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Research article

Overexpression of a stress-responsive MYB transcription factor of *Poncirus trifoliata* confers enhanced dehydration tolerance and increases polyamine biosynthesis

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ABSTRACT

MYBs are an important family of transcription factors that play significant roles in plant development and stress response in plants. However, knowledge concerning the functions of MYBs in the non-model plants and the target genes is still limited. In this study, we isolated a stress-responsive R2R3-type MYB gene from trifoliate orange (*Poncirus trifoliata* (L.) Raf.), designated as *PtsrMYB*. *PtsrMYB* shares the highest degree of identity with *AtMYB109*. Subcellular localization using onion epidermal cells indicates that PtsrMYB is localized in the nucleus. Transcript levels of *PtsrMYB* were up-regulated by abiotic stresses such as dehydration, salt, cold and ABA treatment. Overexpression of *PtsrMYB* in tobacco confers enhanced dehydration tolerance, as indicated by less water loss, lower levels of malondialdehyde and reactive oxygen species. The transgenic tobacco lines displayed higher mRNA levels of two arginine decarboxylase (*ADC*) genes before and after dehydration treatment when compared with the wild type, concurrent with the greater levels of polyamines. Several MYB-recognizing *cis*-acting elements exist on the promoters of *PtADC* gene. Yeast one-hybrid assay demonstrated that *PtsrMYB* predominantly interact with two regions of the promoter, indicating the *PtADC* may be a target gene of *PtsrMYB*. Take together, *PtsrMYB* plays a positive role in dehydration tolerance, which may be, at least in part, due to the modulation of polyamine synthesis by regulating the *ADC* gene.

1. Introduction

Transcription factors (TFs), the significant regulators of diverse biological processes, account for a great proportion of the plant genes. More than 1600 TFs representing nearly 6% of the estimated total number of genes have been identified in the genome of Arabidopsis (*Arabidopsis thaliana*), a well-characterized model plant (Riechmann et al., 2000; Gong et al., 2004). The plants contain a wide range of different TFs, among which MYBs constitute one of the largest TF families, representing an estimated 9% of the total TFs. The MYB proteins are defined by the presence of a conserved MYB domain, located at the N terminus, consisting of 50–53 amino acids that are associated with DNA binding. Based on the number of imperfect adjacent repeats (R1, R2, R3) in the MYB domain, the MYB proteins are primarily classified into three major subgroups, R1R2R3-type MYB (MYB3R, three repeats), R2R3-type MYB (two repeats), and MYB1R or MYB-related (one repeat). The majority of plant MYB genes belong to the R2R3 types, as justified by the existence of 126 and 109 R2R3 MYB proteins in *Arabidopsis* and rice, respectively (Chen et al., 2006). Each of the R1, R2 and R3 repeats contain three helices forming a helix-turn-helix (HTH) motif. The R2R3 is a domain related to sequence-specific DNA binding, while R1 appears to lose interaction with DNA (Ogata et al., 1994, 1995). A large number of studies have provided extensive data revealing that plant MYBs play central roles in an array of physiological and biological processes, such as primary and secondary metabolism (Mellway et al., 2009; Czemmel et al., 2009; Wang et al., 2010; Spitzer-Rimon et al., 2012; Craven-Bartle et al., 2013; Li et al., 2013), cell patterning and tissue differentiation (Mu et al., 2009; Volpe et al., 2013), organ growth and development (Ryu et al., 2005; Müller et al., 2006), cell cycle progression (Mu et al., 2009), stomatal movement (Cominelli et al., 2005; Liang et al., 2005), and hormonal signaling (Seo et al., 2009; Shim et al., 2013). In addition, emerging evidences demonstrate that the MYB genes are closely

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associated with plant responses to abiotic stresses (Agarwal et al., 2006; Dubos et al., 2010; Kim et al., 2013; Wang et al., 2014).

So far, increasing number of MYB genes in a plethora of plants have been identified and functionally elucidated to act as key regulators of responses to diverse abiotic stresses, such as drought, low temperature, salt, and phosphate starvation. The well-characterized MYBs pertinent to abiotic stress response include *AtMYB2*, *AtMYB44*, *AtMYB60*, *MYB96* of *Arabidopsis* (Jung et al., 2008; Seo et al., 2009; Oh et al., 2011; Guo et al., 2013; Baek et al., 2013), *OsMYB2*, *OsMYB3R-2*, and *MYBS3* of rice (Ma et al., 2009; Su et al., 2010; Yang et al., 2012). Involvement of these MYBs in abiotic stress tolerance is largely based on the following experimental evidence. First, transcript levels of the MYB genes were induced, to different extents, by various stresses. Second, overexpression of the MYBs can render the transgenic plants more tolerant to a single or multiple stresses, whereas stress hypersensitivity was observed in the relevant MYB mutants (Zhu et al., 2005).

MYB genes have been shown to function in abiotic stress tolerance by modulating the expression profiles of a large spectrum of stress-responsive genes, either regulatory or functional ones, which are considered as the potential target genes of the MYBs. For example, overexpression of *OsMYB2*, a rice MYB gene involved in salt, cold, and dehydration tolerance, led to up-regulation of genes encoding proline synthase and transporters and other stress-related genes, including *OsLEA3*, *OsRab16A*, and *OsDREB2A* (Yang et al., 2012). In another work, Ma et al. (2009) reported that MYB gene *OsMYB3R-2* took part in cold signaling pathway by means of regulating genes associated with the cell cycle and a putative DREB/CBF. *MYB96* of Arabidopsis has been shown to function in drought and freezing tolerance (Seo et al., 2009, 2011; Guo et al., 2013). Seo et al. (2011) demonstrated that the enhanced drought resistance of *MYB96* might be attributable to the transcriptional activation of cuticular wax biosynthesis through modulating the wax biosynthetic genes. Recently, *LTP3* (lipid transfer protein 3) was revealed to act as a target of MYB96 and accounted for the MYB96-mediated tolerance to freezing and drought stress (Guo et al., 2013).

It is conceivable that the MYBs may achieve their function in stress tolerance via regulating a variety of genes implicated in different metabolic pathways. Although a myriad of stress-responsive genes have been tested so far, the possibility that other candidate genes may be regulated by the MYBs is not fully ruled out. Therefore, it is necessary to examine how an MYB gene can influence the expression of genes involved in other metabolic pathways which have been less investigated. In the present study, we report the functional identification of a R2R3-type MYB gene from trifoliate orange (*Poncirus trifoliata* (L.) Raf.), designated as *PtsrMYB*, in dehydration tolerance. Meanwhile, the effects of *PtsrMYB* overexpression on endogenous polyamine levels and expression of two arginine decarboxylase (ADC) genes, *NtADC1* and *NtADC2*, were investigated. Our data show that overexpression of *PtsrMYB* conferred enhanced dehydration tolerance, up-regulated the expression of *ADC* genes and elevated free polyamine accumulation. Moreover, we also present data indicating that *PtsrMYB* can interact with the promoter of ADC gene of trifoliate orange (*PtADC*), suggesting that *PtADC* might be a potential target gene of *PtsrMYB*.

2. Materials and methods

2.1. Plant material and stress treatments

Tobacco (*Nicotiana tabacum*) were sown on MS medium (Murashige and Skoog, 1962) and incubated at 25 °C in a growth chamber under a 16/8-h light/dark regime. One-month-old trifoliate orange seedlings were used for treatments with abiotic stresses, including dehydration, low temperature, NaCl, and exogenous ABA treatment. For dehydration stress, the seedlings were put on dry filter papers for 0, 0.5, 1, 3, and 6 h at ambient environment (at about 25 °C). For salt treatment, the seedlings were placed in 200 mM NaCl solution for 0, 6, 24, 72, and 144 h. As for cold stress, the seedlings were incubated at 4 °C for 0, 6, 24, 72, and 144 h. ABA treatment was done by incubating the seedlings in 100 μM ABA solution for 0, 6, 12, 24, and 48 h. The leaves were harvested at the designated time points, immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. Gene isolation and sequence analysis

A pair of gene-specific primers (GSP1, Table 1) was used for RT-PCR to amplify *PtsrMYB*. Total RNA was extracted from the leaf samples using TRIZOL reagent (TaKaRa, Dalian, China), according to the manufacturer's instructions. After DNase I treatment, 1 μg of total RNA was used to synthesize first-strand cDNA by the RevertAid™ First Strand cDNA Synthesis Kit (TOYOBO, Japan). PCR reaction mixture, in a total volume of 20 μl, contained 200 ng of cDNA, 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U of *Taq* DNA polymerase (Fermentas, Lithuania) and 0.15 μM of each primer. PCR was performed in a thermocycler (ABI 9700, Applied Biosystems) with a programme consisting of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 56 °C, 2 min at 72 °C, and a 10-min extension at 72 °C. The product was purified, subcloned into pMD18-T vector (Takara, China) and sequenced (Sunny, Shanghai, China). Homology search was done in NCBI, while sequence alignments were conducted using Clustal X and Genedoc software. A phylogenetic tree was constructed using MAGA 5.2 software. Theoretical isoelectric point (*p*I) and molecular weight were predicted in an internet server, ExPASy (Expert Protein Analysis System, http://www.expasy.org/tools).

2.3. Quantitative real time RT-PCR analysis

Quantitative real time RT-PCR (qRT-PCR) was used for analyzing gene expression of *PtsrMYB*, *NtADC1* and *NtADC2*. Total RNA was extracted from the tissues, and reverse transcribed into cDNA as mentioned above. The PCR solution (10 μl) contained 5 μl of 2 × SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara, Japan), 200 ng of cDNA, 0.25 μM of each primer (GSP2, Table 1). The qRT-PCR analysis was performed in a LightCycler 480 Real-time PCR system. An *actin* or ubiquitin gene was amplified as the internal control for *PtsrMYB* and *NtADCs (NtADC1* and *NtADC2)*, respectively, with the same reaction system using specific primers (Table 1). Each sample was assessed in four replicates, and the relative expression levels were analyzed by LightCycler 480 Software 1.5. Expression level at the first time point or wild type was set to 1 and those of other time points or the transgenic plants were accordingly normalized. When the ratios are lower than 1 (i.e. genes repressed), the inverse of the ratio is calculated and shown as negative number (Ochoa-Alfaro et al., 2012).

2.4. Subcellular localization analysis

PCR amplification with primer GSP3 (Table 1) containing *Nco*I and *Spe*I restriction sites was carried out to get a partial open reading frame (ORF). The amplified fragment was cloned into pMD18-T and sequenced, followed by ligation to the N terminal of GFP in the binary vector pCAMBIA 1302 linearized with *Nco*I and *Spe*I. The PtsrMYB-GFP and the control GFP plasmid were separately bombarded into onion epidermal cells as mentioned by Chiu et al. (1996). After culture on MS medium for 16–24 h at 28 °C in