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Research article

Overexpression of *Chrysanthemum lavandulifolium ClCBF1* in *Chrysanthemum morifolium* 'White Snow' improves the level of salinity and drought tolerance

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ABSTRACT

This paper reports the first study on plant *CBF* transcription factors (TF) in salt and drought stress responses in *Chrysanthemum lavandulifolium*. A *CBF* homolog gene, named as *ClCBF1*, from *C. lavandulifolium* was isolated using rapid amplification of cDNA ends (RACE). The deduced peptide is comprised of 210 amino acids (AA) containing an AP2 structural domain characteristic of the AP2 gene family. Quantitative real-time PCR revealed that *ClCBF1* gene exhibit differential expression patterns across root, leaf and stem tissues, and it was strongly induced under salt and drought treatments of *C. lavandulifolium*. Overexpression of *ClCBF1* in *C. morifolium* 'White Snow' resulted in stronger tolerance to salt and drought stresses. The *ClCBF1* expression level, enzymatic activities of superoxide dismutase and peroxidase, and contents of proline and soluble proteins were enhanced in these transgenic lines, they were repressed in the antisense transgenic lines under the same stress conditions. Results indicate that *ClCBF1* represents a promising candidate gene in improving abiotic stress tolerance among ornamental plants.

1. Introduction

During the growth season, plants encounter biotic and abiotic stresses such as low temperature, drought, high salinity and pests and diseases. These suboptimal conditions can directly influence plant growth and development. A generic signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates and reactive oxygen species [ROS]). These secondary messengers under the influence of intracellular Ca^{2+} levels, initiate the protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors (TFs) controlling expression of specific sets of genes (Xiong et al., 2002). Some of these TFs act as master switches and trigger simultaneous expression of a large number of stress-response genes that contribute to the stress-tolerance phenotype.

C-repeat binding factor (*CBF*) is a class of plant specific TFs. Under stress condition, *CBF* TFs can activate downstream CRT/DRE (C-repeat dehydration -responsive cis-element) containing cold-regulated (COR) gene transcription thus increasing plant stress resistance (Morran et al., 2011). In 1997, Stockinger used yeast one hybrid system and isolated a cDNA from *Arabidopsis thaliana*. The protein can recognize and specifically bind to CRT/DRE, it was named *CBF1* (Stockinger et al., 1997).

When plant cells sense drought, salinity and chilling conditions, expression of several TFs upstream *CBF* are activated. These TFs bind to the ICE box in the promoter upstream of *CBF* genes, and thus the stress signals are transmitted to these *CBF* TFs which by regulating the interaction with the DRE/CRT cis-elements activate stress gene expression. Eventually the induced gene expression leads to various physiological and biochemical stress responsive reactions. The binding of CBF protein with CRT/DRE elements downstream of COR promoters induces expression of a large number of resistance genes resulting in enhanced stress resistance (Jaglo et al., 2001). An increasing number of studies have shown that under drought, salinity and chilling conditions, the promoters of these stress responsive genes would activate expression of this class of resistance genes thus increasing plant resistance to the respective stress factors (Chen et al., 2007; Shan et al., 2014; Zhou et al., 2011). Tomato plants overexpressing the *CBF1* gene showed a much stronger cold tolerance than the nontransgenic wild type plants (Hsieh et al., 2002). Furthermore, the same group discovered that signal transduction pathway in these transgenic plants is similar to the Arabidopsis *CBF1* gene. The same study also found that these transgenic

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plants contained a smaller amount of free radicals but a high content of proline and they showed stronger drought resistance and chilling tolerance. This study revealed the potential of using *CBF1* gene in improving plant resistance to abiotic stresses.

Chrysanthemum lavandulifolium is a diploid species in the Asteraceae family, it is a relative of cultivated chrysanthemum (Dai and Wang, 2002). The species has a simple genome structure. In recent years, it has been used as an elite variety for establishing green vegetation on saline-alkali land and therefore it has been widely used in urban landscaping. Plant stress resistance depends on the functional coordination of multiple elements. The *CBF* TFs can regulate expression of multiple genes to increase plant stress resistance. In this study we have cloned a *ClCBF1* gene from *C. lavandulifolium* which has an important role in increasing plant stress resistance. Furthermore, *ClCBF1* transgenic plants of *C. morifolium* 'White Snow' were generated for functional analysis of the TF. We also investigated the expression mode and function of *ClCBF1* in *C. lavandulifolium.* Such information of CBF signaling pathways in *C. lavandulifolium* provided the bases for breeding stress resistant chrysanthemum varieties.

2. Materials and methods

2.1. Plant materials and bacterial strains

Chrysanthemum lavandulifolium seeds were harvested in a nursery at Beijing Foresty University. Seeds were sown in Plant Cultivation Room in the College of Landscape Architecture in Northeast Forestry University. When *C. lavandulifolium* grew to the 8–10 expanded leaf stage, leaves were harvested to extract RNA. *E. coli* strain DH5α and pBI121 vector were used for gene cloning and expression vector construction. *Agrobacterium tumefacien* strain GV3101 was used for transformation of *C. morifolium* 'White Snow'.

2.2. Isolation of ClCBF1 gene

Total leaf RNA was extracted using the Trizol method with minor modification (Huang et al., 2009). The first strand cDNA synthesis was performed via reverse transcription using M-MLV reverse transcirptase following the manufactor's instruction (Promega, USA). Sequences of cDNA fragments previously obtained using RT-PCR of *C. lavandulifolium* were used to design the 3′ gene specific primers (Table 1) which were used in 3′ RACE amplification. The PCR products were separated on a 1% agarose gel. The gel-purified DNA fragments were ligated into pGEM-T vector which were transferred into *E. coli* DH5α competent cells followed by blue-white clonoy screening for the *ClCBF1* clones. Positive clones were sequenced in Beijing Orcrea Technology Limited. DNA fragments were assembled into a full-length gene sequence which were used to design PCR primers (Table 1). PCR was perforemd using the PrimeSTAR® GXL DNA Polymerase (TaKaRa) to amplify gene sequences which were validated using sequence analysis.

Table 1
Primer names and sequences used in this study.

Primer name	Primer sequences(5′-3′)	Primer usage
ClCBF1-F	ACTCCGTGTCCATAGCGTTTC	RT-PCR
ClCBF1-R	CTAGCGGCCAACATCATTTC	RT-PCR
ClCBF1-3-F1	TTATTGTGGGACGACCTACTTTGT	3′RACE
ClCBF1-3-F2	ATCTAGGATATGGCTTGGGACAT	3′RACE
ClCBF1-full-F	CTGTATGCTCCTTCTTCTCACTAC	Full length gene cloning
ClCBF1-ful-R	TTCCTAACTCCTAAAGTAACCAAT	Full length gene cloning
ClCBF1-*Bam*HI-F	TAGGATCCATGGACATGAACAA	Construction of expression vector
ClCBF1-*Sal*I-R	GCGTCGACGTAACTCCATAACG	Construction of expression vector
CmEF1α-F	TTTTGGTATCTGGTCCTGGAG	ACTIN
CmEF1α-R	CCATTCAAGCGACAGACTCA	ACTIN
qRT-*ClCBF1*-F	CCAGTGTACCGTGGAGTACG	qRT-PCR
qRT-*ClCBF1*-R	CGCAGCCTTCCGAATATCCT	qRT-PCR

2.3. Sequence alignment and phylogenetic tree analysis

The DNAman (V.6.0) program was used to determine the similarity of *ClCBF1* peptide sequence with other *CBFs* that have been identified in other plants. The structural domain of *ClCBF1* was determined using NCBI BlastX tool, and the dendrogram was constructed using the MEGA4.0.

2.4. Assay of CICBF1 expression in plant tissues under salinity and drought treatments

Root, stem and leaf tissues were harvested from healthy plants, and those grown under 200 mM NaCl and drought treatment conditions for 0 h, 1 h, 3 h, 6 h, 12 h and 24 h. Total RNA was extracted using the Trizol method, and reverse transcription was performed using the reverse transcription kit (TOYOBO, Japan). Quantitative real-time PCR was performed using SYBR Green (TOYOBO) kit following the manufactor's instruction. In a 20 μl reaction volume, 10 μl SYBR master mix, primers each 0.4 μl, and 0.5 ng of cDNA template were added. PCR reaction mixture was transferred into a 96 well plate (Light Cycler® 96) (Switzerland). The PCR program was 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 15 s and 72 °C for 10 s. Each sample had three technical replicates. The *CmEF1α*(KF305681) was used as an internal reference gene. Relative transcript abundances were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). The expression level of *ClCBF1* in untreated leaves at 0 h was set as 1, and ratio of other tissues and treatments was calculated accordingly.

2.5. Construction of transformation vector and genetic transformation of C. morifolium 'White Snow'

The gene specific primers of *ClCBF1-Bam*HI-F/*CICBF1-Sal*I-R were designed against the *ClCBF1* full length cDNA sequence after the addition of the enzymatic sites for *Bam*HI and *Sal*I (Table 1). PCR products were collected and ligated into PMD18-T vector to produce the construct PMD18-T-CBF1. The PMD18-T-CBF1 and PBI121 plasmids were treated by double enzymatic digestion using *Bam*HI and *Sac*I of plasmids, and then ligated with the PCR products to prepare the binary vector constructs containing the CaMV 35S promoter (PBI121-CBF1). The 35S:*ClCBF1* plasmid was transferred into *Agrobacterium* strain GV3101 using the Agrobacterium-mediated method and it was used for transformation of *C. morifolium* 'White Snow'.

2.6. Confirmation of transgenic plants

Putative transgenic plantlets of 'White Snow' were rooted in MS solid media supplemented with 0.5 mg L^{-1}NAA and 4 mg·L^{-1} kanamycin. Genomic DNA was extracted from leaves on wild type and the antibiotic-resistant plants using the Easy PureTM Plant Genomic DNA kit. Total RNA was extracted using the Trizol method, and cDNA was synthesized using a ReverTra Ace -α-® (TOYOBO, Japan). PCR and RT-PCR using the primer pair of *CICBF1-Bam*HI-F/*CICBF1-Sal*I-R were used to confirm transgenic events. For qRT-PCR analysis, SYBR® Premix Ex Taq™ II (TaKaRa) was used, to determine the *ClCBF1* expression in WT and transgenic plants using the primer pair of qRT-*CICBF1*-F/R. The primer pair of *CmEF1α*-F/R was used to amplify the reference gene *CmEF1a.* The transcript level of target genes (in triplicates) was calculated using the $2^{-\triangle\triangle CT}$ method.

2.7. Assessment of salinity and drought tolerance

After transplanting into pots (peat moss: vermiculite, 1:1), the wild type and transgenic plantlets were grown in a growth chamber, at