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A novel cold-regulated gene from *Phlox subulata*, *PsCor413im1*, enhances low temperature tolerance in Arabidopsis

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ABSTRACT

Low temperature stress adversely affects plant growth, development, and crop productivity. Analysis of the function of genes in the response of plants to low temperature stress is essential for understanding the mechanism of chilling and freezing tolerance. In this study, *PsCor413im1*, a novel cold-regulated gene isolated from *Phlox subulata*, was transferred to Arabidopsis to investigate its function under low temperature stress. Real-time quantitative PCR analysis revealed that *PsCor413im1* expression was induced by cold and abscisic acid. Subcellular localization revealed that PsCor413im1-GFP fusion protein was localized to the periphery of the chloroplast, consistent with the localization of chloroplast inner membrane protein AtCor413im1, indicating that PsCor413im1 is a chloroplast membrane protein. Furthermore, the N-terminal of PsCor413im1 was determined to be necessary for its localization. Compared to the wild-type plants, transgenic plants showed higher germination and survival rates under cold and freezing stress. Moreover, the expression of *AtCor15* in transgenic plants was higher than that in the wild-type plants under cold stress. Taken together, our results suggest that the overexpression of *PsCor413im1* enhances low temperature tolerance in Arabidopsis.

1. Introduction

Low temperature is one of the most common abiotic stresses that adversely affect plant growth, development, and crop productivity [1]. Cold acclimation is the main process involved in enhancing chilling and freezing tolerance of some temperate plant species. During this process, several extensive structural, physiological, biochemical, and molecular changes occur in plants [2,3]. These changes are mediated through the differential expression of many genes that respond to cold stress [1,4]. Upon exposure to cold stress, a set of cold-regulated (*COR*) genes is induced to help plants in adapting to chilling and freezing stress [5]. At present, cold signaling pathways dependent on C-repeat (*CRT*) binding factors (CBFs) or dehydration responsive element (*DRE*) binding proteins (DREBs) are the best-understood regulatory pathway involved in cold acclimation [6,7]. The CBF/DREB proteins can bind to *CRT/DRE* cis-elements and activate transcription of the downstream *COR* genes to increase cold tolerance [8]. Overexpression of *CBF* was reported to activate abundant expression of *COR* genes and enhanced chilling and freezing tolerance in transgenic plants [9]. In Arabidopsis, a large number of *COR* genes have been identified, including *Cor6.6*, *Cor15*, *Cor47*, *Cor78*, and *Cor413* [2,10]. Previous reports have shown that Cor15 is a chloroplast stromal protein that has cryoprotective activity in Arabidopsis [11]. Furthermore, the overexpression of Arabidopsis *Cor15* resulted in enhanced freezing tolerance in transgenic plants [12].

The Cor413 is a protein family, which was initially found to be specific to the plant kingdom. The bioinformatic analyses showed that this protein family is potentially targeted to plasma membrane (Cor413pm) or thylakoid membrane (Cor413tm) [13]. Subsequent studies demonstrated that the Cor413tm proteins are actually located in the inner envelope membrane and were, therefore,

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renamed as Cor413im [14]. In Arabidopsis, *Cor413im* contains two highly similar genes (*Cor413im1* and *Cor413im2*), and both the genes have been reported to be strongly induced by low temperature [13]. Phenotypic analysis of T-DNA insertion mutants revealed that a single copy of *Cor413im* is sufficient to confer normal freezing tolerance to Arabidopsis [14]. As of date, *Cor413im* genes have been cloned from several plant species, including *Triticum aestivum* [13], *Gossypium barbadense* [15], *Phlox subulata* [16], and *Solanum lycopersicum* [17]. However, the mechanisms of the response of *Cor413im* genes to cold stress have yet to be fully characterized.

Phlox subulata is a perennial herbaceous flowering plant and is a member of the Polemoniaceae family. This plant can survive the winter at temperatures as low as −40 °C, and maintains a green phenotype at −12 °C [16]. In the present study, we report the characterization of a novel Cor413-like gene, *PsCor413im1*, from *P. subulata*, along with the *AtCor413im* genes from Arabidopsis. We examined their expression in response to various abiotic stresses. Subcellular localization of PsCor413im1 was performed using green fluorescent protein (GFP) as a marker. Using transgenic Arabidopsis plants, we then investigated the effects of overexpression of *PsCor413im1* on the response of plants to low temperature stress. Our study provides novel data regarding the role of *PsCor413im1* in stress tolerance, and would help in further characterization of the function of Cor413im.

2. Materials and methods

2.1. Identification of PsCor413im1 and sequence analysis

The transcriptome sequencing from a cDNA library of *Phlox subulata* identified a cDNA clone with high sequence similarity to the sequences of the known plant Cor413im genes. This clone was named *PsCor413im1* (GenBank accession number: KT337406.1) on the basis of its sequence similarity to the well-known *Cor413im* genes. The amino acid sequences of PsCor413im1 and its homologs were aligned using Clustal W. The transmembrane domains in PsCor413im1 were predicted by the HMMTOP algorithm (http://www.sacs.ucsf.edu/cgi-bin/hmmtop.py).

2.2. Plant material and growth conditions

Phlox subulata seedlings were grown in pots containing a mixture of turf peat and sand (2:1 v/v). The plants were grown under controlled greenhouse conditions with 70–80% relative humidity, 14-h light, and at an average temperature of 22 °C. All the *Arabidopsis thaliana* plants used in this study belonged to the Columbia-0 (Col-0) ecotype. The seeds were surface sterilized and stratified at 4 °C for 2 days in the dark. The seedlings were then grown on 1/2 strength Murashige and Skoog (MS) medium (3% sucrose, 1% agar, pH 5.8) under a 12-h light/12-h dark photoperiod (100 μmol m^{-2} s^{-1} light intensity) at 22 °C.

For abiotic stress treatments, 2-week-old *P. subulata* and Arabidopsis seedlings were exposed to 4 °C, or 100 μM abscisic acid (ABA) treatments. At least 10 seedlings from each treatment were harvested and pooled at different time points (0, 3, 6, 12, or 24 h after treatment), frozen immediately in liquid nitrogen and stored at −80 °C for RNA preparation.

2.3. RNA extracted and real-time quantitative PCR analyses

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, California, USA), according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA with the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Shiga, Japan). Real-time quantitative PCR analysis was performed using SYBR Green Mix (Agilent Technologies, Palo Alto, CA, USA) in an optical 96-well plate on Mx3000P system (Agilent). Three biological repeats and three technical repeats were performed for each analysis. The primers used in this study are shown in Supplemental Table S1.

2.4. Vector construction and plant transformation

For the construction of pBI121-PsCor413im1, the ORF of *PsCor413im1* was amplified by PCR and cloned at the *Xba*I and *Sac*I sites of pBI121 vector. To construct the GFP fusion genes, the ORF of *PsCor413im1* (or *AtCor413im1*), without the stop codon, was amplified by PCR and cloned at the *Xba*I (or *BamH*I) and *Kpn*I sites of pBI121-GFP vector. For the construction of the N-terminal deletion series (TM1-TM4-GFP, TM2-TM4-GFP, TM3-TM4-GFP, and TM4-GFP), a portion of the *PsCor413im1* was cloned at the *Xba*I and *Kpn*I sites of pBI121-GFP vector. The pBI121-GFP constructs have been described elsewhere [18]. The accuracy of the above constructs was confirmed by sequencing and the specific primers used in this study are shown in Supplemental Table S1.

The constructs were transformed into *Agrobacterium tumefaciens* strain EHA105 for plant transformation. The transient expression in tobacco leaf was performed according to a previously described procedure [19]. Arabidopsis was transformed using the floral dip method [20]. The transgenic plants were selected on half-strength MS medium containing 30 μg mL^{-1} kanamycin. The expression of the *PsCor413im1* in the transgenic lines was assessed by reverse transcription PCR analyses. The T3 generation was used for the analyses.

2.5. Confocal laser scanning microscopy

The leaf epidermis and hypocotyl of tobacco and transgenic Arabidopsis seedlings were peeled to make a temporary squash. The seedlings were visualized by confocal laser scanning microscopy (CLSM; Nikon, A1, Japan). The GFP signals were detected using a 500–530 nm emission filter. The chlorophyll signals were detected using a 620–680 nm emission filter.

2.6. Freezing tolerance assay

The freezing tolerance assay was performed as described [21], with some modifications. In brief, 2-week-old seedlings grown at 22 °C on half-strength MS plates containing 1.0% agar were treated with (NA) or without cold acclimated (CA) at 4°C for 4 d and then placed in a freezing chamber set to −1 °C and programmed to cool at −1°C per hour. The Petri dishes containing the seedlings were removed when the desired temperature (−10°C) was reached. After the freezing treatment, the seedlings were incubated at 4 °C in the dark for 12 h and then transferred to light at 22°C. The survival rates of the seedlings were scored visually after 4 d. The experiment was repeated three times.

2.7. Statistical analyses

All experiments were done with three independent biological replicates and three technical repetitions. The data were analyzed using one-way analysis of variance by SPSS, and statistically significant differences were calculated based on Student's *t*-test, with $P < 0.05$ (*) and $P < 0.01$ (**) as the thresholds for significance.

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