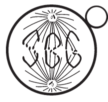
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Research Article

Molecular characterization of the *Jatropha curcas JcR1MYB1* gene encoding a putative R1-MYB transcription factor

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Abstract

The cDNA encoding the R1-MYB transcription factor, designated as *JcR1MYB1*, was isolated from *Jatropha curcas* using rapid amplification of cDNA ends. *JcR1MYB1* contains a 951 bp open reading frame that encodes 316 amino acids. The deduced JcR1MYB1 protein was predicted to possess the conserved, 56-amino acid-long DNA-binding domain, which consists of a single helix-turn-helix module and usually occurs in R1-MYBs. JcR1MYB1 is a member of the R1-MYB transcription factor subfamily. A subcellular localization study confirmed the nuclear localization of JcR1MYB1. Expression analysis showed that *JcR1MYB1* transcripts accumulated in various examined tissues, with high expression levels in the root and low levels in the stem. *JcR1MYB1* transcription was up-regulated by polyethylene glycol, NaCl, and cold treatments, as well as by abscisic acid, jasmonic acid, and ethylene treatment. Analysis of transgenic tobacco plants over-expressing *JcR1MYB1* indicates an inportant function for this gene in salt stress.

Keywords: abiotic stress,gene expression, *Jatropha curcas*, R1-MYB transcription factor.

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Introduction

MYB proteins constitute a large family of transcription factors (TFs), which is functionally diverse and is represented in all eukaryotes (Dubos *et al.*, 2010). MYB proteins are characterized by a highly conserved MYB DNA-binding domain with up to three imperfect repeats, each comprised of an approximately 50-amino acid-long, helix-turn-helix structure (Jin and Martin, 1999). Based on the numbers of adjacent imperfect repeats (R1, R2, and R3) in the DNA-binding domain, MYB TFs are classified into four subfamilies, namely, R2R3-MYB, R1-MYB, R3-MYB, and R4-MYB factors (Dubos *et al.*, 2010). Members of the MYB family have important functions in plant development, metabolism, and stress responses (Allan *et al.*, 2008; Dubos *et al.*, 2010). Most plant MYB TFs belong to the R2R3-type (Du *et al.*, 2009). Compared with R2R3-type MYB TFs, few reports exist on functional studies of MYB TFs with a single helix-turn-helix structure in plants. Potato MybSt1 is the first reported MYB transcription factor possessing such a single module in plants (Wang *et a*l., 1997). In Arabidopsis, CIRCADIAN CLOCK ASSOCIATED (CCA1) is a MYB protein with only a helix-turn-helix motif and functions as a specific activator of phytochrome signal transduction (Wang *et al.*, 1997). Constitutive expression of *CCA1* results in longer hypocotyls and substantially delayed flowering (Wang and Tobin, 1998). In rice, ANTHER INDEHISCENCE1 (AID1) is closely related to other single MYB-like domain TFs in plants. AID1 was identified in a genetic screen as playing a role in partial to complete spikelet sterility (Zhu *et al.*, 2004). *StMYB1R-1* was recently identified as a putative stress-response gene. Over-expression of *StMYB1R-1* in potato plants improved plant tolerance to drought stress (Shin *et al.*, 2011). Growing evidence suggests that R1-MYB TFs have diverse functions in plant growth, development, and stress responses (Lee and Schiefelbein, 1999; Kuno *et al.*, 2003; Rubio-Somoza *et al.*, 2006; Jia *et al.*, 2009; Cheng *et al.*, 2013).

Jatropha curcas L. (physic nut) is a woody oil plant that is found in 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). A draft of the *J. curcas* genome sequence has recently been reported (Sato *et al.*, 2011). The genome-wide MYB genes were identified and described in the physic nut (data not shown). In this work, one member of the physic nut R1-MYB family, designated as *JcR1MYB1*, was investigated for its expression pattern.

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Moreover, the salt tolerance of transgenic *JcR1MYB1* tobacco was 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. The cotyledons were then removed from the seeds and were placed in 100 mL flasks containing 40 mL of Murashige and Skoog (MS) medium and 0.6% (w/v) agar at pH 5.8. After 3 d, the rooted cotyledons were transferred into pots with 1:1 (v/v) vermiculite and peat medium and then incubated at 28 °C with a 16 h light/8 h dark photoperiod for three weeks. Three-week-old light-grown intact plants (with two to three leaves) were used for polymerase chain reaction (PCR) analysis. Chemical treatment was performed as follows: a solution of 200 mM NaCl, 20% polyethylene glycol (PEG), 100 mM ABA, 50 mM ethephon (ET), and 100 mM jasmonic acid (JA) were applied to the surface of solid MS agar medium of the three-week-old seedlings. For cold treatment, the seedlings incubated at 4 °C under continuous light for 1 d. After each treatment, sample seedlings were harvested and immediately frozen in liquid nitrogen until use for real-time quantitative PCR (RT-qPCR).

Isolation of RNA

Total RNA was extracted according to the method by Chang *et al.* (1993). The quality and concentration of the extracted RNA was verified using agarose gel electrophoresis and was measured with a spectrophotometer (DU-70, Beckman, Fullerton, CA).

Cloning of *JcR1MYB1*

Rapid amplification of cDNA ends (RACE) was used to obtain the DNA sequence encoding a putative R1-MYB TF based on the genome sequence at http://www.kazusa.or.jp/jatropha/ (Sato *et al.*, 2011). Moreover, 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 amplified product was purified (Tiangen, China) and cloned into the pGEM-T easy vector (Promega, USA) and then sequenced. The sequences were compared with those in the NCBI database using the basic local alignment search tool (BLAST). Based on the 5' and 3' end cDNA sequences, primers were designed to enable amplification of the entire *JcR1MYB1*. The amplified products were purified (Tiangen, China) and cloned into the pGEM-T easy vector (Promega, USA) and then sequenced.

Table 1 - Primer sequences (Nucleotide sequences from 5'to 3').

3'RACE-PCR primers	
3MYB11	GAATGCCAAGGAATGCTCCCAGTCGAT
3MYB12	TTGCCAGATCGGATTGGTGAATGCTCC
5' RACE-PCR primers	
5MYB11	GGATTGCTTTCCCAGCCTGTATTTCTG
5MYB12	CCTTTACTCCCATTGTCCTCATAATCG
Real time PCR primers	
RF1	AGACCAAGGCTTGCATTTGGT
RF2	TAAATGTCTTTGCCACTCATCC
JcACT specific primers	
AF	CAGTGGTCGACAACTGGTAT
AR	TCCTCCAATCCAGACACTGT

Subcellular localization of JcR1MYB1

The *JcR1MYB1* coding region was fused in frame to the 5' terminus of the gene that encodes green fluorescent protein (GFP) under the control of the CaMV35S promoter in the pCAMBIA1302 vector. The resulting JcR1MYB1-GFP fusion construct was used for transient expression in onion epidermal cells. The location of the introduced gene in the onion cells was observed under an adaptive optics fluorescence microscope with ultraviolet excitation filter.

Expression analysis of *JcR1MYB1*

RT-qPCR was conducted with primers (Table 1). RT-qPCR was performed using the fluorescent dye SYBR-Green (Takara, Dalian, China) and the BIO-RAD CFX96 real-time PCR system (Bio-Rad, USA) using the following protocol: 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 run, and triplicate quantitative assays were performed for each biological replicate. The *actin* gene from *J. curcas* was amplified as internal control. The relative abundance of transcripts was calculated according to the Bio-Rad CFX Manager (Version 1.5.534) of BIO-RAD CFX96.

Plasmid construction and plant transformation

The *JcR1MYB1* coding region was cloned into pBI121, which contains the CaMV 35S promoter fragment. Transgenic tobacco plants were generated by transforming the pBI 121- *JcR1MYB1* constructs into leaves of 6- to 8-week-old tobacco (*Nicotiana tabacum* cultivar Samsun NN) by means of the *Agrobacterium tumefaciens*-mediated leaf disc method. The plant growth conditions, transformation, selection of transformants, and determination of genotyping the T_2 generation were performed as described by Pontier *et al.* (1994). *JcR1MYB1* expression in transgenic

lines was tested by reverse transcription PCR (RT-PCR) assays, using total RNA from transgenic plants amplified with *JcR1MYB1* specific primers (Table 1). *NtACT* used as internal control parallel was amplified with *NtACT* specific primers AF (5'-CAGTGGCCGTACAACAGGTAT-3') and AR (5'-ATCCTCCAAT CCAGACACTGT-3'). PCR assays consisted of a 5 min preheat at 95 °C and 22 cycles of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C, followed by a 10 min final extension at 72 °C. The PCR products were analyzed through agarose gel electrophoresis with ethidium bromide staining.

Tolerance of transgenic tobacco plants to salt stress

Seeds were surface sterilized in 70% ethanol for 10 min, followed by 10% NaClO for 10 min. After rinsing four times with sterile distilled water the seeds were placed in solid Murashige and Skoog (MS) medium containing 200 mM NaCl. Seed germination rate was analyzed after 6 days.

For the detached leaf disc NaCl stress treatments, 10 mm diameter tobacco leaf discs from four-week-old seedlings of the T_2 generation *JcR1MYB1* transgenic plants were soaked in 150 mM NaCl for 4 days. The control plants were treated with H_2O under the same conditions. All plants were treated and incubated under the same conditions at 24 °C ± 2 °C and 65% ± 5% relative humidity during the experiment.

Expression analysis of *JcR1MYB1* in transgenic tobacco plants

The seeds were surface sterilized as described above and, after rinsing, placed in solid Murashige and Skoog (MS) medium. Three-week-old light-grown intact plants were used for RT-qPCR analysis. Chemical treatment was performed as follows: 100 mM abscisic acid (ABA), 50 mM ET, and 100 mM JA were applied to the surface of the solid MS agar medium of the three-week-old seedlings. After 2 h in each treatment, sample seedlings were harvested and immediately frozen in liquid nitrogen until RT-qPCR.

Results

Characterization of *JcR1MYB1*

The full-length cDNA that encodes a putative R1-MYB TF, designated as *JcR1MYB1* (GenBank accession no. KF809956), was cloned via RACE. The 1283 bp full-length cDNA contained a 951 bp open reading frame with a

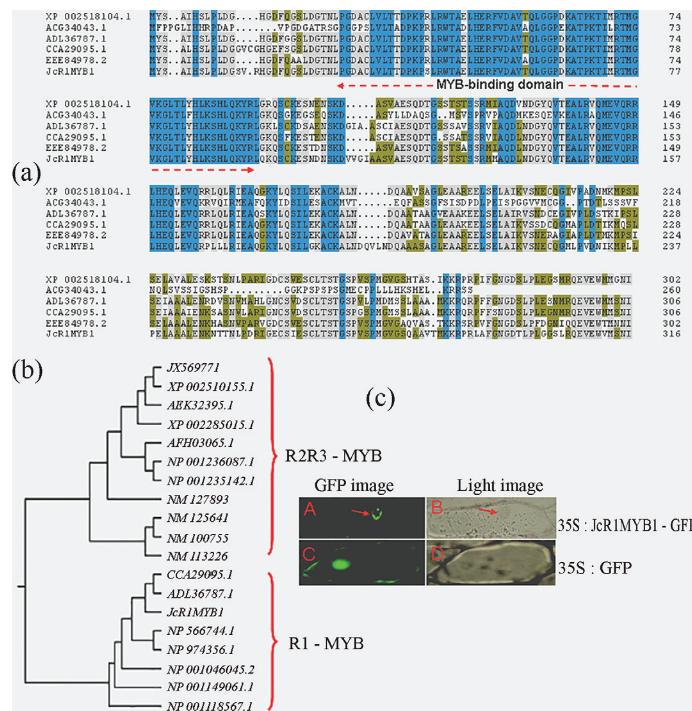

Figure 1 - Characterization of *JcR1MYB1*. (A) Amino acid sequence alignment of JcR1MYB1R1 and other single MYB-like domain proteins. Accession numbers are as follows: *Ricinus communis*, XP005518104.1; *Zea mays*, ACG34043.1; *Malus domestica*, ADL36787.1; *Rosa rugosa*, CCA29095.1; *Populus trichocarpa*, EEE84978.2. Identical amino acid residues are colored. (B) Phylogenetic analysis of JcR1MYB1. The phylogenic tree was constructed with the DNAMAN program using the deduced amino acid sequence. Accession numbers are as follows: *Arabidopsis thaliana,* NP_566744.1; NP_974356.1; NP_001118567.1; NM_127893;NM_100755; NM_113226; *Oryza sativa*, NP_001046045.2; *Zea mays*, NP_001149061.1; *Jatropha curcas*, JX569771.1; *Ricinus communis*,XP_002510155.1; *Citrus sinensis*, AEK32395.1; *Vitis vinifera*, XP_002285015.1; *Epimedium sagittatum*, AFH03065.1; *Glycine max*, NP_001238087.1; NP_001235142.1; *Malus domestica*, ADL36787.1; *Rosa rugosa*, CCA29095.1. (C) Nuclear localization of the JcR1MYB1 -GFP fusion protein in onion epidermal cells. The GFP fluorescence images (GFP image and light image) of onion epidermal cells were compared to show the subcellular localization of 35S-JcR1MYB1-GFP (A) and of 35S-GFP (C).

192 bp 3' UTR downstream from the stop codon and a 137 bp 5' UTR upstream of the start codon. BLAST analysis showed that the *JcR1MYB1* DNA sequence was identical to that of the corresponding full-length cDNA, suggesting that no intron exists in *JcR1MYB1*.

A search for potential motifs identified the conserved, 56-amino acid-long DNA-binding domain, which consists of a single helix-turn-helix module that usually occurs in R1-MYBs. The sequence of the R1 DNA-binding domain of JcR1MYB1 showed 82% to 98% identity with R1MYB from other plants. However, these proteins shared minimal homology outside this domain (Figure 1A). JcR1MYB1 amino acid sequences with R1-MYB and R2R3-MYB from different species were compared, and the result of the phylogenetic tree analysis revealed that JcR1MYB was clearly clustered with R1-MYB of other plants (Figure 1B), which implies that *JcR1MYB1* may code for a typical R1-MYB protein. The subcellular localization of JcR1MYB1 indicates that JcR1MYB1 localizes in the nucleus (Figure 1C), and that the properties of the JcR1MYB1 define it as a TF.

Differential *JcR1MYB1* expression in different organs

Total RNA was isolated from the leaves, stems, and roots of *J. curcas* seedlings and was subjected to RT-qPCR to analyze the *JcR1MYB1* transcription pattern. *JcR1MYB1* was shown to be constitutively expressed in all tested tissues at different levels. The highest transcript levels were seen in stems, followed by the roots and leaves (Figure 2A).

Effects of plant hormones and stress on *JcR1MYB1* expression

The *JcR1MYB1* mRNA accumulation profile was determined under various abiotic stresses by RT-qPCR to determine whether *JcR1MYB1* expression is regulated by multiple factors. Four-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. *JcR1MYB1* expression increased within 0.5 h, reached its maximum at 2 h, and subsequently decreased under ethylene (ET), abscisic acid (ABA), and jasmonic acid (JA) treatment (Figure 2B). *JcR1MYB1* expression increased within 0.5 h under NaCl, PEG, and cold treatments. However under the NaCl treatment, *JcR1MYB1* expression increased within 2 h and then subsequently decreased (Figure 2C).

Phenotypes of transgenic plants under PEG and salt stresses

JcR1MYB1 was overexpressed under the control of the CaMV 35S promoter in tobacco plants. The transgenic tobacco plants that harbored the *JcR1MYB1* gene were selected using RT-PCR (Figure 3A). PCR detection of the T_0-T_2 transgenic lines showed that *JcR1MYB1* was stably inherited. The transcription of *JcR1MYB1* in T_2 transgenic lines was detected by RT-PCR. *JcR1MYB1*was constitutively expressed in all transgenic lines, and these had higher transcript levels compared with WT (transformant host) plants (data not shown).

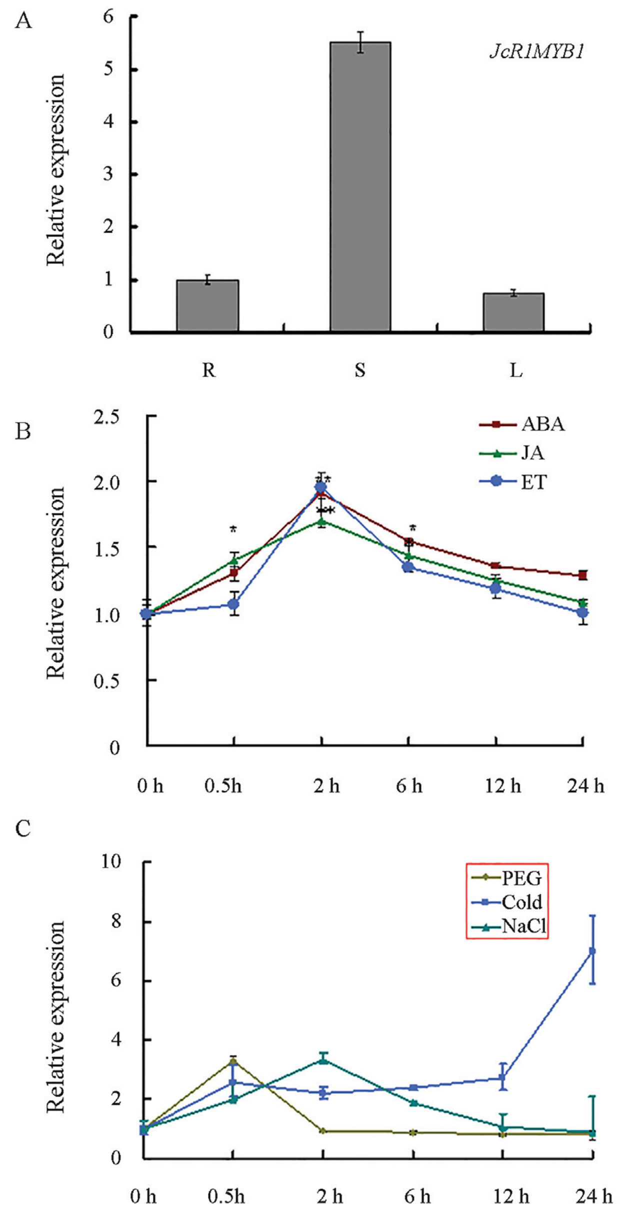

Figure 2 - Expression of *JcR1MYB1*. (A) *JcR1MYB1* expression in the roots (R), stems (S), and leaves (L) of *J. curcas* seedlings. Relative transcript abundances of *JcR1MYB1* were examined using RT-qPCR. Gene-specific primers for *JcR1MYB1* and *JcACT* (internal control) were used. Each point represents the mean of three replicates. Bars indicate standard errors (± SE). The Y-axis is the scale of the relative transcript abundance level. The X-axis refers to the tissues of *J. curcas.* (B) *JcR1MYB1* transcription patterns induced by JA, ET, and ABA treatments. (C) *JcR1MYB1* transcription patterns induced by PEG, cold, and NaCl treatments. Relative transcript abundances of *JcR1MYB1* were examined by RT-qPCR. Gene-specific primers for *JcR1MYB1* and *JcACT* (internal control) were used. Each point represents the mean of three replicates. Bars indicate standard errors (± SE). The Y-axis refers to the scale of the relative transcript abundance level. The X-axis shows the time elapsed after the treatment.

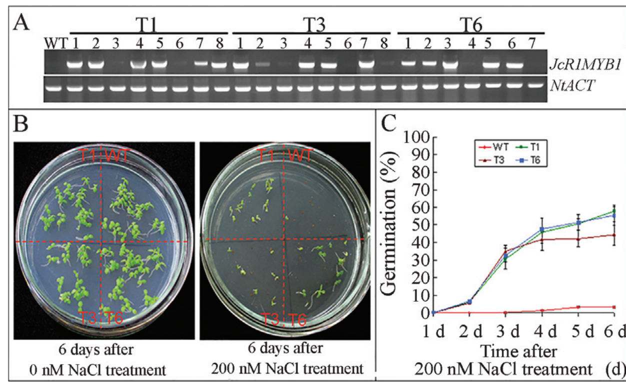

Figure 3 - Characterization of transgenic tobacco plants. (A) Molecular identification of *JcR1MYB1* in T_2 transgenic plants (T1, T3, and T6 lines) by RT-PCR. (B) The seed germination of WT and transgenic plants (T1, T3, and T6 lines) on MS containing 200 mM NaCl. (C) The germination rate of WT and transgenic plants (T1, T3, and T6 lines) on MS containing 200 mM NaCl.

The seeds and leaf discs from the T_2 transgenic tobacco lines were subjected to salt stress to evaluate the response of the *JcR1MYB1* transgenic plants. The seed germination rate of *JcR1MYB1* transgenic plants was significantly higher than that of WT on MS containing 200 mM NaCl (Figure 3B). After treatment, the leaf discs from *JcR1MYB1* transgenic plants exhibited enhanced salt tolerance relative to the WT (Figure 4A). Concomitantly, alterations in chlorophyll content and ion leakage of the leaves under NaCl treatment were also evaluated as reliable indices of photosynthetis and cell membrane damage under NaCl treatment. As shown in Figure 4B, 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 (Figure 4C). These results indicate that *JcR1MYB1* over-expression enhanced tolerance to salt stress. *JcR1MYB1* expression in the

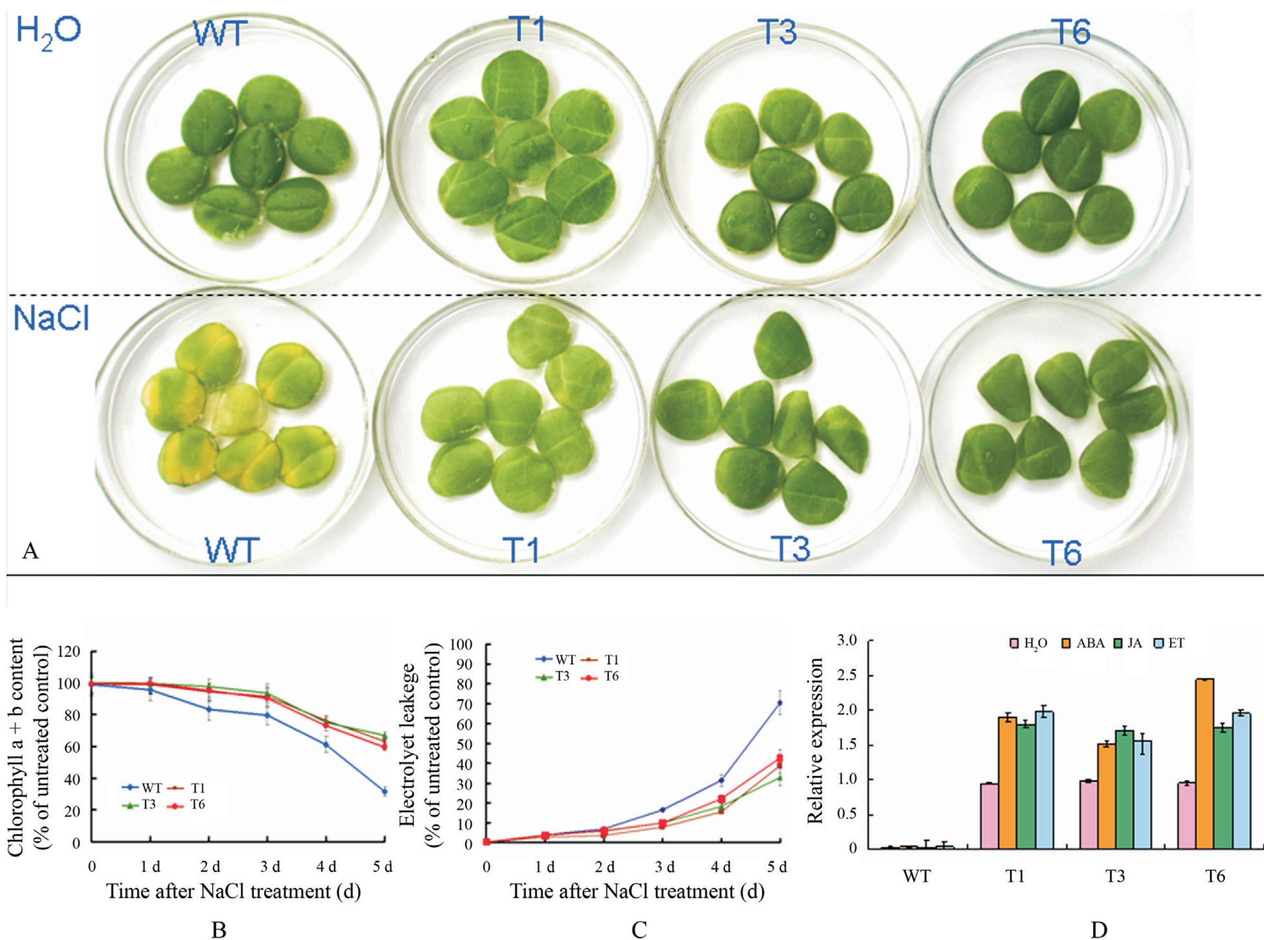

Figure 4 - *JcR1MYB1* transgenic tobacco phenotypes in response to salt. (A) Phenotype of leaf discs from WT and transgenic plants (T1, T3, and T6 lines) after treatment with 200 mM NaCl for 5 d. Detached leaves from WT controls were treated with water under the same conditions. (B) Chlorophyll content of leaf discs from WT and transgenic plants (T1, T3, and T3 lines) after treatment with 200 mM NaCl for 5 d. Error bars show standard deviations for three independent replicates. (C) Electrolyte leakage of leaf discs from WT and transgenic plants (T1, T3, and T3 lines) after treatment with 200 mM NaCl for 5 d. Error bars show standard deviations for three independent replicates. (D). Expression of *JcR1MYB1* in transgenic tobacco. 100 mM ABA, 50 mM ET, and 100 mM JA was applied to the surface of solid MS agar medium of the 3 wk-old seedlings. After 2 h in each treatment, sample seedlings were harvested. Relative transcript abundances of *JcR1MYB1* in transgenic tobacco were examined using RT-qPCR. Gene-specific primers for *JcR1MYB1* and *NtACT* (internal control) were used. Each point represents the mean of three replicates. Bars indicate standard errors of the mean (± SEM). The Y-axis refers to the scale of the relative transcript abundance level. The X-axis denotes the WT and transgenic plants (T1, T3, and T6 lines).

three transgenic lines is regulated by ET, ABA, and JA treatment (Figure 4D). *JcR1MYB1* transgenic tobacco plants affect the signaling pathways related to these three hormones.

Discussion

Compared with R2R3-type MYB TFs, reports on functional studies of single MYB-like domain TFs in plants (Du *et al.*, 2013) are limited. Growing evidence suggests that R-MYB TFs serve vital functions in chromosomal structural maintenance and light cycle response in plants. In Arabidopsis, several R1-MYB proteins, such as EPR1 (Kuno *et al.*, 2003), LHY (Schaffer *et al.*, 1998), and CCA1 (Wang and Tobin, 1998), have been isolated and confirmed as circadian oscillators involved in developmental modulation in plants. In maize, R1-MYB affects developing cells in the root or shoot apex, as well as the gibberellin hormone balance (Klinge *et al.*, 1997). The R1-MYB protein was also reported to participate in the stress response process (Cheng *et al.*, 2013). TFs are rapidly induced during the early phases of environmental stress conditions. Functional roles for single MYB-like domain TFs, such as CCA1, have been identified in light-related and other developmental processes (Wang *et al.*, 1997; Zhu *et al.*, 2004). Nevertheless, other R1-type MYB TFs, such as GmMYB117 in soybean and OsMYBS3 in rice, are enhanced by abiotic and ABA stresses (Liao *et al.*, 2008), as well as cold stress (Su *et al.*, 2010). In this study, we showed that the *JcR1MYB1* transcript levels were significantly up-regulated by PEG, NaCl, and cold treatments, and also in plants treated with ABA and JA. These results suggest that the single MYB-like domain TF JcR1MYB1 is involved in regulating these types of stress responses.

MYB TFs affect stress tolerance in plants. For example, AtMYB2 is induced by dehydration and ABA treatment. Over-expression of AtMYB2 results in increased sensitivity to ABA (Abe *et al.*, 2003). AtMYB44 transgenic plants exhibit enhanced drought tolerance compared with WT plants (Jung *et al.*, 2008). Ectopic expression of GmMYB177 confers salt and freezing tolerance in *Arabidopsis* (Liao *et al.*, 2008). AtMYB41 is expressed in response to drought and salt treatment in an ABA-dependent manner. Furthermore, AtMYB41 has been shown to negatively regulate salt-induced genes, such as *AtDREB2*a and *AtNCED3* (Lippold *et al.*, 2009). Over-expression of *StMYB1R-1* in potato plants improved plant tolerance to drought stress (Shin *et al.*, 2011). In this work, *JcR1MYB1* over-expression improved salt stress tolerance of transgenic tobacco, as demonstrated by alterations in chlorophyll content and ion leakage of the leaves in transgenic plants compared with WT. *JcR1MYB1* transgenic plants also exhibited salt tolerance, as observed by comparing the seed germination rates of WT and transgenic plants in MS medium under high saline conditions. These results indicate that *JcR1MYB1* contributes to salt tolerance in physic nut plants.

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