded, particularly through the large R2R3-MYB family (Du et al. 2012). Based on their well conserved DNA-binding domains, R2R3MYB family have been annotated genome-wide in *Arabidopsis* (126 members), *Populus trichocarpa* (192 members), *Vitis vinifera* (117 members), *Oryza sativa* (102 members) and *Zea mays* (more than 200 members) (Li et al. 2012). The R2R3-MYB genes have been extensively studied and members of the

MYB family are involved in diverse physiologic and biochemical processes, including the regulation of meristem formation, floral and seed development (Higginson et al. 2003; Perez-Rodriguez et al. 2005; Schmitz et al. 2002; Shin et al. 2002), cell shape, organ development (Baumann et al. 2007; Tominaga et al. 2007), the control of cell morphogenesis (Cominelli et al. 2005), the control of the cell cycle (Ito et al. 2001; Araki et al. 2004), and regulation of secondary metabolism (Pierantoni et al. 2010; Shimizu et al. 2011; Almeida et al. 2013). Several members were also involved in hormone signal transduction (Jung et al. 2008; Seo et al. 2009). and environmental stresses (Jung et al. 2010; Gao et al. 2011; Liu et al. 2011; Peng et al. 2011; Qin et al. 2012).

Jatropha curcas L. (physic nut) is a oil woody plant belonging to the Euphorbiaceae family and is found in numerous tropical and subtropical countries. Physic nut produces oil from the seeds, which can be combusted as fuel without prior refining (Openshaw 2000; Fairless 2007; Sato et al. 2011). Recent research on this plant focused on oil extraction, seed composition, and antitumor activities of curcin from the seed (Berchmans and Hirata 2008; Li et al. 2008). Physic nut is a drought-resistant plant and can be used to prevent and/or control erosion and reclaim land (Tang et al. 2007), understanding the molecular response of Jatropha to adverse abiotic environmental factors is relatively rudimentary (Mastan et al. 2012). R2R3-MYB genes are involved in diverse environmental stresses, to our knowledge there isn't report about R2R3-MYB genes from physic nut. A draft of the *J. curcas* genome sequence was reported recently (Sato et al. 2011). The genome-wide of R2R3-MYB genes were identified and described in the physic nut (data not shown). Here, one member of physic nut R2R3-MYB family, designated as *JcMYB1*, was investigated for its expression pattern. Moreover, the functions of transgenic *JcMYB1* tobacco under several types of stresses were evaluated.

Materials and methods

Plant materials, plant hormones, and stress treatments

Mature *J. curcas* seeds were collected from the South China Botanical Garden, Chinese Academy of Sciences, Guangdong Province, China. The seeds were surface sterilized in 70 % ethanol for 10 min, then in 10 % NaClO for 10 min. The seeds were rinsed four times with sterile distilled water. Then, the embryos were removed from the seeds and placed in 100 mL flasks containing 40 mL of Murashige and Skoog (MS) medium and 0.6 % (w/v) agar, pH 5.8. After 3 days, the embryos were transferred into pots with 1:1 (v/v) vermiculite and peat medium and incubated at 28 °C with a 16 h light/8 h dark photoperiod for 3 weeks. Three-week-old light-grown intact plants (with 2–3 leaves) were used for PCR analysis. Chemical treatment was performed as follows: a solution of 250 mM NaCl, 20 % polyethylene glycol (PEG-8000), 100 mM abscisic acid (ABA), 50 mM ethephon, and 100 mM jasmonic acid (JA) was applied to the surface of solid MS agar medium of the 3-week-old seedlings. Drought treatment was applied to 3-week-old seedlings by drying on Whatman 3MM paper according to the method by Jung et al. (2008) or incubation at 4 °C under continuous light (cold treatment). After each treatment, sample seedlings were harvested and frozen immediately in liquid nitrogen until use in real-time PCR.

Isolation of RNA

Total RNA was extracted according to the method of Chang et al. (1993). Total RNA was treated with DNase I according to Invitrogen manufacturer's protocol. The quality and concentration of the extracted RNA was verified using agarose gel electrophoresis and measured with a spectrophotometer (DU-70, Beckman, Fullerton, CA, USA).

Cloning of JcMYB1

Rapid amplification of cDNA ends (RACE) was used to obtain a cDNA encoding putative R2R3-MYB TF based on the genome sequence (Jcr4S04925) at http://www.kazusa.or.jp/jatropha/ (Sato et al. 2011). 3′- and 5′-RACE were conducted using the double-stranded cDNA from *J. curcas* as a template. The primers used for the 3′ RACE and the 5′ RACE were designed based on the sequence (Table 1). The products were purified and cloned into pGEM-T easy vector (Promega, USA) followed by sequencing. Based on the 5′ and 3′ end cDNA sequences, primers were designed to allow amplification of the entire *JcMYB1*. The PCR

Table 1 Primer sequences (nucleic sequences from 5′ to 3′)

3′RACE-PCR primers	
3MYB11	GTTGCAGGCTGAGGTGGATAAATTACT
3MYB12	GTGTTTTAGTGAACAGGAAGAAAGGAT
5′ RACE-PCR primers	
5MYB11	CCTTTGGCAACAAGTGGGCTACCATCGC
5MYB12	GCGATGGTAGCCCACTTGTTGCCAAAGC
Real time PCR primers	
RF1	CTGAGTTCTTGGCAGTGATGC
RF2	GTCGACTCGGCTTGGATCATC
ACT specific primers	
AF	CAGTGGTCGACAACTGGTAT
AR	TCCTCCAATCCAGACACTGT

products were cloned into pGEM-T easy vector and sequenced.

Multiple alignments and phylogenetic analysis

Multiple sequence alignments were performed using ClustalX 1.81 with default parameters, and the alignments were then adjusted manually before phylogenetic tree constructed. A phylogenetic tree was constructed with the aligned JcMYB1 and R2R3 MYB TFs form other plants using MEGA 4.

Subcellular localization of JcMYB1

The complete ORF of *JcMYB1* was amplified by PCR using primer containing an *Nco*I or *Spe*I restriction site. The PCR product, after confirmation by sequencing, was digested with *Nco*I and *Spe*I and cloned into the pCAMBIA1302 vector digested using the same restriction enzymes to create a fusion construct (pCAMBIA1302-JcMYB1-GFP). Both the fusion construct and the control vector (pCAMBIA1302-GFP) were bombarded into onion epidermal cells. After culture on MS medium for 8–24 h at 28 °C in darkness, the transformed cells were visualized using a confocal laser scanning microscope (Zeiss LSM510, Germany).

Expression analysis of JcMYB1

Real-time RT-PCR was conducted with the primers (Table 1). Real-time RT-PCR was performed using the fluorescent dye SYBR-Green (Takara, Dalian, China) and the BIO-RAD CFX96 real-time PCR system (Bio-Rad, USA). The reactions were carried out as follows: denaturation at 95 °C for 30 s, and amplification at 94 °C for 5 s, at 60 °C for 20 s, and at 72 °C for 20 s. Three biological replicates were carried out and triplicate quantitative assays were performed for each replicate. The *Actin* from *J. curcas* was amplified as the internal control. The relative abundance of transcripts was calculated according to the Bio-Rad CFX Manager (Version 1.5.534) of BIO-RAD CFX96. Data are mean values from three independent assays and error bars show the standard deviations of the replicates. Analysis of variance (ANOVA) was used to compare the statistical difference based on Fisher's LSD test, at a significance level of $P < 0.05$, $P < 0.01$.

Plasmid construction and plant transformation

The *JcMYB1* coding region was cloned into pBI121, which contains the CaMV 35S promoter fragment. The *Agrobacterium tumefaciens* strain EHA105 was used in tobacco transformation. Tobacco (*Nicotiana tabacum* cultivar Samsun NN) leaf discs were transformed and the plants were regenerated. The plant growth conditions, transformation, selection of transformants, and determination of T_2 generation genotype were performed as described by Pontier et al. (1994). Transgenic lines were tested by RT-PCR. RT-PCR for the analysis of *JcMYB1* expression was performed using total RNA from transgenic plants, and amplified with *JcMYB1* specific primers (Table 1). The *NtACT* was used as an internal control parallel in the reactions, amplified with *NtACT* specific primers AF (5′-CAGTGGCCGTACAACAGGTAT-3′) and AR (5′-ATCCTCCAATCCAGACACTGT-3′). PCR reaction was carried out in 22 cycles of programmed temperature control for 30 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C with a 5 min preheat at 95 °C and a 10 min final extension at 72 °C. The PCR products were analysed by agarose gel electrophoresis with ethidium bromide staining.

Tolerance of transgenic tobacco plants to drought and salt stress

Transgenic tobacco plants were treated according to the method of Liu et al. (2011). Seeds of untransformed tobacco (WT) and *JcMYB1* transgenic plants in the T_2 generation were sterilized and germinated. After 4 weeks of incubation, the seedlings or the leaf discs cut from the plants were subjected to the following treatments. (1) 10-mm-diameter tobacco leaf discs were soaked in the solution of 20 % PEG for 6 days, 250 mM NaCl for 2 days, control plants were treated with H_2O under the same conditions. (2) The seedlings were irrigated with 20 % PEG-8000 solution or 250 mM NaCl, then not irrigated with water for 40 days. WT control plants watered under the same conditions. All plants were treated and incubated under the same conditions at 24 ± 2 °C and 65 ± 5 % relative humidity during the experiment. Three biological replicates were carried out.

Chlorophyll content and ion leakage measurement

Electrical conductivity of the leaf wash is shown in mS/gFW/h as described by Mitsuhara et al. (1999). For salt resistance assays, leaf discs prepared from fully expanded upper leaves were submerged in 20 % PEG-8000 solution or 250 mM NaCl solution and incubated at 28 °C under continuous light (100 mmol m^{-2} s^{-1}). Leaf discs from wild-type and transgenic lines were treated with 20 % PEG-8000 solution and 250 mM NaCl or with water (as a control). The determination of chlorophyll content was carried out as described by Mitsuhara et al. (1999). All experiments were repeated at least three times.

Chlorophyll fluorescence measurement

Chlorophyll a fluorescence was recorded with a pulse amplitude modulation fluorometer (Mini PAM, Walz, Effeltrich, Germany). Chlorophyll a fluorescence was measured according to the method by Fracheboud et al. (1999).

Results

Characterization of JcMYB1

The full-length cDNA that encodes a putative R2R3 MYB TF, designated as *JcMYB1* (GenBank accession number: JX569771) was cloned via the RACE. The 1,280 bp full-length cDNA contained a 942 bp open reading frame (ORF) with a 266 bp 3′ UTR downstream from the stop codon and a 72 bp 5′ UTR upstream of the start codon. Basic local alignment search tool (BLAST) analysis showed *JcMYB1* DNA sequence was identical to that of the corresponding full-length cDNA, suggesting that there was no intron in *JcMYB1*.

The deduced JcMYB1 protein consisted of 313 amino acid residues with a calculated molecular weight of 33.79 kDa and an isoelectric point of 8.38. BLAST analysis showed that JcMYB1 contains an R2R3 domain and the signature motif specific for the interaction between MYB and basic helix-loop-helix (bHLH) proteins in the R3 domain (Fig. 1). However, the entire JcMYB1 protein sequence has limited identity with other R2R3-MYB proteins, including the putative R2R3-MYB TF in *Citrus sinensis* (GenBank accession number: AEK32395.1, 72 %), *Ricinus communis* (GenBank accession number: XP_002510155, 72 %), *Malus x domestica* (GenBank accession number: ADL36769.1, 66 %), *Glycine max* (GenBank accession number: NP_001235142.1, 62 %), *Medicago truncatula* (GenBank accession number: XP_003611666.1, 61 %), *Coptis japonica* (GenBank accession number: BAJ40867.1, 61 %) and AtMYB44 in *Arabidopsis thaliana* (GenBank accession number:

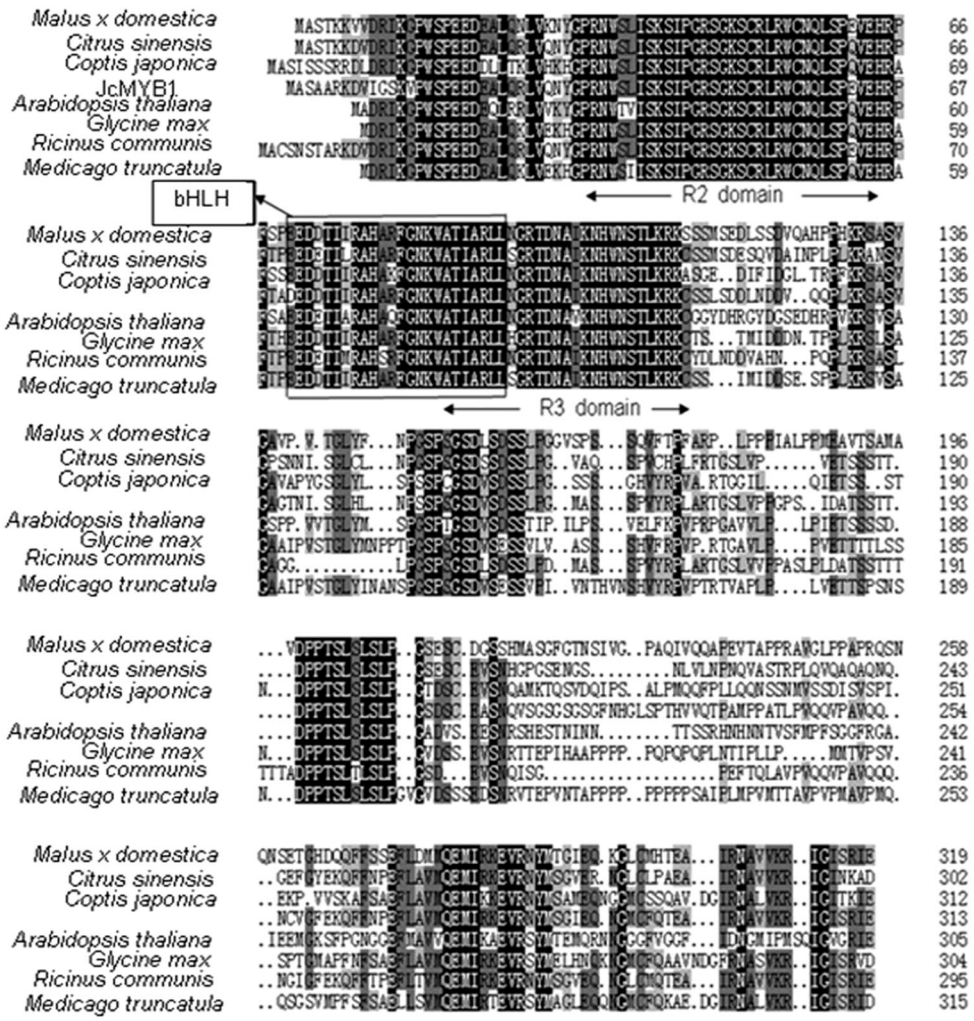

Fig. 1 The deduced amino acid sequence of JcMYB1 is compared with R2R3-MYB proteins of other plant species. Amino acid residues identical in all eight sequences are *black shaded*, whereas well-conserved residues are shaded in *grey*. The R2R3-binding domain is *underlined*. The *box* indicates specific residues forming the motif implicated in bHLH cofactor interactions. The aligned R2R3-MYB TFs were from *C. sinensis* (GenBank accession number: AEK32395.1), *R. communis* (GenBank accession number: XP_002510155), *Malus x domestica* (GenBank accession number: ADL36769.1), *G. max* (GenBank accession number: NP_001235142.1), *M. truncatula* (GenBank accession number: XP_003611666.1), *C. japonica* (GenBank accession number: BAJ40867.1) and *A. thaliana* (GenBank accession number: NP_201531)

NP_201531, 54 %). JcMYB1 maybe a new member of the R2R3-MYB TF subfamily.JcMYB1 amino acid sequences and R2R3-MYB from other different species were compared and a phylogenetic tree showed similarity to the actual relationships among species, which implies that *JcMYB1* may code for a typical R2R3-MYB protein (Fig. 2).

To confirm the subcelluar location of JcMYB1, the fusion proteins 35S::JcMYB1-GFP and 35S::GFP were constructed and transiently expressed in onion epidermal cells. Fluorescence microscopy showed that the JcMYB1-GFP fusion protein was exclusively localized in the nucleus, whereas in the control, GFP was distributed throughout the cell (Fig. 3). Thus, JcMYB1 is a nuclear-localized protein, which is consistent with its predicted function as a TF.

Differential JcMYB1 expression in different organs

The total isolated RNA from leaves, stems, and roots were used to analyze transcript level via real-time PCR. The results indicated that *JcMYB1* was expressed in all tested tissues but at different levels. The highest transcription was in the roots, followed by the leaves and stems (Fig. 4).

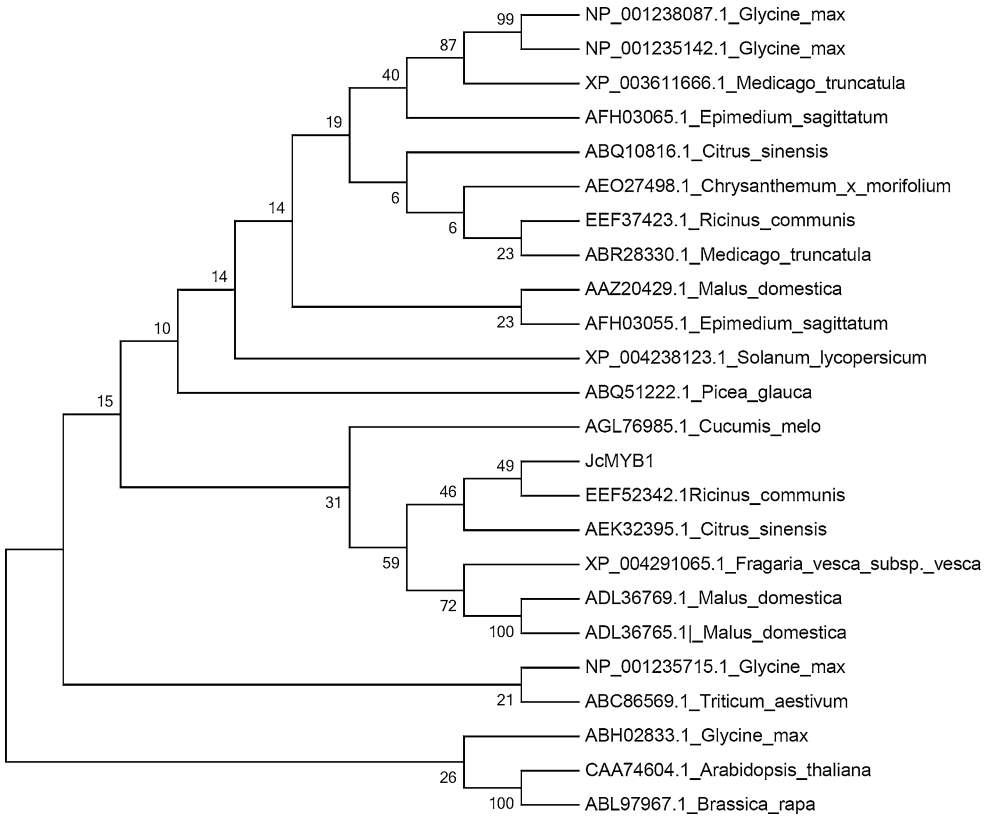

Fig. 2 Phylogenetic tree of JcMYB1 and R2R3-MYB TFs from other plants with indicated GenBank accession numbers

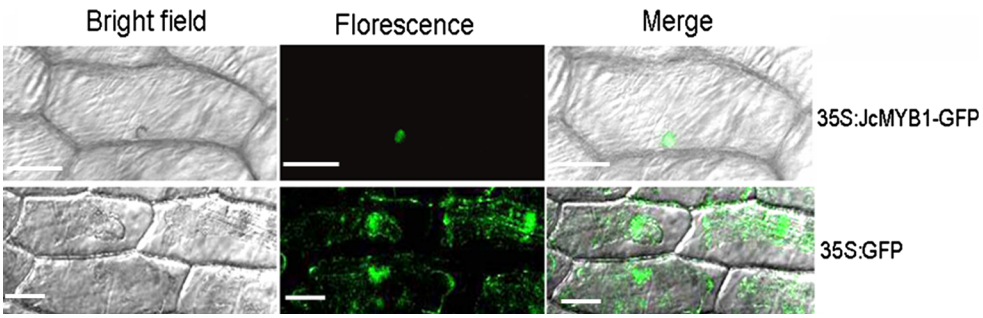

Fig. 3 Nuclear localization of the JcMYB1-GFP fusion protein in onion epidermal cells. The green fluorescent protein (GFP) fluorescence images (GFP image and light image) of onion epidermal cells were compared to show the subcellular localization of 35S:JcMYB1:GFP (the *upper panel*) and of 35S:GFP (the *lower panel*). *Scale bars* 10 μm for the images from the 35S: JcMYB1:GFP (the *upper panel*) and 20 μm for that from the 35S:GFP plant (the *lower panel*), respectively

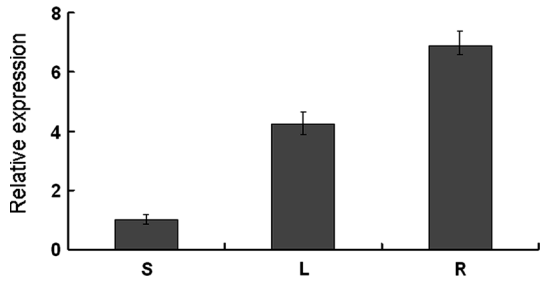

Fig. 4 *JcMYB1* expression in the roots (R), stems (S), and leaves (L) of *J. curcas* seedlings

Effects of plant hormones and stress on JcMYB1 expression

The *JcMYB1* mRNA accumulation profile was determined under various abiotic stresses by real-time RT-PCR to determine whether *JcMYB1* expression is regulated by multiple factors. Three-week-old intact *J. curcas* seedlings were treated with various chemical reagents for various durations, and the transcript levels were monitored at each time point. *JcMYB1* expression significantly increased within 0.5 h and subsequently decreased under the drought, PEG, and cold treatments. However, *JcMYB1* expression significantly increased within 2 h and subsequently decreased under the NaCl treatment (Fig. 5a). *JcMYB1* transcription was significantly up-regulated in plants treated with ABA and JA, whereas *JcMYB1* expression was not induced by ethylene (Fig. 5b).

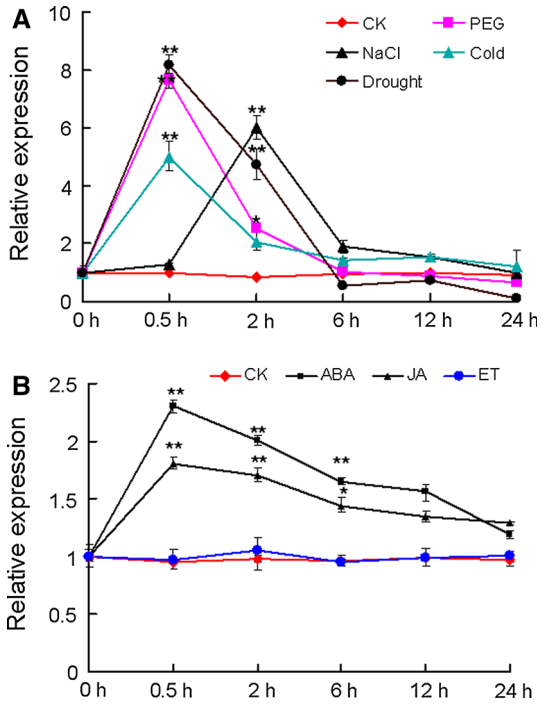

Fig. 5 Effects of plant hormones and stress on *JcMYB1* expression. **a** *JcMYB1* transcription patterns induced by PEG, drought, cold, and NaCl treatments through real-time PCR analysis. **b** *JcMYB1* transcription patterns induced by ABA, JA, and ethylene through real-time PCR analysis. Gene-specific primers for *JcMYB1* and *Actin* (internal control) were used. For each stress, the expression level at time point 0 (the beginning of the relevant treatment) was defined as 1.0, and the expression level at other time points was normalized accordingly. *Error bars* show standard deviations for three independent replicates. The significant difference was assessed by AOVA (* or ** corresponding to $P < 0.05$ and $P < 0.01$)

Phenotype of transgenic plants under drought and salt stresses

JcMYB1 was over-expressed under the control of the CaMV 35S promoter in tobacco plants. The transgenic tobacco plants that harbored the *JcMYB1* gene were selected using semi-quantitative RT-PCR. PCR detection of the T_0–T_2 transgenic lines showed that *JcMYB1* was stably inherited. The transcription of *JcMYB1* in T_2 transgenic lines was detected by semi-quantitative RT-PCR (Fig. 6a). All transgenic lines constitutively expressed higher *JcMYB1* transcript levels were collected to analysis the tolerance to drought and salt stress. Leaf discs from the T_2 transgenic tobacco lines were subjected to drought and salt stress to evaluate the response of the *JcMYB1* transgenic plants transgenic to PEG and salt stress. After treatment, the *JcMYB1* transgenic plants exhibited enhanced PEG and salt tolerance relative to the WT and the control plants (Fig. 6b), respectively. At the same time, alterations in chlorophyll content and ion leakage of the leaves under NaCl and PEG treatment were also evaluated as reliable indices of photosynthetic and cell membrane damage under NaCl and PEG treatment. As shown in Fig. 6c, chlorophyll contents are significantly lower in WT than in the three transgenic lines. The ion leakage in the WT plants is significantly higher than in the three transgenic lines (Fig. 6d). Whole plants were used for the PEG and salt stress assays suggested *JcMYB1* transgenic plants also exhibited enhanced PEG and salt tolerance (Fig. 6e). In vivo chlorophyll fluorescence (Fv/Fm) measurements are commonly used to study the functioning of the photosynthetic apparatus (Fracheboud et al. 1999). The Fv/Fm in the three transgenic lines is significantly higher than in the WT plants under NaCl and PEG treatment (Fig. 6f).

Discussion

In the present study, an MYB TF, designated as *JcMYB1*, was isolated from *J. curcas*. The deduced JcMYB1 protein was predicted to possess a conserved R2R3 domain and the signature motif specific for the interaction between MYB

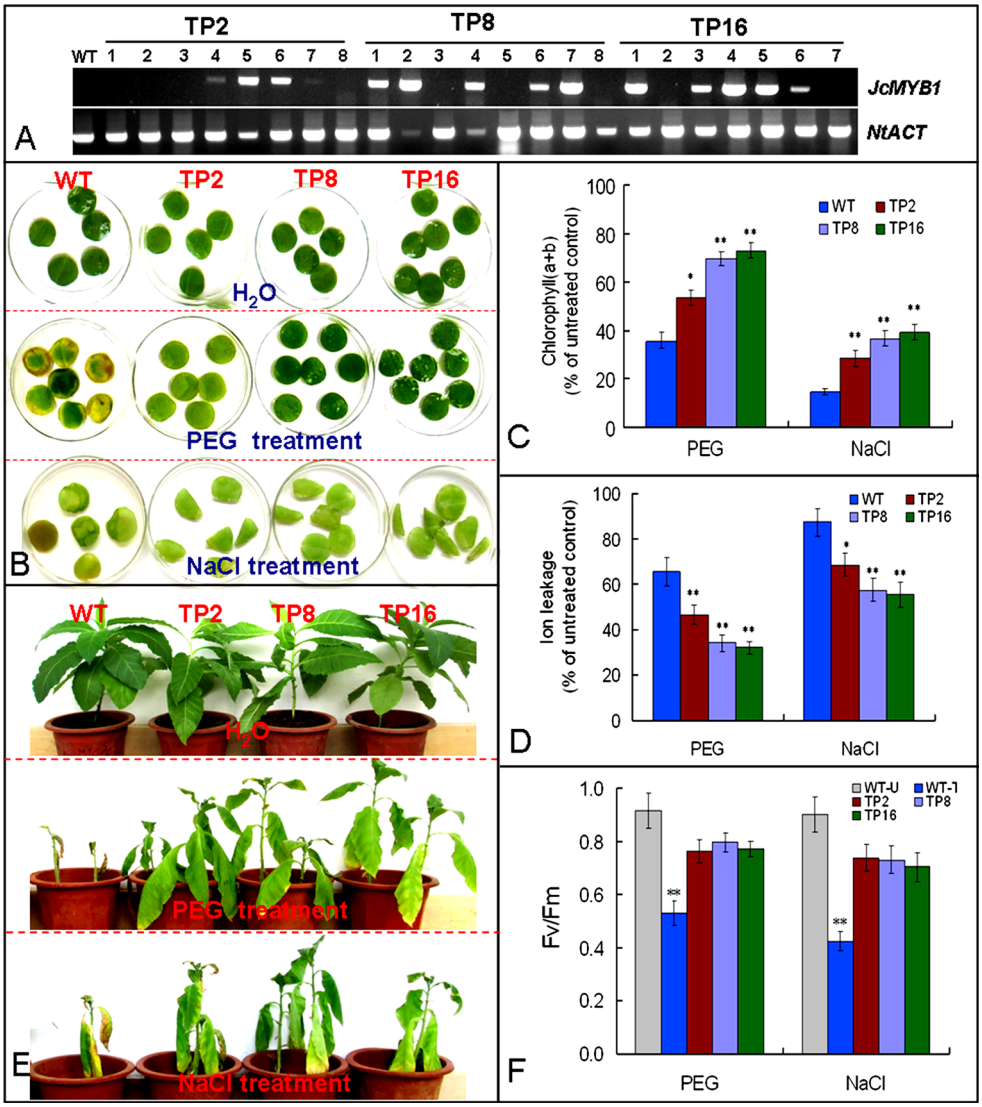

Fig. 6 *JcMYB1* transgenic tobacco phenotypes in response to stress. **a** Molecular identification of *JcMYB1* in T_2 transgenic plants (TP2, TP8 and TP16 lines) by RT-PCR. **b** Phenotype of leaf discs from wild-type (WT) and transgenic plants (TP2, TP8 and TP16 lines) after treatment with 20 % PEG for 6 days or 250 mM NaCl 4 days. **c** Resistance to PEG and NaCl induced chlorophyll degradation in leaf discs from transgenic lines after treatment with 20 % PEG for 6 days or 250 mM NaCl 4 days. Values are an average of triplicate assays, and each SD is within 10 % of the average. **d** Electrolyte leakage from leaf discs after treated with 20 % PEG-8000 for 6 days or 250 mM NaCl 4 days. Values are an average of triplicate assays; *error bars* denote the SD is within 10 % of the average. **e** Phenotype of whole plants from wild-type (WT) and transgenic plants (TP2, TP8 and TP16 lines) after treatment with 20 % PEG-8000 for 6 days or 250 mM NaCl 40 days. **f** Chlorophyll fluorescence (Fv/Fm) of wild-type (WT-T) and transgenic plants (TP2, TP8 and TP16 lines) after treatment with 20 % PEG-8000 for 6 days or 250 mM NaCl 40 days. WT controls (WT-U) were treated with water under the same conditions. *Error bars* show standard deviations for three independent replicates. The significant difference was assessed by AOVA (* or ** corresponding to $P < 0.05$ and $P < 0.01$)

and bHLH proteins in the R3 domain. JcMYB1 is a new member of the R2R3-MYB TF subfamily.

The growing evidence suggests that R2R3-MYB TFs are involved in abiotic responses and plant defense. Diverse R2R3-MYBs participate in the crosstalk between hormone signaling and other stress signaling pathways (Jung et al. 2008). For example, the AtMyb41 gene is responsive to salinity, desiccation, cold, and endogenous ABA (Cominelli et al. 2008). AtMYB96 is responsive to ABA and drought stress and is induced by indole acetic acid (IAA). In addition, MYB44 mRNA is induced in most tissues by treatment with ABA, JA, IAA, ethylene, and gibberellic acid, environmental conditions, and pathogenic infections (Jung et al. 2007, 2008). MYB96 is a molecular convergence point between the ABA signaling and auxin signaling pathways (Seo et al. 2009). In this study, the

transcript level of *JcMYB1* increases after JA and ABA treatment. Furthermore, *JcMYB1* is induced rapidly during the early phase of the response to cold, drought, and salt stress. This is in agreement with the results about regulation of others members of R2R3-MYB-TF showed in previous reports (Jung et al. 2008; Cominelli et al. 2008). Our data also suggest that the over-expressed *JcMYB1* enhanced the drought and salt stress tolerance in transgenic tobacco. These results suggested that *JcMYB1* may participate in the crosstalk between ABA, JA signaling and drought, salt stress signaling pathways. To our knowledge, this is the first report on a Jatropha MYB gene involved in responses to abiotic stresses. This work contributes to an increased understanding of the characteristics and functions of the MYB family in different species.

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