1 **Tittle:**

2 **A Tomato Inducer of *CBF* Expression 1 (SlICE1) is Involved in Cold Stress Signaling**

3

Title:

A Tomato Inducer of *CBF* Expression 1 (*SlICE1*) is Involved in Cold Stress Signaling

Abstract:

Environmental stresses such as cold and salt result in reduction of crop yield through growth retardation and irreversible damage. Recent studies unveiled that Inducer of *CBF* Expression 1 (ICE1) is the master regulator which regulates the expression of dehydration responsive element binding protein / C-repeat binding factor (DREB/CBF)-type transcriptional factors in response to cold and osmotic stress in *Arabidopsis*. To address roles of a tomato ICE homolog in stress signaling involving tomato *CBF* homologs under cold and salt stresses, we raised an antibody crossreacting to a polypeptide epitope specific to an conserved motif among plant ICE1 homologs. An ICE1-related proteins with molecular masses of approximately 55 and 40 kDa is induced in tomato plant under chilling and salt stresses. The expression of *SlICE1* under chilling stress was maintained at a constant level in contrast to the protein 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 up stream sequences in promoter regions of *SlCBF1* and *STPS1*, respectively. These results suggest that tomato ICE1 homolog mediates the expression of *SlCBF1* in a cold-stress-induced transcription factor cascade, leading to induction of cold tolerance by trehalose synthesis.

Key words: CBF, Chilling stress, ICE, tomato, trehalose.

Introduction

Among environmental stresses, cold stress is known to cause the most serious damage on plant growth and crop yield (Hayashi et al. 2009). 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 (Oono et al. 2006; Suwabe and Yano 2008; Phan et al. 2010). 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 (Shinozaki and Yamaguchi-Shinozaki 2000; Zhang et al. 2004). Although various sets of transcriptional factors such as *DREB*/*CBF*, *bZIP*, *MYC*, *MYB*, and *Hsf* (*heat shock factor*), are know to be expressed in specific profiles under osmotic, salt and cold stresses, the whole transcription networks involving the environmental signaling remain to be unresolved yet. 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 (Zarka et al. 2003; Toledo-Ortiz et al. 2003; Zhu et al. 2007). Interestingly, *ICE1* mRNA in itself is scarcely affected by environmental stresses, but ICE1 protein is regulated in complex manner by post-translational modifications (phosphorylation, ubiquitination and sumoylation) (Chinnusamy et al. 2003; Dong et al. 2006; Miura and Hasegawa 2010; Miura et al. 2007). Both monocots and dicots possess *ICE*-related genes (Badawi et al. 2008). Almost dicots have a single *ICE* gene in those geneme sets, while various monocots have two *ICE* homologs encoding about 40 and 55 kDa proteins (Zarka et al. 2003; Wang et al. 2005). As mentioned, ICE1 in Arabidopsis appeaered to be regulated by various post-translational modifications and protein profiles of under cold and salt stress. However, it still remain unclear whether the endogenous ICE1 proteins in tomato are regulated in the same way.

Recent studies unveiled that the gene regulation via *DREB/CBF*, trehalose accumulation and SnRK2-mediated protein phosphorylation implicated in the mechanisms

in acquisition of cold tolerance in tomato (Zhang et al. 2004; Tomikubo et al. 2007; Yuasa et al. 2007). Plant ICE1 homologs could function as a pivotal regulator for *CBF* and trehalose synthesis, and thus of cold tolerance. Here, we show the immunological characterization of tomato ICE-related proteins and their potential roles in aquistion of tolerance to cold and salt stresses via the regulation of *SlCBF1* and *SlTPS1*.

Materials and Methods

After sawing tomato seeds (*Solunam lycoperisicon* L. cv. MicroTom) in 6cm x 6cm x 6cm glass wools, tomato plants were grown for 4 week at 25^{o}C as previously described (Yuasa et al. 2012). The 4 week old-Tomato plants were subjected to cold stress (4°C) or salt stress by spraying with solution of 0.2 M NaCl. After shoot and leaf of plants were harvested at 0, 1, 3, and 5 h, the samples were immediately frozen in liquid nitrogen and stored at -80^{o}C.

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 builded with the deduced amino acid sequences by the alignment program of CLUSTALW (http://align.genome.jp/)t. Specific primer sets for semi-quantitative RT-PCR were designed from *SlICE1*, *SlCBF1*, *SlCBF2*, SlCBF3 and *SlTPS1* (Table 1). Tomato *actin* 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 (Yuasa et al. 2012). 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 94 °C for 2 min; 25 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s; and a final 72 °C for 5 min. The numbers of cycles were as indicated. After electrophoresis in 1.5% agarose gels, ethidium bromide-stained PCR products were visualized by FluorChem Imager (Alpha Innotech, San Leandro, CA, USA).

To prepare glutathione S-transferase (GST)-fused SlICE1, we constructed pGEX-*ICE1*. PCR fragments encoding ΔN-OsICE1 (323–524 aa) and ΔN-OsICE2 (158–381 aa) were amplified with KOD Plus DNA polymerase (TOYOBO, Tokyo, Japan), tomato cDNA, and gene-specific primer sets (Table 1), and then digested with *BamH*I and *Sal*I (*SlICE1*). The resultant *SlICE1* fragment was ligated into *BamH*I-*Sal*I 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* cDNAs was confirmed by sequencing on an ABI Prism 310 DNA sequencer with a Big Dye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystems, Foster City, CA, USA). The recombinant proteins of GST-fused SlICE1 was induced in *E.coli* in the presence of 0.5mM ITPG for 2 h at 37^{o}C after growing in LB/Amp medium over night at 37^{o}C.

Protein extract was prepared from tomato plants (~0.1 g) stored at -80^{o}C 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 mM benzamidine, with or without 1% Triton X-100. The resultant extracts were centrifuged at 10,000 ×*g* for 5 min at 4 °C. Protein concentrations were determined by measuring OD_{595} with a Bio Rad protein assay kit (Bio Rad, Hercules, CA, USA), using 1 mg ml^{-1} bovine serum albumin as a standard.

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 at 10 V cm^{-1} for 2 h. The membranes were then incubated in blocking buffer containing 1× TBS and 2.5% skim milk for 1 h, and then in blocking buffer supplemented with anti–ICE-homolog common peptides primary antibody (1/1000 dilution) and 0.05% Tween 20 for 2 h at 4 °C. 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 h. Immunoreactive signals were

141 visualized by an ECL Plus kit (GE Healthcare Bio-Sciences) and FluorChem.

142

143 **Results and Discussion**

144 A phylogenetic tree of ICE homologs in dicots and monocots, using *Arabidopsis* PIF3 and
145 human c-myc as outgroups, showed that *ICE*-related genes can be classified into dicot and
146 monocot subfamilies as previously reported (Badawi et al. 2008; Nakamura et al. 2011)
147 (Figure 1A). Interestingly, SlICE1 in the phylogenic tree is branched at a root of the two
148 ICE1 subfamily of dicots and monocots even though SlICE1 is possibly classified to the
149 ICE1 family (Fig. 1A). SlICE1 possesses very conserved domain sets including an acidic
150 domain, a Ser-rich domain, a bHLH domain, and a possible zipper region, similar with
151 those of *Arabidopsis* ICE1. SlICE1 and AtICE1 have a little different predicted molecular
152 masses, SlICE1 has similarities of 43% at the amino acid level to *Arabidopsis* ICE1.
153 Compared with MYC-like bHLH transcription factors, the alignments of the plant ICE1
154 homologs revealed a highly conserved motif of 19 amino acids (KMDRASILGDAI(D/E)-
155 YLKELL) that is specific to plant ICE1 homolgs but not to other MYC-like proteins
156 (Toledo-Ortiz et al. 2003: Nakamura et al. 2011) (Fig. 1B).

157 *E. coli* crude extracts containing recombinant GST-SlICE1 was subjected to SDS-
158 PAGE (10% acrylamide) and immunoblot with anti-ICE antibody (Figure 1C).
159 Immunoreactive signals indicate that the anti-ICE1 peptide antibody crossreacted
160 specifically with GST-SlICE1, but not with GST, nor endogenous *E. coli* polypeptides.
161 Immunoblot was performed to assess whether the anti-ICE antibody cross-reacted
162 specifically with endogenous ICE1-related polypeptides in tomato plants subjected to cold,
163 and salt stresses (Fig. 2A). In response to cold stress, immunoreactive signals with
164 molecular masses of about 52 kDa significantly increased at 1 and 3 hr after cold stress, and
165 then decreased to a marginal level at 5 hr, while the immunoreactive signal was stimulated
166 at 1 hr and then maintained at 3 and 5 hr. In contrast to cold and salt stresses, heat stress had
167 no effect on immunoreactive signals of the ICE1-related protein in tomato plants (data not
168 shown). The induction of the endogenous SlICE1 protein (57.6 kDa) under cold stress is

consistent with previous reports that cold stress upregulated a protein level of epitope-tagged *Arabidopsis* ICE1 (Dong et al. 2006; Miura et al. 2007).

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 (Miura and Furumoto 2013). It was reported that phosphorylation of a Ser-rich region in Arabidopsis ICE1 are involved in cold stress signaling (Miura et al. 2011). 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. Cold stress-induced upregulation of ICE1 proteins were suppressed in tomato leaves treated with EGTA, Ca^{2+} chelater, or $LaCl_3$, a Ca^{2+} channel blocker, while cold stress significantly enhanced the tomato ICE1 protein level in the presence of Ca^{2+} as expected (Fig. 2A). In addition, protein kinase inhibitor (K252a) treatment suppressed the cold stress-induced upregulation of the tomato ICE1 proteins (Fig. 2B). 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 (Wan et al. 2007; Martin and Busconi 2001). 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.

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 (Miura et al. 2007). 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 (Dong et al. 2006). 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, right). 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.

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 (Fig. 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, but had no or marginal effect on those of *LeCBF2* nor *LeCBF3* (Fig. 3). 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 (Zhang et al. 2004). *Arabidopsis ICE1* appeared to be upregulated at both the mRNA and protein levels under cold stress (Chinnusamy et al. 2010). Thus, the transcription of *ICE* homologs could be regulated in different manner between tomato and *Arabidopsis*.

The expression profiles of *SlTPS1* and *LeCOR413* under cold were examined. Tomato *TPS1* is induced in response to cold and salt stress (Tomikubo et al. 2007). *OsTPP1* appeared to be induced under cold stress and trehalose treatment alleviates chilling damage of rice (Nakamura et al. 2011). These observations suggest that trehalose synthesis is a key step for acquisition of cold trelance 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 (Breton et al. 2003). *SlCBF1* expression increased greatly from 1 to 3 and 5 h In contrast, the expression of *SlTPS1* and *LeCOR413* increased at 5 h. Based on data of immunoblot and RT-PCR, Increase of SlICE1 protein and then induction of *LeCBF1* expression were followed by upregulatiuon of *SlTPS1* and *LeCOR413* in an apparently sequential manner after cold stress treatment (Fig. 2A and Fig3). 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

225 *LeCOR413*.

226 Analysis of the cis-element genes which interact with Arabidopsis ICE1 revealed the
227 presence of MYC recognition sequences (CANNTG) in their promoters (Chinnusamy et al.
228 2003). When we focused on a tomato CBF gene cluster region (locus AY497899), at least 5
229 different possible MYC binding sequences of the "CANNTG" core were identified in the
230 promoter region ranging -650 to + 150 bp in locus encoding LeCBF1 by "PLACE" (A
231 Database of Plant Cis-acting Regulatory DNA Elements:
232 http://www.dna.affrc.go.jp/PLACE/signalscan.html). Thus, it is reasonable to assume that
233 tomato ICE1 regulates the expression of *LeCBF1* by binding to the MYC core sequences in
234 the promoter region of *LeCBF1*.

235 It has been reported that the accumulation of trehalose enhances cold acclimation of
236 rice and tomato, according to induction of *OsTPP1* and *SlTPS1* under cold stress,
237 respectively (Pramanik and Imai 2005; Tomikubo et al. 2007). Putative cis-elements in the
238 promoter region of *SlTPS1* were predicted by "PLACE", based on whole genome data of
239 tomato genome. There is a site of C-repeat element (CCGAC or RYCGAC), potentially
240 recognized by DREB/CBF type transcription factor in the *SlTPS1* promoter (Liu et al. 1998).
241 In addition, at least 4 sites of possible MYC binding consensus are alsoidentified. Thus, it is
242 reasonable to assume that the cold-responsive transcriptional factors (*SlICE1* and *SlCBF1*)
243 are implicated in cold acclimation of tomato *via* trehalose synthesis.

244 Our present data of tomato ICE1 homolog and cold stress-related genes indicate that
245 cold stress increased the levels of *SlICE1* proteins but did not enhance the expression of
246 their genes. Thus, SlICE1 are regulated mainly by post-translational mechanisms such as
247 ubiquitination and proteasome system, in a manner similar to that of *Arabidopsis* ICE1.
248 Furthermore, the increase of the tomato ICE1-related protein under cold stress was followed
249 by the sequential upregulation of *SlCBF1*and *SlTPS1*. Originally, *Arabidopsis* ICE1 was
250 identified to bind specifically to *cis*-elements in the promoter region of *DREB/CBF* and to
251 be a master regulating transcription factor for induction of *DREB/CBF* in response to cold
252 stress (Zarka et al. 2003; Chinnusamy et al. 2003). 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 genes by gel-shift assays and chromatin-immunoprecipitation assays and in transgenic tomato plants.

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Figure legends

Figure 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 (AK24172), *Solanum lycopersicum*; AtICE1 (At3g26740), AtPIF3 (At1g09530), *Arabidopsis thaliana*; CapsellaICE1 (AY504806), *Capsella bursa-pastoris*; BrassicaICE1 (HQ902162), *Brassica napus*; GmICE1 (FJ393223), *Glycine max*; PopulusICE (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 and5 μg protein per lane, respectively) of *E. coli* containing pGEX4T-1 empty and pGEX-SlICE1-CT.

Figure 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 (4^{o}C, left) and salt stress (0.2 M NaCl, right). The predicted molecular masses of SlICE1 are 57.6 kDa. (B) Effects of Ca^{2+} antagonists on upregulation of tomato *ICE1* protein levels in response to chold stress. Leaves of 4-week-old tomato plants grown at

25°C were preincubated in 10 mM $CaCl_2$, 10mM EGTA and 10mM $LaCl_3$ for 2 hr and then subjected to cold stress (4°C). (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 25°C were preincubated in 5 μM K252a (left) or 50 μM MG132 (right) for 2 hr and then subjected to cold stress (4°C).

Figure 3. Cold stress induced cold-related genes in tomato. The expression of *SlICE*-homolog and cold stress-related genes was analyzed by semi-quantitative RT-PCR. Four-week-old tomato plants grown at 25 °C were subjected to cold stress (4 °C) and harvested at the indicated times for preparation of total RNA.

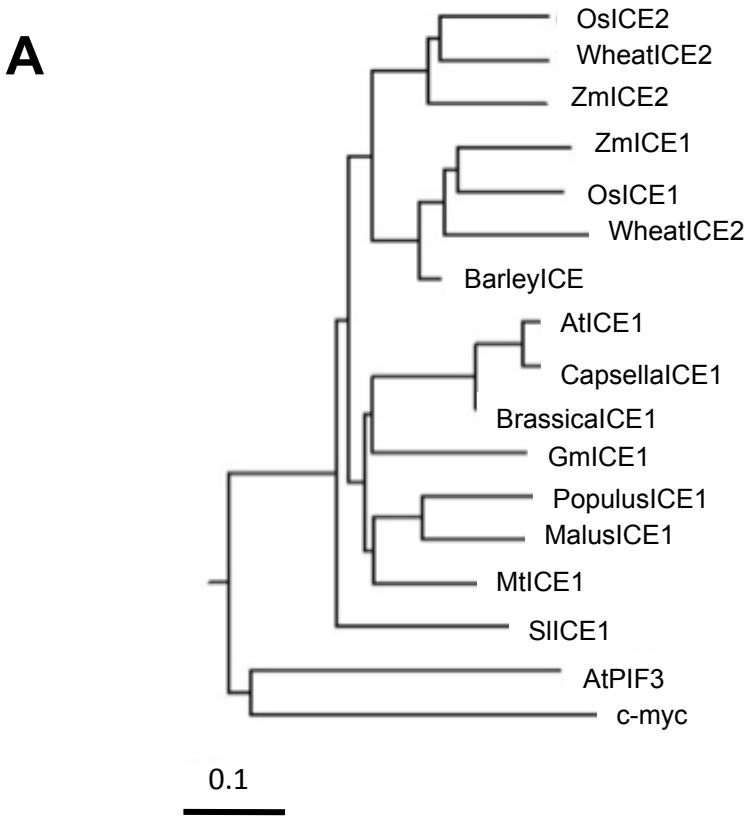
Table 1. Primers used for RT-PCR analysis and construction of expression plasmids

Gene	Accession number and/or KTU contig	Primer set
SlICE1-F	AK24172	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	KTU3 Contig23669	5- ATGGGTAGGATGGATTATTTGGCTATG -3
SiCOR413-R		5- TCAGACGGCTCGAAGAACCAGAGC-3
SlUbi-F	BT012698	5-ACGTGGATCCATGCAAATCTTTGTGAAGAC-3
SlUbi-R		5-AAAGTCGACTAACCACCACGGAGACGGAGG-3

Introduced restriction sites are show 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



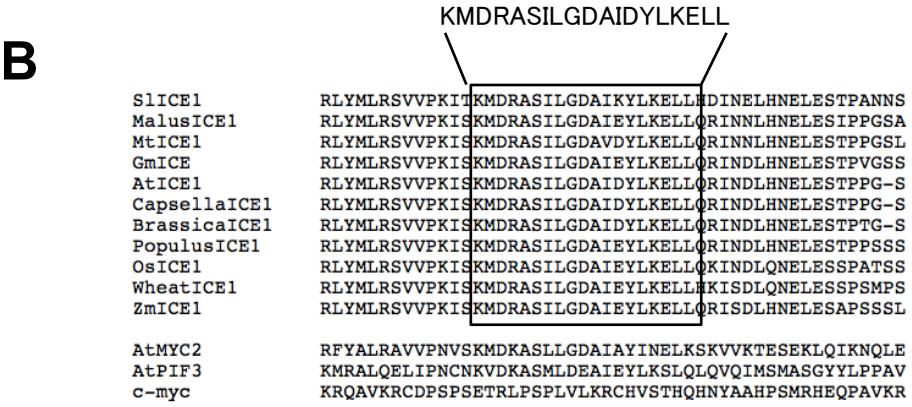


Fig. 1AB

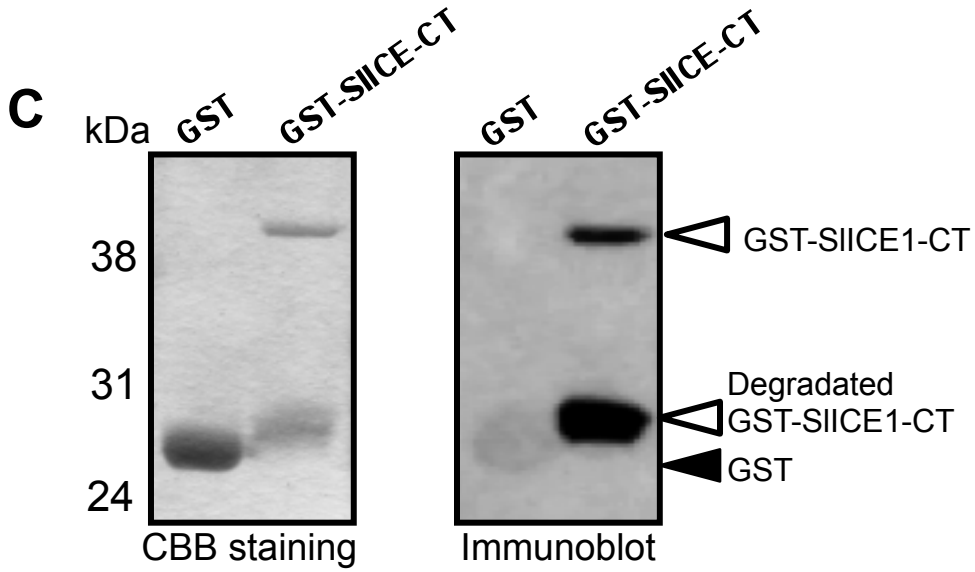


Figure 1. Phylogenic tree of ICE (Inducer of CBF Expression) homologs. (A) ICE homolog sub-families are apparently classified into two groups of dicots and monocots. A group of plant ICE homologs has weak similarity in the bHLH domain to those of human c-myc and *Arabidopsis* PIF3 (AtPIF3). (B) Alignments of plant ICE1 homolog proteins. A conserved amino acid motif (KMDRASILGDAI-KYLKELL) present in the carboxyl-half region of the bHLH region was used as an epitope for raising anti-ICE1-specific antibody. SlICE1 (AK24172), *Solanum lycopersicum*; AtICE1 (At3g26740), AtPIF3 (At1g09530), *Arabidopsis thaliana*; CapsellaICE1 (AY504806), *Capsella bursa-pastoris*; BrassicaICE1 (HQ902162), *Brassica napus*; GmICE1 (FJ393223), *Glycine max*; PopulusICE (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 and5 μg protein per lane, respectively) of *E. coli* containing pGEX4T-1 empty and pGEX-SlICE1-CT.

Fig. 1C

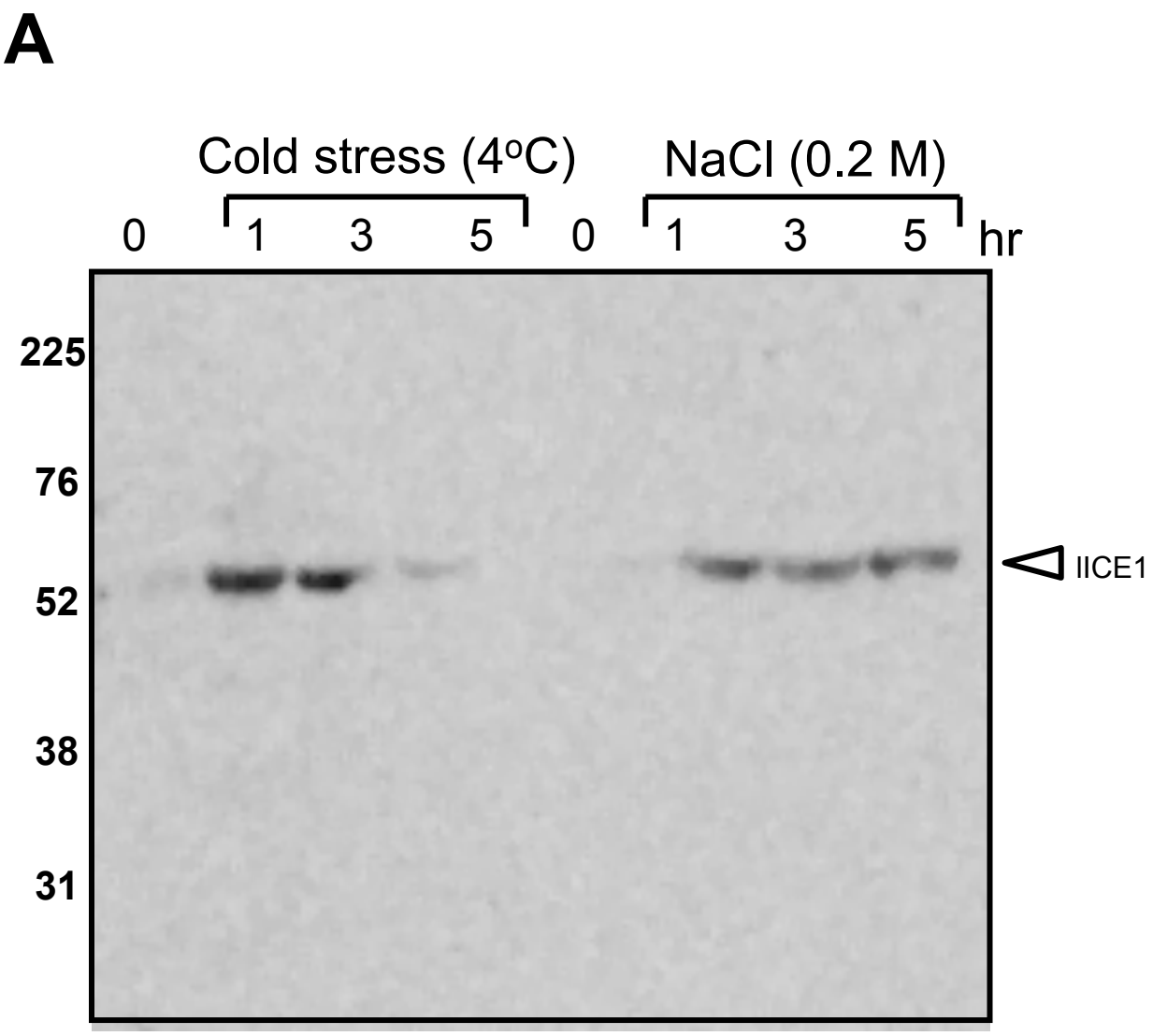


Fig. 2A

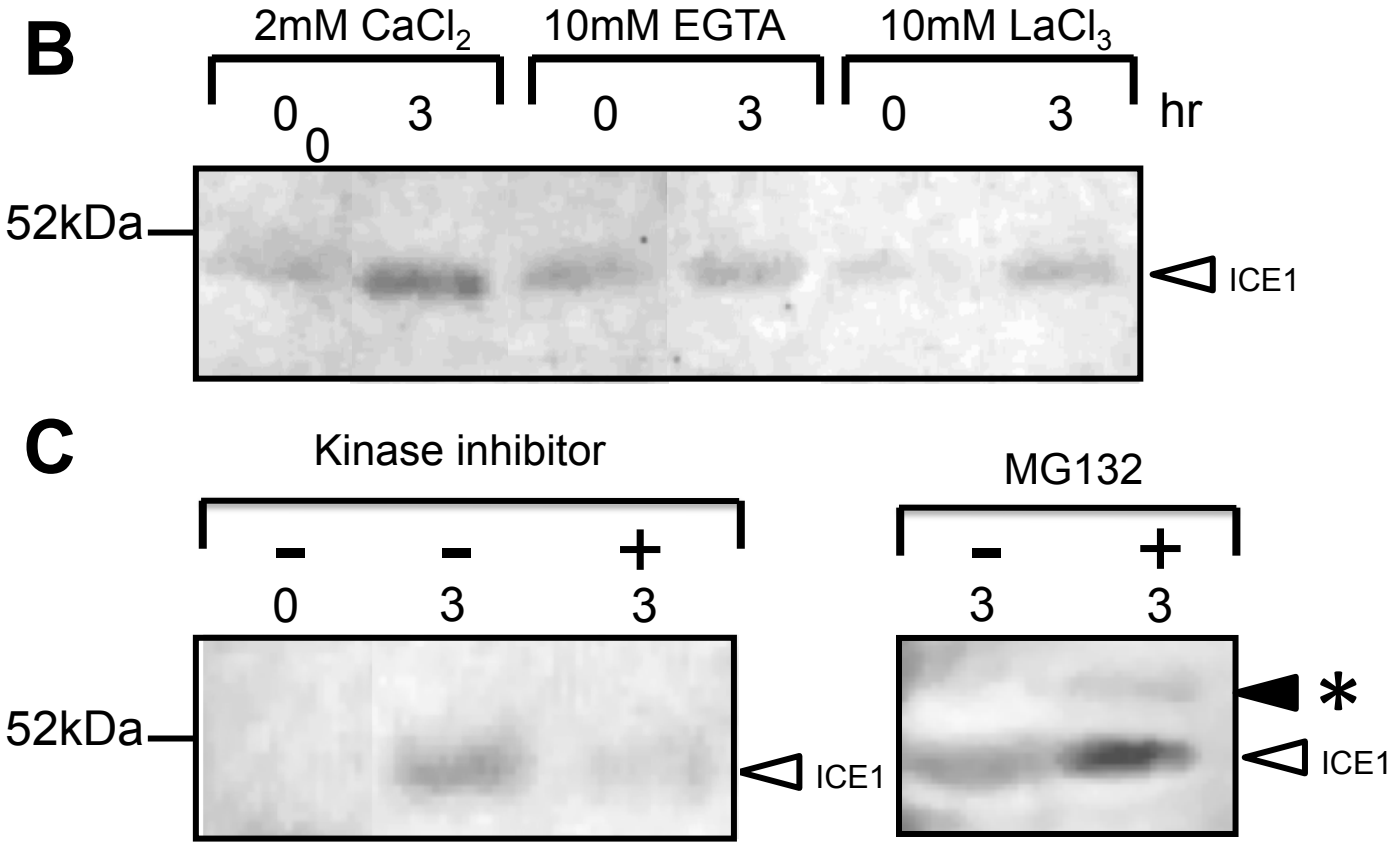


Figure 2. Imunochemical detection of tomato ICE1-related proteins in various conditions. (A) ICE-related polypeptides in tomato were detected by immunoblot with the anti-ICE specific peptide antibody. Four week-old tomato plants were subjected to cold stress (4°C, left) and salt stress (0.2 M NaCl, right).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

Fig. 2BC

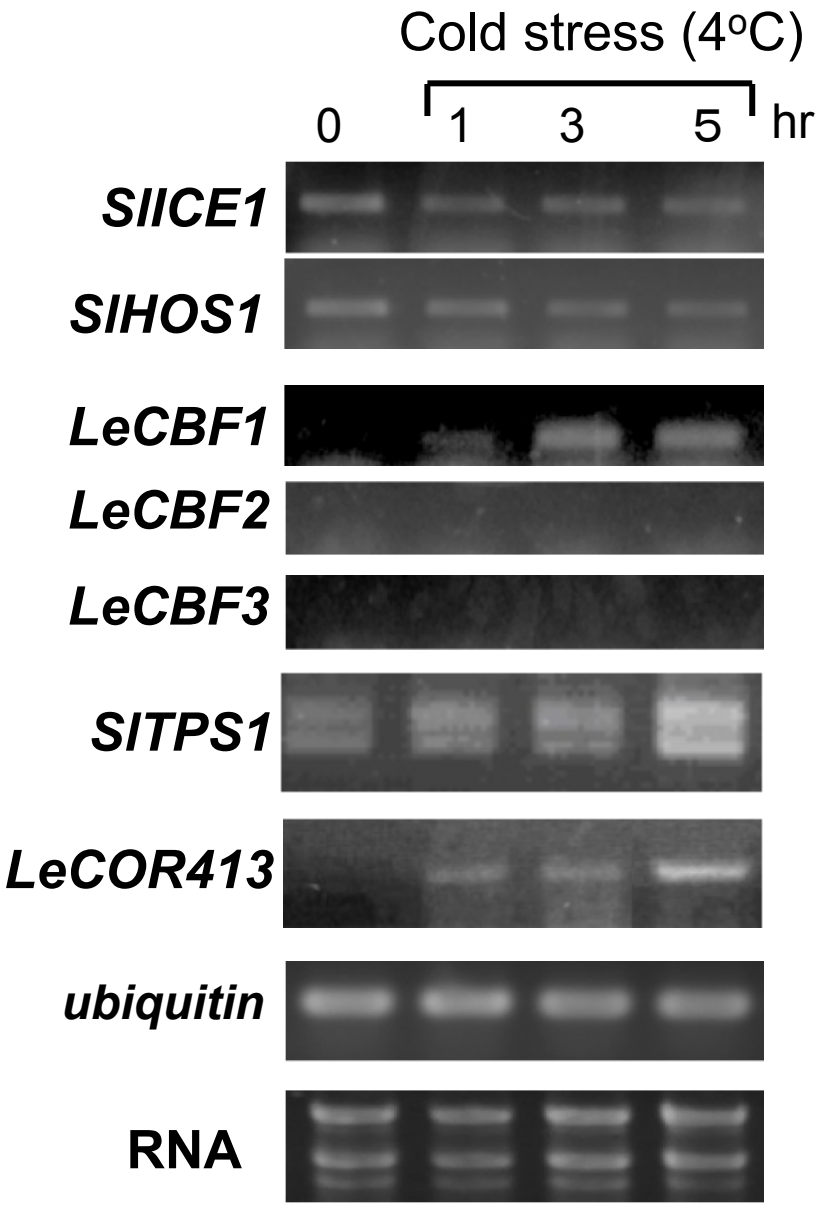


Figure 3. Cold stress induced cold-related genes in tomato. The expression of *SlICE*-homolog and cold stress-related genes was analyzed by semi-quantitative RT-PCR. Four-week-old tomato plants grown at 25 °C were subjected to cold stress (4 °C) and harvested at the indicated times for preparation of total RNA.

Fig. 3