ICE1 Ser403 is necessary for protein stabilization and regulation of cold signaling and tolerance.
Title:

ICE1 Ser403 is necessary for protein stabilization and regulation of
cold signaling and tolerance

Kenji Miuraa,b,1,2, Masaru Ohtaa,1, Machiko Nakazawaa, Michiyuki Onoa, and Paul M. Hasegawab

aGraduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba
305-8572, Japan
bCenter for Plant Environmental Stress Physiology, Purdue University, West Lafayette,
IN 47907, USA
1K.M. and M.O. contributed equally to this work

2Corresponding author: Kenji Miura

Address: Graduate School of Life and Environmental Sciences, University of Tsukuba,
Tsukuba 305-8572, Japan
Email, kmiura@gene.tsukuba.ac.jp; TEL & Fax, +81-29-853-6401

Running title: Stabilization and regulation of ICE1 by serine 403

Key words: cold tolerance, cold signaling, transcription factor, stabilization,
ubiquitylation, substitution

Word count: 5685
Summary
ICE1, a MYC-type transcription factor, has an important role in the induction of
CBF3/DREB1A for the regulation of cold signaling and tolerance. In this study, we
reveal that serine 403 of ICE1 is involved in regulating the transactivation and stability
of the ICE1 protein. Substitution of serine 403 to an alanine enhanced the
transactivational activity of ICE1 in Arabidopsis protoplasts. The overexpression of
ICE1(S403A) conferred more freezing tolerance than ICE1(WT) in Arabidopsis, and the
expression of cold-regulated genes such as CBF3/DREB1A, COR47, and KIN1 was
enhanced in ICE1(S403A)-overexpressing plants. Furthermore, the ICE1(S403A)
protein level was not changed after cold treatment, whereas the ICE1(WT) protein level
was reduced. Interestingly, polyubiquitylation of the ICE1(S403A) protein in vivo was
apparently blocked. These results demonstrate that serine 403 of ICE1 has roles in both
the transactivation and the cold-induced degradation of ICE1 via the ubiquitin/26S
proteasome pathway, suggesting that serine 403 is a key residue for the attenuation of
cold stress responses by HOS1 mediated degradation of ICE1.

Abbreviations: bHLH, basic helix-loop-helix; CBF, CRT-binding factor; CRT,
C-repeat; DRE, dehydration responsive element; DREB, DRE-binding protein; HOS1,
high expression of osmotically responsive genes 1; ICE1, inducer of CBF expression 1;
SUMO, small ubiquitin-related modifier
Introduction

Plants need to adapt to changes in the environment for their survival. When the temperature decreases to below the freezing point, some plants native to temperate zones have the ability to tolerate the freezing temperature. The freezing tolerance of temperate plants is not a constant property but it is induced by exposure to non-freezing low temperatures. This process is known as cold acclimation (Guy, 1990; Thomashow, 1999). Global transcription profiling analyses have revealed that more than 10% of the genes in the Arabidopsis genome are regulated during cold acclimation (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002). These cold-responsive genes include key metabolic enzymes, late embryogenesis-abundant proteins, and detoxification enzymes (Thomashow, 1999).

Many cold- and dehydration-responsive genes contain one or more copies of C-repeat (CRT)/dehydration responsive element (DRE) cis-elements in their promoters. The CRT/DRE cis-element has a core sequence, CCGAC, and is able to respond to cold, dehydration and salinity. (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). A family of AP2-type transcription factors, known as the CBF (CRT-binding factor)/DREB (DRE-binding protein) family, binds to these cis-elements and induces expression of the cold- and dehydration-responsive genes (Stockinger et al., 1997; Liu et al., 1998). Thus, the ectopic expression of CBF/DREB confers improved tolerance to freezing, dehydration and salinity (Jaglo-Ottosen et al., 1998; Liu et al., 1998). Because the expression of CBF/DREB itself is induced by cold (Stockinger et al., 1997; Liu et al., 1998; Gilmour et al., 1998), other transcription factors also control the induction of CBF/DREB in response to low temperature.

Several factors involved in the regulation of CBF/DREB expression have been
identified genetically in *Arabidopsis* (Chinnusamy et al., 2007; Lissarre et al., 2010). The *ice1* mutant has been identified by a genetic screen of mutants impaired in the expression of the *CBF3-LUC* reporter gene (Chinnusamy et al., 2003). Expression of *CBF3/DREB1A* and its downstream target genes was shown to be down-regulated in the *ice1* mutant, and, consequently, *ice1* plants display significantly reduced chilling and freezing tolerance (Chinnusamy et al., 2003). *ICE1* (inducer of CBF expression 1) encodes a MYC-like bHLH (basic helix-loop-helix) transcription factor that can bind directly to the canonical MYC cis-elements (CANNTG) in the *CBF3/DREB1A* promoter (Chinnusamy et al., 2003). A genome-wide transcription profile revealed that the expression of 204 of the 939 cold-regulated genes is affected in the *ice1* mutant (Lee et al., 2005). Although *ICE1* mainly affects *CBF3/DREB1A* expression (Chinnusamy et al., 2003), *ICE2*, a homolog of *ICE1* (At1g12860), primarily influences the expression of *CBF1/DREB1B*, but not of *CBF3/DREB1A* (Fursova et al., 2009). Thus, *ICE1* and *ICE2* have pivotal roles in the transcriptional regulation of *CBF/DREBs*. Interestingly, *ICE1/SCREAM* is also involved in stomatal differentiation (Kanaoka et al., 2008), suggesting that *ICE1* links the transcriptional regulation of environmental adaptation and stomatal development in plants.

The recessive mutant *hos1* causes the enhanced expression of *CBF/DREB* and cold-responsive genes in cold conditions (Ishitani et al., 1998; Lee et al., 2001). *HOS1* (high expression of osmotically responsive genes 1) encodes a RING finger-type E3 ligase for the ubiquitylation of ICE1, which results in the cold-induced degradation of ICE1 (Lee et al., 2001; Dong et al., 2006). ICE1 is also post-translationally regulated by SUMO (small ubiquitin-related modifier) E3 ligase SIZ1-mediated sumoylation (Miura et al., 2007a; Miura et al., 2007b; Miura and Hasegawa, 2010). Protein
phosphorylation is the most common mechanism controlling gene expression, however, it is unknown whether ICE1 is also regulated by this modification.

In this study, we evaluated the serine/threonine residues in the regulation of ICE1. The substitution of S403 to alanine in ICE1 [ICE1(S403A)] enhanced GAL4-mediated transactivation in protoplasts, the expression of cold-regulated genes, such as CBF3/DREB1A, COR47, and KIN1, and cold tolerance in transgenic plants. Furthermore, the ICE1(S403A) protein level was not changed after cold treatment, whereas the ICE1(WT) level was reduced. Polyubiquitylation of the ICE1(S403A) protein was blocked in vivo. These results demonstrate that S403 of ICE1 plays a role in the regulation of both the transactivation and the cold-induced degradation of the transcription factor via the ubiquitin/26S proteasome pathway, probably mediated by HOS1. Thus, the enhanced transactivation and impaired degradation of ICE1(S403A) may enhance the expression of CBF3/DREB1A and its regulon genes in ICE1(S403A)-overexpressing plants.
Results

Serine 403 to alanine substitution increases the transactivation activity of ICE1 and the freezing tolerance of Arabidopsis

Previously, we have demonstrated that the SIZ1-mediated sumoylation of ICE1 at K393 is necessary for cold signaling and tolerance (Miura et al., 2007a) and that the flanking region of the sumoylation site, including serine and threonine residues, is highly conserved among orthologs. Several reports have also demonstrated relationship between phosphorylation and sumoylation (Mohideen et al., 2009). Consequently, separate variant proteins with S to A or T to A mutations were generated; T387A, S389A, S399A, S400A or S403A (Fig. 1A). These ICE1 variants were fused with the DNA-binding domain of the yeast GAL4 transcription factor (Fig. 1B; Ohta et al., 2000; Tiwari et al., 2003). Each GAL4-ICE1 variant was co-introduced into Arabidopsis leaf protoplasts with the GAL4-GUS reporter and 35S-LUC (Fig. 1B), and the protoplasts were incubated at 23°C. Because of the moderate temperature, we presumed that ICE1 variant would not have been degraded by the proteasome (Dong et al., 2006). The transactivation activity was measured as GUS activity and LUC activity was used for plasmid uptake normalization between samples (Tiwari et al., 2003; Yoo et al., 2007). GAL4-ICE1(S403A) and GAL4-ICE1(S399A, S400A, S403A) activated GAL4-mediated transactivation ~2.8-fold higher than GAL4-ICE1(WT) did (Fig. 1C; Supplemental Fig. S1). In contrast, the transactivation activities of ICE1(T387A), ICE1(S389A), and ICE1(S399A or S400A) were similar to that of ICE1(WT) (Supplemental Fig. S1). These results indicate that S403 is an important residue in the negative regulation of ICE1 transactivational activity.
In order to assess the biological role of S403 in ICE1, we produced transgenic plants overexpressing \textit{ICE1(S403A)}. Transcript abundance of the \textit{ICE1(S403A)} transgene was evaluated by semi-quantitative RT-PCR analysis (Supplemental Fig. S2), which indicated that transgenic gene expression was similar in \textit{ICE1(WT)} (line #12; Miura et al., 2007) or \textit{ICE1(S403A)} (#5, 12, 13) transgenic plants. Non-acclimated three-week-old wild-type (Col-0), a vector control, \textit{ICE1(WT)}-overexpressing (Miura et al., 2007), and \textit{ICE1(S403A)}-overexpressing plants were subjected to -4°C for 4 h. \textit{ICE1(WT)}-overexpressing plants exhibited higher survival rates (9.38% ± 6.0) than wild-type (3.13% ± 1.8) and vector control (all died under this condition) plants after one week of recovery at room temperature (Fig. 2A and 2C). The \textit{ICE1(S403A)}-overexpressing plants were more freezing tolerant than the \textit{ICE1(WT)}-overexpressing plants (survival rates ranging from 34.4% to 45.3%, with statistical significance at \( P < 0.05 \) against the survival of \textit{ICE1(WT)}-overexpressing plants; Fig. 2C). Similar results were also observed when cold-acclimated plants were subjected to -8°C for 4 h (Fig. 2B and 2D). Electrolyte leakage analysis was performed on detached leaves from the non-acclimated plants. At -3°C, the percentages of electrolyte leakage were 41.4 ± 4.6% for the vector control, 38.5 ± 2.5% for the \textit{ICE1(WT)}-overexpressing, and 28.2 ± 2.2% for the \textit{ICE1(S403A)}-overexpressing plants (Fig. 2E), indicating that the leaves of the \textit{ICE1(S403A)}-overexpressing plants were less susceptible to freezing (Fig. 2E). These results demonstrate that the serine403 substitution to alanine enhanced freezing tolerance in \textit{Arabidopsis}.

Overexpression of \textit{ICE1(WT)} or \textit{ICE1(S403A)} did not affect plant morphology (Fig. 3A, 3B), fresh weight (Fig. 3C), leaf area (Fig. 3D), or stomatal development (Fig. 4A).
or density (Fig. 4B). SCREAM (SCRM)/ICE1 and SCREAM2 (SCRM2)/ICE2 are paralogous genes whose proteins interact with and regulate critical determinants of stomatal differentiation (Kanaoka et al., 2008). Interestingly, ICE1(WT) or ICE1(S403A) expression could suppress ice1-2 scrm-2 double mutant phenotypes of reduced stomatal density (not shown) and dwarfism (Fig. 3E; Kanaoka et al., 2008). ICE1(S403A) was functionally redundant to ICE1(WT).

**ICE1(S403A) overexpression enhanced induction of cold-regulated gene expression**

Because ICE1 controls the expression of CBF3/DREB1A and the regulon genes, such as COR47 and KIN1 (Chinnusamy et al., 2003), transcript abundance of these cold-regulated genes was monitored by quantitative RT-PCR analysis. CBF3/DREB1A transcript was greater in ICE1(WT)-overexpressing plants than in the vector control plants (Fig. 5), confirming results described previously (Chinnusamy et al., 2003; Miura et al., 2007a). ICE1(S403A) overexpression increased CBF3/DREB1A expression to a greater extent, which was most evident after 12 h of cold treatment (Fig. 5). Transgenic ICE1 and native ICE1 expression was also investigated in ICE1(WT) and ICE1(S403A) transgenic plants. Interestingly, native ICE1 expression was induced substantially by ICE1(WT) and, to greater extent, by ICE1(S403A) (Fig. 5), even prior to cold induction, probably because of the three canonical MYC cis-elements (CANNTG) present in 2 kb of the ICE1 promoter. Transgene ICE1 expression, which was driven by the cassava vein promoter, was similar in both ICE1(WT) and ICE1(S403A) (Fig. 5). Even though ICE1 transcript was accumulated under normal condition, induction of CBF3/DREB1A expression is required for cold treatment (Fig. 5), suggesting that ICE1 protein may be activated by cold and S403 is not a major residue for ICE1 activation. Transcript
accumulation of *CBF3/DREB1A* regulon genes *COR47* and *KIN1*, which are evident 12 h after exposure of the plants to low temperatures (Gilmour et al., 1998), was induced by overexpression of *ICE1(WT)* or *ICE1(S403A)* but to a greater extent by the later (Fig. 5). These results suggest that a prolonged expression of *CBF3/DREB1A* leads to higher levels of cold-regulated gene expression, probably resulting in the higher freezing tolerance of *ICE1(S403A)*-overexpressing plants.

Non-acclimated *ICE1(WT)* or *ICE1(S403A)* plants exhibited more tolerance to freezing stress (Figs. 2A, 2C). Low temperatures of 10°C or less induce *CBF/DREB1* expression within 15 min, and transcript accumulation increases with low temperature (Jaglo-Ottosen et al., 1998; Chinnusamy et al., 2003). In our conditions for freezing experiments, plants were kept at 4°C for 30 min and 0°C for 1 h, and then the temperature was successively decreased at -1°C h⁻¹, as described in Experimental procedures. Therefore, it took 3.5 h to reach the desired temperature (-3°C; Fig. 2A). Because we observed the expression of *CBF3/DREB1A* at 3 h of cold treatment to be up-regulated in *ICE1(S403A)* (Fig. 5), we suggest that the plants may have been partially acclimated to the cold. Thus, the *ICE1(S403A)* transgenic plants were able to survive better than the *ICE1(WT)* transgenic or wild-type plants at -3°C (Fig. 2A, 2C).

**Serine 403 to alanine substitution increased stability of ICE1 against cold-induced proteasome degradation**

Low temperature induces ICE1 degradation after cold treatment through the ubiquitin/proteasome pathway, a process that is mediated by the ubiquitin E3 ligase HOS1 (Dong et al., 2006). To investigate whether the S403A substitution affects the
stability of ICE1, ICE1 protein abundance was determined before and after cold treatment using western blot analysis. ICE1(WT) and ICE1(S403A) were expressed as T7-ICE1 fusion proteins, allowing for detection with anti-T7 tag antibody. Because the ice1-2 scrm-2 double mutation was complemented by T7-ICE1(WT) and T7-ICE1(S403A) (Fig. 3E), T7-tag did not disturb biological function of ICE1. A 24 h low temperature (4°C) treatment results in substantial reduction in the T7-ICE1(WT) protein but affected T7-ICE1(S403A) protein abundance to a lesser degree (Fig. 6A). To confirm the stability of the ICE1(S403A) protein in vivo, we observed the fluorescence of GFP-ICE1 fusion proteins. The level of nuclear GFP-ICE1(WT) protein detected after cold treatment was reduced to a greater extent than GFP-ICE1(S403A) protein (Fig. 6B). These results indicate that the S403A substitution increases the stability of the ICE1 protein against cold-induced degradation.

The substitution of serine 403 to alanine blocked the polyubiquitylation of ICE1 in vivo

The proteasome inhibitor MG132 was used to assess whether low temperature-induced degradation of ICE1 proteins was due to ubiquitylation. High-molecular weight polypeptide bands corresponding to the polyubiquitylated forms of T7-ICE1 were detected in the immunoprecipitated T7-ICE1 proteins obtained from ICE1(WT)-overexpressing plants (Fig. 7A). However, the polyubiquitylated forms of T7-ICE1 were not detected in the immunoprecipitated T7-ICE1 proteins from ICE1(S403A)-overexpressing plants (Fig. 7A). These results suggest that the substitution of S403A inhibits the polyubiquitylation of ICE1 and, consequently, results in greater stability of the ICE1 protein against cold-induced degradation.
Nonlysine residues such as serine, threonine, and cysteine are ubiquitylation sites in MHC-1 and the Bcl-2 family member Bid (Cadwell and Coscoy, 2005; Tait et al., 2007; Wang et al., 2007). Thus, an in vitro ubiquitylation assay of ICE1 proteins was assessed using HOS1 as an E3 ligase as described (Dong et al., 2006). After the addition of HOS1, a high molecular weight band was detected in both ICE1(WT) and ICE1(S403A) recombinant proteins (Fig. 7B), a pattern that is similar to another report (Dong et al., 2006). Thus, serine 403 is not the main target residue for ubiquitylation, but it inhibits the polyubiquitylation of ICE1 in vivo.

The effect of the S403A substitution on the ICE1 sumoylation status was determined. T7-ICE1(WT) or T7-ICE1(S403A) was transiently expressed in Nicotiana benthamiana leaves (Voinnet et al., 2003) and immunoprecipitated with anti-T7 antibody. Immunoblot analysis was then performed using anti-SUMO1 antibodies (Supplemental Fig. S3; Miura and Ohta, 2010). No significant difference was apparent, suggesting that sumoylation is not involved in regulation of ICE1 through S403.
Discussion

S403A substitution in ICE1 enhanced \textit{GAL4}-mediated transactivation in \textit{Arabidopsis} protoplasts (Fig. 1C), and expression of cold-regulated genes, such as \textit{CBF3/DREB1A}, \textit{COR47}, and \textit{KIN1} in plants (Fig. 3), and cold tolerance (Fig. 2). Furthermore, the ICE1(S403A) protein level did not undergo cold-induced polyubiquitylation mediated by the ubiquitin E3 ligase HOS1 or proteasome degradation \textit{in vivo} to the extent of ICE1(WT) (Figs. 6, 7), but ICE1(S403A) can be polyubiquitylated \textit{in vitro} (Fig. 7B), suggesting that ICE1(S403A) facilitates protein stability \textit{in vivo} even though S403 is not a major site of ubiquitin conjugation. Greater ICE1 stability caused by the S403A allele likely enhances expression of \textit{CBF3/DREB1A} and its regulon genes such as \textit{COR47} and \textit{KIN1} (Fig. 5).

Regulation of ICE1 transactivational activity

Substitution of S403A in ICE1 enhanced the \textit{GAL4}-mediated transactivation in \textit{Arabidopsis} protoplasts (Fig. 1C), indicating the region around S403 has a role in the negative regulation of the transcriptional activity of ICE1. S403A substitution appears to repress this negative synergy. MYB15, a negative regulation of \textit{CBF} expression, binds to the C terminus of ICE1 (amino acids 395 to 494) and it is possible that MYB15 interaction can tranrepress ICE1 transcriptional activity (Agarwal et al., 2006). It is feasible that the transcriptional repression of \textit{CBF3/DREB1A} expression by MYB15 is suppressed to some extent by S403A mutation. In this scenario, the S403A mutation may inhibit the association of MYB15 with ICE1, thereby allowing for the ICE1-activated transcription of \textit{CBF3/DREB1A} in response to cold.
The expression of *CBF3/DREB1A* was not expressed before cold treatment, as previously described (Chinnusamy et al., 2003; Miura et al., 2007), even though *ICE1(WT)* or *ICE1(S403A)* was overexpressed (Fig. 5). These results support the notion that low temperature is required for ICE1 transcriptional activation of *CBF3/DREB1A* in vivo, although the mechanism remains unelucidated.

**ICE1 stability caused by the S403 allele**

ICE1(S403A) was substantially more stable than ICE1(WT) to low-temperature-induced proteasomal degradation (Fig. 6). It is postulated that greater ICE1 abundance is responsible for the more pronounced and sustained induction of *CBF3/DREB1A* expression, which was most evident 12 h after cold treatment, a time when the *CBF3/DREB1A* transcript abundance had decreased substantially in vector control plants (Fig. 5). ICE1(S403A) protein abundance was increased after low temperature treatment, because cold causes the ubiquitylation of ICE1 protein by a RING finger ubiquitin E3 ligase HOS1 that leads to proteasomal degradation of the transcription factor (Dong et al., 2006). The expression of *CBF3/DREB1A* in the hos1 mutant was enhanced and therefore the ICE1 level was higher after cold treatment (Lee et al., 2001), as in the *ICE1(S403A)*-overexpressing plants (Fig. 5). S403A substitution apparently attenuates HOS1-mediated ubiquitylation and proteasomal degradation that is induced by low temperature (Fig. 7A). However, S403 is not principal ubiquitylation site for HOS1 but regulates the activity of the E3 ligase to ubiquitylate ICE1 (Fig. 7B).

The T7-ICE1(WT) polypeptide was the same molecular weight as the
T7-ICE1(S403A) polypeptide (Fig. 6), and there was no additional band at a higher molecular weight, which may have corresponded to phosphorylated T7-ICE1, in either WT or S403A (Fig. 6). Furthermore, the substitution of S403 to aspartate (S403D) in ICE1 also increased the transactivational activity, as did the ICE1(S403A) substitution (Supplemental Fig. S1). These results suggest that S403 is not a major phosphorylation site.

Hydroxyl-oxygen side chains in serine and threonine residues may be subjected to O-linked glycosylation. An increased number of proline residues is present near the O-glycosylated serine or threonine residues, in comparison with nonglycosylated serines or threonines (Christlet and Veluraja, 2001). Because serine 403 in ICE1 is also in close proximity to proline residues (Fig. 1A), it is plausible that O-glycosylation occurs via S403. Because the recombinant ICE1 protein, expressed in E.coli, would not have had any glycosylation, the substitution of S403 may not have affected ubiquitylation (Fig. 7B). However, ICE1(S403A) may have blocked glycosylation in vivo, resulting in a different poly-ubiquitylation pattern (Fig. 7A). Among several O-linked glycosylations, such as O-N-acetylgalactosamine and O-mannose, O-N-acetylglucosamine (O-GlcNAc) modification is an important posttranslational modification that modulates the function of many nuclear and cytoplasmic proteins, regulation of protein-protein interaction, competition with phosphorylation, nucleo-cytoplasmic shuttling, and modulation of transcription factor activity (Hu et al., 2010; Özcan et al., 2010). Because increasing O-GlcNAc levels enhances ubiquitylation and the RNAi of O-GlcNAc transferase reduced ubiquitylation (Guinez et al., 2008), O-GlcNAC and ubiquitylation may be linked. It is possible that the glycosylation of
ICE1 at S403 regulates ubiquitylation and cold signaling.

**Neither ICE1(WT) nor ICE1(S403A) overexpression affect stomatal development**

*ICE1/SCREAM* has also been shown to be involved in stomatal differentiation (Kanaoka et al., 2008). This indicates that *ICE1* links the transcriptional regulation of environmental adaptation and development in plants and that the mechanisms of transcriptional regulation by ICE1 might overlap between cold adaptation and stomatal development processes. However, there were no significant differences in either stomatal development or number among wild-type, *ICE1(WT)*-overexpressing and *ICE1(S403A)*-overexpressing plants (Fig. 4). The hypothesis is that ICE1 may act as an integrator of cold signaling and stomatal development. Normally, MYC-type transcription factors form dimers for transactivation. For example, mammalian Myc/Max/Mad can form dimers in multiple combinations through interactions mediated by their helix-loop-helix leucine zipper dimerization interfaces (Davis and Halazonetis, 1993; Ferré-D’Amaré et al., 1993; Luscher and Larsson, 1999). However, different members of the Myc/Max/Mad family have distinct biological functions. As described (Kanaoka et al., 2008), ICE1/SCRM can interact with three closely related bHLH transcription factors, SPCH, MUTA, and FAMA, for stomatal development, the entry into the stomatal cell lineage, the transition from meristemoid to guard mother cells, and the terminal differentiation of guard cells (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). It is assumed that ICE1/SCRM and other MYC-type proteins, as well as SPCH, MUTA, and FAMA, can form several combinations to control cold signaling or stomatal development. The stabilization of ICE1 conferred by the S403A substitution did not affect the stomatal development (Fig. 4), suggesting that
the cold-induced degradation of ICE1 mediated by HOS1 is specific to cold adaptation but not to stomatal development.

The overexpression of ICE2 also conferred a freezing tolerance in Arabidopsis (Fursova et al., 2009). Expression of ICE2/SCRM2(R203H), which corresponds to the ice1 mutation (R236H), phenocopied the constitutive stomatal differentiation of ice1/scrm in the epidermis (Kanaoka et al., 2008). These results demonstrate that ICE2/SCRM2 is functionally related to ICE1 and that ICE2/SCRM2 may exert a similar mechanism of transcriptional regulation as ICE1. The amino acid sequence surrounding S403 in ICE1 is highly conserved in ICE2/SCRM2 (Supplemental Fig. S4). It is possible that S359 of ICE2, which corresponds to S403 of ICE1, is likely to be involved in a similar mechanism as the S403 residue of ICE1.

The overexpression of CBF/DREB1s improves the freezing tolerance of plants but results in severe growth retardation under normal conditions (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al. 2004). ICE1(S403A)-overexpressing plants were also more tolerant to freezing temperatures, but they grew similar to WT plants under normal conditions, even though the expression of ICE1(S403A) was driven by a constitutive cassava vein mosaic virus promoter (Fig. 3). ICE1 homologs have also been identified in barley and wheat (Skinner et al., 2006; Tondelli et al., 2006; Badawi et al., 2008). The overexpression of ICE1(S403A) can provide a potent strategy for improving the freezing tolerance of crop plants, thereby increasing their productivities and potentially expanding their global distribution.
Experimental procedures

Transient expression assay

*Arabidopsis* protoplasts were prepared from two-week-old wild-type seedlings with the Cellulase Ononza R-10 and Macerozyme R-10 (Yakult Pharmaceutical, http://www.yakult.co.jp/pi/en/index.html), as described (Tiwari et al., 2003; Yoo et al., 2007). Plasmid DNA of a reporter *GAL4-GUS* (Tiwari et al., 2003) and an effector *GAL4DB-ICE1(WT)* or its variants was introduced into *Arabidopsis* protoplasts by polyethylene glycol-mediated transfection (Tiwari et al., 2003; Yoo et al., 2007). For each transfection, 5 μg of the reporter and 4 μg of the effector plasmid DNA were used. After transfection, the protoplasts were incubated at 23°C in dark for 48 h. One μg of the reference plasmid DNA, *35S-LUC*, was used to normalize the efficiency of each transfection. GUS activity was measured as described (Yoo et al., 2007). A luciferase assay was performed with the Luciferase Reporter Assay system (Promega, http://www.promega.com) using a luminescence reader (Gene Light 55, Microtech Nichion, http://nition.com/en/).

Plant freezing assay

The whole plant freezing assay was performed essentially as described, with slight modifications (Miura et al., 2007a; Miura and Ohta, 2010). Wild-type (Col-0), vector control (#8), *ICE1(WT)*-overexpressing (#12), and *ICE1(S403A)*-overexpressing plants (in this study) were grown at 23°C for 3 weeks under constant illumination in soil. For cold acclimation, 3-week-old plants were incubated at 4°C for 7 d. Non-acclimated plants were incubated at 4°C for 30 min and then at 0°C for 1 h. Next, the temperature was successively decreased 1°C per hour until -4°C and held at -4°C for 4 hr in a
programmed incubator (IN602, Yamato, http://www.yamatokikai.com). Cold-acclimated plants were incubated at 0°C for 1 h, and the temperature was successively decreased 2°C per hour until -8°C and held at -8°C for 4 hr in a programmed incubator. To facilitate uniform nucleation, crushed ice was spread over the plants at 0°C. After cold acclimation, the plants were kept overnight at 4°C and then transferred to 23°C. The survival ratio was determined one week after the freezing treatment.

**RNA preparation and quantitative RT-PCR**

Vector control (#8), *ICE1(WT)*-overexpressing (#12) (Miura et al., 2007a), and *ICE1(S403A)*-overexpressing (#13) plants (in this study) were grown on half-MS agar plates at 23°C for 10 days under a long-day photoperiod (16 h light/8 h dark). The plants were then subjected to cold treatments at 4°C. Isolation of the total RNA, cDNA synthesis, and quantitative RT-PCR was performed as described (Miura et al., 2009). Primers used for the real-time PCR are listed in Supplemental Table S1.

**Construction of plasmids**

Mutations in the *ICE1* coding regions were introduced by PCR-based methods using the PrimeSTAR DNA polymerase (Takara Bio, http://www.takara-bio.com) and pairs of primers (Supplemental Table S2). The mutated *ICE1* coding regions were amplified by the PrimeSTAR DNA polymerase with ICE1-GDF and ICE1-HAR primers and then digested with *Xma*I and *Xho*I. The digested PCR products were introduced into the plant expression vector 35S-GAL4 DB digested with *Xma*I and *Sal*I (Ohta et al., 2000) to produce 35S-GAL4-ICE1(mutant) fusion genes. To construct pCsV-T7-ICE1(S403A), the *ICE1(S403A)* coding region was amplified by the
PrimeSTAR DNA polymerase (Takara Bio) with ICE1-expF and ICE1-EGR primers (Supplemental Table S2), and the PCR product was introduced into pCsV1300-T7 as described (Miura et al., 2007a; Verdaguer et al., 1996).

The ICE1 or ICE1(S403A) coding region was amplified by the PrimeSTAR DNA polymerase with primers, ICE1-expF and ICE1-HAR, and then digested with BamHI and SalI. The digested PCR product was cloned into an *E.coli* expression vector, pCold ProS2 (Takara Bio). The resulting plasmids were named as pCold-ICE1 and pCold-ICE1(S403A). Each plasmid was transformed into *E.coli* Origami 2(DE3) pLysS cells (Novagen, http://www.emdchemicals.com/life-science-research).

**Plant transformation**

pCsV-ICE1(S403A) was transformed into wild-type *Arabidopsis* plants (Col-0 background) by *Agrobacterium*-mediated transformation (Miura et al., 2007a; Miura et al., 2009). Hygromycin-resistant plants were selected, and semi-quantitative RT-PCR was carried out with the primers ICE1S403AF and NOS-transR (Supplemental Table S2) to check the expression levels of the transgenes (Supplemental Fig. S2).

**Agroinfiltration procedure**

Agroinfiltration assay was performed as described (Voinnet et al., 2003). Briefly, *Agrobacterium tumefaciens* strain GV3101 containing pCsV-T7-ICE1(WT), pCsV-T7-ICE1(S403A), or p19 (Voinnet et al., 2003) was grown at 28°C in L-broth supplemented with 10 mM MES, 20 μM acetosyringone, 50 μg ml⁻¹ kanamycin, 30 μg ml⁻¹ gentamycin, and 30 μg ml⁻¹ rifampicin to stationary phase. Bacteria were sedimented by centrifugation at 5,000 g for 15 min at room temperature, suspended in
10 mM MgCl₂, 10mM MES and 100 μM acetosyringone, and left for more than 3 h. Equal volume of bacteria containing pCsV-T7-ICE1(WT) or pCsV-T7-ICE1(S403A) and p19 was mixed. The mixture was infiltrated into the abaxial sides of 3-4-week-old *Nicotiana benthamiana* leaves. After 3-4 days incubation, plants were ground in extraction buffer as described (Murtas et al., 2003).

**Western blot analysis and Immunoprecipitation**

Ten day-old seedlings of *ICE1(WT)-overexpressing (#12)* and *ICE1(S403A)-overexpressing (#13)* plants were ground in extraction buffer, as described (Murtas et al., 2003). Total protein (20-40 μg) was separated by SDS-PAGE, and western blot analysis was performed as described (Miura et al., 2005; Miura et al., 2009).

To detect ubiquitylated ICE1, ten-day-old *ICE1(WT)-overexpressing (#12)* and *ICE1(S403A)-overexpressing (#13)* plants were pretreated with 50 μM proteasome inhibitor (MG132) for 24 h and then treated at 4°C for 15 h. Total protein extract (10 mg protein) was immunoprecipitated with T7 antibodies coupled to agarose beads (Novagen) for 4 h at 4°C, and the beads were recovered by centrifugation, washed 5 times with the extraction buffer, and eluted according to the manufacturer’s instructions. The eluted samples were separated by SDS-PAGE and immunoblot analysis with monoclonal antibody against ubiquitin (P4D1, Santa Cruz Biotechnology Inc, http://www.scbt.com) was performed.

**Ubiquitylation assay**

The ICE1 or ICE1(S403A) coding region was cloned into pCold ProS2 (Takara Bio).
When the *E. coli* cells grew to mid-log phase, IPTG was added to the medium at a final concentration of 0.4 μM. The culture was incubated at 15°C for 24 h. The bacterial cell walls were disrupted by the BugBuster Protein Extraction Reagent with rLysosome and Benzonase Nuclease (Novagen). The recombinant proteins, His-ProS2-ICE1(WT) and His-ProS2-ICE1(S403A), were purified with the HIS-Select Nickel Affinity Gel (Sigma, http://www.sigmaaldrich.com), according to the manufacturer’s instructions. MBP-HOS1 (Dong et al., 2006) was purified using amylase resin (New England Biolabs, http://www.neb.com), as instructed. The ubiquitylation assay was performed using the Ubiquitylation Kit (Enzo Life Sciences, http://www.enzolifesciences.com) with UbcH5b for E2, according to the instructions.

**Microscopic observation**

The coding region of *ICE1(S403A)* was excised from the pCsV-ICE1(S403A) plasmid DNA by digestion with *BamHI*. The resulting DNA fragment was introduced into the *BamHI* site of the binary vector pEGAD to generate GFP-ICE1(S403A). The direction of the insert was confirmed by sequencing, and the plasmid DNA with a sense insert orientation was used for further experiments. This GFP-ICE1(S403A) plasmid was introduced into *Agrobacterium* strain GV3101 and transformed into wild-type *Arabidopsis* plants (Col-0). T2 transgenic lines resistant to Basta (glufosinate) were chosen for the analysis of GFP expression. Fluorescence of the GFP-ICE fusion proteins was observed with a confocal laser-scanning microscope (TCS SP2, Leica, http://www.leica.com).
Acknowledgments

We thank Dr. Jian-Kang Zhu and Dr. Keiko Torii for providing GFP-ICE1 transgenic plants, MBP-HOS1 plasmids, and the ice1-2 scrm-2 mutant. We also thank members of the Miura Lab for their critical comments on the manuscript. This work was supported in part by grants from the Special Coordination Funds for Promoting Science and Technology from the Japanese Ministry of Education, Culture, Sports, Science and Technology; from a Grant-in-Aid for Young Scientists (B, no. 21770032); and by the U.S. Department of Agriculture-National Research Initiative Competitive Grants Program (no. 2008-35100-04529).
References


Dong C.H., Agarwal M., Zhang Y., Xie Q. and Zhu J.K. (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the


**Fowler S. and Thomashow M.F. (2002)** Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell, 14, 1675-1690.


**Hu P., Shimoji S. and Hart G.W. (2010)** Site-specific interplay between


Seki, M., Narusaka, M., Ishida, J. *et al.* (2002) Monitoring the expression profiles of


**Verdaguer B., de Kochko A., Beachy R.N. and Fauquet C.** (1996) Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus


Short legends for Supporting Information

**Supplemental Table S1.** Primers used for mutagenesis and construction.

**Supplemental Table 2.** Primers used for real-time PCR and RT-PCR analyses.

**Supplemental Fig. S1.** Identification of serine/threonine residues affecting the transactivation activity of ICE1.

**Supplemental Fig. S2.** *ICE1(WT)* or *ICE1(S403A)* expression in transgenic plants.

**Supplemental Fig. S3.** The sumoylation status of ICE1 was not altered by substitution of the serine 403 to alanine.

**Supplemental Fig. S4.** Sequence alignment of the flanking regions of K393 and S403 in ICE1 and the corresponding region in ICE2.
Figure legends

Fig. 1. Substitution of serine 403 to alanine enhances the transactivation activity of ICE1. (A) Amino acid sequence near the sumoylation site, K393. Serine or threonine residues substituted to alanine are underlined, and lysine 393 is boxed. (B) Schematic diagram of the reporter and effector plasmids used in the transient expression assay. Wild-type and mutated ICE1 open-reading frames were inserted into the effector plasmid fused with GAL4 DB, the DNA-binding domain of the yeast transcription factor, GAL4. GUS encodes β-glucuronidase, which is used as a reporter. NOS is the termination signal of the nopaline synthase gene. Ω is the translational enhancer of the tobacco mosaic virus. (C) Relative GUS activities after transfection with the reporter, GAL4-GUS, and the effector plasmid, 35S-GAL4-ICE1 or 35S-GAL4-ICE1(S403A). Firefly luciferase activity (35S-LUC) was used for normalization; GUS activity is expressed in arbitrary units relative to the activities of firefly luciferase. The values are averages of three independent experiments, and error bars indicate standard deviations (SD).

Fig. 2. ICE1(S403A) overexpression improved the freezing tolerance of non-acclimated and cold-acclimated plants. (A) Non-acclimated plants and (B) cold-acclimated plants were treated at -4°C and -8°C, respectively. Photographs are of representative wild-type (a), vector control (b), ICE1-overexpressing [line #12 (c)], and ICE1(S403A)-overexpressing plants [line #5 (d), #12 (e), and #13 (f)]. (C) Quantification of the survival rates for non-acclimated plants after a freezing treatment at -4°C and (D) for cold-acclimated plants after a freezing treatment at -8°C. Data shown are mean values with standard error (n = 4). The tolerance of the
ICE1(S403A)-overexpressing plants was significantly different from the wild-type and vector control plants (T test, $P < 0.01$) and from the ICE1-overexpressing plants (T test, $P < 0.05$). (E) Electrolyte leakage at -3°C. The electrolyte leakage of the ICE1(S403A)-overexpressing plants was significantly lower than that of the vector control and ICE1-overexpressing plants (T test, $P < 0.05$).

Fig. 3. Growth resulting from the overexpression of ICE1(WT) and ICE1(S403A). Photographs are representative of vector control, ICE1(WT), and ICE1(S403A) transgenic plants grown for 4 weeks (A) or 8 weeks (B). Fresh weight of 4-week-old plants and leaf area of the fifth rosette leaf of 8-week-old plants were measured. (H) A representative photograph of a heterologous ice1-2/+ scrm-2 and ice1-2/- scrm-2 double mutant and an ice1-2/- scrm-2 double mutant harboring ICE1(WT) and ICE1(S403A). Bars = 1 cm (A), or 5 cm (B).

Fig. 4. Stomatal development of ICE1(WT) and ICE1(S403A)-overexpressing plants. A. Abaxial leaf epidermis of WT (a), ICE1(WT) (b) and ICE1(S403A) (c)-overexpressing plants. Bar indicates 20-μm length. B. Mean number of stomata on the abaxial leaf epidermis. Mean ± SE (n = 16).

Fig. 5. Induction of cold regulated genes is enhanced by the overexpression of ICE1(S403A). Relative mRNA transcript levels of transgenic ICE1, endogenous ICE1, CBFI/DREB1A, COR47, and KIN1 in vector control (#8), ICE1-overexpressing (#12) and ICE1(S403A)-overexpressing (#13) seedlings were determined by quantitative RT-PCR.
analyses. Ten-day-old seedlings grown at 23°C were incubated at 4°C for the indicated time. Data are means with SD (n = 3) from a representative experiment of three independent experiments.

**Fig. 6.** The S403A substitution stabilizes ICE1 against cold-induced degradation. (A) Detection of the T7-ICE1 protein by western blot analysis. Fourteen-day-old *ICE1(WT)*- and *ICE1(S403A)*-overexpressing plants were examined before (0 h) or after (24 h) treatment at 4°C for 24 h. The T7-ICE1 protein was detected with anti-T7 antiserum. Histone H3 was detected as a loading control. (B) Visualization of GFP-ICE1 fusion proteins. Ten-day-old seedlings of *GFP-ICE1(WT)* and *GFP-ICE1(S403A)* transgenic plants grown on agar plates were treated (4°C for 24 h) or not treated (23°C) with cold stress. Roots of the seedlings were observed immediately after cold stress under a confocal microscope. Scale bars = 150 μm.

**Fig. 7.** The S403A substitution inhibits the polyubiquitylation of ICE1 *in vivo*. (A) Polyubiquitylation status *in vivo*. Ten-day-old *ICE1(WT)*- and *ICE1(S403A)*-overexpressing plants were treated with the proteasome inhibitor MG132 (50 μM) for 24 h and then subjected to cold treatment at 4°C for 15 h. Total protein extract (10 mg protein) was immunoprecipitated with T7 antibodies coupled to agarose beads and analyzed by immunoblotting with antibody against ubiquitin (upper panel). The lower panel indicates the immunoblot analysis of the input total protein extract with T7 antibody as a loading control. (B) *In vitro* ubiquitylation assay. His-ProS2-ICE1(WT), His-ProS2-ICE1(S403A), and MBP-HOS1 recombinant proteins were prepared. Without HOS1, the ubiquitylation of ICE1 was not detected even
though ubiquitin, E1, and E2 proteins were added. Addition of HOS1 enhanced the ubiquitylation of both ICE1(WT) and ICE1(S403A). The blot was incubated with anti-ProS2 antibody to detect His-ProS2-ICE1(WT) or His-ProS2-ICE1(S403A). The arrowhead indicates polyubiquitylated ICE1(WT) and ICE1(S403A).
Figure 1
Figure 2
Figure 3

A. Comparison of vector (vec), ICE1(WT), and ICE1(S403A) treatments.

B. Comparison of vector (vec), ICE1(WT), and ICE1(S403A) treatments.

C. Graph showing fresh weight (mg) for vector (vec), ICE1(WT), and ICE1(S403A).

D. Graph showing leaf area (mm²) for vector (vec), ICE1(WT), and ICE1(S403A).

E. Visual comparison of ice1-2/+ scrm-2, ice1-2/- scrm-2, ICE1(WT), and ICE1(S403A).
Figure 4
Figure 5
Figure 6
Figure 7

A

ICE1

ICE1 (WT) (S403A)

(ub)n-T7

ICE1

T7-ICE1

WB: anti-Ub

IP: anti-T7

B

ICE1 (WT)    ICE1 (S403A)

HOS1 - + - + (kDa)

WB: anti-ProS2

ProS2-ICE1

87

117
Supplemental Figure S1

[Bar chart showing relative GUS activity for different genotypes]
Supplemental Figure S2
Supplemental Figure S3
ICE1  TPQTLSCRVEELCP-SSLPPKGQQARVE
************* * ********** ***
ICE2  TPQTLSCRVEELYPSSLSPLPGQQPRVE
348     359

Supplemental Figure S4
**Supplementary Table S1.** Primers used for mutagenesis and construction.

<table>
<thead>
<tr>
<th>Name of primer for mutagenesis of ICE1</th>
<th>DNA sequence (5′-to-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE1S403AF</td>
<td>CCCTCTTCTTTACCAGCTCCTAAAGGCC</td>
</tr>
<tr>
<td>ICE1S403AR</td>
<td>GGCCTTTAGGAGCTGGTAAAGAGGG</td>
</tr>
<tr>
<td>ICE1S403DF</td>
<td>CCCTCTTCTTTACCAGATCCTAAAGGCC</td>
</tr>
<tr>
<td>ICE1S403DR</td>
<td>GGCCTTTAGGATCTGGTAAAGAGGG</td>
</tr>
<tr>
<td>ICE1SSSSAAAF</td>
<td>GTCCCGGCTGCTTTACCAGCTCCTAAAGG</td>
</tr>
<tr>
<td>ICE1SSSSAAR</td>
<td>CTTTTAGGAGCTGGTAAAGCAGCGGGAC</td>
</tr>
<tr>
<td>ICE1S389AF</td>
<td>CGCGAAGACTTTCTTTGTGTGTAAGGG</td>
</tr>
<tr>
<td>ICE1S389AR</td>
<td>CTTTGACAGCAAGCAAGAGGTTTGG</td>
</tr>
<tr>
<td>ICE1T387AF</td>
<td>CCTACACCAGCTTCTTTCTTGTCTG</td>
</tr>
<tr>
<td>ICE1T387AR</td>
<td>CACGACAAGAGAAGGCTNGGCTGTAG</td>
</tr>
<tr>
<td>ICE1S399AAF</td>
<td>AAGAGTTTGTGTCCCCTGCTTACCAAG</td>
</tr>
<tr>
<td>ICE1S399AAR</td>
<td>CTGGTTAAGCAGCGGGACACAACTCTT</td>
</tr>
</tbody>
</table>

for construction of pGD-ICE1

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>DNA sequence (5′-to-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE1-GDF</td>
<td>CACGCCCCGGGATGGGTCTTGACGGAAACAA</td>
</tr>
<tr>
<td>ICE1-HAR</td>
<td>CCGCTCGAGTCAGATCACCAGCATACCC</td>
</tr>
</tbody>
</table>

for construction of pCsV-ICE1

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>DNA sequence (5′-to-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE1-expF</td>
<td>CGGGATCCATGGGTCTTGACGGAAACAA</td>
</tr>
<tr>
<td>ICE1-EGR</td>
<td>CGGGATCCTCAGATCACCAGCATACCC</td>
</tr>
</tbody>
</table>
Supplementary Table S2. Primers used for Real-time PCR and RT-PCR analyses.

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>DNA sequence (5‘-to-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DREB1A/CBF3</td>
<td>5’-GATGACGACGTATCGTTATGGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TACACTCGTTTCTCAGTTTTACAAAC-3’</td>
</tr>
<tr>
<td>COR47</td>
<td>5’-CAGTGTCGGAGAGTGTGGTG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-ACAGCTGGTGGAATCCTCTGC-3’</td>
</tr>
<tr>
<td>KIN1</td>
<td>5’-TGGAGCTGGAGCACAACA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GACCCGAATCGCTACTTTGTTTCC-3’</td>
</tr>
<tr>
<td>ACTIN2</td>
<td>5’-TAACAGGGGAAGATGACTCAGATCA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AAGATCAAGACGAAGGATAGCATGAG-3’</td>
</tr>
</tbody>
</table>

To detect expression of ICE1 transgene

ICE1S403AF  CCCTCTTCTTTACCAGCTCCTAAAGGCC
NOS-transR  GCCAAATGTTTGAACGATCGGGA