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Original Paper

SlICE1 encoding a MYC-type transcription factor controls cold tolerance in tomato, *Solanum lycopersicum*

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Abstract Many abiotic and biotic stresses can reduce plant growth and development. Low temperature is one of the most harmful abiotic stresses, particularly for plants that are tropical or subtropical in origin. To elucidate the molecular mechanisms underlying the cold-stress response, components involved in the signal transduction of cold stress have been characterized. In this study, we characterized a basic helix–loop–helix (bHLH) transcription factor encoding gene, *SlICE1*, from tomato (*Solanum lycopersicum*), which shows similarity with *Arabidopsis ICE1*. The expression of *SlICE1* was observed in younger leaves, flowers, and green and red fruits. To characterize the function of *SlICE1*, overexpressing tomato lines were produced. *SlICE1*-overexpressing tomatoes exhibited chilling tolerance, and *SlICE1* enhanced the expression of cold-responsive genes, such as *SlCBF1* and *SlDRCi7*, as well as accumulation of ascorbic acid. The SlICE1 protein was degraded after cold treatment. These results indicate that *SlICE1* enhances cold tolerance in tomatoes.

Key words: Cold tolerance, signal transduction, transactivation activity.

Abiotic stresses, such as cold, drought, salinity, and heat, significantly affect plant growth, productivity, and the quality of crops. One-third of the total land area of the planet is free of ice, and approximately 40% of this land regularly experiences temperatures below −20°C (Juntilla and Robberecht 1999). Low temperature is a major factor that limits the productivity and geographical distribution of cold-sensitive plant species, particularly in crops such as cucumbers and tomatoes, which originate from tropical regions. Such plants are unable to tolerate freezing and suffer chilling injury when exposed to temperatures in the range of 0–12°C. In contrast, plants from temperate regions, such as wheat and *Arabidopsis*, exhibit tolerance to chilling and freezing stresses (Thomashow 1999).

To adapt to low temperatures, plants develop mechanisms that mitigate cold stress. Several types of genes, such as cold-regulated genes (*CORs*), are controlled in response to cold adaptation (Verlues et al. 2006; Lissarre et al. 2010). The cold hardiness of plants is correlated with the expression level of *COR* genes (Pearce et al. 1996). Moreover, some COR proteins act as protectants by preventing protein aggregation (Nakayama et al. 2008). Many *COR* genes are regulated by *CBF*/*DREB1* (C-repeat binding factor/dehydrin responsive element binding protein 1) (Jaglo-Ottoson et al. 1998; Liu et al. 1998). The expression of *Arabidopsis AtCBF1*/*DREB1B* in tomato plants improves cold tolerance (Singh et al. 2011) and induces several oxidative stress-responsive genes, such as *CAT1* (which encodes catalase), to protect the plants from cold stress (Hsieh et al. 2002). The overexpression of *Arabidopsis AtCBF1*/*DREB1B* in tobacco plants induces the activity of Cu/Zn superoxide dismutase (Yang et al. 2010). The ectopic expression of tomato *SlCBF1* in *Arabidopsis* promotes tolerance to freezing (Zhang et al. 2004).

AtICE1 (inducer of CBF expression) has been identified as a positive regulator of *AtCBF3*/*DREB1A* and its regulon expression. And *AtICE1* positively controls cold tolerance in *Arabidopsis* (Chinnusamy et al. 2003; Lee et al. 2005). *AtICE1* encodes a MYC-type basic helix–loop–helix (bHLH) transcription factor and can bind to MYC recognition elements in the *AtCBF3*/*DREB1A* promoter (Chinnusamy et al. 2003). Overexpression of *AtICE1* in *Arabidopsis* plants enhances tolerance to freezing (Chinnusamy et al. 2003; Miura et al. 2007).

Abbreviations: bHLH, basic helix–loop–helix; CBF, C-repeat binding factor; COR, cold-regulated; DREB, dehydrin responsive element binding protein.

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In wheat, the ICE1 homologs *TaICE141* and *TaICE187* activate the wheat *CBF* group IV genes, which are associated with freezing tolerance. Overexpression of *TaICE141* and *TaICE187* in *Arabidopsis* enhances *CBF*-dependent cold-responsive gene expression and cold tolerance after cold acclimation (Badawi et al. 2008). Overexpression of *AtICE1* in cucumbers improves chilling tolerance, and leads to the accumulation of soluble sugars and proline, but results in dwarf phenotypes being exhibited (Liu et al. 2010). In rice, chilling stress induces OsICE1 and OsICE2 proteins, and sequentially upregulates *OsDREB1B*, *OsHsfA3* (rice heat shock factor A3), and *OsTPP1* (rice trehalose-6-phosphate phosphatase), suggesting that OsICE homologs function in transcriptional regulation for cold acclimation (Nakamura et al. 2011).

It has not yet been established whether tomatoes possess an upstream transcription factor that is similar to *Arabidopsis* AtICE1. In the present study, we report on the identification of tomato *SlICE1*, which controls the expression of cold-responsive genes and cold tolerance in tomato *Solanum lycopersicum* cv. Micro-Tom. *SlICE1*-overexpressing plants exhibited enhanced cold tolerance and expression of cold-responsive genes, such as *SlCBF1* and its regulon gene dehydrin Ci7 homolog *SlDRCi7*. These results indicate that *SlICE1* plays an important role in the regulation of cold signaling and tolerance in tomatoes.

Materials and methods

Plant materials, transformation, and growth conditions

The tomato (*Solanum lycopersicum*) cultivar Micro-Tom (accession number TOMJPF00001) was selected for genetic transformation. The plant material was provided by the University of Tsukuba through the National BioResource Project of MEXT, Japan. *SlICE1* cDNA was synthesized from the Micro-Tom tomato RNA. The *SlICE1* ORF was amplified with the following primers: SlICE1-KYLX-F (5′-GAAAGCTTA TGATAACTGGAGTGAAT-3′) and SlICE1-KYLX-R (5′-GAC TCGAGTTATATCGTCCCCCC-3′). The PCR product was digested with *Hin*dIII and *Xho*I, and was cloned into pKYLX71 (http://www.uky.edu/~aghunt00/kylx.html). The pKYLX71-SlICE1 vector was then mobilized into *Agrobacterium tumefaciens*, strain GV2260 (Deblaere et al. 1985) using electroporation. Transgenic tomatoes overexpressing *SlICE1* were generated by *Agrobacterium*-mediated transformation, as described previously (Sun et al. 2006; Sun et al. 2007). The kanamycin-resistant tomato plants were grown at 25°C in soil under fluorescent light with a 16/8 h (light/dark) photoperiod. Tomatoes were watered with Hyponex nutrient solution (Hyponex Japan, Osaka, Japan).

Cold treatment and electrolyte leakage from leaves

The cold treatment consisted of 3-week-old tomato plants being incubated at 4°C. The number of wilted shoots was then counted 12 days later. To determine the amount of electrolyte leakage from the leaves, all leaflets from tomato plants exposed to the cold treatment were washed and immersed in 15 ml of milli-Q water (Millipore, Billerica, MA, USA), and incubated for 2 h with shaking. The conductivity of the solution (Ca) was determined with a CD-4302 (Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan) conductivity meter. The tube was then autoclaved and cooled to room temperature, following which the conductivity of the solution (Cb) was remeasured. Electrolyte leakage was calculated as Ca/Cb (Miura and Ohta 2010). The conductivities of 4 leaflets were also measured at different time points.

Transactivation activity

Transactivation activity was examined in *Arabidopsis* protoplast. Protoplasts were prepared from 2-week-old wild-type *Arabidopsis thaliana* seedlings using Cellulase Onozuka R-10 and Macerozyme R-10 (Yakult Pharmaceutical, Tokyo, Japan), as described previously (Yoo et al. 2007). Plasmid DNA of the reporter *GAL4-GUS* (Tiwari et al. 2003) and the effector *GAL4DB-SlICE1* were introduced and transiently expressed into *Arabidopsis* protoplasts by polyethylene glycol-mediated transformation (Yoo et al. 2007). For each transfection, 5 μg of the reporter plasmid DNA, 4 μg of the effector plasmid DNA, and 1 μg of the reference plasmid DNA, *35S-LUC*, were used. Following transfection, the protoplasts were incubated at 23°C in the dark for 48 h. GUS and LUC activities were measured as described previously (Miura et al. 2011a), and LUC activity was used to normalize the efficiency of each transformation.

RNA isolation and quantitative RT-PCR analysis

To compare the level of expression of the *SlICE1* transgene between individual transgenic lines, total RNA was isolated from samples taken from each line. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate the total RNA, according to the manufacturer's instructions, and 4 μg of total RNA was used as a template for first-strand cDNA synthesis using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Semi-quantitative RT-PCR was performed using the primers SlICE1-1 (5′-GGA AGGAAAAGCGGTGAAC-3′) and rbcS-seqR (5′-AAACTG ATGCATTGAACTTG-3′), as described previously (Miura et al. 2010; Miura et al. 2011a). As an internal control, *UBI3* (ubiquitin) expression was monitored using the primers UBI3-F (5′-CACCAAGCCAAAGAAGATCA-3′) and UBI3-R (5′-TCAGCATTAGGGCACTCCTT-3′). Three lines (#34, #70, and #74) were used for further investigation.

Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and the gene-specific primers SlICE1-1 and SlICE1-2 (5′-AACACATCC AACACAAACCC-3′) for *SlICE1*; SlCBF1-F (5′-TTCATCGTC

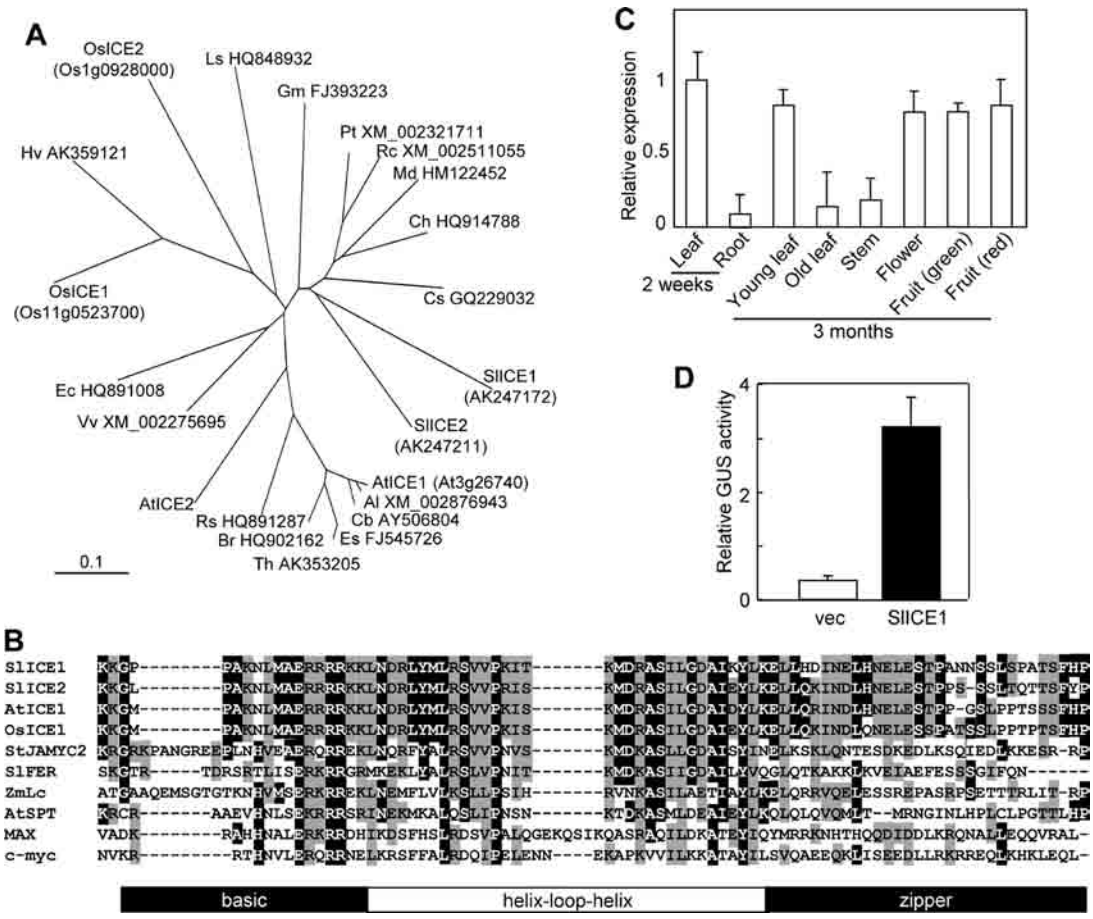

Figure 1. Phylogenetic tree of ICE1 and predicted ICE1 homologs. (A) Phylogram of proteins constructed using the CLUSTALW program (http://clustalw.ddbj.nig.ac.jp/top-j.html). The species identifying code of each protein is as follows: At, *Arabidopsis thaliana*; Al, *Arabidopsis lyrata*; Br, *Brassica rapa*; Cb, *Capsella bursa-pastoris*; Ch, *Corylus heterophylla*; Cs, *Camellia sinensis*; Ec, *Eucalyptus camaldulensis*; Es, *Eutrema salsugineum*; Gm, *Glycine max*; Hv, *Hordeum vulgare*; Ls, *Lactuca sativa*; Md, *Malus* × *domestica*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; Rc, *Ricinus communis*; Rs, *Raphanus sativus*; Sl, *Solanum lycopersicum*; Th, *Thellungiella halophile*; Vv, *Vitis vinifera*. The numbers represent GenBank accession numbers. (B) Sequence alignment of the basic helix–loop–helix (bHLH) domains, and possible zipper (ZIP) regions of ICE1 and other plant and animal bHLH transcription factors. Identical and similar residues are shown in black and gray, respectively. DDBJ/EMBL/GenBank accession numbers, with amino acid numbers in parentheses, are as follows: SlICE1, AK247172 (337–417); SlICE2, AK247211 (334–413); AtICE1, AAP14668 (301–380); OsICE1, BAF28350 (330–410); *Solanum tuberosum* StJAMYC2, CAF74710 (503–590); SlFER, NP_001234654 (109–186); *Zea mays* ZmLc, NP00105339 (402–489); AtSPT, BAF001131 (194–273); human MAX, P52161 (21–107); and human c-myc, 1001205A (354-435). (C) Quantification of the *SlICE1* transcript in various tissues. RNA from 2-week-old or 3-month-old plants was used as a template for cDNA synthesis. Relative mRNA level of *SlICE1* was determined by quantitative RT-PCR analysis. Values represent means±SD (n=3). (D) *SlICE1* transactivation activity. Relative GUS activity after transfection with the *GAL4-GUS* reporter and the effector plasmid *35S-GAL4-SlICE1* into *Arabidopsis* protoplasts (Miura et al. 2011a) was investigated. Luciferase activity (*35S-LUC*) was used for normalization. Values represent means±SD from 3 independent experiments.

ATCGTCGTTTTCT-3′) and SlCBF1-R (5′-TCCTCTTCCTGATTCCCCTGT-3′) for *SlCBF1* (Zhao et al. 2009); SlDRCi7-SGF (5′-TTGTGTTTCTGTGTTGTTTTGG-3′) and SlDRCi7-SGR (5′-GCACATACATATGCACTTACATACAG-3′) for *SlDRCi7*; and UBI3-F and UBI3-R (see above for primer sequence information) for *UBI3*, as an internal control. PCR products were detected by Thermal Cycler Dice Real Time System (Takara Bio Inc., Kyoto, Japan). Relative differences in expression were calculated as described previously (Miura et al. 2011b; Miura et al. 2011c). Briefly, relative transcript abundance was calculated using the comparative C_T method (User Bulletin 2 for ABI PRISM 7700 sequence detection systems). The change in C_T (ΔC_T) was then calculated ($C_{T,\text{interesting gene}} - C_{T,UBI3}$), following which $\Delta\Delta C_T$ ($\Delta C_T - \Delta C_{T,\text{control}}$) was also calculated. The relative expression level was represented as $2^{-\Delta\Delta CT}$, with the $2^{-\Delta\Delta CT}$ value for the control having been normalized to 1 ($2^{-(\Delta CT,\text{control} - \Delta CT,\text{control})} = 20 = 1$).

Immunoblot analysis

Tomato leaves (second leaves) were ground by mortar in liquid nitrogen. Lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.2 mM sodium orthovanadate, 100 mM NaF, 10% glycerol, 0.2% Triton X-100, 5 mM DTT, and 1× protein inhibitor cocktail [Roche Applied Science, Penzberg, Germany]; Miura and Ohta 2010) was then added and incubated on ice with shaking. The samples were spun and the concentration of

proteins in the supernatant was determined, following which 30 μg of total protein was loaded onto an SDS-PAGE gel. The blot was probed with anti-ICE1 antibody and detected using ImmunoStar LD (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Ascorbate measurement

Two-week-old tomato plants were either incubated at 25°C (control) or treated with cold stress (4°C). Shoots of these plants were harvested and homogenized with mortar in liquid nitrogen. The resulting powder (1 g) was mixed with 5% (w/v) metaphosphoric acid (4 ml). Following centrifugation at 12,000×*g* for 5 min, the supernatant was used as a crude extract. Total ascorbic acid content was measured by Ascorbic Acid Test (Merck, Darmstadt, Germany) using RQ Flex plus 10 (Merck, Darmstadt, Germany).

Results

Isolation of tomato SlICE1

AtICE1 (At3g26744), a MYC-type transcription factor, plays an important role in the regulation of cold signaling and tolerance in *Arabidopsis thaliana* (Chinnusamy et al. 2003). A TBLASTN search in the nucleotide collection database using the AtICE1 amino acid sequence as the query resulted in the identification of ICE1 homologs in several plant species (Figure 1A). The AtICE1 homologs were conserved in tomatoes (*Solanum lycopersicum*) (GenBank accession nos. AK247172 and AK247211, which contain the full-length ORF of *SlICE1* and *SlICE2,* respectively). The bHLH domain of SlICE1 shows high amino acid similarity to those of AtICE1 and known MYC-related bHLH transcription factors (Figure 1B). *SlICE1* was expressed in younger leaves, flowers, and both green and red fruits of tomato plants, but the expression was reduced in roots, older leaves, and stems (Figure 1C).

To confirm whether *SlICE1* harbors transactivation activity similar to *AtICE1* (Chinnusamy et al. 2003; Miura et al. 2007), the full-length coding region of the *SlICE1* gene was fused to the DNA-binding domain of the yeast GAL4 transcription factor (Tiwari et al. 2003). The effector plasmid was co-introduced into *Arabidopsis* leaf protoplasts with the *GAL4-GUS* reporter and *35S-LUC*, and the protoplasts were incubated at 23°C. Transactivation activity was measured by GUS activity, and LUC activity was used to normalize plasmid uptake levels between samples (Tiwari et al. 2003). It was found that *GAL4-SlICE1* was able to activate *GAL4*-mediated transactivation (Figure 1D), indicating that *SlICE1* acts as transcriptional activator in a similar manner to *AtICE1*.

Effect of SlICE1 on chilling tolerance and expression of cold-responsive genes

To investigate the biological functions of *SlICE1*, transgenic *SlICE1* tomato plants were produced. The open reading frame region of *SlICE1* was cloned into the binary vector pKYLX71 (http://www.uky.edu/~aghunt00/kylx.html), in which *SlICE1* expression was driven by the 35S promoter (Figure 2A). The vector was transformed into tomatoes *Solanum lycopersicum* cv. Micro-Tom. The transcript abundance of the *SlICE1* transgene was evaluated by semi-quantitative RT-PCR analysis (Figure 2B). Among 10 transgenic lines, 3 lines (#34, 70, and 74) were used for further investigation.

The levels of SlICE1 protein in the wild-type and transgenic tomato lines were investigated. The *SlICE1* transgene, which is driven by the 35S promoter, was expressed in the transgenic tomato plants (Figure 2B). Consequently, overexpressing plants contained a higher level of SlICE1 protein than wild-type tomatoes in non-stressed conditions (Figure 2C). In *Arabidopsis*, ICE1 protein degradation through the ubiquitin/proteasome pathway was observed more than 16 h after cold treatment (Dong et al. 2006). Similarly, SlICE1 protein levels were substantially lower 24 h after cold treatment, but protein levels were still higher in cold-treated transgenic plants (particularly #70) than in cold-treated wild-type tomatoes (Figure 2C). These results suggest that *SlICE1* functions in a similar manner to *AtICE1* in the regulation of cold tolerance.

Homozygous *SlICE1*-overexpressing plants had relatively smaller shoots than wild-type plants (Figure 2D). Similarly, 3-week-old *SlICE1*-overexpressing plants had lower fresh weights than wild-type plants (0.52±0.06 g, 0.38±0.02 g, and 0.38±0.03 g for #34, #70, and #74, respectively and 0.72±0.05 g for wild-type plants; Figure 2E) and smaller fruit (Figure 2F).

Three-week-old tomato plants were incubated at a chilling temperature (4°C) for 2 weeks. Following this chilling treatment, most of the wild-type plants had wilted leaves (Figure 3A), whereas transgenic plants retained non-wilted, healthy leaves (Figures 3A, B). Wild-type tomato plants also had higher levels of electrolyte leakage than *SlICE1*-overexpressing lines (Figure 3C). These data indicate that overexpression of *SlICE1* confers plants with improved tolerance to chilling stress.

Among 3 *CBF* genes in tomato, the *SlCBF1* transcript is transiently expressed following cold treatment, and overexpression of *SlCBF1* in *Arabidopsis* enhances freezing tolerance (Zhang et al. 2004). Thus, the expression of *SlCBF1* and the *SlCBF1*-dependent gene *SlDRCi7* (Zhang et al. 2004) was investigated in wild-type and *SlICE1*-overexpressing tomatoes (Figure 3D). The expression of these genes was up-regulated in *SlICE1*-overexpressing plants. The accumulation of ascorbate was also measured, because this important antioxidant protects plants against reactive oxygen species that are produced by several stresses, including exposure to low

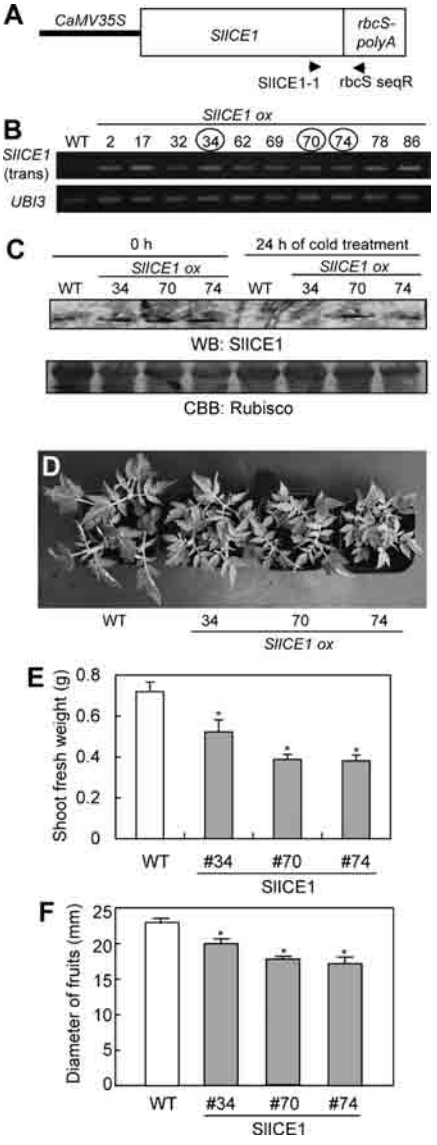

Figure 2. *SlICE1* overexpression in tomatoes. (A) Diagram of the binary vector for expression of *SlICE1*, driven by the Cauliflower Mosaic Virus promoter (CaMV35S). Arrowheads indicate the locations at which primers (SlICE1-1 and rbcS seqR) annealed for PCR amplification. (B) *SlICE1* transcript abundance in independently transformed tomato plants. Total RNA was prepared from the second leaves of each transgenic plant. Semi-quantitative RT-PCR analysis was performed using primers SlICE1-1 and rbcS seqR, which detect mRNA produced by the transgene but not native *SlICE1*. The numbers above the columns indicate the independent transgenic lines; circled numbers identify lines used for phenotypic analyses. (C) SlICE1 protein degradation following cold treatment. Two-week-old transgenic tomatoes were treated at 4°C for 24 h. The second leaves were harvested and the crude extract was separated by SDS-PAGE. Immunoblot analysis with anti-SlICE1 antibody was performed. As loading control, a large subunit of Rubisco is shown by Coomassie Brilliant Blue (CBB)-staining. (D) Growth characteristics of transgenic tomato plants overexpressing *SlICE1*. Three-week-old wild type (WT) plants and 3 different transgenic tomatoes grown in a 16-h photoperiod condition at 25°C are shown. (E, F) Fresh weight of wild-type and *SlICE1*-overexpressing tomato shoots grown for 3 weeks, and diameter of red fruits harvested from 45 to 52 days after anthesis. Values represent means±SE (n≥14). Asterisks indicate significant difference from wild-type ($p<0.05$).

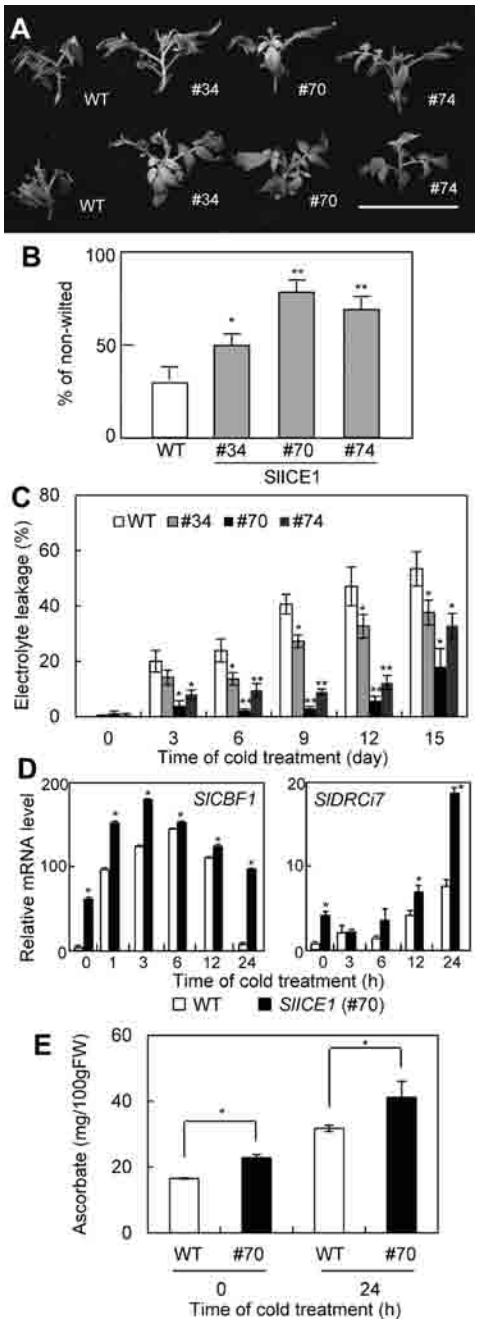

Figure 3. Cold tolerance of *SlICE1*-overexpressing tomato plants. (A) Representative shoots from wild-type or *SlICE1*-overexpressing tomato plants after 2 weeks of cold treatment (3-week-old plants were subjected to cold treatment at 4°C for 2 weeks). (B) Percentage of non-wilted leaves following cold treatment. Values represent means±SE (n=12) from 3 independent experiments. (C) Electrolyte leakage from wild-type or *SlICE1*-overexpressing tomato plants after exposure to low temperature (4°C) for the indicated days. Values represent means±SE (n=4 leaflets). (D) The relative mRNA transcript levels of *SlCBF1* and *SlDRCi7* in wild-type and *SlICE1*-overexpressing leaves, as determined by quantitative RT-PCR analyses. Two-week-old plants grown at 25°C were incubated at 4°C for the indicated time. Values represent means±SD (n=3). (E) The content of ascorbate before and after cold treatment in 2-week-old wild-type and *SlICE1*-overexpressing (#70) tomato shoots. Values represent means±SE (n≥5). Asterisks indicate significant difference from wild-type (WT) (*, $p<0.05$; **, $p<0.01$).

temperatures (Conklin 2001), and the accumulation of ascorbate is increased by exposure to the cold and overexpression of *AtCBF3*/*DREB1A* in *Arabidopsis* (Cook et al. 2004). Following the cold treatment, both wild-type and *SlICE1*-overexpressing tomato shoots contained greater concentrations of ascorbate (Figure 3E). On the athor hand, *SlICE1*-overexpressing shoots accumulated more ascorbate than wild-type shoots (Figure 3E). These results suggest that *SlICE1* is involved in the regulation of *SlCBF1*-dependent cold signaling and cold tolerance.

Discussion

In the present study, we identified that *SlICE1* in tomato (*Solanum lycopersicum*) is a transcription factor that regulates cold signaling and tolerance. *SlICE1*-overexpressing tomato plants exhibited increased chilling tolerance, enhanced expression of cold-responsive genes, and cold-induced accumulation of ascorbate (Figure 3).

Tomato exhibits the basic components of a CBF-dependent cold-response pathway, as the overexpression of *SlCBF1* or *AtCBF3* in transgenic tomatoes resulted in the activation of cold-regulated genes, which have putative CRT/DRE (C-repeat/dehydrin responsive element) *cis*-elements in their promoters (Zhang et al. 2004). It has also been shown that the overexpression of *Arabidopsis AtCBF1* in tomatoes improves chilling tolerance (Singh et al. 2011; Zhang et al. 2011). In the present study, *SlICE1* enhanced the expression of *SlCBF1* and the cold-regulated *SlDRCi7* gene (Figure 3D), and promoted cold tolerance (Figures 3A–C). These results suggest that ICE1-dependent cold signaling is conserved in a tomato. The high expression of *SlCBF1* in *SlICE1*-overexpressing tomato plants may cause retardation of growth, *SICBF1*-with overexpressed plants exhibiting dwarf-like phenotypes under normal growth conditions (Zhang et al. 2004). This is similar to findings for *Arabidopsis* plants overexpressing *AtCBF* genes at high levels (Gilmour et al. 2000; 2004). The growth retardation of *AtCBF1*-overexpressing plants is partly caused by the accumulation of DELLA proteins, a family of nuclear growth-repressing proteins (Archard et al. 2008). It has been shown that the application of gibberellin to *AtCBF1*-overexpressing *Arabidopsis* plants rescues normal growth (Archard et al. 2008), as gibberellin degrades the DELLA proteins (Dill et al. 2004). Therefore, it may also be possible to suppress the growth retardation of *SlICE1*-overexpressing tomato plants through the application of gibberellin.

The bHLH-type transcription factors constitute a large family of genes that play important roles in eukaryotic growth and development. Genes encoding 162 and 70 bHLH have been identified in *Arabidopsis* (Bailey et al. 2003) and tomato (http://planttfdb.cbi.edu.cn/family.php?sp=Sly&fam=bHLH), respectively. Some bHLH genes have been functionally characterized in tomato, including FER, which controls root physiology and development in response to iron supply (Ling et al. 2002); the *fer* mutant fails to activate iron reduction, Fe(II) transport, and root hair proliferation.

The tomato genome project has progressed, and the current project has added to this with the discovery of *SlICE2*, which is similar to *AtICE1* (Figure 1A). In *Arabidopsis*, *AtICE1* mainly controls the expression of *AtCBF3*/*DREB1A* (Chinnusamy et al. 2003), *AtICE2* controls the expression of *AtCBF1*/*DREB1B* (Fursova et al. 2009), and both genes control *AtCBF*/*DREB1*-dependent cold signaling and cold tolerance. The functional role of *SlICE2* is still unknown, but it is likely that *SlICE2* may control cold signaling and cold tolerance.

In summary, this study indicated that one of the bHLH genes, *SlICE1*, plays an important role in the regulation of cold signaling and chilling tolerance (Figure 3), as well as the regulation of antioxidant activity in tomatoes (Miura et al. 2012).

Acknowledgments

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References

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. *Plant Cell* 20: 2117–2129

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat ICE (inducer of CBF expression) genes. *Plant Cell Physiol* 49: 1237–1249

Bailey PC, Martin C, Toledo-Ortiz G, Quail PH, Huq E, Heim MA, Jakoby M, Werber M, Weisshaar B (2003) Update on the basic helix-loop-helix transcription factor gene family in *Arabidopsis thaliana*. *Plant Cell* 15: 2497–2502

Boter M, Ruíz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. *Genes Dev* 18: 1577–1591

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev* 17: 1043–1054

Conklin PL (2001) Recent advances in the role and biosynthesis of ascorbic acid in plants. *Plant Cell Environ* 24: 383–394

Cook D, Fowler S, Fiehn O, Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. *Proc Natl Acad Sci USA* 101: 15243–15248

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucleic Acids Res* 13: 4777–4788

Dill A, Thomas SG, Hu J, Steber CM, Sun TP (2004) The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *Plant Cell* 16: 1392–1405

Dong CH, Agarwal M, Zhang Y, Xie Q, Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc Natl Acad Sci USA* 103: 8281–8286

Fursova OV, Pogorelko GV, Tarasov VA (2009) Identification of *ICE2*, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. *Gene* 429: 98–103

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol* 124: 1854–1865

Gilmour SJ, Fowler SG, Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol Biol* 54: 767–781

Hsieh TH, Lee JT, Yang PT, Chiu LH, Charng YY, Wang YC, Chan MT (2002) Heterology expression of the Arabidopsis C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. *Plant Physiol* 129: 1086–1094

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science* 280: 104–106

Juntilla O, Robberecht R(1999) Ecological aspects of cold-adapted plants with a special emphasis on environmental control of cold hardening and dehardening. In: Margesin R, Schinner F. (eds), *Cold-adapted organisms—ecology, physiology, enzymology and molecular biology*. Springer-Verlag, Berlin, Germany, pp 57–77

Lee BH, Henderson DA, Zhu JK (2005) The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell* 17: 3155–3175

Ling HQ, Bauer P, Bereczky Z, Keller B, Ganal M (2002) The tomato *fer* gene encoding a bHLH protein controls iron-uptake responses in roots. *Proc Natl Acad Sci USA* 99: 13938–13943

Lissarre M, Ohta M, Sato A, Miura K (2010) Cold-responsive gene regulation during cold acclimation in plants. *Plant Signal Behav* 5: 948–952

Liu L, Duan L, Zhang J, Zhang Z, Mi G, Ren H (2010) Cucumber (*Cucumis sativus* L.) over-expressing cold-induced transcriptome regulator *ICE1* exhibits changed morphological characters and enhances chilling tolerance. *Sci Hortic* (Amsterdam) 124: 29–33

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10: 1391–1406

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ, Hasegawa PM (2007) SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* expression and freezing tolerance in *Arabidopsis*. *Plant Cell* 19: 1403–1414

Miura K, Lee J, Miura T, Hasegawa PM (2010) *SIZ1* controls cell growth and plant development in Arabidopsis through salicylic acid. *Plant Cell Physiol* 51: 103–113

Miura K, Ohta M (2010) SIZ1, a small ubiquitin-related modifier ligase, controls cold signaling through regulation of salicylic acid accumulation. *J Plant Physiol* 167: 555–560

Miura K, Ohta M, Nakazawa M, Ono M, Hasegawa PM (2011a) ICE1 Ser403 is necessary for protein stabilization and regulation of cold signaling and tolerance. *Plant J* 67: 269–279

Miura K, Lee J, Gong Q, Ma S, Jin JB, Yoo CY, Miura T, Sato A, Bohnert HJ, Hasegawa PM (2011b) *SIZ1* regulation of phosphate starvation-induced root architecture remodeling involves the control of auxin accumulation. *Plant Physiol* 155: 1000–1012

Miura K, Sato A, Ohta M, Furukawa J (2011*c*) Increased tolerance to salt stress in the phosphate-accumulating *Arabidopsis* mutants *siz1* and *pho2*. *Planta* 234: 1191–1199

Miura K, Sato A, Shiba H, Kang SW, Kamada H, Ezura H (2012) Accumulation of antioxidants and antioxidant activity in tomato, *Solanum lycopersicum*, are enhanced by the transcription factor *SlICE1*. *Plant Biotechnol*, 29: 261–269

Nakamura J, Yuasa T, Huong TT, Harano K, Tanaka S, Iwata T, Phan T, Iwaya-Inoue M (2011) Rice homologs of inducer of *CBF* expression (OsICE) are involved in cold acclimation. *Plant Biotechnol* 28: 303–309

Nakayama K, Okawa K, Kakizaki T, Inaba T (2008) Evaluation of the protective activities of a late embryogenesis abundant (LEA) related protein, Cor15am, during various stresses *in vitro*. *Biosci Biotechnol Biochem* 72: 1642–1645

Pearce RS, Dunn MA, Rixon JE, Harrison P, Hughes MA (1996) Expression of cold-induced genes and frost hardiness in the crown meristem of young barley (*Hordeum vulgare* L. cv. Igri) plants grown in different environments. *Plant Cell Environ* 19: 275–290

Singh S, Rathore M, Goyary D, Singh RK, Anandhan S, Sharma DK, Ahmed Z (2011) Induced ectopic expression of *At-CBF1* in marker-free transgenic tomatoes confers enhanced chilling tolerance. *Plant Cell Rep* 30: 1019–1028

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiol* 47: 426–431

Sun HJ, Kataoka H, Yano M, Ezura H (2007) Genetically stable expression of functional miraculin, a new type of alternative sweetener, in transgenic tomato plants. *Plant Biotechnol J* 5: 768–777

Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* 50: 571–599

Tiwari SB, Hagen G, Guilfoyle T (2003) The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* 15: 533–543

Verlues PE, Agarwal M, Kariyar-Agarwal S, Zhu J, Zhu JK (2005) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J* 45: 523–539

Yang JS, Wang R, Meng JJ, Bi YP, Xu PL, Guo F, Wan SB, He QW, Li XG (2010) Overexpression of *Arabidopsis CBF1* gene in transgenic tobacco alleviates photoinhibition of PSII and PSI during chilling stress under low irradiance. *J Plant Physiol* 167: 534–539

Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* 2: 1565–1572

Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, Thomashow MF (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *Plant J* 39: 905–919

Zhang YJ, Yang JS, Guo SJ, Meng JJ, Zhang YL, Wan SB, He QW, Li XG (2011) Over-expression of the *Arabidopsis CBF1* gene improves resistance of tomato leaves to low temperature under low irradiance. *Plant Biol* (Stuttg) 13: 362–367