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Research article

Cold-regulated protein (SlCOR413IM1) confers chilling stress tolerance in tomato plants

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A B S T R A C T :

Chilling stress severely affects the growth, development and productivity of crops. Chloroplast, a photosynthesis site, is extremely sensitive to chilling stress. In this study, the functions of a gene encoding a cold-regulated protein (*SlCOR413IM1*) under chilling stress were investigated using sense and antisense transgenic tomatoes. Under chilling stress, *SlCOR413IM1* expression was rapidly induced and the sense lines exhibited better growth state of seedlings and grown tomato plants. Overexpression of *SlCOR413IM1* alleviated chilling-induced damage to the chloroplast membrane and structure, whereas suppression of *SlCOR413IM1* aggravated the damage to chloroplast. Moreover, the net photosynthetic rate (Pn), maximum photochemical efficiency of photosystem II (PSII) (Fv/Fm), actual photochemical efficiency of PSII (ΦPSII) and the activities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and stromal fructose-1, 6-bisphosphatase (sFBPase) were higher in the sense lines than those in the antisense lines. Hence, the inhibition of photosynthetic capacity was less severe in the sense lines but more severe in the antisense lines compared with that in wild-type (WT) plants. Taken together, overexpression of *SlCOR413IM1* enhanced the chilling stress tolerance, whereas suppression of this gene increased the chilling sensitivity of tomato plants.

1. Introduction

Cold stress (< 20 °C) is a major environmental factor that influences the growth, development and geographical distribution of plants (Agarwal et al., 2006; Chinnusamy et al., 2006). Cold stress including chilling stress (0 - 20 °C) and freezing stress (< 0 °C) (Chinnusamy et al., 2010; Shi et al., 2014) can reduce crop productivity, particularly in chilling-sensitive crops (Zhou et al., 2011). Many plants can enhance their cold stress tolerance after exposure to low but non-freezing temperatures. This mechanism of adaptation, referred to as cold acclimation, involves numerous changes related to gene expression in plants (Guy, 1990; Steponkus, 1984; Thomashow, 1999). Altered gene expression is a major response to cold stress. Numerous cold-responsive genes, such as cold-regulated (*COR*) genes, have been cloned and characterized (Thomashow, 1999).

COR genes, such as *COR47*, *COR78*, *COR15A* and *COR6.6*, which were first found in *Arabidopsis thaliana*, are rapidly induced by cold stress treatment. Among these genes, *AtCOR15A* is the best characterized (Lin and Thomashow, 1992a). Overexpression of *AtCOR15A* improves the freezing stress tolerance in *A. thaliana* (Artus et al., 1996; Nakayama et al., 2008; Thalhammer et al., 2014). Most COR genes have C-repeat/dehydration responsive (CRT/DRE) *cis*-elements in their promoters. CBF/DREB1 can bind to CRT/DRE *cis*-elements to regulate expression of *COR* genes (Liu et al., 2012; Maruyama et al., 2004). COR413 is a special protein family found in plants (Breton et al., 2003). Previous studies have shown that COR413 may be classified into three distinct groups, namely, COR413-plasma membrane (COR413PM) proteins, COR413-thylakoid membrane (COR413TM) proteins and COR413-inner membrane (COR413IM) proteins (Breton et al., 2003; Ma et al., 2017; Okawa et al., 2008). Although numerous *COR413* genes have been isolated and investigated in various plants, including *A. thaliana*, wheat (*Triticum aestivum*), rice (*Oryza sativa*), *Sorghum*, *Chrysanthemum* and *Gossypium barbadense*, the functions of the *COR413* genes responding to chilling stress have been rarely studied (Breton

Abbreviations: CaMV35 S, cauliflower mosaic virus 35 S; DAB, 3,3′ diaminobenzidine; Fv/Fm, the maximal photochemical efficiency of PSII; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; MS, Murashige-Skoog; NBT, nitroblue tetrazolium; $O_2^{\bullet-}$, superoxide anion radical; Pn, net photosynthetic rate; PSII, photosystem II; qRT-PCR, quantitative real-time polymerase chain reaction; REC, relative electronic conductance; ROS, reactive oxygen species; sFBPase, stromal fructose-1,6-bisphosphatase; SlCOR413IM1, *Solanum lycopersicum* cold-regulated protein inner membrane 1; WT, wild-type; Φ PSII, actual photochemical efficiency of PSII

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et al., 2003; Chen et al., 2014; Okawa et al., 2008; Wang et al., 2007). Okawa et al. (2008) speculated that AtCOR413IM may stabilize the inner envelope membrane of the chloroplast or function as metabolite transporters under cold stress. However, these predicted mechanisms should be further evaluated and confirmed.

A chloroplast is an organelle where photosynthesis and metabolic reactions occur. The structure and metabolic reactions of chloroplasts are highly susceptible to chilling stress (Kimball and Salisbury, 1973; Kratsch and Wise, 2000). This type of stress can destroy the main processes of photosynthesis, including thylakoid electron transport, Calvin cycle and stomatal conductance (Allen and Ort, 2001). PSII, a large pigment-protein complex in the thylakoid membrane, is involved in the main reactions of photosynthesis and is sensitive to chilling stress (Duan et al., 2012; Huang et al., 2010; Li et al., 2010; Shi et al., 2012). Under chilling stress, the imbalance between the capacity to harvest and dissipate light energy causes excessive PSII excitation pressure (Asada, 2006; Cheng et al., 2016; Foyer and Shigeoka, 2011; Miura and Furumoto, 2013; Mubarakshina et al., 2010). Moreover, chilling stress restrains enzyme activities in the Calvin cycle, thereby reducing the utilisation of absorbed light energy for CO_2 assimilation (Allen and Ort, 2001; Gururani et al., 2015; Mignolet-Spruyt et al., 2016). The excessive PSII excitation pressure and inhibition of carbon fixation lead to the overproduction of reactive oxygen species (ROS). Excessive amounts of ROS produced can cause oxidative damage to various cellular components, including membrane lipids, structural proteins, chlorophylls, nucleic acids, and enzymes, and result in inhibition of plant growth and development (Jaspers and Kangasjarvi, 2010; Miller et al., 2008). Therefore, maintaining the photosynthetic capacity is particularly important for normal plant growth and metabolism under chilling stress conditions.

Plant species, such as tomato, rice and maize, which originate from tropical and subtropical regions, are chilling-sensitive plants and cannot resist chilling injury when exposed to temperatures ranging from 0 °C to 12 °C (Barrero-Gil et al., 2016; Hu et al., 2015; Ntatsi et al., 2017; Zhang et al., 2004). Tomato (*Solanum lycopersicum* L.) is a major vegetable crop worldwide. The growth, development and production of this crop can be severely impaired by chilling stress (Liu et al., 2012; Ré et al., 2017). Thus, it is particularly important to enhance the chilling stress tolerance of tomato. There are several lines of evidence that chloroplast envelope membrane proteins may have significant functions in the temperature response of plants. For example, the amount of digalactocyl diacylglycerol in the chloroplast envelope increases during cold acclimation, implying that some proteinaceous factors might be involved in this process (Uemura and Steponkus, 1997). OEP16, a channel protein of the outer envelope membrane protein, is strongly induced by cold stress (Baldi et al., 1999; Drea et al., 2006). However, few envelope proteins implicated in the temperature response of plants have been studied in detail. In this study, we investigated the function of a gene encoding the chloroplast-targeted protein (*SlCOR413IM1*) by using sense and antisense transgenic tomatoes. The expression of *SlCOR413IM1* was rapidly upregulated by chilling treatment. Overexpression of *SlCOR413IM1* in tomato improved the chilling tolerance by alleviating the damage to the chloroplast, whereas suppression of this gene increased the chilling sensitivity of tomato.

2. Materials and methods

2.1. Plant growth conditions and treatment

Wild-type (WT) tomatoes (*S. lycopersium* cv. L-402), sense T_2 transgenic lines (S1, S2 and S5) and antisense T_2 transgenic lines (A6, A7 and A21) were used as plant materials. Tomato seeds were sterilized, sown on Murashige-Skoog (MS) medium, and incubated in the illuminated incubation chamber with a 16 h/8 h (light/dark) photoperiod, a photon flux density of 200 μmol $m^{-2} s^{-1}$ and a relative humidity of 70% at 25 °C for 7 days. The seedlings were then planted in soil and grown at 25/22 °C (day/night) with a 16 h/8 h (light/dark) photoperiod, a photon flux density range of 500–600 μmol $m^{-2} s^{-1}$ and a relative humidity range of 60–70% in a greenhouse. WT, sense and antisense lines were irrigated two times a week, alternately with tap water and 1 × Hoagland's nutrient solution (Kong et al., 2014; Wang et al., 2014) to ensure the normal growth of tomato plants.

To investigate the expression patterns of *SlCOR413IM1* under chilling stress, six-week-old WT tomato plants were treated in an illuminated incubation chamber (PERCIVAL, E-36L) at 12 °C, 8 °C and 4 °C, respectively, for 0, 0.5 1, 3, 6, 9, 12, and 24 h. For each temperature treatment, we used three chamber simultaneously. And the three chambers were set to the same temperature. WT plants with the same growth period and the same number were placed in the same location of each chamber and the treatment time points (0, 0.5 1, 3, 6, 9, 12, and 24 h) were also the same for the three chambers. Treatment of each chamber was considered as a biological replicate. To ensure that all plants are under the same conditions, the plants were placed in the middle of the chamber. At every time point, three tomato plants were randomly selected and sampled, and then the three plants were abandoned. The sample was frozen in liquid nitrogen and stored at −80 °C. Therefore, we needed 24 tomato plants per replicate. The 24 tomato plants were grown in eight pots with three in one pot and placed in one big box when they were treated.

2.2. RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from tomato leaves by using a simple total RNA kit (Tiangen, Beijing, China). RT-PCR analysis (Tiangen, Beijing, China) was conducted to produce cDNA. The qRT-PCR was performed on a Bio-Rad CFX96TM real-time PCR system (Bio-Rad, USA) by SYBR Real Master Mix (Tiangen, Beijing, China). The PCR thermal cycle conditions were as follows: 95 °C for 5 min, 42 cycles at 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 20 s. *EF-1α* (GenBank accession number LOC544055) was used as reference gene in tomato. The results were repeated three times with three independent samples.

2.3. Plant transformation and transgenic tomato identification

The coding sequence of *SlCOR413IM1* (GenBank accession number: AK321304.1) was cloned into *Xba* I and *Sal* I restriction sites of the binary vector pBI121, behind the promoter of cauliflower mosaic virus 35S (CaMV 35S). The recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain LBA4404 and tested by PCR and sequencing analyses. The recombinant vector was introduced into tomato leaf through leaf dish transformation mediated by *A. tumefaciens*.

After obtaining T_0 kanamycin-resistant plants, DNA was extracted from the resistant and WT plants. Then these kanamycin-resistant plants were identified by PCR. T_1 transgenic lines were selected by qRT-PCR and Western blot. All primers were synthesized by the Beijing Genomics Institute (Tiangen, Beijing, China), and their sequences for PCR and qRT-PCR analyses are listed in Supplemental Table S1. Total proteins were extracted from the leaves of WT and transgenic lines as described by Kong et al. (2014). For Western blot, the extracted proteins were separated by using SDS-PAGE Gel Kit (Kangwei, Beijing, China) and were electroblotted to a polyvinylidene fluoride (PVDF) membrane. The protein was detected with antibodies. The SlCOR413IM1 protein antibody was rabbit polyclonal antibodies synthesized by Abmart (Abmart, Shanghai, China) and was used at a dilution of 1:500. Peroxidase-conjugated goat anti-rabbit IgG was utilised as the secondary antibody at a dilution of 1:5000. The same total proteins were separated by SDS-PAGE gel and then stained with Coomassie brilliant blue R250 to qualify as a loading control for Western blot.

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