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Research article

Isolation and functional characterization of the *ShCBF1* gene encoding a CRT/DRE-binding factor from the wild tomato species *Solanum habrochaites*

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ABSTRACT

Plant growth and productivity are greatly affected by low ambient temperature. Complex cascades of gene expression in cold stress response are regulated by transcription factors. In this study, a cDNA clone, named *ShCBF1*, was isolated from *Solanum habrochaites* seedlings (a wild relative of cultivated tomato). It was classified as one of CBF family members based on multiple sequence alignment. The expression analysis confirmed that *ShCBF1* was induced by low temperature, high salinity and drought stress. Experiments of subcellular localization in tobacco leaf cells indicated that it was localized in nucleus. Transient expression assay using onion epidermal cells revealed that the ShCBF1 protein could function similarly to AtCBF1 in activating the expression of reporter genes with a CRT/DRE element in their promoter. Moreover, ectopic overexpression of *ShCBF1* in *Arabidopsis* enhanced freezing and high salinity tolerance of transgenic plants by improving the expression levels of some stress-responsive marker genes. Taken together, our results suggest that ShCBF1 behaves as a typical plant CBF transcription factor and might be involved in plant response to various environmental stresses.

1. Introduction

Plants as sessile organisms must be able to interpret and respond to unfavorable environmental conditions. Therefore, plants have developed unique mechanisms to cope with adverse environmental factors such as cold, drought and high salinity stresses. Cold stress accounts for significant reductions in the yields of many economically important crops whose agricultural distribution is limited by their maximum freezing tolerance capacity (Chinnusamy et al., 2007; Pino et al., 2008). Plants have adapted to respond to cold stress at the molecular and cellular levels as well as at the physiological and biochemical levels, thus enabling them to survive (Yamaguchi-Shinozaki and Shinozaki, 2006).

An important advance in the field of plant resistance to cold stress was the finding of the C-repeat (CRT)/dehydration responsive element (DRE) *cis*-acting DNA regulatory element. The element, which has a 5-bp core sequence of CCGAC, is present in the promoters of many cold and dehydration responsive genes (Stockinger et al., 1997; Thomashow, 1999; Baker et al., 1994). The cDNAs encoding CRT/DRE-binding proteins, CBF/DREB1 (C-repeat Binding Factor/DRE Binding protein 1), and DREB2, were isolated using yeast one-hybrid screening (Liu et al., 1998). CBF/DREB1 transcription factors could recognize the regulatory CRT/DRE element present in the promoters of many cold-inducible genes. However, the expression of the DREB1/CBF genes could be induced by cold, but not by dehydration and high-salinity stresses (Liu et al., 1998; Gilmour et al., 1998). By contrast, expression of the *DREB2* genes and one member of the DREB1 family, *CBF4*, are induced by dehydration and high-salinity stresses but not by cold stress (Liu et al., 1998; Nakashima et al., 2000; Haake, 2002).

In *Arabidopsis*, three major *CBF/DREB1* genes which are organized in tandem on chromosome 4 play a critical role in the regulation of many cold-stress related genes (Liu et al., 1998; Gilmour et al., 1998; Medina et al., 1999). Conserved signature motifs are present in the

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three CBF homologs. The CBF/DREB1 protein differs from the other DREB proteins by the presence of "signature sequences" (PKK/RPAGRxKFxETRHP and DSAWR) flanking the AP2 DNA-binding domain (Jaglo et al., 2001). In addition, PKK/RPAGRxKFxETRHP motif was proved to play a role not only in nuclear targeting of CBF proteins, but also in helping CBF1 proteins recognize and bind to the CRT/DRE element (Canella et al., 2010; Wang et al., 2005).

Constitutive overexpression of each CBF in *Arabidopsis* results in similar expression of CRT-containing target genes and increased freezing tolerance under control conditions (Stockinger et al., 1997; Gilmour et al., 2000; Kasuga et al., 1999; Gilmour et al., 2004; Jaglo-Ottosen et al., 1998). The negative regulation of CBF2 on the expression of CBF1 and CBF3 in *Arabidopsis* guarantees the proper induction of downstream genes and optimal responses to freezing and related stresses (Novillo, 2004). With the development of genomic technologies including methods for gene expression profiling, many downstream regulons of CBF transcription factors were identified and other cold regulatory pathways were proved to exist beyond the CBF cold response pathway (Fowler and Thomashow, 2002; Maruyama et al., 2004; Seki et al., 2002). The CBF regulatory network has been best understood in *Arabidopsis* and the positive role of *Arabidopsis* CBF/DREB1 genes in improving stress tolerance has promoted the isolation and functional characterization of the homologous genes from a wide variety of plants such as rice, *Brassica napus*, barley, cherry, and *Solanum lycopersicoides* (Jaglo et al., 2001; Dubouzet et al., 2003; Skinner et al., 2005; Owens et al., 2002; Zhang et al., 2013).

Wild relatives of the cultivated tomato (*Solanum lycopersicum* L.) are major sources of new genetic diversity for tomato improvement (Hanson et al., 2007). Wild species can adapt harsh environments and soil conditions and have rich stress resistance genes worth to explore. *Solanum habrochaites* (*S. habrochaites*, LA1033) is a self-incompatible, green-fruited accession well known to tomato breeders. Grumet et al. (1981) identified *S. habrochaites* (then, *Lycopersicon hirsutum*) as having particularly unusual properties. It can grow and develop well on poor-quality land, and it also can withstand extreme low temperature stress (Vidavsky and Czosnek, 1998). This highly unusual low temperature tolerance suggested that *S. habrochaites* may be a good source of genes that modify cold response pathway. We therefore initiated screens of *S. habrochaites* genome for genes that are responsible for the cold resistant property.

In this study, we isolated a full-length *CBF* gene from *S. habrochaites* treated with low temperature which was designated as *ShCBF1*. Also we investigated its expression pattern, subcellular localization and transcription activation ability. Moreover, the functions of transgenic *Arabidopsis* overexpressing *ShCBF1* under several types of stresses were evaluated.

2. Results

2.1. Phenotypic responses of *S. habrochaites* and cultivated tomato (CM) under cold stress

Wild relatives of the cultivated tomato (*S. lycopersicum*, Fig. 1B) are major sources of new genetic diversity for tomato improvement. *S. habrochaites* (Fig. 1A) is a wild tomato species that is found on the Western slopes of the Andes from Central Ecuador to Central Peru (http://solgenomics.net/organism/938/view).

Cultivated tomato is sensitive to low temperatures (~0–15 °C), but *S. habrochaites* is more cold tolerant than the cultivated tomato. To gain deeper insights into the mechanism underlying cold tolerance of *S. habrochaites*, we first evaluated the phenotypic responses of *S. habrochaites* and cultivated tomato seedlings under low temperature (4 °C). After 8 h of cold treatment, *S. habrochaites* seedlings showed strong tolerance to cold stress and no obvious external damage could be observed (Fig. 1C). However, CM exhibited wilting phenotypes, although not very severe, and was relatively hypersensitive to cold stress compared to *S. habrochaites* (Fig. 1D).

Leaves of *S. habrochaites* are interrupted imparipinnate covered with a mixture of uniseriate trichomes which are absent on the leaves of cultivated tomato (Fig. 1E and F). The significance of this difference and how it relates to the cold resistance are of interest.

2.2. Multiple sequence alignment and phylogenetic analysis of *ShCBF1* and other CBFs

A pair of degenerate primers was designed based on the conserved regions of *CBF1* nucleotide sequences from *Arabidopsis thaliana*, *Solanum tuberosum*, *S. lycopersicum*, and *Solanum commersonii*. A cDNA fragment was obtained using degenerate PCR and subsequently, the full length gene was obtained by RACE method and designated as *ShCBF1* (GenBank Accession No. GU129699). The full-length *ShCBF1* clone is 931 bp containing an open reading frame of 669 bp and encoding a putative protein of 222 amino acids with a predicted molecular mass of 24.6 kDa and an isoelectric point of 4.85. To investigate whether the *ShCBF1* possessed introns, we isolated its genomic sequence. Alignment of the *ShCBF1* cDNA with its genomic counterpart revealed that the *ShCBF1* gene does not have introns interrupting its ORF. As shown in Fig. 2A, the ShCBF1 protein is very similar to other CBFs with highly conserved AP2-DNA binding domain and CRT/DRE *cis*-element recognition region (also known as the nuclear localization signal) and other relatively conserved signature motifs marked with thick lines (Fig. 2A). However, the selected CBFs shared relatively low homology with each other

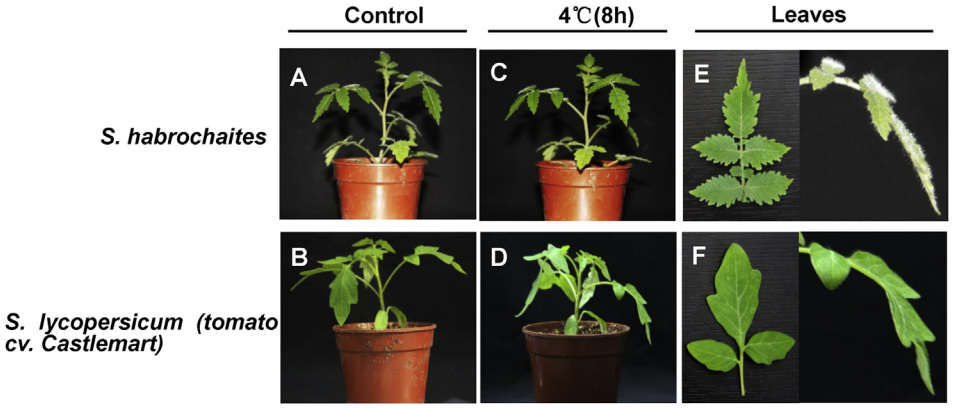

Fig. 1. Difference in cold tolerance between *S. habrochaites* and *S. lycopersicum* (CM). (A–D) Seedlings of *S. habrochaites* and CM in control (A, B), treated at 4 °C for 8 h (C, D). Four-week-old plants were used in this experiment. (E, F) Leaf characteristics of *S. habrochaites* (E) and CM (F).