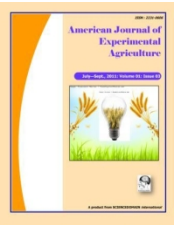

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Tomato Inducer of CBF Expression 1 (SlICE1) is Involved in Cold and Salt Stress Signaling

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Authors' contributions

This work was carried out in collaboration between all authors. Author TY designed the study, performed the experimental analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JN and YI helped the analyses of the study. Authors TY and MII managed the research funding grants for this study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Inducer of CBF Expression 1 (ICE1), which is one of basic Helix-Loop-Helix type transcription factors, has important roles in regulation of cold stress-indiced genes of plants. Sample: To investigate functions of tomato ICE1 in cold and salt tolerance, c index (SI), immunochemical assay of endogenous ICE1 protein and RT-PCR of cold-inducible genes were conducted with tomato plants.
Methodology: Tomato plants grown for 4 weeks were subjected to cold (4ºC) and salt (0.2 M NaCl) in the presence or the absence of cell signaling inhibitors. An antibody was raised against ICE1 specific epitoipe. Immunoblot with the anti-ICE1 antibody was carried out with extractions of tomato plants treated by cold and salt stresses. The expression profiles of tomato ICE1 (SlICE1) and other cold-inducible genes including LeCBF1/2/3 and SlTPS1 were analyzed by semi-quantitative RT-PCR.
Results: An ICE1-related protein with molecular masses of approximately 55 is induced in tomato plant under chilling and salt stresses. The expression of a tomato ICE1 gene (SlICE1) under chilling stress was maintained at a constant level in contrast to the protein

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level. Chilling stress sequentially upregulated tomato CBF homolog, *SlCBF1* and trehalose-6-phosphate synthase (SlTPS1). Based on the whole genome database of tomato, cis-elements potentially binding to ICE1 and CBF were located in upstream sequences in promoter regions of SlCBF1 and SlTPS1, respectively.
Conclusion: Tomato ICE1 homolog mediates the expression of *SlCBF1*in a cold-stress-induced transcription factor cascade via binding to ICE1-specific cis-elements, leading to induction of cold tolerance by trehalose synthesis.

Keywords: CBF; cold stress; ICE1; tomato; transcription factor; trehalose.

ABBREVIATIONS

CBF: C-box binding factor; DREB: Drought-responsive element binding factor; EGTA: Ethylene glycol tetraacetic acid; GST: Glutathione S-transferase; ICE: Inducer of CBF expression; RT-PCR: Reverse Transcription Polymerase Chain Reaction; SDS: sodium dodecyl sulfate; TPP: Trehalose 6-phosphate phosphatase; TPS: Trehalose 6-phosphate synthase.

1. INTRODUCTION

Among environmental stresses, cold stress is known to cause the most serious damage on plant growth and crop yield [1]. When plants are subjected to cold stress, the expression of cold-regulated genes such as the synthesis of osmolytes (galactinol and trehalose) and antifreezing proteins, leading to cold acclimation [2,3,4]. Molecular genetic studies focusing on cold-stress-induced genes unveiled that dehydration-responsive-element–binding protein (DREB)/C-repeat–binding factor (CBF) genes are key transcription factors which are responsible for gene regulation network under cold and drought stresses [5,6,7]. Various sets of transcriptional factors such as *DREB*/*CBF*, *bZIP*, *MYC*, *MYB* and *Hsf* (*heat shock factor*) are known to be expressed in specific profiles under osmotic, salt and cold stresses. It is hypothesized that the environmental stress-stimulated transcription networks is involved in synthesis of osmolytes, such as proline, trehalose and galactinol etc [6]. Based on molecular genetic studies using *Arabidopsis*, Inducer of *CBF* Expression 1 (ICE1), a MYC-like transcription factors possessing basic helix-loop-helix (bHLH) domain, appeared to function at the upstream of *DREB*/*CBF* genes by binding to specific cis-element of promoter regions in those genes [8,9,10]. Recent studies unveiled that trehalose accumulation in tomato is essential for acquisition of cold tolerance and that the gene regulation via DREB/CBF and SnRK2-mediated protein phosphorylation are potentially implicated in the mechanisms of the trehalose synthsis in tomato under cold stress [7,11,12].

Several studies in Arabidopsis have unveiled a set of post-translational modifications, such as phosphorylation, ubiquitination and sumoylation and improvement of cold tolerance by ectopically expressed ICE1 [13]. But there is little information about whether the endogenous ICE1 proteins in tomato are regulated in the same way. It is interesting if a tomato ICE1 homolog functions as a pivotal regulator for *CBF* at upstream of induction trehalose synthesis, leading to acquisition of cold tolerance. In this study, we show the immunological characterization of tomato ICE-related proteins and their potential roles in acquisition of tolerance to cold and salt stresses via the regulation of *SlCBF1* and *SlTPS1*.

2. MATERIALS AND METHODS

2.1 Materials and Growth Conditions

After sowing tomato seeds (*Solanum lycoperisicum* L. cv. MicroTom) in 6cm x 6cm x 6cm glass wools, tomato plants were grown for 4 week in phytotron (25ºC) at glass house in Kyushu University as previously described [14].

2.2 Stress Treatments

The 4 week old-tomato plants were subjected to cold stress (4ºC) or salt stress by spraying with solution of 0.2 M NaCl. To examine the effects of calcium antagonists on ICE1 protein profiles, mature leaves of 4 week old-tomato plants were preincubated in 10 ml of 2 mM $CaCl_2$ (as control), 10 mM EGTA-Na or 10 mM $LiCl_3$ in petridish for 2 hr and then subjected to cold stress (4ºC). To examine the involvement of protein kinase and proteasome on ICE1 protein stability, mature leaves of 4 week old-Tomato plants were preincubated in 10 ml of 5 mM K252a (protein kinase inhibitor) or 50 mM MG132 (proteasome specific inhibitor) in petridish for 2 hr, and then were subjected to cold stress (4ºC). After shoot and leaf of plants were harvested at 0, 1, 3 and 5 hr, the samples were immediately frozen in liquid nitrogen and stored at -80ºC.

2.3 Bioinformatic Analysis

BLAST searching with the nucleotide sequence of *Arabidopsis ICE1* in the plant gene index in the Dana-Faber Cancer Institute Plant Gene Index (DFCI) (http://compbio.dfci.harvard.edu/tgi/) identified several*ICE* gene homolog candidates among dicots and monocots (Fig. 1A). A phylogenic tree of plant ICE1 homologs was buildt with the deduced amino acid sequences by the alignment program of CLUSTALW (http://align.genome.jp/). For prediction of ICE1-recognition sites, consensus motives in the promoter regions in tomato gene locus for specific cis-element interacting to transcription factors by “PLACE” (A Database of Plant Cis-acting Regulatory DNA Elements: http://www.dna.affrc.go.jp/PLACE/signalscan.html).

2.4 RNA Extraction and Semi-quantitative RT-PCR

Total RNA was extracted from tomato tissue frozen in liquid N_2 by the SDS/phenol/LiCl method. Specific primer sets for semi-quantitative RT-PCR were designed from *SlICE1*, *SlCBF1*, *SlCBF2*, SlCBF3 and *SlTPS1* (Table 1). Tomato *ubiquitin* was used as a standard gene. RT-PCR was carried out with total RNA from tomato plants by using ReverTra Ace reverse transcriptase (TOYOBO, Tokyo, Japan) and GoTaq Green Master Mix (Promega, Tokyo, Japan) as previously described [14]. PCR reaction was performed with a PC-816 thermal cycler (ASTEC Co., Fukuoka, Japan) in a 20-µl reaction mixture under the following thermal cycle conditions: an initial 94ºC for 2 min; 25 cycles of 94ºC for 20 s, 60ºC for 20 s, and 72ºC for 30 s and a final 72ºC for 5 min. After electrophoresis in 1.5% agarose gels, ethidium bromide-stained PCR products were visualized by FluorChem Imager (Alpha Innotech, San Leandro, CA, USA).

Table 1. Primers used for RT-PCR analysis and construction of expression plasmids

Gene	Accession number and/or KTU contig	Primer set
SlICE1-F	AK247172	5-CTGGGATCCGTTGTCCCAAAGATAACCAAG-3
SlICE1-R		5-GTTCGGTCGACCATGGTCTGTAACCATGTA-3
SlHOS1-F	BP882690	5-GCGGCTCTGAAGGAAGCCTGTCAACTTCTC -3
SlHOS1-R		5-CTTCCTATGGGCGTTGAAGGATCCTCGGCA-3
LeCBF1-F	AY034473	5-TCAGGATCCATGAATATCTTTGAAACCTAT-3
LeCBF1-R		5-TTAGATAGAATAATTCCATAAAGTTATACT -3
LeCBF2-F	AY497899	5-CATGGATATCTTTGAATCCTATTATTCAAA -3
LeCBF2-R		5-TTAGATAGAATAATCCCATAAGGGCAT-3
LeCBF3-F	AAS77819	5-ATGTTTTATTCGGACCCACGTATAGAATCT-3
LeCBF3-R		5-TATAGAATAGCTCCATAAAGGCATATCATC-3
SlTPS1-F	AB368491	5-GGTACCTGCAGACACTGAGTGGAA-3
SlTPS1-R		5-CTGTCGACTATACAAAGGATGCATGATTCTTAAC-3
SlCOR413-F	Contig23669	5-ATGGGTAGGATGGATTATTTGGCTATG -3
SiCOR413-R		5-TCAGACGGCTCGAAGAACCAGAGC-3
SlUbi-F	BT012698	5-ACGTGGATCCATGCAAATCTTTGTGAAGAC-3
SlUbi-R		5-AAAGTCGACTAACCACCACGGAGACGGAGG-3

Introduced restriction sites are showed by underlines. KTU3 contigs were referred to Micro-Tom EST database (MiBASE) and Kazusa Tomato Unigene ver. 3 (KTU3). URL: http://www.kazusa.or.jp/jsol/microtom/indexj.html.

2.5 Construction of pGEX-SlICE1 and Expression of Recombinant Proteins

To prepare a recombinant SlICE1 protein, we constructed pGEX-SlICE1-carboxyl terminus (SlICE1-CT). PCR fragments encoding SlICE1-CT region (367-535) were amplified with KOD plus DNA polymerase (TOYOBO, Tokyo, Japan), tomato cDNA and specific primer sets of SlICE1-F and SlICE1-R (Table 1) and then digested with BamHI and SalI (SlICE1). The resultant SlICE1 fragment was ligated into BamHI-SalI sites of pGEX4T-1 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) by DNA Ligation Kit v. 2 (TaKaRa Bio Inc., Tokyo, Japan). The cloned SlICE1 cDNA was confirmed by sequencing on an ABI Prism 310 DNA sequence with a Big Dye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystems, Foster City, CA, USA). The recombinant proteins of GST-fused SlICE1-CT was induced in E. coli in the presence of 0.5 mM ITPG for 2 hr at 37ºC after growing in LB/Amp medium over night at 37ºC. The recombinant proteins of GST and GST-SlICE1-CT were purified with glutathione Sepharose 4B (GE LifeScience) as in manufacture's manual.

Protein extract was prepared from tomato plants (~0.5 g) stored at -80ºC by homogenization in liquid nitrogen and mixed with 500 µl lysis buffer containing 1× TBS, 10 mM EDTA, 5% glycerol, 0.2% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), 10 µg ml–1 leupeptin (a cysteine protease inhibitor) and 1 mMbenzamidine, with or without 1% Triton X-100. The resultant extracts were centrifuged at 10,000 ×g for 5 min at 4ºC. Protein concentrations were determined by measuring OD595 with a Bio Rad protein assay kit (Bio Rad, Hercules, CA, USA) using 1 mg ml–1 bovine serum albumin as a standard.

2.6 Protein Extraction and Immunoblot

For immunoblot, polypeptides separated in 10% acrylamide gel by SDS-PAGE were electro-transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in blotting buffer containing 25 mM Tris-base, 0.05% SDS, and 20% methanol at10 Vcm–1 for 2 hr. The membranes were then incubated in blocking buffer containing 1× TBS and 2.5% skim milk for 1 hr and then in blocking buffer supplemented with anti–ICE-homolog common peptides primary antibody (1/1000 dilution) and 0.05% Tween 20 for 2 hr at 4ºC. After washing in 1× TBS containing 0.05% Tween 20, the membrane was incubated in blocking buffer supplemented with horseradish peroxidase–labeled antibody (1/5000 dilution, v/v; GE Healthcare Bio-Sciences) for 1 hr. Immunoreactive signals were visualized by an ECL Plus kit (GE Healthcare Bio-Sciences) and FluorChem.

3. RESULTS

3.1 Phylogenic Analysis and ICE1 Specific Epitope

A phylogenetic tree of ICE homologs in SlICE1 (AK247172), other dicots and monocots, using *Arabidopsis* PIF3 and human c-mycas outgroups, showed that *ICE*-related genes can be classified into dicot and monocot subfamilies as previously reported [15,16,17] (Fig. 1A). Both monocots and dicots possess ICE-related genes [16]. Almost dicots have a single ICE gene in those genome sets, while various monocots have two ICE homologs encoding about 40 and 55 kDa proteins [8,18]. Interestingly, SlICE1 in the phylogenic tree is branched at a root of the two ICE1 subfamily of dicots and monocots even though SlICE1 is possibly classified to the ICE1 family (Fig. 1A). SlICE1 possesses very conserved domain sets including an acidic domain, a Ser-rich domain, a bHLH domain and a possible zipper region, similar with those of *Arabidopsis* ICE1. SlICE1 and AtICE1 have a little different predicted molecular masses, SlICE1 has similarities of 43% at the amino acid level to *Arabidopsis* ICE1. Compared with MYC-like bHLH transcription factors, the alignments of the plant ICE1 homologs revealed a highly conserved motif of 19 amino acids (KMDRASILGDAI(D/E)-YLKELL) that is specific to plant ICE1 homolgs but not to other MYC-like proteins [9,17] (Fig. 1B).

3.2 Immunoblot of ICE1-Related Proteins

E. coli crude extracts containing recombinant GST-SlICE1 was subjected to SDS-PAGE (10% acrylamide) and immunoblot with anti-ICE antibody (Fig. 1C). Immunoreactive signals indicate that the anti-ICE1 peptide antibody crossreacted specifically with GST-SlICE1 but not with GST, nor endogenous *E. coli* polypeptides. Immunoblot was performed to assess whether the anti-ICE antibody cross-reacted specifically with endogenous ICE1-related polypeptides in tomato plants subjected to cold and salt stresses (Fig. 2A). The putative molecular mass of the ICE1-releted polypeptide with 52 kDa was closed to an expected molecular mass of 57.6 kDa of SlICE1. In response to cold stress, immunoreactive signals with molecular masses of about 52 kDa significantly increased at 1 and 3 hr after cold stress, and then decreased to a marginal level at 5 hr, while the immunoreactive signal was stimulated at 1 hr and then maintained at 3 and 5 hr. In contrast to cold and salt stresses, heat stress had no effect on immunoreactive signals of the ICE1-related protein in tomato plants (data not shown). The induction of the endogenous SlICE1 protein with the relative molecular mass of 52 kDa under cold stress is consistent with previous reports that cold stress upregulated a protein level of epitope-tagged *Arabidopsis*ICE1 [19,20].

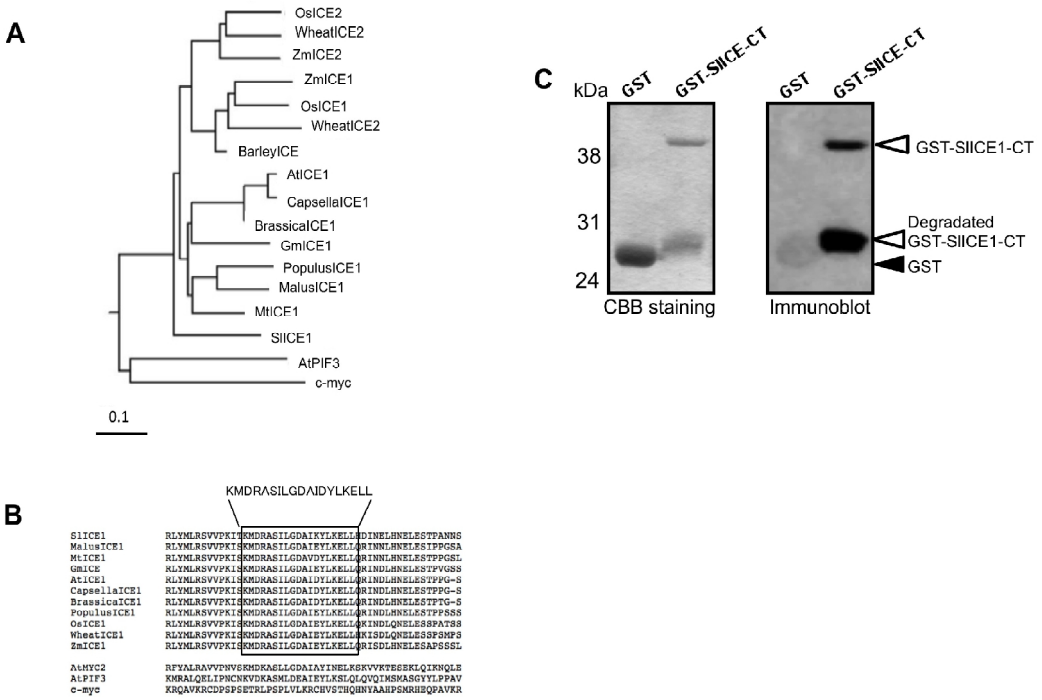

Fig. 1. Phylogenic tree of ICE (Inducer of CBF Expression) homologs

(A) ICE homolog sub-families are apparently classified into two groups of dicots and monocots. A group of plant ICE homologs has weak similarity in the bHLH domain to those of human c-myc and Arabidopsis PIF3 (AtPIF3). (B) Alignments of plant ICE1 homolog proteins. A conserved amino acid motif (KMDRASILGDAIKYLKELL) present in the carboxyl-half region of the bHLH region was used as an epitope for raising anti-ICE1-specific antibody. SlICE1 (AK247172), Solanum lycopersicum; AtICE1 (At3g26740), AtPIF3 (At1g09530), Arabidopsis thaliana; CapsellaICE1 (AY504806), Capsella bursa-pastoris; BrassicaICE1 (HQ902162), Brassica napus; GmICE1 (FJ393223), Glycine max; Populus ICE (XV000793), Populus trichocarpa; MalusICE1 (HM122452), Malus×domestica; MtICE1 (Tentative consensus TC174139 in DFCI plant gene index), Medicago truncatula; OsICE1 (Os11g0523700), OsICE1 (Os1g0928000), Oryza sativa; WheatICE1 (EU562184), WheatICE2 (EU562183), Triticum aestivum; ZmICE1 (DV024434), ZmICE2 (Tentative consensus T348661 in DFCI plant gene index), Zea mays; BarleyICE1 (AK359121), Hordeum vulgare; c-myc (HS06259), Homo sapiens. (C) Anti-ICE1 specific peptide antibody cross-reacted with recombinant proteins of GST-SlICE1-CT but not GST. Immunoblot was conducted with purified proteins of GST and GST-SlICE1-CT (10 and 5 mg protein per lane, respectively) of E. coli containing pGEX4T-1 empty and pGEX-SlICE1-CT

3.3 Effects of Ca^{2+} Antagonists and Inhibitors for Cell Signaling on ICE1

A line of evidence indicates that cold stress signaling in higher plants is tightly connected with Ca^{2+} mobilization and Ca^{2+}-stimulated protein phosphorylation [13]. It was reported that phosphorylation of a Ser-rich region in Arabidopsis ICE1 are involved in cold stress signaling [21]. Accordingly, effects of Ca^{2+} antagonists on the tomato ICE1-related protein profiles in tomato under cold stress were examined with Ca^{2+} antagonist and inhibitors for cell signaling. When tomato leaves were subjected to cold stress, induction of ICE1 protein levels was not observed in the presence of EGTA (Ca^{2+}chelater) and weak signal was induced in the presence of $LaCl_3$ (a Ca^{2+} channel blocker). On the other hand, cold stress

significantly enhanced the tomato ICE1 protein level in the presence of Ca^{2+} as expected (Fig. 2B). In addition, protein kinase inhibitor (K252a) treatment suppressed the cold stress-induced up regulation of the tomato ICE1 proteins (Fig. 2C). Previous studies demonstrated that cold stress enhances expression of genes for Ca^{2+}-dependent protein kinases (OsCDPKs) in rice and enzymatic activities of CDPK in rice [22,23]. The present data in the experiments with Ca^{2+} antagonists and protein kinase inhibitor suggests that crosstalk between Ca^{2+}signaling and protein phosphorylation play important roles in upregulation of the ICE1 protein and cold stress signaling in tomato.

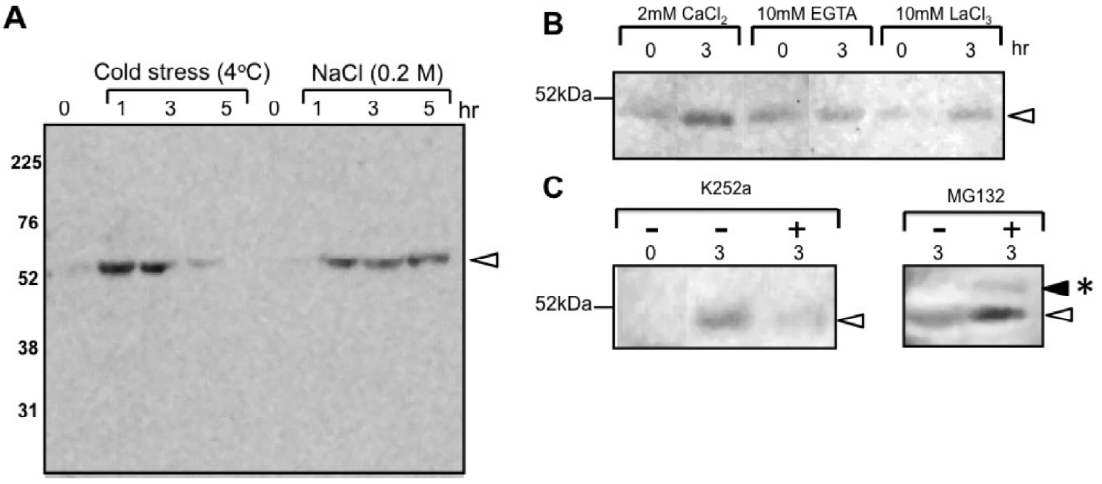

Fig. 2. Imunochemical detection of tomato ICE1-related proteins in various conditions
(A) ICE-related polypeptides in tomato were detected by immunoblot with the anti-ICE specific peptide antibody. Four week-old tomato plants were subjected to cold stress (4ºC, left) and salt stress (0.2 M NaCl, right). The predicted molecular mass of SlICE1 is 57.6 kDa. (B) Effects of Ca^{2+} antagonists on upregulation of tomato ICE1 protein levels in response to cold stress. Leaves of 4-week-old tomato plants grown at 25ºC were preincubated in 10 mM $CaCl_2$, 10 mM EGTA and 10 mM $LaCl_3$ for 2 hr and then subjected to cold stress (4ºC). (C) Effects of protein kinase inhibitor and proteasome inhibitor on upregulation of tomato ICE1 protein levels in response to cold stress. Tomato leaves grown at 25ºC were preincubated in 5 μM K252a (left) or 50 μM MG132 (right) for 2 hr and then subjected to cold stress (4ºC)

Increasing numbers of molecular biological and biochemical studies of *Arabidopsis* ICE1 indicate that E3 ligases, HOS1-dependent ubiquitination and SIZ1-dependent sumoylation play pivotal roles in the degradation and regulation of Arabidopsis ICE1 proteins in response to cold stress [20,21]. Thus, to examine if ubiquitin-proteasome system is implicated in regulation of tomato ICE1 protein, an effect of MG132 in protein status of the tomato ICE1 protein was analyzed [19]. In the presence of MG132, cold stress significantly enhanced the immunoreactive signal of tomato ICE1-related protein at 52 kDa, compared to that in the absence of MG132 (Fig. 2C). Interestingly, a 60 kDa band appeared in addition of the major signal at 52 kDa in the presence of MG132. The difference of the relative sizes between 60 and 52 kDa of the ICE1-related protein is consist to that of ubiquitin monomer (about 8 kDa), suggesting that MG132 suppresses proteasome-mediated degradation of the ubiquitinated ICE1 protein.

3.4 Expression Profiles of Cold Stress-Inducible Genes

In the next, the expression profiles of *SlICE1,* and cold stress-stimulated genes were examined by RT-PCR. The expression of *SlICE1* under cold was maintained at constant level even though the protein is upregulated (Figs. 2B and 3). The expression of tomato HOS1 homolog (*SlHOS1*), which possibly mediates ubiquitination of tomato ICE1 protein, was maintained under cold stress, suggesting that cold stress-mediated increase of the tomato ICE1 protein is not regulated by transcription level of *SlHOS1*. In the contrast, cold stress enhanced the expression of *SlCBF1* significantly at 1 and 5 hr (Fig. 3). At the same PCR cycles as that for *LeCBF1*, increase in PCR signals of *LeCBF2* nor *LeCBF3* was not observed. This observation on the difference of induction profiles among tomato CBF homologs under cold stress is consistent to data of RNA blot of *LeCBF1-3* as described previously [7].

Trehalose 6-phosphate synthase (TPS) is a key enzyme for trehalose synthesis pathway to catalayze UDP-galactose and glucose to trehalose 6-phosphate. Then trehalose phosphate phosphatase (TPP) catalyzes dephosphorylation of trehalose 6-phosphate to generate trehalose. It was reported that one of tomato TPS-related genes, SlTPS1, is induced in response to both cold and salt stresses [11]. The expression profiles of *SlTPS1* and *LeCOR413* under cold were examined. RT-PCR analysis indicated that the expression of SlTPS1 and LeCOR413 increased significantly at 5 hr after cold stress following upregulation of *LeCBF*1 at 3 hr after cold stress (Fig. 3). *OsTPP1* appeared to be induced under cold stress and trehalose treatment alleviates chilling damage of rice [17]. These observations suggest that trehalose synthesis is a key step for acquisition of cold tolerance in tomato and rice. *LeCOR413* is a homolog of Arabidopsis cold-responsive gene 413 (AtCOR413) involved in freezing tolerance and was analyzed as a positive control [24]. *SlCBF1* expression increased greatly from 1 to 3 and 5 hr. In contrast, the expression of *SlTPS1* and *LeCOR413* increased at 5 hr. Based on data of immunoblot and RT-PCR, increase of SlICE1 protein and then induction of *LeCBF1* expression were followed by up regulation of *SlTPS1* and *LeCOR413* in an apparently sequential manner after cold stress treatment (Figs. 2A and 3). This result suggests that tomato ICE1 regulates the cold-stimulated transcription cascade composing of *SlCBF1* leading to induction of cold acclimation-related genes, such as *SlTPS1* and *LeCOR413*.

3.5 Putative Cis-Elements Interacting to SlICE1

Analysis of the *cis*-element genes which interact with Arabidopsis ICE1 revealed the presence of MYC recognition sequences (CANNTG) in their promoters [25]. When we focused on a tomato CBF gene cluster region (locus AY497899), at least 5 different possible MYC binding sequences of the “CANNTG” core were identified in the promoter region ranging - 650 to + 150 bp in locus encoding *LeCBF1* by “PLACE” (A Database of Plant Cis-acting Regulatory DNA Elements). Thus, it is reasonable to assume that tomato ICE1 regulates the expression of *LeCBF1* by binding to the MYC core sequences in the promoter region of *LeCBF1*.

Our previous studies demonstrated that the expression of *OsTPP1* is regulated at 3 hr after the treatment and the maximum expression is 24 hr after cold stress and that various stresses including cold, salt and heat stress up regulated the expression of *SlTPS1* [11,17]. It has been reported that the accumulation of trehalose enhances cold acclimation of rice and tomato, according to induction of *OsTPP1* and *SlTPS1* under cold stress, respectively

[11,26]. Putative *cis*-elements in the promoter region of *SlTPS1* were predicted by "PLACE", based on whole genome data of tomato genome. There is a site of C-repeat element (CCGAC or RYCGAC), potentially recognized by DREB/CBF type transcription factor in the *SlTPS1* promoter [27]. In addition, at least 4 sites of possible MYC binding consensus are also identified. Thus, it is reasonable to assume that the cold-responsive transcriptional factors (*SlICE1* and *SlCBF1*) are implicated in cold acclimation of tomato *via* trehalose synthesis.

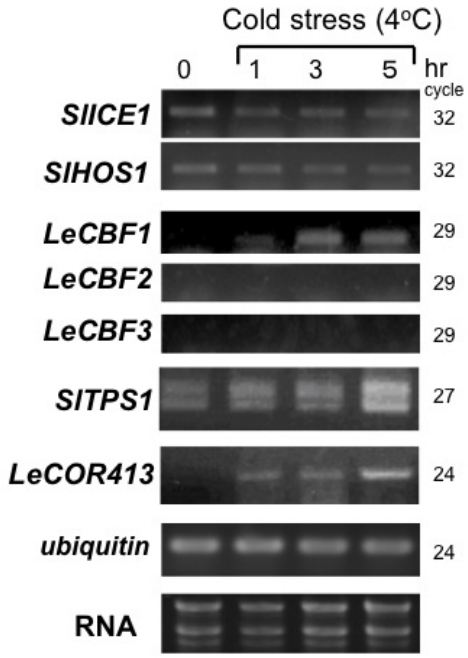

Fig. 3. Cold stress induced cold-related genes in tomato

The expression of SlICE-homolog and cold stress-related genes was analyzed bysemi-quantitative RT-PCR. Four-week-old tomato plants grown at 25ºC were subjected to cold stress (4ºC) and harvested at the indicated times for preparation of total RNA. Cycles of RT-PCR were indicated

4. DISCUSSION

Our present data of tomato ICE1 homolog and cold stress-related genes indicate that cold stress increased the levels of *SlICE1* proteins but did not enhance the expression of their genes. Interestingly, *Arabidopsis ICE1* mRNA in itself is scarcely affected by environmental stresses, but the ICE1 protein is regulated in complex manner by post-translational modifications (phosphorylation, ubiquitination and sumoylation) [18,20,25,28]. As mentioned, *Arabidopsis* ICE1 protein appeared to be regulated by various post-translational modifications and protein profiles of under cold and salt stress. Thus, SlICE1 are regulated mainly by post-translational mechanisms such as ubiquitin-proteasome system, phosphorylation and Ca^{2+} signaling, in a manner similar to that of *Arabidopsis* ICE1. *Arabidopsis ICE1* appeared to be upregulated at both the mRNA and protein levels under cold stress [25]. Thus, the transcription of *ICE* homologs could be regulated in different manner between tomato and *Arabidopsis*.

Furthermore, the increase of the tomato ICE1-related protein under cold stress was followed by the sequential upregulation of *SlCBF1*and *SlTPS1*. Originally, *Arabidopsis* ICE1 was identified to bind specifically to *cis*-elements in the promoter region of *DREB/CBF* and to be a master regulating transcription factor for induction of *DREB/CBF* in response to cold stress [8,25]. Trehalose 6-phosphate synthase (TPS) is a key enzyme for trehalose synthesis pathway to catalayze UDP-galactose and glucose to trehalose 6-phosphate. Then trehalose phosphate phosphatase (TPP) catalyzes dephosphorylation of trehalose 6-phosphate to generate trehalose. It was reported that one of tomato TPS-related genes, SlTPS1 is induced in response to both cold and salt stresses [11]. Thus, it is conceivable that the tomato ICE1 homologs induces tomato *CBF1* and a set of genes related to cold acclimation such as *SlTPS1*, on consideration of the similarity of the biochemical properties between *Arabidopsis* ICE1 and the SlICE1 and the expression profiles of the cold-inducible genes *SlCBF1*and *SlTPS1* (Fig. 3).

It still remains uncertain whether SlCBF1 functions directly at upstream of *SlTPS1*, leading to cold acclimation by mediating trehalose synthesis. To investigate this issue, it will be necessary to analyze the interactions among SlICE1, LeCBF1 and *cis*-element of cold responsive genesby gel-shift assays and chromatin-immunoprecipitation assays (ChiP) and in transgenic tomato plants.

5. CONCLUSION

In this study, we report that an ICE1-related proteins with molecular masses of approximately 55 is induced in tomato plant under cold and salt stresses and that cold stress sequentially upregulated tomato CBF homolog, SlCBF1 and trehalose-6-phosphate synthase (SlTPS1). Promoter regions of SlCBF1 and SlTPS1 possess cis-elements potentially binding to ICE1 and CBF, respectively. These results indicate that tomato ICE1 homolog functions in transcriptional regulation of LeCBF1 and SlTPS1 in response to cold stress, resulting in acquisition of cold tolerance via induction of trehalose synthesis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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