Biochemical and Biophysical Research Communications 394 (2010) 1018–1023

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# A single-repeat R3-MYB transcription factor *MYBC1* negatively regulates freezing tolerance in *Arabidopsis*

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ARTICLE INFO

*Article history:*
Received 9 March 2010
Available online 21 March 2010

*Keywords:*
R3-MYB
MYBC1
Transgenic plant
Mutant
Freezing
*Arabidopsis thaliana*

ABSTRACT

We had previously identified the *MYBC1* gene, which encodes a single-repeat R3-MYB protein, as a putative osmotic responding gene; however, no R3-MYB transcription factor has been reported to regulate osmotic stress tolerance. Thus, we sought to elucidate the function of *MYBC1* in response to osmotic stresses. Real-time RT-PCR analysis indicated that *MYBC1* expression responded to cold, dehydration, salinity and exogenous ABA at the transcript level. *mybc1* mutants exhibited an increased tolerance to freezing stress, whereas 35S::*MYBC1* transgenic plants exhibited decreased cold tolerance. Transcript levels of some cold-responsive genes, including *CBF*/*DREB* genes, *KIN1*, *ADC1*, *ADC2* and *ZAT12*, though, were not altered in the *mybc1* mutants or the 35S::*MYBC1* transgenic plants in response to cold stress, as compared to the wild type. Microarray analysis results that are publically available were investigated and found transcript level of *MYBC1* was not altered by overexpression of *CBF1*, *CBF2*, and *CBF3*, suggesting that *MYBC1* is not down regulated by these CBF family members. Together, these results suggested that *MYBC1*is capable of negatively regulating the freezing tolerance of *Arabidopsis* in the CBF-independent pathway. In transgenic *Arabidopsis* carrying an *MYBC1* promoter driven β-glucuronidase (GUS) construct, GUS activity was observed in all tissues and was relatively stronger in the vascular tissues. Fused *MYBC1* and GFP protein revealed that *MYBC1* was localized exclusively in the nuclear compartment.



## 1. Introduction

Low temperature is one of the more serious environmental stressors that elicits adverse effects on the growth and productivity of crops. Plants, being sessile, have evolved efficient mechanisms to sense and rapidly adapt to low temperature conditions. Many temperate plants have the potential to increase their freezing tolerance after a prior exposure to nonfreezing low temperatures, a process known as cold acclimation [1–3]. At the molecular level, expression of a variety of genes is induced in response to low temperature in order to facilitate plants ability to survive under chilling and freezing stress [4,5]. The proteins encoded by these genes function not only in stress tolerance but also in downstream regulation of gene expression and signal transduction in stress response pathways [6,7].

The promoters of many of these cold-responsive genes are characterized by a 9-bp conserved sequence, TACCGACAT; named as the DRE/CRT/LTRE (dehydration responsive element/C-repeat/low temperature responsive element). This sequence comprises a *cis* element necessary and sufficient for gene transcription under cold stress conditions [8,9]. *CBF*/*DREB1* (C-repeat Binding Factor/DRE Binding protein 1) and *DREB2* specifically bind to the DRE/CRT *cis* element through a conserved DNA-binding domain, whereby they activate the transcription of cold-response genes. The CBF pathway is a central component of cold response. Several studies have shown that ectopic overexpression of some CBFs was able to activate the expression of target genes and enhance tolerance of transgenic plants against freezing, high salt, or dehydration [10–12]. In addition to the CBF pathway, some recent studies have revealed the presence of parallel pathways associated with cold acclimation [13,14] and defined some important components mediating cold tolerance through such CBF-independent pathways [15,16].

The phytohormone abscisic acid (ABA) has been shown to be involved in mediating responses to a number of environmental stresses [17]. Exogenous application of ABA induced a number of genes that are known to respond to dehydration and cold stress [18]. ABA was able to induce the expression of CBF genes and activate the expression of cold-regulated genes via the CRT promoter element [19]. Recently, *ADC1* and *ADC2*, key genes in the biosynthesis of the polyamine putrescine, were shown to mediate cold acclimation and freezing tolerance by altering ABA levels in *Arabidopsis* [20]. Taken together, these observations have contributed to the hypothesis that ABA may play a role in low temperature signaling.

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.03.114

In our previous study, we had examined the *cis*-regulatory motifs known to regulate gene expression in conditions of osmotic stress and we constructed an artificial neural network model in order to identify other functionally relevant genes involved in the same process. *MYBC1* was identified as a putative osmotic responding gene [21], and selected for further study. The *MYBC1* gene is known to encode a single-repeat R3-MYB protein; yet, to date, no R3-MYB transcription factor has been reported to regulate osmotic stress tolerance. Thus, we sought to elucidate the functions of *MYBC1* under osmotic stress conditions in this study. We generated transgenic plants that ectopically expressed the *MYBC1* gene, and identified a homozygous T-DNA mutant that was subsequently used to study the effects of *MYBC1*. The transcript levels of cold-response genes were examined in the *mybc1* mutants and the 35S::*MYBC1* transgenic plants. Furthermore, the publically available microarray data was searched to augment our analysis of the role of *MYBC1*.

## 2. Materials and methods

### 2.1. Plant materials and treatments

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used throughout this study. Seeds of the *mybc1* T-DNA insertion line (SALK_072083) were obtained from The European Arabidopsis Stock Centre (NASC). Plants were grown in growth bottles on filter-paper-beds soaked in one-half strength Murashige and Skoog liquid medium, and maintained at 22–24 °C with 60% relative humidity under long-day conditions (16-h light/8-h dark cycle).

Stress treatments were applied to 2-week-old seedlings. Rosette leaf samples were taken as three biological replicates at 0 min, 30 min, 3 h, and 6 h after treatment. Stress applications were carried out as described below. Salt/ABA treatment: the liquid medium was removed from the growth bottle and replaced with fresh Murashige and Skoog liquid medium (one-half strength) supplemented with 150 mM NaCl/50 μM ABA. Plants were further cultivated in a standard phytochamber until harvest. Cold treatment: 2-week-old seedlings were transferred from growth bottles to new filter-paper-beds that had been pre-incubated at 4 °C. The seedlings were, then, incubated at 4 °C under continuous light until harvest. Dehydration treatment: 2-week-old seedlings were removed from the growth bottles and exposed to a stream of air in a sterile bench for 15 min in order to cause desiccation (approximately 40% loss of fresh weight). The seedlings were immediately transferred back to the growth bottles, and plants were returned for further cultivation in a standard phytochamber until harvest.

### 2.2. Quantitative real-time PCR

Total RNA was isolated using an RNeasy plant mini-kit (Qiagen, Germany). The isolated RNA was then subjected to reverse transcription by using the SuperScript™ III Reverse Transcriptase kit. Quantitative real-time PCR was performed on each cDNA sample with the SYBR Green Master Mix on an ABI 7500 sequence detection system (Applied Biosystems, USA). The measured $C_t$ values were converted to relative copy-numbers using the $\Delta\Delta C_t$ method. Amplification of *Actin2* was used as an internal control to normalize all data. Primers used were *MYBC1*(At2g40970)-F 5′-AAGGCGGGAACGGTAAC-3′ and *MYBC1*-R 5′-CTCTAATGGCGGCATCAAG-3′; *Actin2* (At3g18780)-F 5′-TTACCCGATGGGCAAGTC-3′ and *Actin2*-R 5′-GCTCATACGGTCAGCGATAC-3′; CBF1-F 5′-GTCGCTGCATTAGCCCTC-3′ and CBF1-R 5′-TGATTCGTGGTCGTCGTATC-3′; CBF3-F 5′-AACTTGCGCTAAGGACATCC-3′ and CBF3-R 5′-CTCGGCATCTCAAACATCG-3′; RD29A-F 5′-GGCGTAACAGGTAAACCTAGAG-3′ and RD29A-R 5′-TCCGATGTAAACGTCGTCC-3′; COR47-F 5′-CGTTGATTGCATTTGATCC-3′ and COR47-R 5′-TCACCAAACGTAAGAGTGAGTAT-3′. *ADC1* and *ADC2* primers were described previously [22]. *CBF2* primers were described previously [23] and *NCED3* [19]. To enable statistical analysis, three fully independent biological replicates were obtained and subjected to real-time PCR run in triplicate. Raw data were standardized as described previously [24].

### 2.3. Histochemical GUS assay

To investigate *MYBC1* gene expression, a 2939 bp fragment upstream from the translation initiation codon was amplified by PCR from genomic DNA using the following primers: F: 5′-TCTAGCTGCGGTGATAGTGACG-3′ and R: 5′-CTTGTCCCACATCAGGATTCAG-3′. The PCR product was cloned into the pCAMBIA 3301 vector at the PstI and NcoI sites upstream from the *GUS* gene. The resulting construct was then transformed into *Arabidopsis*. GUS staining was performed as described by previously [25]. Plants were then washed in a graded ethanol series (30%, 50%, 70%, and 100% [v/v]) for 30 min in each solution at 60 °C. Tissues were visualized using a stereomicroscope (Olympus, Japan).

### 2.4. Subcellular localization

For subcellular localization, the cDNA fragment containing the *MYBC1* coding region without stop codon was amplified by PCR using primers: F: 5′- GTCACTATCACCGCAGCTAGAATG-3′ and R: 5′- AGATCTACCATATAATTTCCGGCAG-3′. The PCR product was then inserted downstream from the CaMV 35S promoter and in-frame with the 5′ terminus of the GFP gene in the pCAMBIA1302 vector to obtain the *MYBC1*:GFP fusion plasmid. pCAMBIA1302 or pCAMBIA1302-*MYBC1*:GFP were introduced into the *Agrobacterium tumefaciens* strain EHA105. The leaves of 4-week-old *Nicotiana benthamiana* plants were infiltrated with *A. tumefaciens* as described previously [26]. Three days after infiltration, *MYBC1*:GFP expression was detected in the leaves by visualization under confocal laser scanning microscope (SP5; Leica, Germany).

### 2.5. Genotyping and RT-PCR identification of mybc1 mutants

PCR tests were used for genotyping to identify homozygous T-DNA inserted plants. Genotyping was performed by using the gene-specific primer pair, F: 5′-TCACTATCACCGCAGCTAGAAT-3′ and R: 5′-TTTCATTAATTTCCGGCAGG-3′ and gene-specific primer and T-DNA-specific primer pair F: 5′-TACCAAAAGATCCACCTCG-3′ and R: 5′-TGTTATTAAGTTGTCTAAGCGTC-3′. After confirmation of homozygous T-DNA insertion, gene knock-out was confirmed by RT-PCR of *MYBC1* with gene-specific primers.

### 2.6. MYBC1 overexpression

*MYBC1* was PCR amplified with 5′-TCACTATCACCGCAGCTAGAAT-3′ and 5′-TTTCATTAATTTCCGGCAGG-3′ and cloned into the NcoI/SpeI sites of the pFGC1008 vector. *MYBC1* expression was driven by a CaMV 35S promoter. The resulting vector was mobilized into the *A. tumefaciens* strain LBA4404. Transformation of *Arabidopsis* plants was carried out by floral-dipping method. The $T_1$ transgenic plants were selected on Murashige and Skoog medium containing 30 mg/L hygromycin. Seeds from each $T_1$ plant were individually collected. Selected $T_2$ plants were propagated, and homozygous lines of overexpressing plants were confirmed by RT-PCR analysis.

### 2.7. Freezing tolerance assays

Ion-leakage test after freezing was carried out essentially as described previously [27]. The ion leakage experiment was repeated