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Title:

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

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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 *ICE1(S403A)*. Transcript abundance of the *ICE1(S403A)* transgene was evaluated by semi-quantitative RT-PCR analysis (Supplemental Fig. S2), which indicated that transgenic gene expression was similar in *ICE1(WT)* (line #12; Miura et al., 2007) or *ICE1(S403A)* (#5, 12, 13) transgenic plants. Non-acclimated three-week-old wild-type (Col-0), a vector control, *ICE1(WT)*-overexpressing (Miura et al., 2007a), and *ICE1(S403A)*-overexpressing plants were subjected to -4°C for 4 h. *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 *ICE1(S403A)*-overexpressing plants were more freezing tolerant than the *ICE1(WT)*-overexpressing plants (survival rates ranging from 34.4% to 45.3%, with statistical significance at $P < 0.05$ against the survival of *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 *ICE1(WT)*-overexpressing, and 28.2 ± 2.2% for the *ICE1(S403A)*-overexpressing plants (Fig. 2E), indicating that the leaves of the *ICE1(S403A)*-overexpressing plants were less susceptible to freezing (Fig. 2E). These results demonstrate that the serine403 substitution to alanine enhanced freezing tolerance in *Arabidopsis*.

Overexpression of *ICE1(WT)* or *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 10ºC 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 *GAL4*-mediated transactivation in *Arabidopsis* protoplasts (Fig. 1C), and expression of cold-regulated genes, such as *CBF3/DREB1A, COR47,* and *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 *in vivo* to the extent of ICE1(WT) (Figs. 6, 7), but ICE1(S403A) can be polyubiquitylated *in vitro* (Fig. 7B), suggesting that ICE1(S403A) facilitates protein stability *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 *CBF3/DREB1A* and its regulon genes such as *COR47* and *KIN1* (Fig. 5).

Regulation of ICE1 transactivational activity

Substitution of S403A in ICE1 enhanced the *GAL4*-mediated transactivation in *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 *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 *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 *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 is 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 Ononzuka R-10 and Macerozyme R-10 (Yakult Pharmaceutical, http://www.yakult.co.jp/ypi/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 *Bam*HI and *Sal*I. 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 $MgCl_2$, 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 rLysozyme 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 *Bam*HI. The resulting DNA fragment was introduced into the *Bam*HI 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

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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)*.

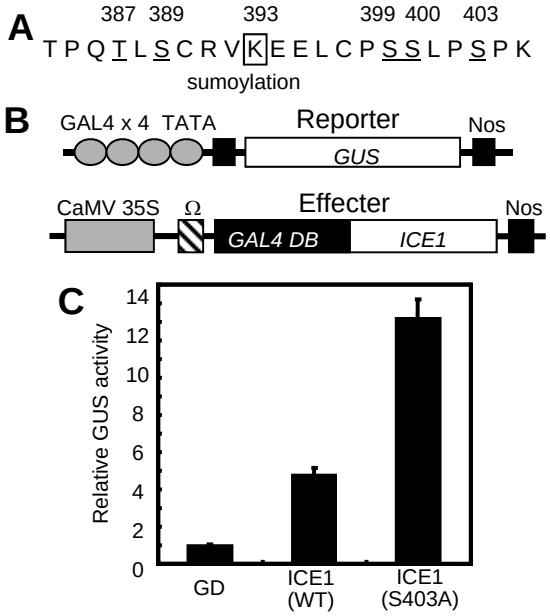
Relative mRNA transcript levels of transgenic *ICE1,* endogenous *ICE1*, *CBF3/DREB1A, COR47,* and *KIN1* in vector control (#8), *ICE1*-overexpressing (#12) and *ICE1(S403A)*-overexpressing (#13) seedlings were determined by quantitative RT-PCR

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).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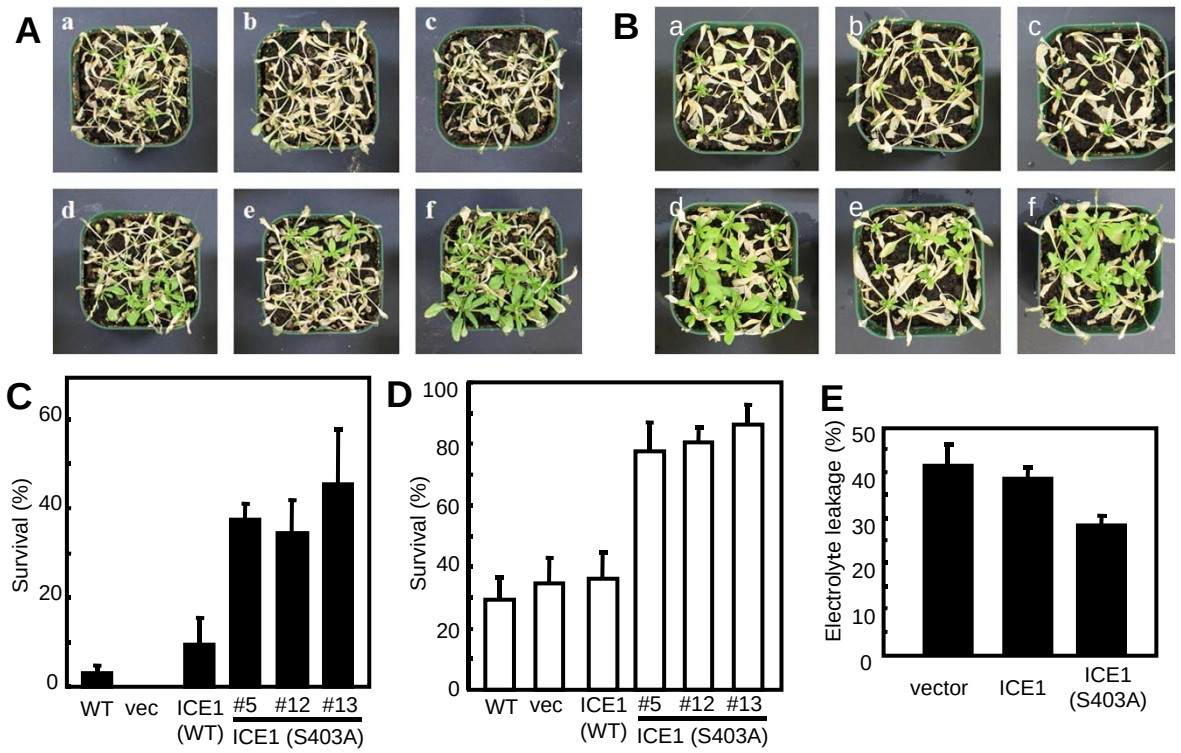
sumoylation
B
GAL4 x 4 TATA
Reporter
Nos
GUS
CaMV 35S
Ω
Effecter
Nos
GAL4 DB
ICE1
C
Relative GUS activity
0
2
4
6
8
10
12
14
GD
ICE1 (WT)
ICE1 (S403A)

Figure 1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c
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e
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B
a
b
c
d
e
f
C
Survival (%)
60
40
20
0
WT
vec
ICE1 (WT)
#5
#12
#13
ICE1 (S403A)
D
Survival (%)
100
80
60
40
20
0
WT
vec
ICE1 (WT)
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E
Electrolyte leakage (%)
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30
20
10
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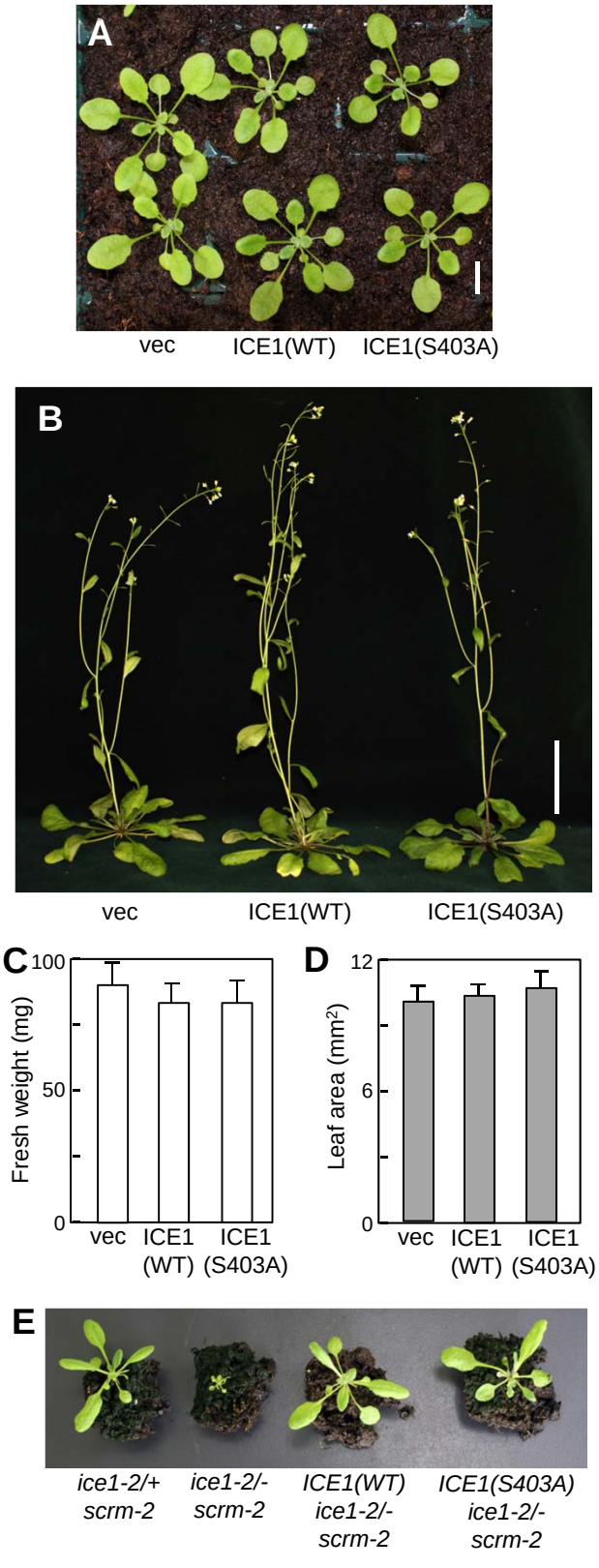
vector
ICE1
ICE1 (S403A)

Figure 2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vec
ICE1(WT)
ICE1(S403A)
B
vec
ICE1(WT)
ICE1(S403A)
C
Fresh weight (mg)
100
50
0
vec
ICE1 (WT)
ICE1 (S403A)
D
Leaf area (mm2)
12
6
0
vec
ICE1 (WT)
ICE1 (S403A)
E
ice1-2/+ scrm-2
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ICE1(S403A) ice1-2/- scrm-2

Figure 3

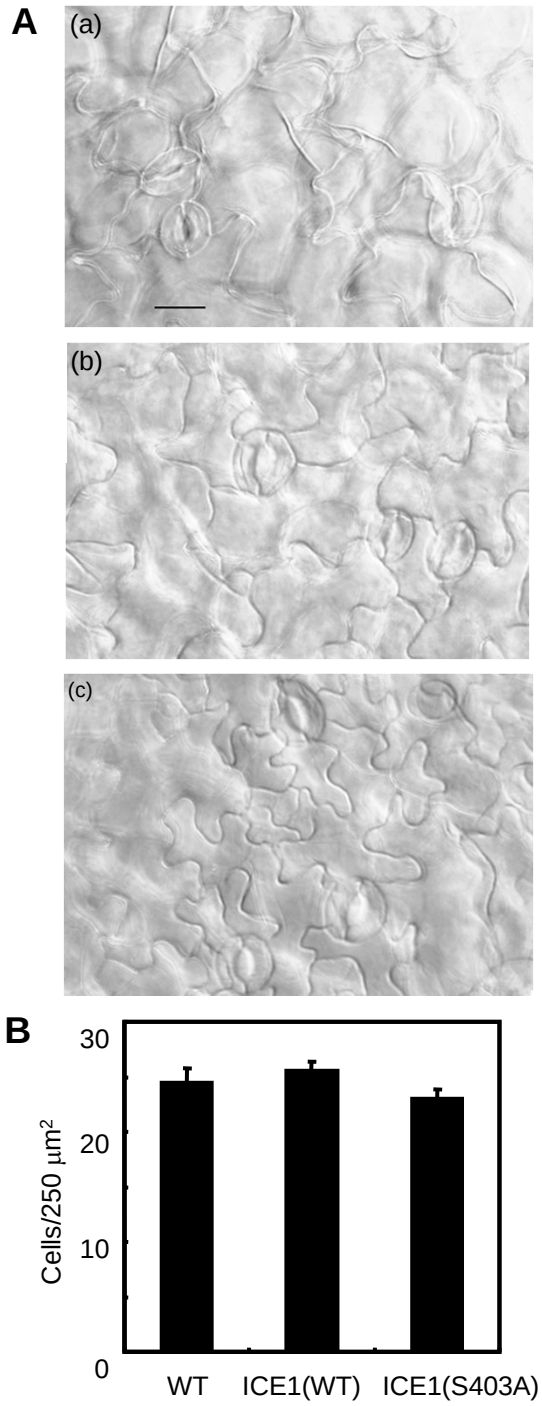
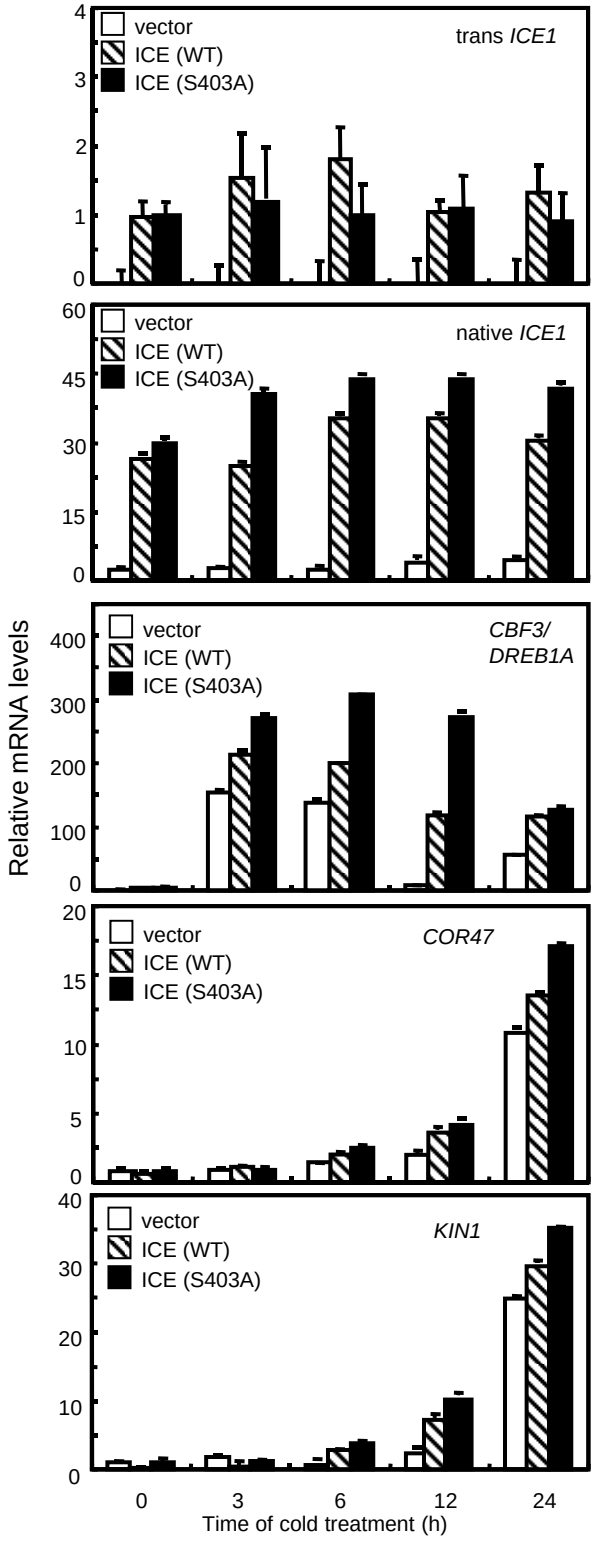


Figure 4


vector
ICE (WT)
ICE (S403A)
trans ICE1
native ICE1
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DREB1A
COR47
KIN1
Relative mRNA levels
Time of cold treatment (h)
0
3
6
12
24

Figure 5

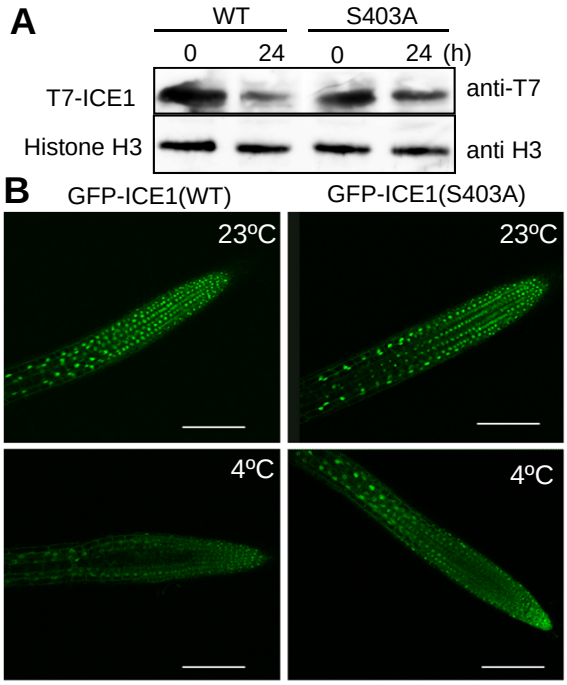

A
WT
S403A
0
24
0
24
(h)
T7-ICE1
anti-T7
Histone H3
anti H3
B
GFP-ICE1(WT)
GFP-ICE1(S403A)
23ºC
23ºC
4ºC
4ºC

Figure 6

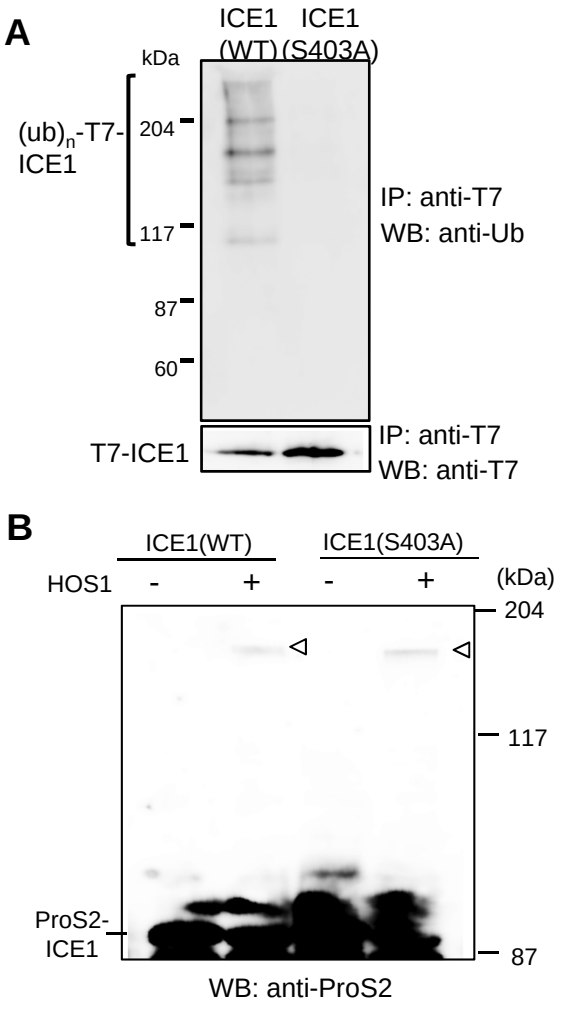
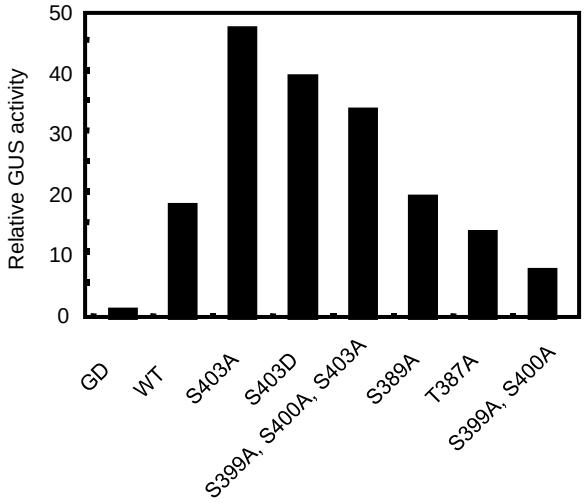
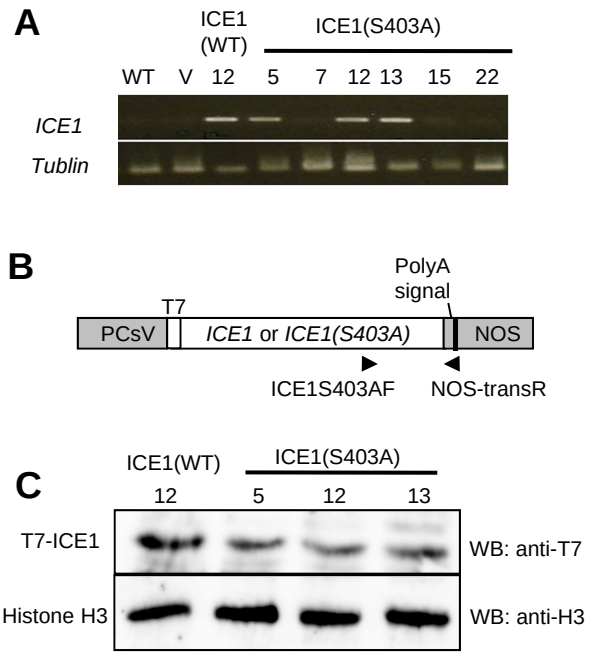

A
ICE1 (WT)
ICE1 (S403A)
kDa
(ub)n-T7-ICE1
204
117
87
60
IP: anti-T7
WB: anti-Ub
T7-ICE1
IP: anti-T7
WB: anti-T7
B
ICE1(WT)
ICE1(S403A)
HOS1
-
+
-
+
(kDa)
204
117
87
ProS2-ICE1
WB: anti-ProS2

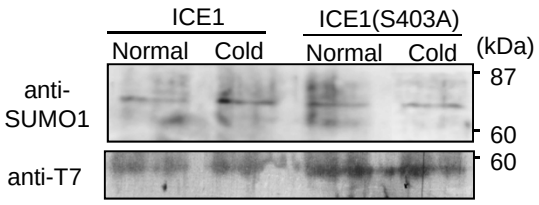
Figure 7



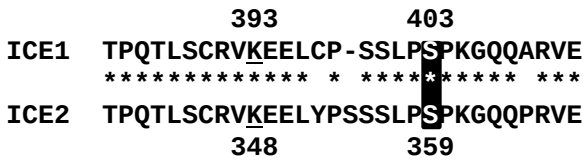
Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3



Supplemental Figure S4

Supplementary Table S1. Primers used for mutagenesis and construction.

Name of primer	DNA sequence (5'-to-3')
for mutagenesis of ICE1	
ICE1S403AF	CCCTCTTCTTTACCAGCTCCTAAAGGCC
ICE1S403AR	GGCCTTTAGGAGCTGGTAAAGAAGAGGG
ICE1S403DF	CCCTCTTCTTTACCAGATCCTAAAGGCC
ICE1S403DR	GGCCTTTAGGATCTGGTAAAGAAGAGGG
ICE1SSSAAAF	GTCCCGCTGCTTTACCAGCTCCTAAAGG
ICE1SSSAAAR	CCTTTAGGAGCTGGTAAAGCAGCGGGAC
ICE1S389AF	CCGCAAACTCTTGCTTGTCGTGTCAAGG
ICE1S389AR	CCTTGACACGACAAGCAAGAGTTTGCGG
ICE1T387AF	CCTACACCGCAAGCTCTTTCTTGTCGTG
ICE1T387AR	CACGACAAGAAAGAGCTTGCGGTGTAGG
ICE1SS399AAF	AAGAGTTGTGTCCCGCTGCTTTACCAAG
ICE1SS399AAR	CTTGGTAAAGCAGCGGGACACAACTCTT
for construction of pGD-ICE1	
ICE1-GDF	CACGCCCGGGGATGGGTCTTGACGGAAACAA
ICE1-HAR	CCGCTCGAGTCAGATCATACCAGCATACCC
for construction of pCsV-ICE1	
ICE1-expF	CGGGATCCATGGGTCTTGACGGAAACAA
ICE1-EGR	CGGGATCCTCAGATCATACCAGCATACCC

Supplementary Table S2. Primers used for Real-time PCR and RT-PCR analyses.

Name of primer	DNA sequence (5'-to-3')
DREB1A/CBF3	5’-GATGACGACGTATCGTTATGGA-3’
	5’-TACACTCGTTTCTCAGTTTTACAAAC-3’
COR47	5’-CAGTGTCGGAGAGTGTGGTG-3’
	5’-ACAGCTGGTGAATCCTCTGC-3’
KIN1	5’-TGGAGCTGGAGCACAACA-3’
	5’-GACCCGAATCGCTACTTGTTC-3’
ACTIN2	5'-TAACAGGGAGAAGATGACTCAGATCA-3'
	5'-AAGATCAAGACGAAGGATAGCATGAG-3'
To detect expression of ICE1 transgene	
ICE1S403AF	CCCTCTTCTTTACCAGCTCCTAAAGGCC
NOS-transR	GCCAAATGTTTGAACGATCGGGAA