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Research Article

A tomato dynein light chain gene *SlLC6D* is a negative regulator of chilling stress

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ABSTRACT

Dynein light chain (DLC) proteins are an important component of dynein complexes, which are widely distributed in plants and animals and involved in a variety of cellular processes. The functions of *DLC* genes in plant chilling stress remain unclear. In this study, we isolated a *DLC* gene from tomato, designated *SlLC6D*. Promoter analysis revealed many *cis*-elements involved in abiotic stress in the *SlLC6D* promoter. Expression of *SlLC6D* was induced by heat and salt stress, and inhibited by polyethylene glycol and chilling stress. Knockdown of *SlLC6D* in tomato exhibited low relative electrolyte leakage, malondialdehyde content, and reactive oxygen species (ROS) accumulation under chilling stress. The content of proline and activities of superoxide dismutase and peroxidase in knockdown lines were higher than in the wild type and overexpression lines during chilling stress. The high transcript abundances of three cold-responsive genes were detected in knockdown lines in response to chilling stress. Seedling growth of knockdown lines was significantly higher than that of the wild type and overexpression lines under chilling stress. These results suggest that *SlLC6D* is a negative regulator of chilling stress tolerance, possibly by regulating ROS contents and the ICE1–CBF–COR pathway.

1. Introduction

Plants are sessile and inevitably exposed to environmental stresses, such as extreme temperature, salt, drought, and flooding stress. Diverse environmental factors affect plant geographical distribution, growth, and yield [1]. Low temperature is the most important environmental stresses, which can cause damage to many tissues and influence the biological yield and quality of a crop at harvest [2]. Plants alter their morphology, physiology, development, metabolism, and molecular mechanisms to adapt to low temperature stress [3]. Low temperature stress is classified into two categories: chilling stress (0–15 °C) and freezing stress (< 0 °C). As a result, plants can show enhanced tolerance of chilling after exposure to chilling stress, which is a process termed cold acclimation [4].

Plant response to chilling stress is a complex process that involves changes to many metabolic pathways, membrane fluidity, and gene regulation [5]. Membranes can sense the chilling stress signals. In addition, chilling stress affects the stability of cell membranes, which leads to leakage of free ions [6]. Ion leakage rates are elevated under chilling stress [7]. Likewise, chilling stress induces the production of excessive reactive oxygen species (ROS), including superoxide anion (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2), which influences the yield and quality of crops [2]. To avoid ROS-induced damage, plants generally activate the enzymatic antioxidant defense system to scavenge excessive ROS [8]. The ROS-scavenging enzymes include peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) [9]. Overexpression of *CuZnSOD* and *APX* in sweet potato increases SOD and APX activity and enhances salt stress tolerance [10]. Many studies have shown that ROS-scavenging enzyme activity increases under chilling stress. For example, the activities of SOD, POD, and CAT in tomato and apple are enhanced during chilling stress [11,12]. Therefore, the antioxidant defense system is an important mechanism to improve chilling stress.

The molecular basis of the plant response to chilling stress has been

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extensively studied. The INDUCER OF CBF EXPRESSION 1–C-repeat binding factors–Cold-responsive genes (ICE1–CBF–COR) pathway is a low-temperature signal transduction pathway [13]. ICE1 belongs to the MYC-like basic helix–loop–helix (bHLH) transcription family, which recognizes and binds to the sequence CANNTG in the promoter of *CBF* genes and activates their expression [14–16]. ICE1 is ubiquitinated by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), which promotes ICE1 degradation by the 26S proteasome pathway [17]. In addition, ICE1 is sumoylated by the SUMO E3 ligase SlZ1 and phosphorylated at Ser278 by OPEN STOMATA 1 (OST1), resulting in ICE1 stability and transcriptional activity, which can enhance chilling stress tolerance [18,19]. ICE1 is phosphorylated by MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3)/MPK6 resulting in reduced ICE1 stability [20]. Similarly, overexpression of *SlICE1* in tomato enhances chilling stress tolerance [21]. Ectopic expression of *VaICE1* in tobacco increases cold tolerance by improving the activities of ROS-scavenging enzymes [22]. These data suggest that ICE1 can improve the tolerance of chilling stress in plant.

The CBF transcription factors belong to the APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/ERF) family, which can recognize and bind to the *cis*-element G/ACGAC in the promoter of *COR* genes resulting in regulation of their expression under chilling stress [23,24]. In Arabidopsis, three *CBF* genes (*CBF1*, *CBF2*, and *CBF3*) are rapidly induced in response to chilling stress [25,26]. Overexpression of *CBF3* enhances tolerance of freezing stress [27]. The CBF pathway is highly conserved in other flowering plants and performs a similar function. For example, ectopic expression of *SlCBF1* in Arabidopsis enhances tolerance of freezing stress [28]. Overexpression of *GmDREB1B;1/CBF* in soybean protoplasts positively regulates the expression of many soybean-specific stress-responsive genes under abiotic stress [29]. These data imply that the ICE1–CBF–COR pathway plays an important role in response to chilling stress.

Dynein light chain (DLC) was identified in the flagella of *Chlamydomonas* [30]. DLC proteins are an important component of dynein complexes, which are widely distributed in plants and animals and involved in a variety of cellular processes [31,32]. DYNEIN LIGHT CHAIN 1 (LC8) can bind to tubulin and promotes microtubule assembly, resulting in increased stability of microtubules in *Drosophila* [33]. Deletion of the dynein light chain gene *Dyn2p* can affect peroxisomal matrix protein import and alter peroxisome morphology, resulting in change in peroxisome function and biogenesis in yeast [34]. DLC-1 binds to the cell fate regulator GLD-1, resulting in prevention of ectopic germ cell proliferation and facilitating gametogenesis in *Caenorhabditis elegans* [35]. These data suggest that DLC proteins affect many processes involved in microtubule stability and cell fate in animals. However, the function of DLC proteins in flowering plants remains unclear. The plant DLC protein LC8 can interact with the Sirevirus Gag protein, which affects the conformation of the Gas protein in rice and soybean [36]. The transcript abundance of the *AtDLC* and *OsDLC* genes is induced by abiotic stress and phytohormone [32].

Tomato (*Solanum lycopersicum* L.) is a cold-sensitive plant that originated in tropical and subtropical areas. However, tomato is widely cultivated around the world. Low temperature can damage many tissues, such as the leaves, flowers, and fruit, which influences fruit yield and quality. Many breeders aim to breed tomato cultivars with enhanced cold resistance. In this study, we isolated a DLC gene, *SlLC6D*, which was suppressed by chilling stress. Overexpression of *SlLC6D* in tomato was sensitive to low temperature. Conversely, knockdown of *SlLC6D* enhanced tolerance of chilling stress. We showed that *SlLC6D* negatively regulates the tolerance of chilling stress, possibly by modulating the accumulation of ROS under chilling stress.

2. Materials and methods

2.1. Plant growth conditions and treatments

Tomato 'Ailsa Craig' (AC) was used as the transgene recipient in this study. Seeds of AC and the *SlLC6D* transgenic lines were sown in plastic pots containing soil, perlite, and vermiculite (v/v/v = 2:1:1) under the following nonstress conditions: 25 °C, 60 %–65 % relative humidity, and 16 h/8 h (day/light) photoperiod. One-month-old plants were used to the subsequent experiments for abiotic stress. For treatment with chilling stress, the plants were transferred to a climate chamber maintained at 4 °C for 0, 0.5, 1, 2, 4, 6, 12, and 24 h. For heat treatment, the tomato plants were exposed to 42 °C in the climate chamber for 0, 0.5, 1, 2, 4, 6, 12, and 24 h. For indole-3-acetic acid (IAA) and abscisic acid (ABA) treatment, the tomato plants leaves were sprayed with 50 μM/L IAA and 50 μM/L ABA for 0, 0.5, 1, 2, 4, 6, 12, and 24 h. For salt and polyethylene glycol (PEG) treatment, the one-week-old seedlings were transferred to half-strength Murashige and Skoog medium plates supplemented with 100 mM NaCl or 20 % PEG and placed in the climate chamber for 0, 0.5, 1, 2, 4, 6, 12, and 24 h.

For chilling stress treatment of seedlings, seeds of AC and the *SlLC6D* transgenic lines were surface-sterilized and sown on half-strength Murashige and Skoog medium plates under the nonstress condition for 7 days. Six plates were transferred to a climate chamber maintained at 4 °C. Six plates were grown under the nonstress condition in a separate climate chamber. After 7 days, we measured the fresh weight, primary root length, and hypocotyl length of the seedlings.

2.2. Vector construction and tomato transformation

The full-length CDS of *SlLC6D* and RNA interference (RNAi) fragment were generated by PCR using the Q5® Hot Start High-Fidelity DNA Polymerase (NEB, USA) from tomato cDNA using gene-specific primers with 5′-attB1 and 5′-attB2 extensions on forward and reverse primers, respectively (Table S1). The PCR product was constructed onto the pDONR221 vector (Invitrogen, USA) using the BP recombination reaction. Then, the *SlLC6D* CDS and RNAi fragment were cloned into the plant expression vector pGWB402 and pHellsgate8 by the LR recombination reaction, respectively. Finally, the recombinant vector was introduced into tomato of cv. Alisa Craig (AC) by Agrobacterium-mediated transformation. The positive transgenic lines were identified by PCR using genomic DNA.

2.3. RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from tomato leaves using TRIzol™ Reagent (Invitrogen, USA). The first-strand cDNA was synthesized from the purified RNA using the M-MLV Reverse Transcriptase kit (Toyobo, Japan). qPCR was performed using the GoTaq® Probe qPCR Master Mix (Promega, USA) following the manufacturer's instructions. Primer sequences are listed in Table S1. The *Actin* gene (GenBank accession no. BT013524) was used as the internal reference gene.

2.4. Gene structure and conserved domains analysis

The structure of *SlDLC* genes were analyzed using GSDS 2.0 (Gene Structure Display Server, http://www.mybiosoftware.com/gsds-2-0-gene-structure-display-server.html). The conserved domains of SlDLC proteins were predicted using the online MEME server (http://meme-suite.org/). The parameters were set as follows: maximum numbers of different motifs, 10; minimum width, 6; and maximum width, 50.

A phylogenetic tree was constructed with MEGA7.0 using the neighbor-joining method. The parameters were set as follows: bootstrap, 1000 replicates; p-distance; and pairwise deletion.

Table 1
Summary of physiological and biochemical properties of tomato dynein light chain (SlDLC) proteins.

Gene ID	Animo acids	MW (kDa)	pI	GRAVY	Instability index	Aliphatic index
Solyc01g005460	130	14.68	8.68	−0.243	42.68	82.54
Solyc01g066590	119	13.06	8.59	−0.482	23.27	67.98
Solyc03g113540	257	28.61	9.61	−0.456	39.31	67.90
Solyc03g120690	94	10.67	7.76	−0.034	30.13	74.68
Solyc06g071180	269	30.18	9.75	−0.543	38.09	66.65
Solyc07g063180	147	16.33	9.84	−0.161	67.31	77.69
Solyc07g063610	142	16.14	4.83	−0.064	27.34	78.45
Solyc12g006630	224	25.28	9.74	−0.426	32.59	73.04
Solyc12g044240	94	10.50	6.04	0.056	37.59	77.87

MW, Molecular weight of the amino acid sequence; pI, theoretical isoelectric point; GRAVY, grand average of hydropathicity.

2.5. Relative electrolyte leakage (REL) assay

The REL was measured using a method described previously [11]. After chilling treatment for 0 and 4 days, ten leaf discs (each of 0.5 cm in diameter) from each line were harvested and put into a 15 mL glass tube containing 10 mL of deionized water. Then, the tube was shaken at 100 cycles/min for 30 min under normal conditions. The initial electrolyte leakage (C1) was measured with a conductivity meter (Mettler Toledo FE30, Switzerland) at 25 °C. Then, the glass tube was boiled for 30 min and cooled to 25 °C. The final electrolyte leakage (C2) was measured at 25 °C. The blank control (C0) was the electrolyte leakage of deionized water. The REL was calculated as followed by: (C1-C0)/(C2-C0) × 100 %.

2.6. Measurement of malondialdehyde and proline contents

The MDA content was measured using the thiobarbituric acid method as previously described [11]. The proline content was measured using the acid ninhydrin method as previously described [11].

2.7. Histochemical staining

Hydrogen peroxide was detected by 3,30-diaminobenzidine (DAB) staining as previously described [11]. Superoxide anion was detected by nitro blue tetrazolium (NBT) staining as previously described [11].

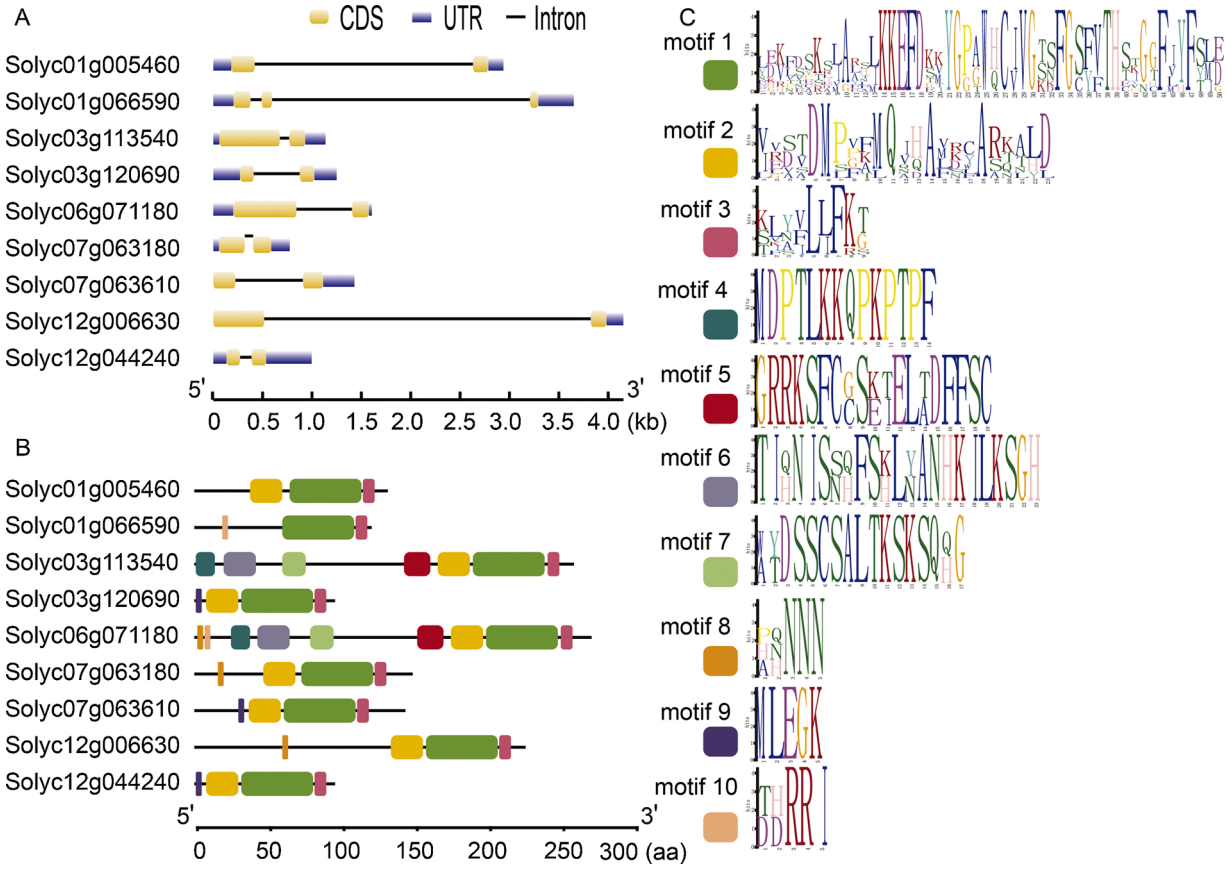

Fig. 1. Genomic structure and conserved motifs of *DLC* genes in tomato. (A) Exon–intron structure of *SlDLC* genes, where a yellow box indicates an exon, a black line indicates an intron, and a blue box indicates an untranslated region. (B) The distribution of conserved motifs in SlDLC proteins, where the conserved motifs are indicated by a colored box. (C) Sequences of the 10 conserved motifs in the SlDLC proteins identified in this study (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

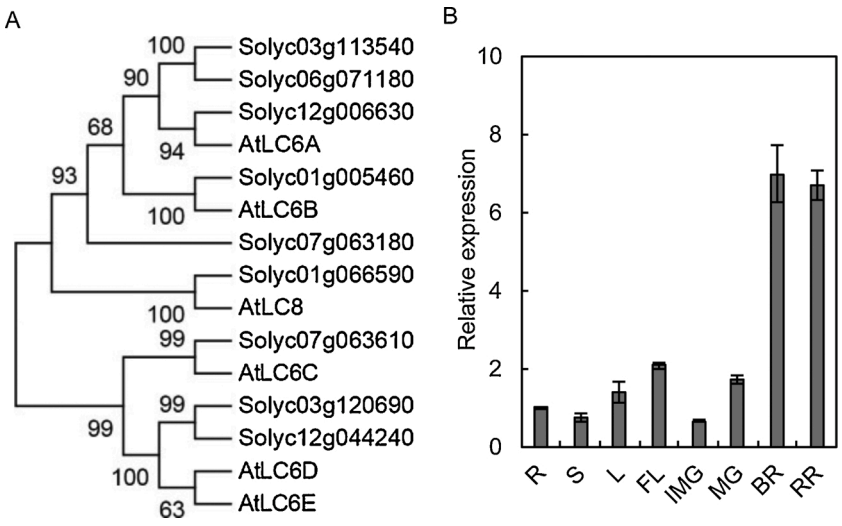

Fig. 2. Sequence analysis of SlLC6D. (A) Phylogenetic tree of SlLC6D and *Arabidopsis thaliana* DLC proteins. The phylogenetic tree was constructed with MEGA7.0 using the neighbor-joining method. DLC protein sequences from tomato (Solyc01g005460, Solyc01g066590, Solyc03g113540, Solyc03g120690, Solyc06g071180, Solyc07g063180, Solyc07g063610, Solyc12g006630, and Solyc12g044240) and *Arabidopsis thaliana* (AtLC6B: At1g23220, AtLC6D: At3g16120, AtLC8: At4g15930, AtLC6C: At4g27360, AtLC6A: At5g20110, and AtLC6E: At1g52245) were used. The accession numbers refer to the SGN (https://solgenomics.net/) and TAIR (https://www.arabidopsis.org/) databases, respectively. (B) Transcript abundance of *SlLC6D* in different tomato organs. R: Root; S: Shoot; L: Leaf; Fl: Flower; IMG: Immature fruit; MG Mature fruit; BR: Breaker fruit; RR: Red ripe fruit. Values are represented as means ± SE (n = 3), with three biological replicates in the experiment (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

2.8. Measurement of peroxidase (POD) and superoxide dismutase (SOD) activities

Leaf samples (approximately 0.2 g per sample) were ground in 1 mL of ice precooling 100 mM PBS (pH 7.0) containing 1 mM EDTA (ethylenediaminetetraacetic acid), 0.1 % (v/v) Triton X-100, and 1% (w/v) PVP (polyvinylpolypyrrolidone). The mixture was then centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatants were used for measurement of the activities of the antioxidant enzymes SOD and POD. The activities of POD and SOD were measured as previously described [11].

3. Results

3.1. Identification and characterization of DLC genes in tomato

Nine putative *DLC* genes have been detected in the tomato genome [32]. The physiological and biochemical properties of the DLC proteins were analyzed using the Prosite database (https://prosite.expasy.org/prosite.html). Information on the nine SlDLC proteins is presented in Table 1. The length of the SlDLC proteins ranged from 94 (Solyc03g120690 and Solyc12g044240) to 269 (Solyc06g071180) amino acids. The predicted molecular weights ranged from 10.50 to 30.18 kDa. The theoretical isoelectric point (pI) ranged from 4.83 to 9.84. The grand average of hydropathicity (GRAVY) ranged from −0.543 to 0.056. The instability index ranged from 23.27 to 67.31, and the aliphatic index ranged from 66.65 to 82.54.

Gene structure analysis revealed that the *SlDLC* genes contained two or three exons (Fig. 1A). The majority of *SlDLC* genes consisted of two exons, whereas Solyc01g066590 contained three exons. This suggests that exon loss and gain have both occurred in the *DLC* gene family. The distribution of conserved domains in the SlDLC proteins was predicted using the online MEME server. Ten putative conserved motifs in the SlDLC proteins were identified, and the number of conserved domains in SlDLC proteins ranged from two to eight (Fig. 1B). The length of conserved domains ranged from 6 to 50 (Fig. 1C). Analyses of Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) showed that motif 1 belonged to the domain of dynein light chain type 1, while the other motifs were uncharacterized. Motif 1 and motif 2 were found in all SlDLC proteins. Motif 3 was presented in 8 of the 9 SlDLC proteins. The remaining seven motifs were unique in part of these proteins, like motif 4 in Solyc06g071180 and Solyc03g113540, and motif 8 in Solyc07g063180 and Solyc12g006630 (Fig. 1B). These data suggested that the SlDLC proteins displayed extreme divergence during the evolutionary process.

Table 2
Predominant cis-elements detected in the promoter of *SlLC6D*.

Factor or site name	Signal sequence	site	Function
ABRE	ACGTG	2252 (-)	cis-acting element involved in the abscisic acid responsiveness
MYB	CAACAG TAACCA	848 (-); 920 (-); 1021 (-)	MYB binding site
WUN-motif	CCATTTCAA	64 (-)	wound-response element
TC-rich repeats	ATTCTCTAAC	91 (-)	cis-acting element involved in defense and stress responsiveness
G-box	TAACACGTAG TACGTG	952 (+); 2252 (-)	cis-acting regulatory element involved in light responsiveness
ARE	AAACCA	411 (+); 455 (+)	cis-acting regulatory element essential for the anaerobic induction
TGA-element	AACGAC	2864 (-)	auxin-responsive element
CAT-box	GCCACT	575 (+)	cis-acting regulatory element related to meristem expression
GARE-motif	TCTGTTG	847 (+)	gibberellin-responsive element
MRE	AACCTAA	566 (-)	MYB binding site involved in light responsiveness
W box	TTGACC	308 (+)	WRKY binding site
AT1-motif	AATTATTTTTTATT	1774 (-)	part of a light responsive module
I-box	TGATAATGT	782 (+)	part of a light responsive element
GT1-motif	GGTTAAT GGTTAA	921 (+); 1022 (+); 2728 (+)	light responsive element
TCT-motif	TCTTAC	1540 (-)	part of a light responsive element
HD-Zip I	CAAT(A/T)ATTG	372 (+)	element involved in differentiation of the palisade mesophyll cells
circadian	CAAAGATATC	1388 (+)	cis-acting regulatory element involved in circadian control
Box II	TGGTAATAA	2373 (-)	part of a light responsive element
GATA-motif	AAGATAAGATT AAGGATAAGG	1471 (-); 1813 (+)	part of a light responsive element

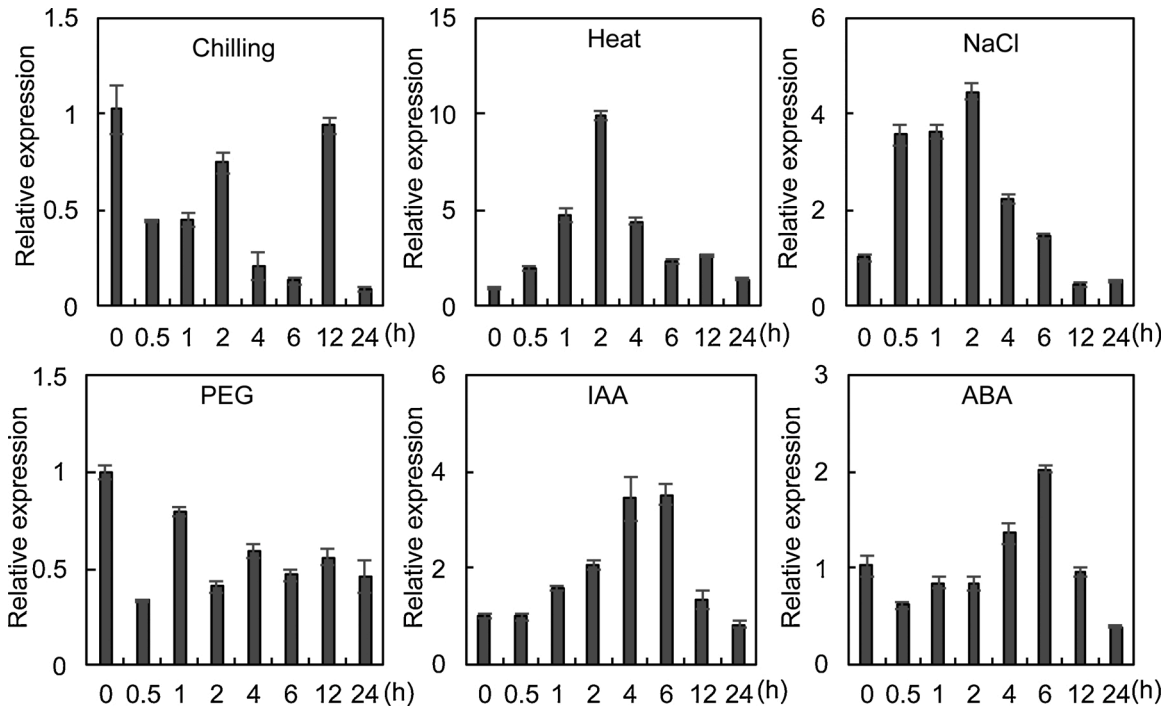

Fig. 3. Quantitative real-time PCR analysis of *SlLC6D* transcript abundance in tomato leaves following abiotic stress treatment (chilling, heat, NaCl, or polyethylene glycol [PEG]) or plant hormone treatment (indole-3-acetic acid [IAA] or abscisic acid [ABA]). Values are represented as means ± SE (n = 3), with three biological replicates in the experiment.

3.2. Sequence analysis of SlLC6D

We isolated the full-length cDNA of *SlLC6D* (SGN accession no. Solyc03g120690) with a length of 285 bp that encodes a deduced protein of 94 amino acids. The *SlLC6D* gene contained two exons and three introns (Fig. 1A). A phylogenetic analysis of six DLC proteins from Arabidopsis [32] and the nine SlDLC proteins from tomato indicated that the amino acid sequence of SlLC6D showed high identity with AtLC6D and AtLC6E (Fig. 2A). Therefore, this gene was named *SlLC6D*.

We estimated the transcript abundance of *SlLC6D* using quantitative real-time PCR (qPCR) analyses of RNA extracted from the root, stem, leaf, flower, immature fruit, mature fruit, breaker fruit, and red ripe fruit of tomato. The transcript level of *SlLC6D* was highest in red ripe fruit and lowest in immature fruit, whereas a moderate transcript abundance was detected in the root, leaf, and flower (Fig. 2B).

3.3. Promoter analysis of SlLC6D

Cis-elements are recognized and bound by transcription factors, resulting in regulation of gene expression. To analyze the *cis*-elements of the *SlLC6D* promoter, the 3 kb sequence upstream from the *SlLC6D* start codon was analyzed using the PlantCARE database. Many *cis*-elements detected were involved in abiotic stress response, including ABRE (ABA-responsive element), GT1-motif (light-responsive element), GARE-motif (GA-responsive element), and TGA-element (auxin-responsive element) (Table 1). In addition, we detected two LTR (low-temperature-responsive) *cis*-elements involved in low-temperature stress in the *SlLC6D* promoter (Table 2). These data suggested that the expression of *SlLC6D* might be induced by abiotic stresses.

3.4. Expression of SlLC6D is induced under abiotic stress in tomato

We investigated the transcript abundance of *SlLC6D* in response to a variety of abiotic stress treatments in tomato. Expression of *SlLC6D* was substantially up-regulated at an early stage with a 3.3-fold increase after 0.5 h, and decreased after 6 h, when plants exposed to chilling (4 °C) stress (Fig. 3). Similar to the chilling stress treatment, *SlLC6D* expression increased at an early stage, and decreased after 4 h, in plants treated with heat (42 °C) and salt (100 mM NaCl) stress (Fig. 3). Conversely, the transcript abundance of *SlLC6D* was rapidly decreased under simulated drought (20 % polyethylene glycol, PEG) treatment (Fig. 3). These data suggested that *SlLC6D* may be involved in the responses to various abiotic stresses in tomato. In addition, we investigated the *SlLC6D* transcript level in response to exogenous phytohormones (indole-3-acetic acid, IAA; and abscisic acid, ABA). Expression of *SlLC6D* was increased at 1 h, and peaked after 6 h, in response to IAA treatment. Expression of *SlLC6D* was slightly decreased within 2 h, and thereafter increased peaking at 6 h, in response to exogenous ABA (Fig. 3).

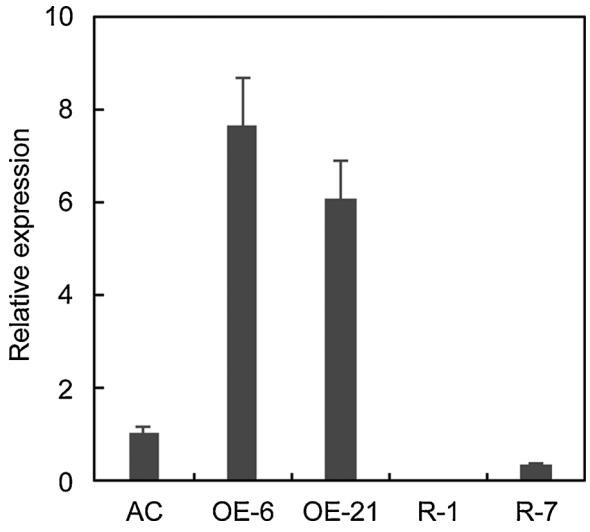

Fig. 4. Quantitative real-time PCR analysis of *SlLC6D* transcript abundance in tomato leaves of transgenic lines. Values are represented as means ± SE (n = 3), with three biological replicates in the experiment.

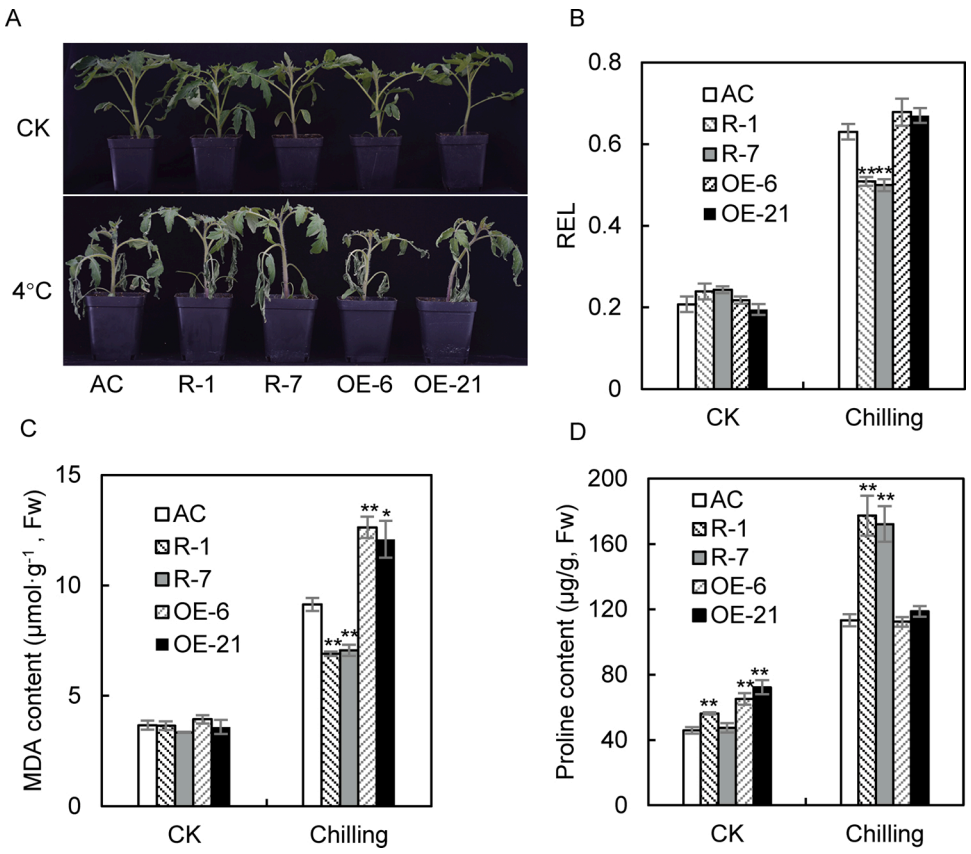

Fig. 5. Growth of *SlLC6D* transgenic lines and the wild type under control (CK) and chilling stress treatments. (A) Phenotype, (B) relative electrolyte leakage (REL), (C) malondialdehyde (MDA) content, and (D) proline content of the wild type ('Ailsa Craig'; AC) and *SlLC6D* transgenic lines (RNAi lines R-1 and R-7; overexpression lines OE-6 and OE-21) under chilling stress. Values are represented as means ± SE (n = 4), with three biological replicates in the experiment. Asterisks denote significant differences from the wild type (AC) as indicated by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

3.5. Transformation and characterization of transgenic tomato plants

To investigate the function of *SlLC6D* in response to chilling stress in tomato, we produced an overexpression (OE) construct (pBI121) containing the full-length cDNA of *SlLC6D* under the control of the *Cauliflower mosaic virus* 35S promoter, and generated two *SlLC6D*-OE tomato lines (OE-6 and OE-21). In addition, we produced an RNA interference (RNAi) vector (pHellsgate 2) and generated two RNAi lines (R-1 and R-7). We examined the transcript level of *SlLC6D* in the transgenic plants using qPCR assays. The *SlLC6D* transcript level in leaves of the OE lines was higher than that in the wild type ('Ailsa Craig', AC), whereas transcript abundance was lower in leaves of the RNAi lines (Fig. 4).

3.6. SlLC6D is a negative regulator of chilling stress in tomato

To investigate the role of *SlLC6D* in the chilling stress response, 5-week-old transgenic (OE and RNAi lines) and control plants were exposed to chilling stress (4 °C) for 4 days. No significant differences in growth and morphology between the transgenic and control plants were observed under the nonstress treatment (CK). Under chilling stress, the leaves of all plants wilted. The degree of wilting was weaker in RNAi lines than the control and OE lines (Fig. 5A). In addition, we analyzed the relative electrolyte leakage (REL) and the contents of malondialdehyde (MDA) and proline in leaves. The REL and MDA content were both dramatically lower in the RNAi lines compared with those of the wild type (AC) and OE lines (Fig. 5B, C). The proline content in the RNAi lines was notably higher than that in AC and the OE lines (Fig. 5D). These data implied that chilling stress damage was reduced in the RNAi lines compared with that in AC and the OE lines.

3.7. SlLC6D might affects ROS accumulation under chilling stress

Previous studies have suggested that chilling stress induces the production of excessive ROS, which causes damage to membranes and promotes programmed cell death [37]. We determined the accumulation of H_2O_2 and O_2^- by staining with 3,30-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively, in leaves of tomato plants exposed to chilling stress (4 °C). No significant difference in H_2O_2 content between AC and *SlLC6D* transgenic lines was observed under the CK treatment. After chilling stress for 3 days, the intensity of brown staining with DAB was noticeably weaker in the RNAi lines than in AC and the OE lines (Fig. 6A). Similarly, the intensity of blue staining with NBT in RNAi lines was weaker than that in AC and the OE lines under chilling stress for 3 days (Fig. 6B). These results implied that suppression of *SlLC6D* can mitigate the contents of H_2O_2 and O_2^- under chilling stress.

To avoid ROS damage, plants have the capacity to scavenge excessive ROS through promotion of the enzymatic antioxidant defense system, which includes POD, CAT, SOD, and APX. These enzymes play crucial roles in scavenging excessive ROS and regulating the balance of ROS in plant cells. We measured the activities of POD and CAT in AC and the transgenic lines under the CK and chilling stress treatments. No significant difference in activities of POD and SOD were observed between AC and the transgenic lines under the CK treatment. The activities increased rapidly under chilling stress for 3 days. The activities of POD and SOD were higher in the RNAi lines than in AC and the OE lines (Fig. 6C, D). In addition, the transcript levels of *SlSOD* and *SlPOD* in the RNAi lines were higher than those in AC and the OE lines under chilling stress (Fig. 6E, F). These data suggested that *SlLC6D* improved the ability to remove excessive ROS through the enzymatic antioxidant defense system under chilling stress.

3.8. SlLC6D affects the tolerance of seedlings to chilling stress

To investigate the function of *SlLC6D* in the response to chilling stress at the seedling stage, we analyzed the growth of 10-day-old seedlings under the CK and chilling stress treatments for 7 days. The seedlings of

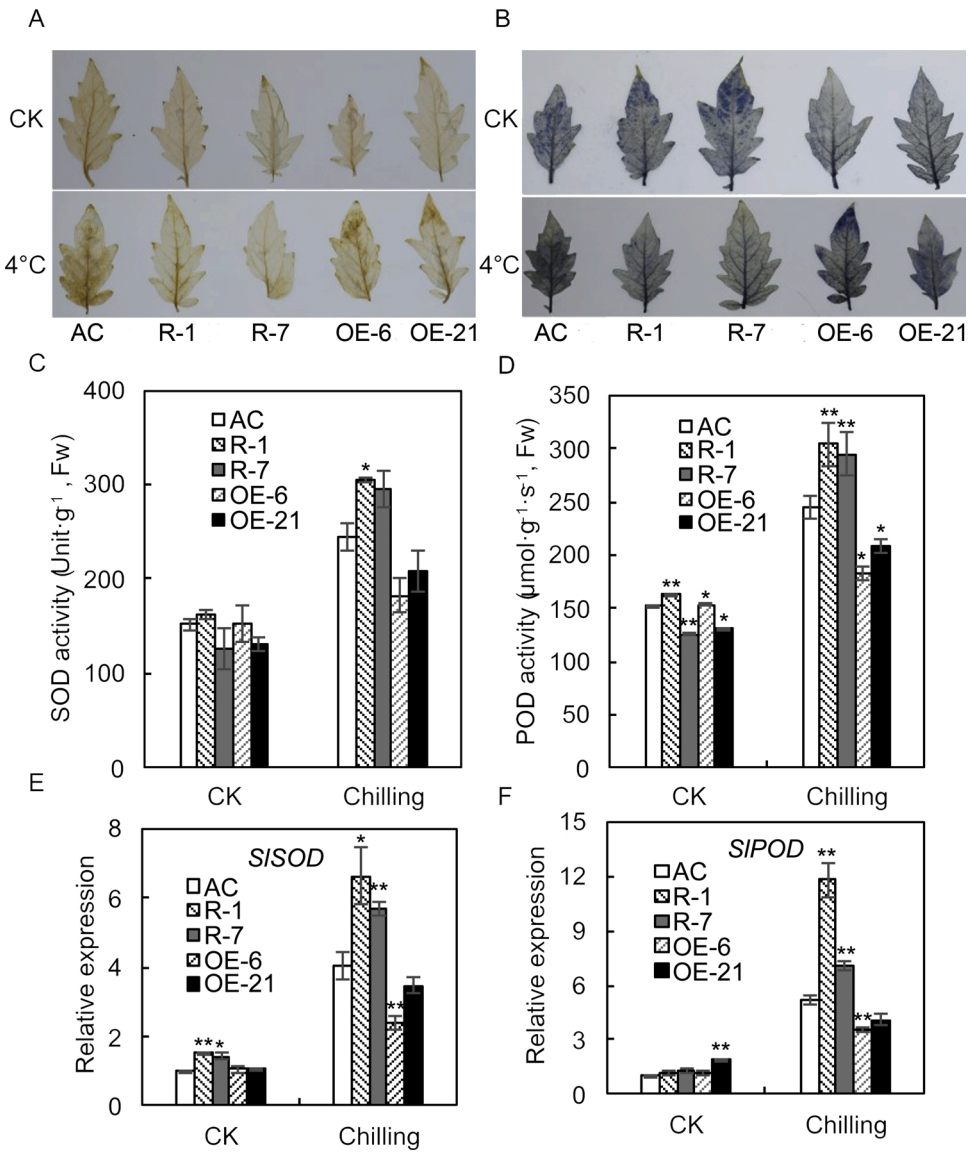

Fig. 6. Involvement of *SlLC6D* in reactive oxygen species (ROS) scavenging in tomato plants under chilling stress. (A) DAB staining for H_2O_2 in leaves under chilling stress. (B) NBT staining for O_2^- in leaves of the wild type ('Ailsa Craig'; AC) and *SlLC6D* transgenic lines (RNAi lines R-1 and R-7; overexpression lines OE-6 and OE-21) under chilling stress. (C) Superoxide dismutase (SOD) activity and (D) peroxidase (POD) activity in the leaf. (E) Relative expression level of *SlSOD* and (F) relative expression level *SlPOD* in the leaf. Values are represented as means ± SE (n = 4 in C and D, 3 in E and F), with three biological replicates in the experiment. Asterisks denote significant differences from the wild type (AC) as indicated by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

AC and the transgenic lines grew well and showed no significant differences under the CK treatment. The growth of AC and *SlLC6D* transgenic seedlings was suppressed under chilling stress. The growth of the OE lines was more severely depressed compared with that of AC and the RNAi lines (Fig. 7A). The seedling fresh weight and lengths of the primary root and hypocotyl in AC and the transgenic lines showed no significant differences under the CK treatment (Fig. 7B). The seedling fresh weight and hypocotyl length of the OE lines were lower than those of AC and the RNAi lines (Fig. 7D). However, the primary root length of all lines showed no significant differences (Fig. 7C). On the basis of these data, we concluded that suppression of *SlLC6D* expression enhanced the tolerance of seedlings to chilling stress.

3.9. Expression of chilling stress-related genes

To investigate the molecular mechanism of *SlLC6D* function in response to chilling stress, we used qPCR to analyze the transcript abundance of genes in the ICE–CBF–COR pathway. The transcript abundances of *SlICE1*, *SlCBF1*, and *SlDRCi7* differed slightly in AC and the *SlLC6D* transgenic lines under the CK treatment. After chilling stress, the transcript abundances of *SlICE1* and *SlCBF1* were lower in the OE lines and higher in the RNAi lines compared with those of AC. The transcript level of *SlDRCi7* was significantly higher in the RNAi lines than in AC and the OE lines (Fig. 7). These results suggested that *SlLC6D* may be involved in promoting the ICE–CBF–COR pathway under chilling stress in tomato.

4. Discussion

The DLC protein family is an important component of the dynein complexes involved in a variety of cellular functions. In animals, DLC1 interacts with tubulin and promotes microtubule assembly, which increases the stability of microtubules [33]. DYNLL1 belongs to the DLC family, which interacts with a microtubule-associated adaptor that regulates spindle orientation in humans [38]. DLC1 can bind to the Bim protein, which promotes the assembly of large Bim complexes on mitochondria in human cells [39]. DLC-1 can bind to GLD-1, which is a cell fate regulator protein involved in meiosis and germ cell differentiation in *Caenorhabditis elegans* [35]. However, the functions of DLC proteins in plants remain unclear. Nine *SlDLC* genes were detected in the tomato genome (Table 1). The transcription of *DLC* genes are induced by abiotic stresses [32]. In the present research, we identified a DLC gene, *SlLC6D*, which could improve the tolerance of chilling stress in tomato. We speculated that the function of *SlLC6D* was associated with chilling

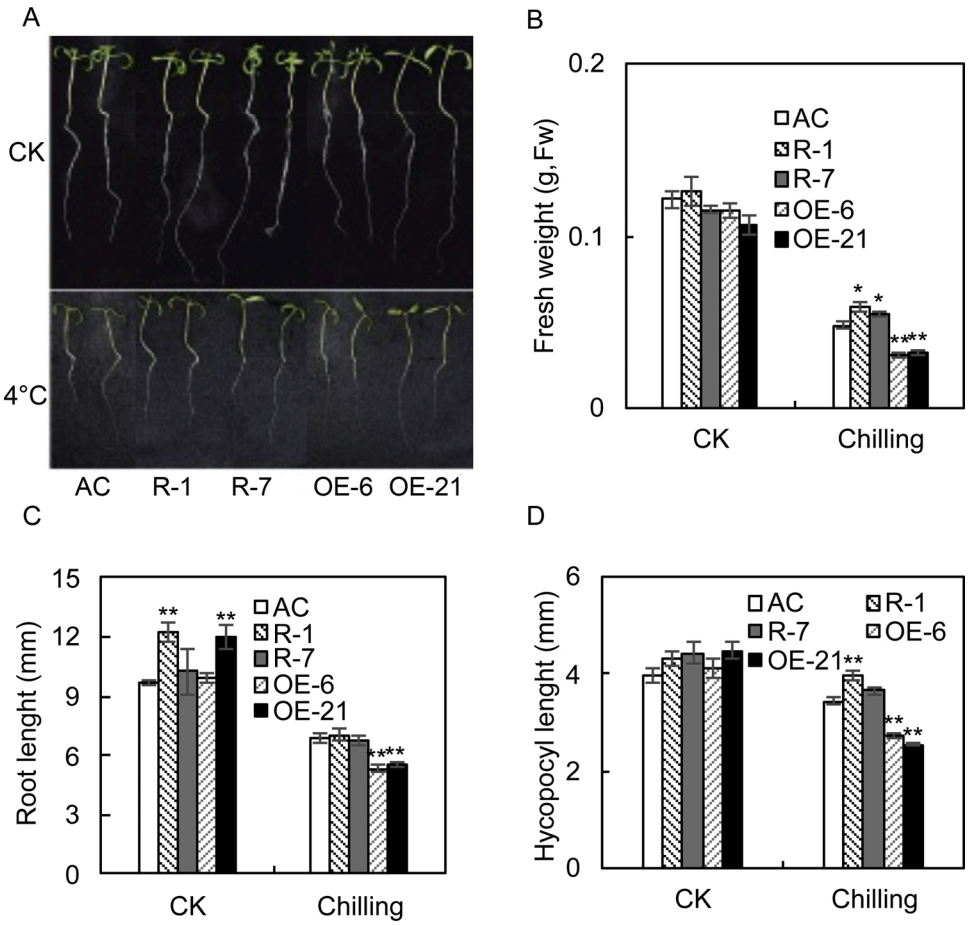

Fig. 7. Growth of tomato seedlings under chilling stress. (A) Seedling phenotype, (B) fresh weight, (C) primary root length, and (D) hypocotyl length of the wild type ('Ailsa Craig'; AC) and *SlLC6D* transgenic lines (RNAi lines R-1 and R-7; overexpression lines OE-6 and OE-21) under chilling stress. Values are represented as means ± SE (n = 6), with three biological replicates in the experiment. Asterisks denote significant differences from the wild type (AC) as indicated by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

stress because *SlLC6D* expression was suppressed by chilling stress. To determine the function of *SlLC6D* in response to low temperature, we created loss-of-function and potential gain-of-function transgenic tomato plants using RNAi technology and overexpression, respectively. The RNAi lines were insensitive to chilling stress, which suggested that *SlLC6D* is a negative regulator of chilling stress tolerance.

Plant cell membranes play a crucial role in sensing and transmitting environmental stimulation signals in response to abiotic stress [40]. The structure of cell membranes is damaged when plants are exposed to low temperature [41]. The composition of membrane lipids changes under chilling stress, which leads to increase in the REL [42]. The REL is higher under chilling stress compared with a nonstress condition [43]. In addition, MDA is produced as a result of membrane lipid peroxidation during exposure to low temperature [44]. The present data showed that the REL and MDA content in the RNAi lines were lower than those in AC and the OE lines under chilling stress (Fig. 5). Proline is a soluble amino acid that can protect cell membranes and scavenge ROS [41]. The proline content was significantly higher in the RNAi lines compared with that of AC and the OE lines under chilling stress (Fig. 5). These results indicated that *SlLC6D* is a negative regulator of tolerance to chilling stress.

Chilling stress can induce the production of large amounts of ROS, which damages DNA, proteins, and membranes as a result of oxidative stress [45]. The present results showed that the contents of H_2O_2 and O_2^- in RNAi lines were significantly lower than those of AC and the OE lines during chilling stress (Fig. 6). In plants the enzymatic antioxidant

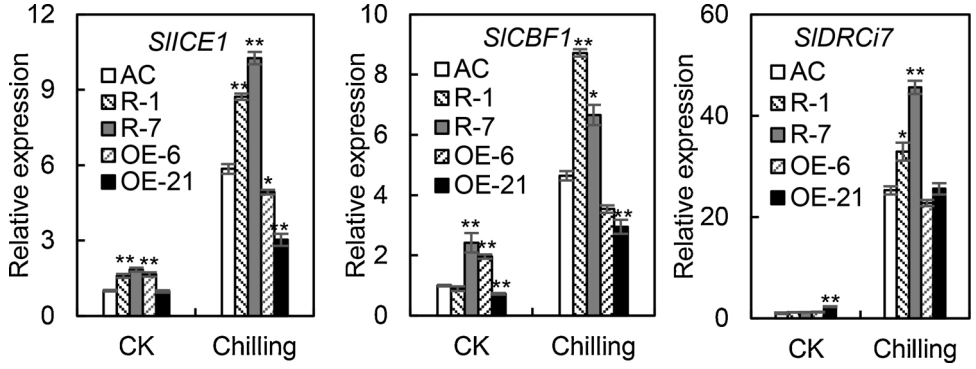

Fig. 8. Relative expression of three cold-responsive genes in the wild type ('Ailsa Craig'; AC) and *SlLC6D* transgenic lines (RNAi lines R-1 and R-7; overexpression lines OE-6 and OE-21) under chilling stress. Values are represented as means ± SE (n = 3), with three biological replicates in the experiment. Asterisks denote significant differences from the wild type (AC) as indicated by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

defense system, including SOD and POD, is promoted to scavenge excessive ROS under abiotic stress [37]. In the present study, the activities of SOD and POD in the RNAi lines were significantly higher compared with those of AC and the OE lines during chilling stress (Fig. 6). In addition, expression of *SlSOD* and *SlPOD* was upregulated in the RNAi lines compared with that of AC and the OE lines under chilling stress (Fig. 6). These data indicated that *SlLC6D* improved chilling tolerance in tomato by promoting the enzymatic antioxidant defense system.

The ICE1–CBF–COR pathway plays an important role in the response to chilling stress in plants [13]. ICE1 binds to the promoter of *CBF1* and regulates the expression level of CBF1 during cold stress [15]. In Arabidopsis three CBF genes play crucial roles in response to chilling stress [6]. CBF1 binds to the C-repeat/DRE DNA regulatory element resulting in activation of COR genes expression under cold stress [24]. The *cbf2* mutant shows higher tolerance of freezing compared with that of the wild type and increased expression levels of *CBF1* and *CBF3* [46]. The *CBF* genes in tomato perform similar functions. Ectopic expression of *SlCBF1* in Arabidopsis enhances freezing tolerance [28]. *SlICE1* improves tolerance of chilling stress and affects the transcript abundance of *SlCBF1* and *SlDRCi7* [21]. The expression levels of *SlCBF1* and *SlCBF2* are promoted by chilling stress [47]. The present data showed that SlLC6D affected the expression levels of *SlICE1*, *SlCBF1*, and *SlDRCi7* under chilling stress in tomato (Fig. 8). These results suggested that SlLC6D may regulate the tolerance of chilling stress in tomato plants via the ICE–CBF–COR pathway.

5. Conclusions

In the present study, we cloned tomato *SlLC6D*, which is a member of the dynein light chain gene family. Our data showed that *SlLC6D*-overexpression lines are more sensitive to chilling stress, whereas *SlLC6D*-silenced lines show enhanced tolerance of chilling stress. SlLC6D negatively affects the ROS scavenging system, which affects the tolerance of chilling stress. In addition, *SlLC6D* affects the transcript levels of genes in the ICE–CBF–COR pathway. Elucidation of the regulatory mechanisms of SlLC6D will be helpful to understand the mechanism by which plants respond to chilling stress.

CRediT authorship contribution statement

Tixu Hu: Conceptualization, Writing - original draft, Writing - review & editing. **Shufeng Wang:** Conceptualization, Experimentation, Writing - original draft, Writing - review & editing. **Qi Wang:** Experimentation, Data curation. **Xin Xu:** Experimentation, Data curation. **Qiqi Wang:** Data curation. **Xiangqiang Zhan:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110753.

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