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Research article

A tomato transcription factor, SlDREB3 enhances the tolerance to chilling in transgenic tomato

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ABSTRACT

The dehydration response factor (DREB) transcription factor (TF) family can function in response to multiple cues around environment in plants. Nevertheless, the functions of dehydration response factor (DREB protein) in plant cold tolerance, especially in tomatoes (*Solanum lycopersicum*), have been rarely studied. In this study, the functions of tomato DREB TF (SlDREB3) in cold resistance were studied using transgenic tomatoes. The level of transcripts revealed that *SlDREB3* was triggered by H_2O_2 and 4 °C treatments, indicating that SlDREB3 participates in response to cold stress in plants. *SlDREB3*-overexpressing plants exhibited high fresh mass, chlorophyll content, Fv/Fm, and O_2-evolving activity; low membrane damage; and reactive oxygen species accumulation under chilling stress. Furthermore, the high expression levels of late embryogenesis-abundant genes *SlLEA9* and *SlLEA26* were detected in transgenic plants in response to cold stress. These findings revealed that *SlDREB3* overexpression improved the tolerance to cold stress in transgenic plants possibly by upregulating *SlLEAs* expression.

1. Introduction

In life cycle, plants are inevitably threatened by environmental stimuli, such as salt, drought, and extreme temperature stresses. Tropical or subtropical vegetable crops, such as tomatoes (*Solanum lycopersicum*), cucumbers (*Cucumis sativus*), and bell peppers (*Capsicum annuum*), are sensitive to low temperatures. Cold stress is generally divided into chilling and freezing stresses. These cold-sensitive vegetables are vulnerable to chilling stress, thereby resulting in a large yield reduction. As a result, plants have evolved various mechanisms to enhance chilling tolerance after exposure to chilling stress, which is known as cold acclimation (Miura and Furumoto, 2013).

Plant response to cold stress is a complex process involving multiple gene regulation and different metabolic pathways and intercellular connections (Hannah et al., 2005). A series of genes encoding transcriptional regulators, such as NAC (NAM, ATAF1/2 and CUC1/2) and dehydration response factor/C-repeat binding factor (DREB/CBF), is induced to adapt to cold stress during cold acclimation, and the encoded proteins activate downstream genes, causing cold responses. DREB transcription factors (TFs) are a subfamily member of APETALA2 (AP2)/ethylene-responsive factor (ERF) family. DREB TFs were divided into six subfamilies named A-1 to −6 on the basis of the sequence similarities of the AP2 domains (Sakuma et al., 2002). DREB TFs play a significant role in plant growth and development and in response to abiotic stresses. DREB TFs are involved in leaf expansion and internode elongation, adventitious root development, vertical root development, cell elongation, and hypocotyl development (Li et al., 2012, 2018; Xiu et al., 2016; Liao et al., 2017; Upadhyay et al., 2017; Kudo et al., 2017). DREB TFs found in rice, soybean, wheat, corn, and other crops can also participate in abiotic stress responses, such as in low temperature, high temperature, salt, and drought stresses (Herath, 2016; Kidokoro et al., 2015; Shavrukov et al., 2016; Gu et al., 2016; Wu et al., 2018).

Up to now, the research results have shown that all DREB/CBF transcription factors cloned and isolated from plants can interact with DRE/CRT cis-acting elements (Rehman and Mahmood, 2015). TFs DREB2 and DREB1A/B/C in groups A-1 and A-2 are involved in stress

Abbreviations: ABA, abscisic acid; COR, cold stress response; DAB, 3,3′-diaminobenzidine; DREB, dehydration response factor; EGFP, enhanced green fluorescent protein; H_2O_2, hydrogen peroxide; LEA, late embryogenesis abundant; NBT, nitroblue tetrazolium; $O_2^{\cdot-}$, superoxide anion radical; PSII, photosystem II; qPCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SlDREB3, *Solanum lycopersicum* DREB protein 3; SOD, superoxide dismutase

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responses independent of ABA, respectively. Other transcription factors of DREB/CBF induced by stresses, such as AtDREB1D/AtCBF4, GmDREBa, GmDREB2, ZmDBP2, GhDBP2 and GhDBP3, are involved in the ABA-dependent dehydration response (Guttikonda et al., 2014; Li et al., 2005; Chen et al., 2007; Wang et al., 2011; Huang et al., 2008; Huang and Liu, 2006). These results suggesting that DRE/CRT cis-elements are involved in stress responses via two kinds of signaling pathways, dependent on ABA and independent on ABA, respectively. The two kinds of pathways closely link with each other by the specific component, constituting a complex signal transduction network, therefore making the plants improve the stress tolerance.

Chilling stress triggers DREB1/CBF TF transcription, thereby activating numerous cold stress-responsive genes (*CORs*), which transcriptional cascade pathway is a widely studied cold acclimation signal transduction pathway (Thomashow, 1999; Chinnusamy et al., 2007; Knight and Knight, 2012). However, other cold-related factors, such as cold-induced (KIN), low temperature-induced (LTI), and late embryogenesis-abundant (LEA) factors (Erpen et al., 2017), can also play prominent roles in plant's response to chilling stress. LEA proteins belong to a great and highly plentiful family of hyper-hydrophilic proteins, which firstly found in seeds of cotton (*Gossypium hirsutum*) (Dure et al., 1981). Some LEA proteins stabilize membranes, where they have been related to anionic phospholipid vesicle-stabilizing model membranes at freezing temperatures (Kosová et al., 2007). The overexpression of the rice *OsDREB1A* gene in transgenic *Arabidopsis* also increases the LEA protein expression by twofold (Dubouzet et al., 2003). Nonetheless, whether or not tomato DREB TFs can regulate the LEA protein expression under chilling stress remains unclear and must be further elucidated.

SlDREB3 is a gene that contains the AP2 domain (Liu et al., 2016). In this study, *SlDREB3* was induced by chilling stress, which was consistent with previous studies (Islam and Wang, 2009). The overexpression of *SlDREB3* showed obvious resistance to chilling stress in transgenic tomatoes. This study provides a new perspective for plant cold signal transduction and network regulation.

2. Materials and methods

2.1. Plant growth and treatments

Wide type tomato (WT, *S. lycopersicum* cv. L-402) and three T_2 generation overexpressed lines, namely, OE2, OE4, and OE7, were sown in nutritional soil under the following conditions: 25 °C, 55%–65% relative humidity, 250 μmol·m^{-2}·s^{-1} photon flux density (PFD) and 16 h/8 h (light/dark). These plants were watered with Hoagland's nutrient solution twice a week. After six weeks of growth, the plants were placed into a light incubator GPJ-400 (Dongpeng Instruments, Jiangshu, China) for 2 days before treatment.

To investigate *SlDREB3* expression under chilling stress, we treated the six-week-old WT tomatoes at 4 °C for 24 h under a 16 h/8 h (light/dark) condition, and samples were taken at 0, 3, 6, 9, 12 and 24 h. For the ABA treatment, the plant leaves were sprayed with 100 μM ABA, whereas the control plants were only sprayed with water. For the H_2O_2 treatment, the roots of WT plants were steeped in Hoagland's nutrient solution supplement with 20 mM H_2O_2 for 24 h, whereas the control plants were only steeped in Hoagland's nutrient solution, and samples were collected at 0, 3, 6, 9, 12 and 24 h. At the 4 °C treatment, we treated WT and transgenic seedlings for 5 days, and WT and transgenic mature plants for 24 h and 48 h in a light incubator with approximately 250 μmol·m^{-2}·s^{-1} PFD for physiological parameter detection.

2.2. SlDREB3 cloning and sequencing

Specific primers (forward, 5′-ATGAATTCCCAAATCTTTT-3′ and reverse, 5′-TTATAGAGAGGCCCAATCAAT-3′) were designed according to the mRNA sequences (GenBank no. AF506825), and the coding sequences (CDS) of *SlDREB3* were amplified by PCR using cDNA from WT tomato leaves as the template. Afterward, PCR products were ligated into pMD19-T vector (TaKaRa, Beijing, China) and sequenced.

2.3. SlDREB3 subcellular localization

The CDS of *SlDREB3* was amplified with specific primers (forward: 5′-TCTAGAATGAATTCCCAAATCTTTT-3′ and reverse: 5′-GGTACCGTT AGAGAGGCCCAATCAATTT-3′). Subsequently, CDS of *SlDREB3* was ligated into the binary vector PBI121 using *Xba*I *Kpn*I sites, which generated a SlDREB3-GFP recombinant plasmid. SlDREB3::GFP and GFP alone respectively were introduced into *Agrobacterium tumefaciens* strain LB4404. *A. tumefaciens* was then infiltrated into tobacco (*Nicotiana benthamiana*) mesophyll cells and onion (*Allium cepa*) epidermal cells, and finally, the laser confocal microscopy (Leica, Heidelberg, Germany) was used to detect fluorescence.

2.4. Transcriptional activation analysis in yeast

SlDREB3 transactivation was examined by a yeast system. The complete CDS of *SlDREB3* was amplified by primers attached to *Nco*I and *Pst*I restriction sites and ligated into pGBKT7 vector (Clontech, San Francisco, USA) to generate pGBKT7-SlDREB3 construct. pGBKT7-SlDREB3 (recombinant plasmids), pGBKT7-53 + pGADT7-RecT (positive control) and pGBKT7 (negative control) were transformed into the yeast strain AH109. Then, these transformants were plated on SM-W (without Trp) medium at 30 °C for 3 days. Finally, the transformants were spread on SM-WHA (without Ade, Trp, and His) medium at 30 °C for 3 days, and the activity of β-galactosidase (X-Gal) was estimated as described previously (Duttweiler, 1996).

2.5. Tomato genetic transformation and identification

The CDS of *SlDREB3* was amplified using the primers (forward 5′-CCATGGATGAATTCCCAAATCTTTT-3′, *Nco*I site underlined; reverse 5′- CACGTGGTGGTGGTGGTGGTGTAGAGAGGCCCAATCAATTT-3′, *Pml*I site and the His epitope tag sequences are underlined) and inserted into pCAMBIA3301 vector controlled by the cauliflower mosaic virus (CaMV) 35 S promoter. The *SlDREB3*-pCAMBIA3301 was introduced into *A. tumefaciens* strain LBA4404. The *A. tumefaciens*-mediated leaf disk method was used to produce the T_0 phosphinothricin-resistant transgenic plants, and then PCR was performed to further identify the T_0 transgenic plants. T_2 transgenic homozygotes were selected for subsequent experiments.

2.6. Real-time qPCR analysis

Total RNA was isolated from WT and transgenic plant leaves by using Total Plant RNA Extraction Kit (Solarbio, Beijing, China). qPCR was performed as described by Zhang et al. (2009). *EF-1α* (GenBank no. LOC544055) was used as internal standards. The qPCR primers used were listed in Table S1.

2.7. Protein extraction and Western blot analysis

Total protein extraction was performed following as the method described previously (Kong et al., 2014). Western blot analysis was performed as described by Wang et al. (2015).

2.8. Growth performances of WT and transgenic plants under chilling stress

In order to analyze the growth of seedlings, the sterilized WT and transgenic plant seeds were planted on Murashige and Skoog medium and transferred to the light incubator of a 16 h (light)/8 h (dark) regime at 25 °C with approximately 250 μmol·m^{-2}·s^{-1} PFD. For growing 10-old-day plants, one half of the seedlings remained in the original light

incubator as the control. The other half was transferred into another light incubator with the same conditions, but, at 4 °C. After 5 days, the seedlings were photographed. The control plants were transferred into quartz sands for further culture in the greenhouse. After WT and transgenic plants grew for 4 weeks, they were placed for 48 h under 4 °C and photographed.

2.9. Physiological parameter measurements

The cold resistance index and growth inhibition were determined as the method Wang described (Wang et al., 2018). The relative electrical conductivity (REC), malondialdehyde (MDA) and chlorophyll contents in leaves were determined in accordance with the methods (Kong et al., 2014). The crude enzyme used for the determination of antioxidant enzyme activities was extracted from leaves following the method described by Wang et al. (2018). The catalase (CAT, EC1.11.1.6) and superoxide dismutase (SOD, EC1.12.1.11) activities were determined according to the methods by Zong et al. (2009).

2.10. ABA content determination

For abscisic acid (ABA) extraction, 2 g leaves were ground homogenously in 10 mL of ice-cold 80% (v/v) methanol, and stood overnight at 4 °C. The mixture was filtered through four gauze layers. After the filtrate was centrifuged at 10000 *g* for 15 min, the supernatant was adjusted to a pH of 3, and then the supernatant was extracted three times by adding ethyl acetate of the same volume. The organic phase containing ABA was vacuum evaporated, and the ABA left in the tube after evaporation was dissolved in 1 mL of methanolic TBS buffer (10% methanol) and then kept at 4 °C overnight. Phytodetek ABA Test Kit (Agdia, Indiana, USA) was used to measure ABA content by indirect ELISA following the manufacturer's protocol.

2.11. Chlorophyll fluorescence and O_2-evolving activity measurement

The chlorophyll fluorescence was determined by a Handy PEA (Hansatech Instruments, Norfolk, UK) as the methods described in Ma et al. (2013). The Fv/Fm of PSII was calculated as follows: Fv/Fm = (Fm − Fo)/Fm. O_2-evolution rates of plants was detected by a modified Clarke type O_2 electrode unit (Hansatech, Kings Lynn, UK) as the methods described by Ma et al. (2013).

2.12. Histochemical staining and measurements of H_2O_2 and $O_2^{\cdot -}$

The 6-week-old overexpressed lines and WT plants were proceeded for 24 h under 4 °C. By contrast, the control plants were concurrently placed at 25 °C for the same time. H_2O_2 and $O_2^{\cdot -}$ were stained with 3′,3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) as described by Pan et al. (2012). The H_2O_2 and $O_2^{\cdot -}$ contents in WT and transgenic plant leaves were detected as the method described by Kong et al. (2014).

2.13. Statistical analysis

SigmaPlot 12.5 (Systat Software, San Jose, CA, USA) and SPSS18.0 (Chicago, IL, USA) were used for statistical analysis. The mean value ± SD of at least three replicates are presented. *p < 0.05 and **p < 0.01 indicate significant differences in comparison with control.

3. Result

3.1. SlDREB3 subcellular localization

ProtComp 9.0 database (http://linux1.softberry.com/cgi-bin/programs/proloc/protcomppl.pl) predicted that SlDREB3 may be localized in the nucleus. To prove the prediction, *Agrobacterium*-mediated transient transformation of onion epidermal cells were performed and showed that the green signal of the 35S::SlDREB3-GFP fusion protein mainly appeared in the nuclei, while the green fluorescence of 35S::GFP predominantly appeared in both cytoplasm and nucleus (Fig. 1A and B). The transient transformation of tobacco leaf mediated by *Agrobacterium* also showed that SlDREB3-GFP mainly existed in the nucleus (Fig. 1 C). These results indicated that SlDREB3 is localized in the nucleus.

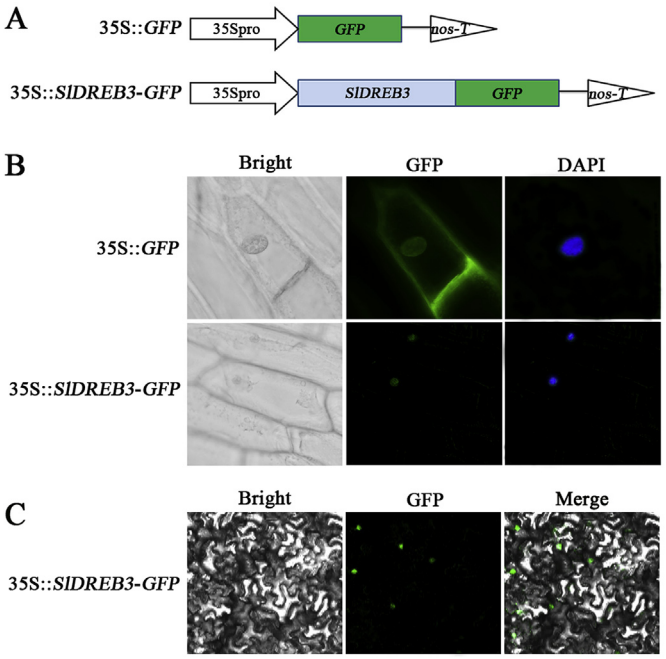

Fig. 1. Subcellular localization of SlDREB3 protein. (**A**) Structure pattern of GFP fusion protein. (**B**) Transient expression of 35S::GFP (upper layer) and 35S::SlDREB3-GFP (under layer) in onion epidermal cells. (**C**) Transient expression profiles of 35S::SlDREB3-GFP in tobacco leaf cells.

3.2. SlDREB3 protein was a typical TF

TFs generally have nuclear localization and transcriptional activation activity. Except for nuclear localization, the transactivation activity of SlDREB3 assay was undergone in a yeast system. In the medium lacking tryptophan, histidine and adenine, SlDREB3 could fully promote the growth of yeast cells, show the activity of X-β-gal (Fig. 2 A), and increase the activity of β-galactosidase (Fig. 2 B). However, pGBKT7 transformed yeast cells in the control group did not grow normally.

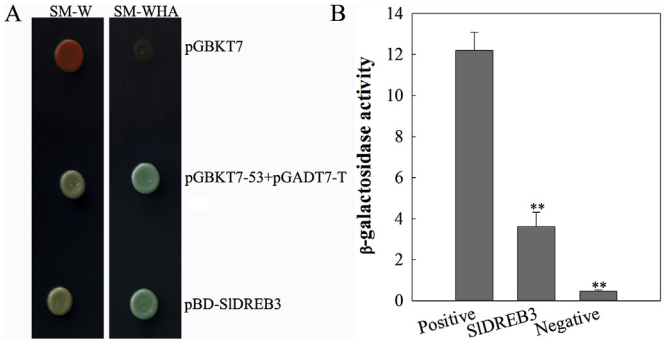

Fig. 2. Transactivation activity of SlDREB3 protein. (**A**) SlDREB3 and GAL4 DB domain fusion proteins were expressed in AH109 yeast cells. (**B**) β-Galactosidase assay. The vectors pGBKT7 and pGBKT7-53 + pGADT7-RecT were expressed in yeast cells as the negative and positive controls, respectively. The yeast cells were cultured on SM-W and SM-WHA.

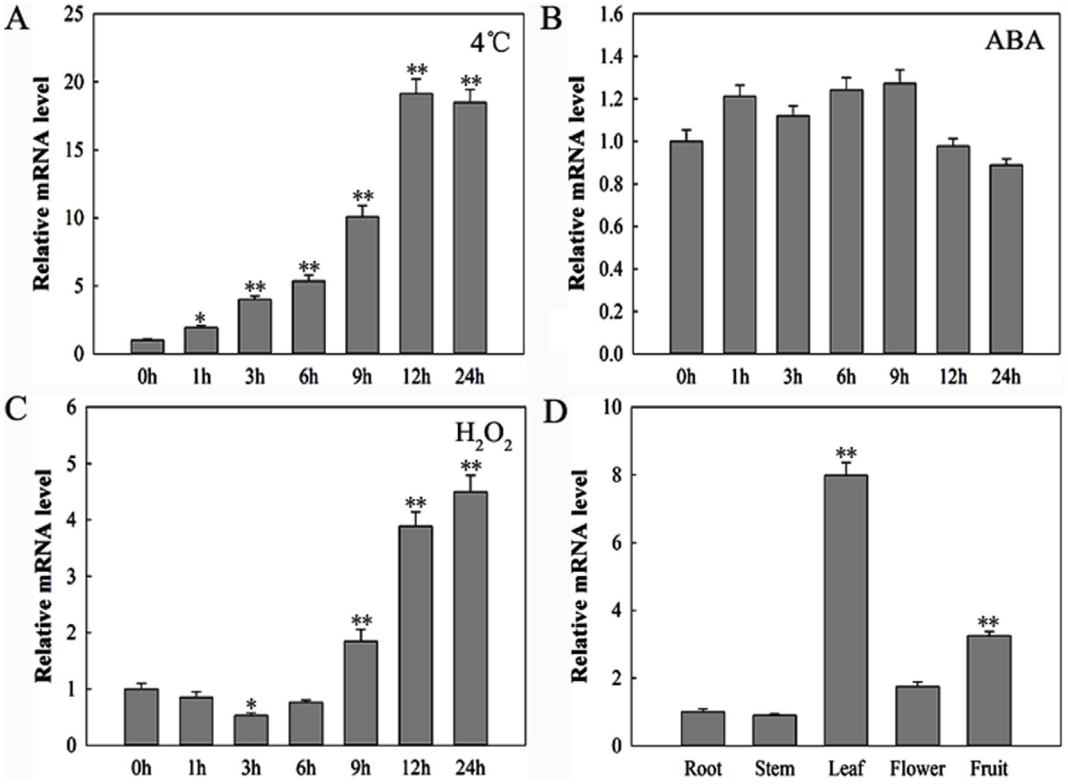

Fig. 3. Quantitative PCR analysis of *SlDREB3* expression patterns in tomato: (**A**) 4 °C, (**B**) 100 μM ABA, (**C**) 20 mM H_2O_2, and (**D**) *SlDREB3* expression in different tomato tissues.

3.3. SlDREB3 induction by chilling stress

SlDREB3 expression in response to 4 °C, abscisic acid (ABA) and H_2O_2 was investigated using qPCR. Fig. 3 shows that the expression of *SlDREB3* was induced by 4 °C and H_2O_2 but not by ABA. With the treatment of 4 °C, the transcripts level of *SlDREB3* reached the maximum at 12 h and then decreased. Then, 20 mM H_2O_2 increased *SlDREB3* expression by 4.6-fold compared with the control after 24 h (Fig. 3 A and C). *SlDREB3* expression patterns in the different tomato tissues were also analyzed by qPCR. As shown in Fig. 3 D, the *SlDREB3* expression was the highest in leaves, followed by the fruits.

3.4. Transgenic plant identification

A total of 15 tomato transgenic lines with phosphinothricin resistance were generated from tissue culture. Eight T_2 lines were screened by qPCR. The relative mRNA levels of *SlDREB3* in overexpressed lines were increased by 10.8-, 6.6-, 14.6-, 18.8-, 12.4-, 9.2-, 16.0-, and 8.4-fold compared with those of the WT plants (Fig. 4 A). Among them, OE2, OE4, and OE7 were selected for western blot analysis. The protein level of SlDREB3 was coincident as that of *SlDREB3* mRNA level (Fig. 4 B). Hence, OE2, OE4, and OE7 were chosen for subsequent experiments.

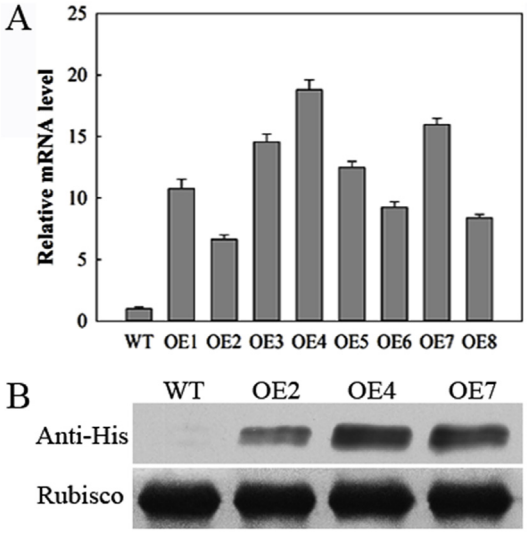

Fig. 4. Transgenic tomato identification by qPCR and Western blot analysis. (**A**) qPCR analysis of the expression levels of *SlDREB3* in WT and the transgenic plants. (**B**) The protein levels of SlDREB3 in WT and different transgenic plants analyzed using Western blot. The loading control is the large subunit of Rubisco.

3.5. SlDREB3 overexpression enhanced chilling stress

Given that *SlDREB3* expression level can be evidently increased by 4 °C treatment, we observed the growth phenotypes of 10-day-old seedlings and 6-week-old plants under chilling stress. Under normal growth circumstances, both seedlings and mature plants grew well, and the difference in growth phenotype and physiological indexes between WT and transgenic plants was insignificant (Fig. 5). After five days of chilling stress, the growth of all seedlings was inhibited at different levels, but the inhibition degree of WT was significantly higher than that of overexpressed plants (Fig. 5 A). The cold resistance index and growth inhibition degree of seedlings was also calculated and investigated under chilling stress (Fig. 5B and C). Leaf withering was less severe in the mature transgenic lines than that in WT plants after chilling stress for 48 h (Fig. 5 D). Therefore, the transgenic lines exhibited higher chlorophyll content and fresh weight than WT lines (Fig. 5E and F). The above results showed that *SlDREB3* overexpression can enhance the resistance to chilling stress. The ABA levels were

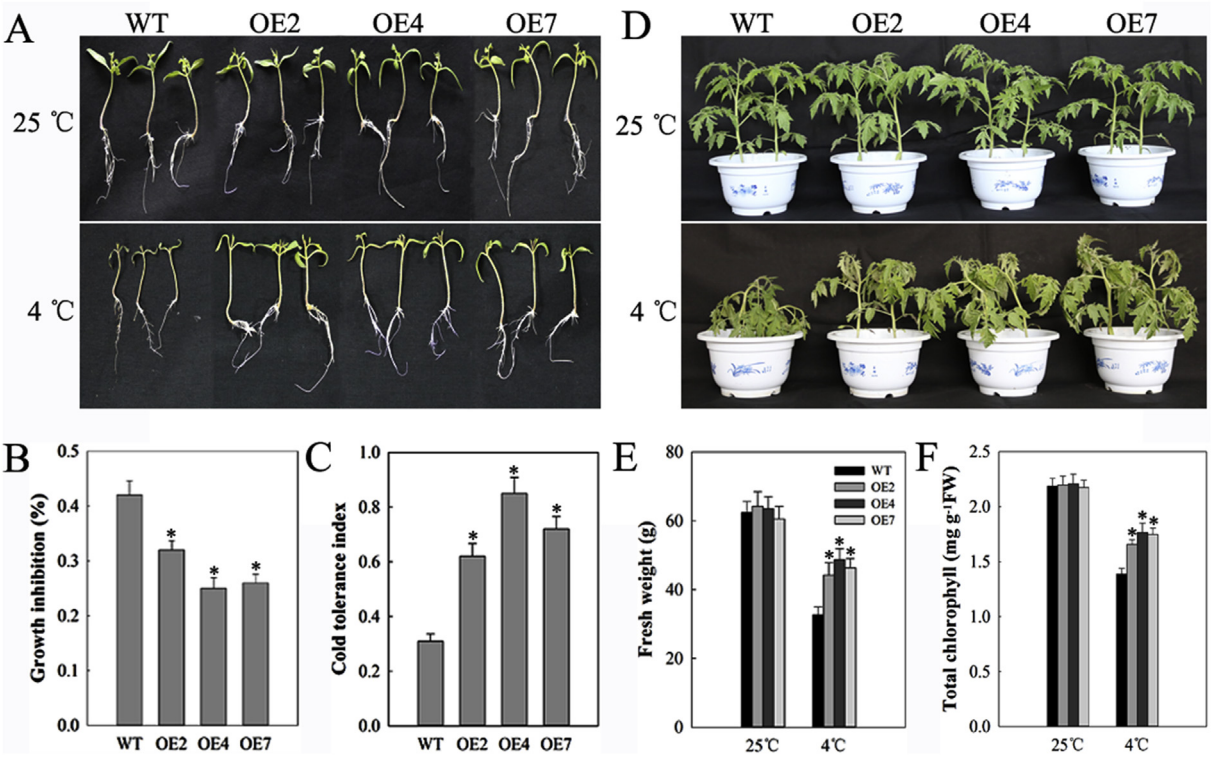

Fig. 5. Cold tolerance of 10-day-old seedlings and 6-week-old plants. (**A**) Phenotype of 10-day-old seedlings under 25 °C and 4 °C for 5 days. (**B**) Growth inhibition. (**C**) Cold resistance index. (**D**) Phenotype of 6-week-old plants under 25 °C and 4 °C for 48 h. (**E**) Fresh weight of grown plants. (**F**) Total chlorophyll content in grown plants.

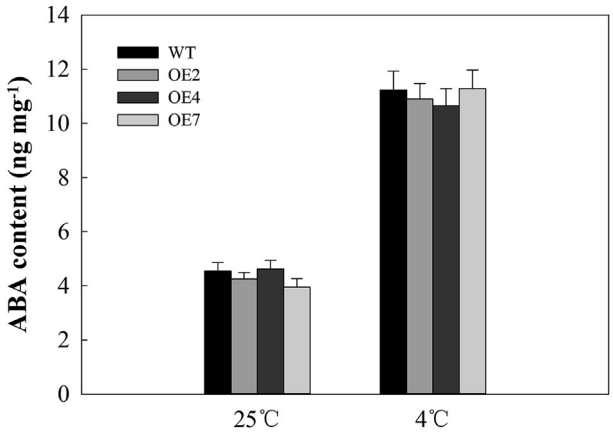

Fig. 6. ABA levels in WT and transgenic plant analyses.

detected in WT and overexpressed lines. The difference in the ABA content between WT and overexpressed lines under both natural conditions and chilling stress was insignificant. However, the ABA content increased significantly in all of the strains under low temperature stress (Fig. 6). This result indicates that *SlDREB3* overexpression did not depend on ABA to enhance chilling stress resistance.

3.6. SlDREB3 overexpression alleviated ROS accumulation in transgenic plants

Chilling stress generally causes ROS generation. Thus, the DAB and NBT staining analyzed the H_2O_2 and $O_2^{\bullet -}$ levels, respectively. Under natural conditions, H_2O_2 and $O_2^{\bullet -}$ accumulations were relatively low, and the difference between WT and overexpressed lines was insignificant. After 24 h of chilling stress, the amounts of brown polymerization (DAB staining) and blue polymerization products (NBT staining) were more in WT than that in overexpressed lines (Fig. 7A and B). The quantitative determination of H_2O_2 and $O_2^{\bullet -}$ contents showed the same results (Fig. 7 C and D). These findings suggest that *SlDREB3* overexpression alleviated H_2O_2 and $O_2^{\bullet -}$ accumulation.

Plants maintain intracellular ROS balance by scavenging excess ROS through the antioxidant enzyme system (Wang et al., 2019). Hence, the activities of CAT and SOD were analyzed. Under normal conditions, the difference in the activities of CAT and SODbetween WT and overexpressed plants was insignificant. After 24 h of chilling stress, CAT and SOD activities increased at varying degrees, and there was a more significant increase in overexpressed plants than in WT plants (Fig. 7 E and F). To investigate the reason for high enzyme activities in transgenic plants, we detected the *SlCAT* and *SlCnZnSOD* expression levels by using qPCR (Fig. 7 G and H). The gene expression level of overexpressed plants induced by chilling stress was significantly higher than that of WT plants. Thus, *SlDREB3* overexpression alleviates ROS accumulation in transgenic plants possibly by up-regulating the expression of the target antioxidative genes under chilling stress.

3.7. SlDREB3 overexpression alleviated chilling-induced cell damage

Cell membrane system was the primary site of cold damage. Plant cell membrane changed from liquid crystal state to gel state at low temperature, and this change caused metabolic changes and plant cell dysfunction and eventually cold damage to plants. Trypan blue staining was used to assess the degree of cell death. All plants showed similar blue staining levels under normal growth conditions. However, WT plants exhibited darker blue stains than *SlDREB3*-overexpressing plants under chilling stress (Fig. 8 A). As physiological indicators of cell damage, REC and MDA content were detected to confirm these results. Under natural conditions, the differences in REC and MDA contents were insignificant between WT and *SlDREB3*-overexpressing plants. After 4 °C treatment, both REC and MDA contents was accelerated, and that the increase was more apparent in WT than that in *SlDREB3*-

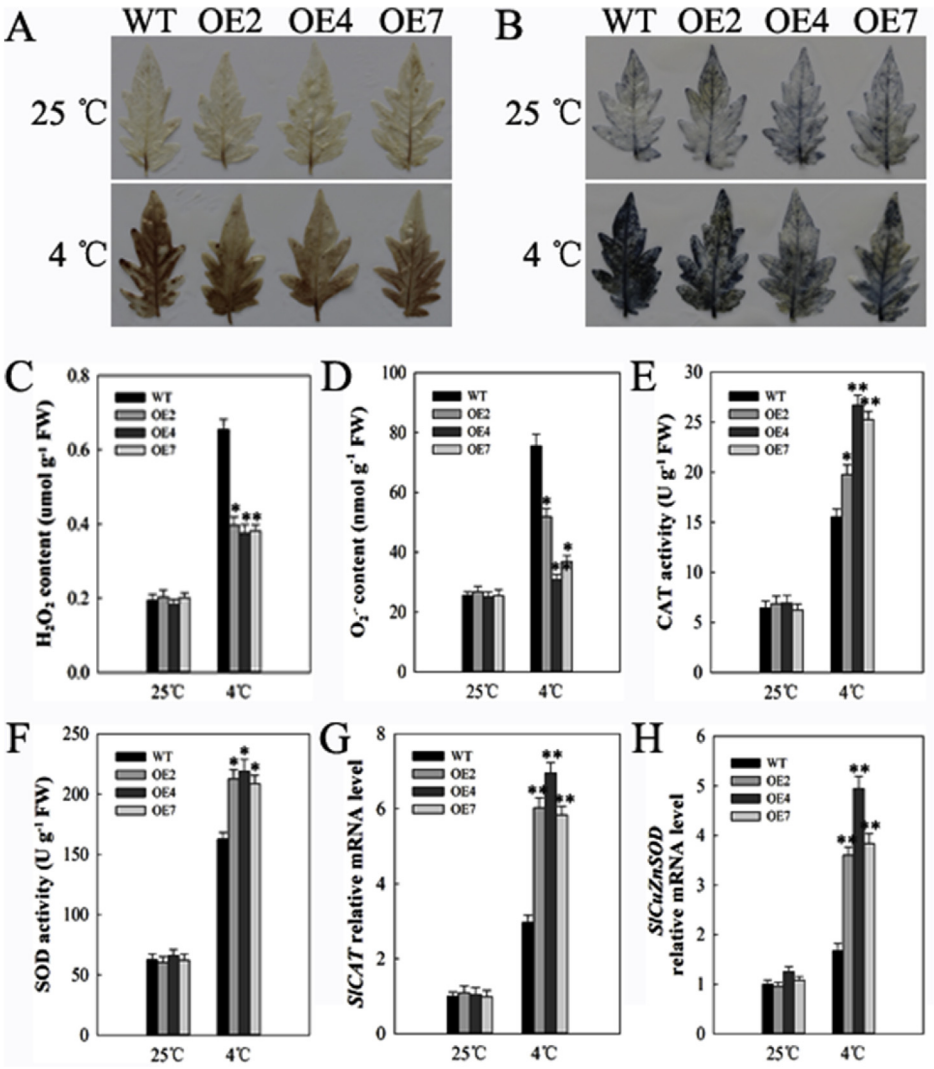

Fig. 7. ROS correlation analysis in 6-week-old WT and transgenic lines. (**A**) DAB staining analysis for H_2O_2. (**B**) NBT staining analysis for $O_2^{\cdot -}$. The upper layer represents plants grown at 25 ℃, and the under layer refers to plants subjected to 4 ℃ for 24 h. (**C**) H_2O_2 content. (**D**) $O_2^{\cdot -}$ content. (**E**) CAT activity. (**F**) SOD activity. (**G**) *SlCAT* expression. (**H**) *SlCuZnSOD* expression.

overexpressing plants (Fig. 8 B and C). The above results revealed that *SlDREB3* overexpression protects the cell from damage under chilling stress.

3.8. SlDREB3 overexpression alleviated PSII photoinhibition under chilling stress

Fv/Fm represented the maximum photochemical efficiency of PSII, and the O_2-evolving activity indicated the stability of the O_2-evolving complex. Both of these indices can reflect the damage and repair degree of PSII. Upon the treatment of 4 ℃ for 24 h, both O_2-evolving activity and Fv/Fm decreased in WT and transgenic plants, while O_2-evolving activity and Fv/Fm decreased slowly in transgenic plants. When transferring the plants to the 25 ℃ condition, Fv/Fm and O_2-evolving activity recovered, but those in WT plant was slower than that in *SlDREB3*-overexpressing plants and ultimately did not return to the initial value (Fig. 9A and B). These results indicated that the PSII damage of *SlDREB3*-overexpressing plants is less in comparison of WT plants under chilling stress.

3.9. SlDREB3 regulated SlLEA genes

To explore how SlDREB3 can enhance the low-temperature resistance of transgenic tomatoes, we used qPCR to analyze the expression of several cold stress-responsive factors. As shown in Fig. 10 A, the expression levels of *SlCBF1*, *SlCBF2*, *SlCOR413*, and *SlCOR518* were insignificantly different in WT and overexpressed plants after 4 ℃ treatment but higher than that under 25 ℃. These results suggested that SlDREB3 may not be directly involved in the CBF cold acclimation signaling pathway. Cao and Li (2015) reported that six *LEA* genes (*SlLEA3*, *SlLEA9*, *SlLEA10*, *SlLEA13*, *SlLEA22*, and *SlLEA26*) in tomato are able to respond to chilling stress. Therefore, the expression levels of *SlLEA3*, *SlLEA9*, *SlLEA10*, *SlLEA13*, *SlLEA22*, and *SlLEA26* were also detected using qPCR. Under natural conditions, the difference in the expression of these genes between WT and overexpressed plants was insignificant. After 4 ℃ treatment, the expression levels of *SlLEA9* and *SlLEA26* in transgenic plants were significantly higher than that in WT plants (Fig. 10 B). These findings revealed that *SlDREB3* overexpression contributes to resisting to low-temperature stress for the transgenic plants, which may be mainly achieved by upregulating *SlLEA* expression.

4. Discussion

DREB TFs is an important TF in plants, which can respond to various abiotic stresses (Erpen et al., 2017). Although numerous *DREB* genes

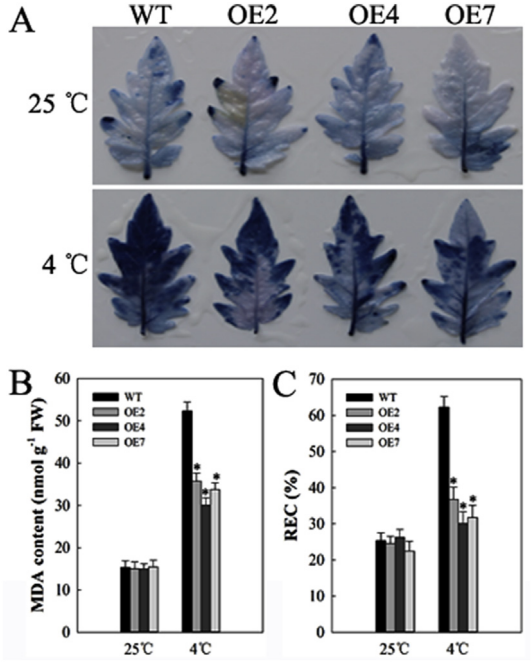

Fig. 8. Cell injury assays in 6-week-old WT and transgenic lines. (**A**) Trypan blue staining. The upper layer represents the plants placed at 25 °C, and the under layer refers to the plants subjected to 4 °C for 24 h. (**B**) MDA content. (**C**) REC.

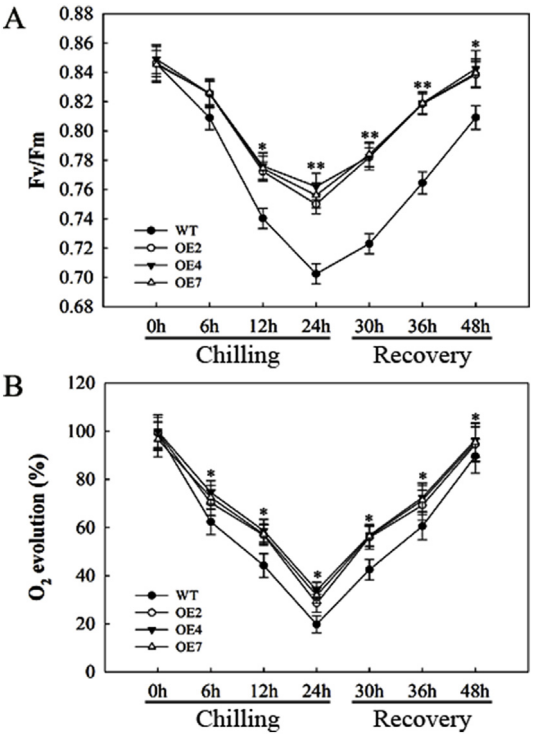

Fig. 9. Fv/Fm and O_2-evolving activities of 6-week-old transgenic and WT plants under chilling stress and recovery stage. (**A**) Fv/Fm. (**B**) O_2-evolving activities.

have been isolated and cloned, and their roles have been studied in *Arabidopsis*, rice, wheat and maize, only a few of these DREB TFs in tomato has been extensively studied in their biological functions, e.g. SlDREB plays a vital role in positively regulating drought stress and in negatively regulating GA biosynthesis (Li et al., 2012), SlDREB1 in

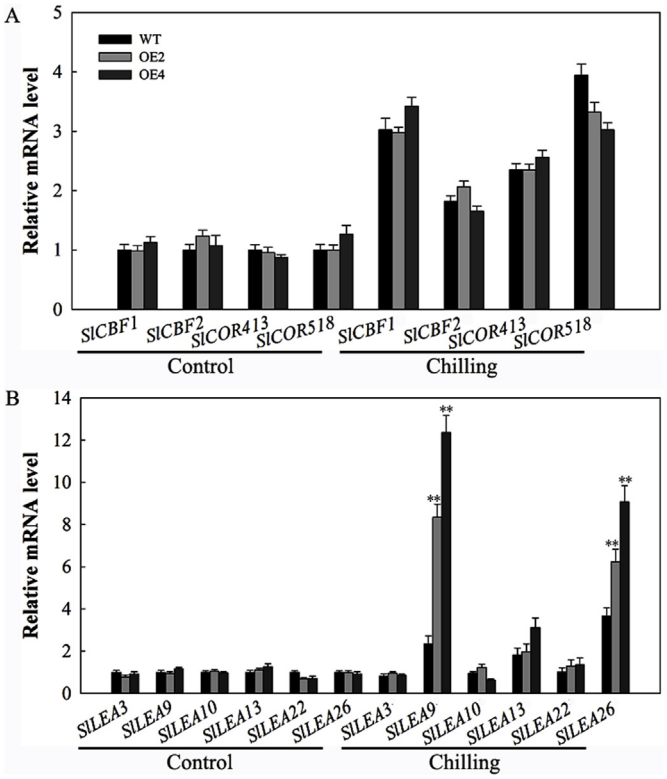

Fig. 10. Cold-related gene expression of 6-week-old transgenic and WT plants. (**A**) *SlCBF1*, *SlCBF2*, *SlCOR413*, and *SlCOR518* expression. (**B**) *SlLEA3*, *SlLEA9, SlLEA10, SlLEA13, SlLEA22*, and *SlLEA26* expression.

enhancing drought tolerance (Jiang et al., 2017), and SlDREB2 in positively regulating salt stress (Hichri et al., 2016). In our study, a cDNA of *SlDREB3* was isolated from tomato. SlDREB3 was located in the nucleus and exhibited the activity of transcriptional activation in yeast cells (Figs. 1 and 2), thereby indicating that SlDREB3 is an activating TF. The phylogenetic tree analysis showed that SlDREB3 belongs to the A-6 subgroup (Fig. S1). Islam and Wang (2009) reported that SlDREB3 shares 60% similarity with a RAP2.4 in *Arabidopsis thaliana*. *RAP2.4* can response to drought stress and participate in the regulation of a variety of developmental processes, such as flowering time, root elongation, root hair formation, and chloroplast peroxidase activity (Lin et al., 2008; Rudnik et al., 2017). Upadhyay et al. (2017) reported that the ectopic expression of *SlDREB3* affects photosynthesis, transpiration, germination, and lateral root growth by reducing ABA levels. However, the function of SlDREB3 under chilling stress remains unreported. The *SlDREB3* expression was induced by oxidative and chilling stresses (Fig. 3 A and C). After the chilling treatment, the phenotype of seedlings and mature transgenic plants and related physiological indicators revealed that *SlDREB3* overexpression promoted the chilling resistance of transgenic tomatoes (Fig. 5). These results indicated that SlDREB3 played a significant function in plant responses to cold stress.

ABA is a major hormone in plants that can regulate abiotic stress through its own activities (Fujita et al., 2011). In the process of DREB TFs responding to abiotic stress, two signaling pathways, namely, ABA-dependent and -independent pathways, which are closely related through some components of the same nature and together constitute a complex signal transduction network and ultimately make plants tolerant to stress, were observed. Our studies showed insignificant difference in ABA content between WT and transgenic lines, except that ABA content increased after chilling stress (Fig. 6). This result indicated that SlDREB3 may respond to chilling stress through ABA-independent signaling pathway.

Various stresses, including chilling stress, may generate excessive

ROS accumulation, cause oxidative damage to cells, and disrupt cellular function. Thus, plants maintain low ROS levels by removing excess ROS production (Mittler, 2002; Miller et al., 2010; Suzuki et al., 2012). After chilling stress, H_2O_2 and $O_2^{\bullet -}$ were determined, and the content of H_2O_2 and $O_2^{\bullet -}$ in the leaves of transgenic plants was lower than that of WT plants (Fig. 7 A, B, C and D). Plants have evolved a set of ROS detoxification system to maintain the intracellular ROS homeostasis, wherein several enzymes, such as CAT and SOD, play essential roles (Miller et al., 2010). In this study, the CAT and SOD activities were higher in transgenic plants than those in WT plants, and the increased activities of these enzymes in transgenic plants were caused by the high *SlCAT* and *SlZnCuSOD* gene expression levels (Fig. E, F, G and H). Cell membrane systems were the major target of chilling injury and particularly sensitive to ROS-induced lipid peroxidation. After chilling stress, *SlDREB3*-overexpressing lines showed less membrane damage in relative to WT plants (Fig. 8). These findings consumingly suggested that *SlDREB3* overexpression could protect plants from ROS-initiated cellular injury under cold stress. The O_2-evolving activities and Fv/Fm of PSII also declined, and the photoinhibition of PSII was observed. However, the extent of photoinhibition in transgenic lines was less compared with that in WT plants (Fig. 9), thereby indicating that *SlDREB3* overexpression alleviated PSII photoinhibition and enhanced the chilling tolerance of plants.

AtDREB1 overexpression can induce the high expression levels of cold-related genes, including *COR6.6/KIN2*, *COR15A*, and *COR47/RD17* (Liu et al., 1998; Nakashima et al., 2009). This study showed that insignificant differences observed in the expression levels of *SlCOR413*, *SlCOR518*, *SlCBF1*, and *SlCBF2*, between transgenic lines and WT plants with or without chilling stress (Fig. 10 A). This finding suggested that SlDREB3 may not be involved in the CBF–COR signaling pathway. Nonetheless, Dubouzet et al. (2003) reported that *OsDREB1A* overexpression in transgenic *Arabidopsis* causes the high *LEA* gene expression. The expression levels of *SlLEA9* and *SlLEA26* in transgenic lines were significantly higher relative with that in WT plants treatment with 4 °C (Fig. 10 B). The findings revealed that *SlDREB3* overexpression improved the tolerance of transgenic lines to cold stress by activating the *SlLEA* expression.

In summary, *SlDREB3* took participated in plant responses to chilling stress and possibly through the ABA-independent signaling pathway. *SlDREB3* overexpression reduced the excess accumulation of H_2O_2 and $O_2^{\bullet -}$, which can alleviate PSII photoinhibition under chilling stress. *SlDREB3* overexpression improved the chilling tolerance of transgenic tomatoes possibly by up-regulating the expression of *SlLEAs*, a detailed molecular mechanism still requires further research.

Conflicts of interest

The authors declare no conflict of interest.

Author Contributions

Guodong Wang performed most of the experiments and wrote the manuscript. Xinping Xu and Hao Wang analyzed the data, Qi Liu, Xiaotong Yang and Lixiang Liao helped obtain the experimental data. Guohua Cai designed the overall study. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.017.

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