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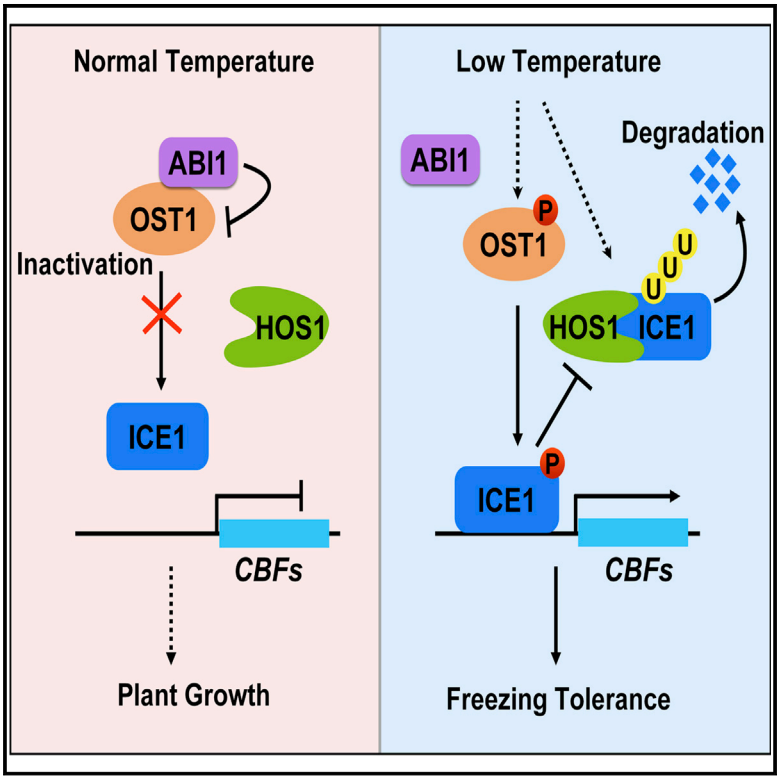
OST1 Kinase Modulates Freezing Tolerance by Enhancing ICE1 Stability in *Arabidopsis*

Graphical Abstract



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In Brief

The ICE1 transcription factor integrates different signals to regulate cold tolerance in *Arabidopsis*. Ding et al. demonstrate that the protein kinase OST1, a key component in ABA signaling, is also activated by cold stress. In response to cold, OST1 phosphorylates and stabilizes ICE1, promoting the expression of downstream cold-tolerance genes.

Highlights

- OST1 is a positive regulator in CBF-dependent cold signaling
- OST1 interacts with and phosphorylates the ICE1 protein
- OST1 interferes with the interaction between ICE1 and HOS1
- OST1 suppresses HOS1-mediated ICE1 degradation

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OST1 Kinase Modulates Freezing Tolerance by Enhancing ICE1 Stability in *Arabidopsis*

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SUMMARY

Cold stress is a major environmental factor that limits plant growth and development. The C-repeat-binding factor (CBF)-dependent cold signaling pathway is extensively studied in *Arabidopsis*; however, the specific protein kinases involved in this pathway remain elusive. Here we report that OST1 (OPEN STOMATA 1), a well-known Ser/Thr protein kinase in ABA signaling, acts upstream of *CBF*s to positively regulate freezing tolerance. The *ost1* mutants show freezing hypersensitivity, whereas transgenic plants overexpressing *OST1* exhibit enhanced freezing tolerance. The OST1 kinase is activated by cold stress. Moreover, OST1 interacts with both the transcription factor ICE1 and the E3 ligase HOS1 in the CBF pathway. Cold-activated OST1 phosphorylates ICE1 and enhances its stability and transcriptional activity. Meanwhile, OST1 interferes with the interaction between HOS1 and ICE1, thus suppressing HOS1-mediated ICE1 degradation under cold stress. Our results thus uncover the unexpected roles of OST1 in modulating CBF-dependent cold signaling in *Arabidopsis*.

INTRODUCTION

Global warming has caused climate changes with extreme temperatures in different areas that greatly restrain plant growth, development, and geographic distribution. Plants have evolved sophisticated mechanisms to adapt to the extreme temperatures. Upon exposure to cold stress, a set of cold-regulated (*COR*) genes are induced to help plants adapt to chilling and freezing stress (Chinnusamy et al., 2007; Thomashow, 1999). The transcription factors known as C-repeat (CRT)-binding factors (CBFs) or dehydration-responsive element (DRE)-binding proteins (DREBs) can bind to CRT/DRE *cis* elements and activate transcription of the downstream *COR* genes to increase cold tolerance (Liu et al., 1998; Stockinger et al., 1997; Thomashow, 1999). Inducer of CBF expression 1 (ICE1) and calmodulin binding transcription activator 3 (CAMTA3) are positive regulators of *CBF* genes (Chinnusamy et al., 2003; Doherty et al., 2009), whereas MYB15 and ethylene insensitive 3 (EIN3) are negative regulators of *CBF* genes (Agarwal et al., 2006; Shi et al., 2012). ICE1 is a bHLH transcription factor that binds to the *CBF3* promoter and activates its expression (Chinnusamy et al., 2003). ICE1 has been shown to be ubiquitinated by the E3 ligase HOS1 (high expression of osmotically responsive gene 1) and degraded by the 26S-proteasome pathway, whereas sumoylation of ICE1 mediated by SIZ1 (SAP and Miz) stabilizes ICE1 (Dong et al., 2006; Miura et al., 2007). A recent study has shown that JA ZIM-domain 1/4 (JAZ1/4) interacts with ICE1 to repress its transcriptional activity (Hu et al., 2013). Therefore, ICE1 is not only a central component in cold signaling, but also serves as a convergence point integrating cold and other signaling pathways.

Protein phosphorylation plays a pivotal role in plant responses to cold stress. Several Ca^{2+}-dependent protein kinases (CPKs), such as OsCPK13, AtCPK1, and CBL-interacting protein kinase3 (AtCIPK3), are involved in cold responses of plants (Böhmer and Romeis, 2007; Kim et al., 2003; Komatsu et al., 2007). In addition, the mitogen-activated protein kinase (MAPK) cascade is also implicated in cold signaling. MKK2 enhances freezing tolerance by activating *COR* expression (Teige et al., 2004). The Ca^{2+} binding calcium/calmodulin-regulated receptor-like kinase CRLK1 regulates *CBF* regulons and freezing tolerance by modulating MAPK kinase activity (Yang et al., 2010a, 2010b). However, the mechanism of these kinases underlying cold signaling remains largely unknown.

OPEN STOMATA 1 (OST1)/SnRK2.6/SnRK2E is a Ser/Thr protein kinase in ABA signaling (Mustilli et al., 2002). When ABA accumulates under stress conditions, the PYR/PYL/RCAR ABA receptors bind to ABA and subsequently interact with and inhibit PP2C phosphatases, thus releasing the inhibition of OST1 by PP2Cs (Hao et al., 2011; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Yoshida et al., 2006). Activated OST1 can phosphorylate ABRE-binding proteins/factors (AREBs/ABFs) to regulate the expression of stress-responsive genes (Furihata et al., 2006) and phosphorylate S-type anion channel SLAC1 to control stomatal movement in plants (Geiger et al., 2009).

In this study, we report that OST1 interacts with and phosphorylates ICE1, which in turn stabilizes ICE1 and promotes its transcriptional activity under cold stress. Moreover, OST1 interferes with the interaction between HOS1 and ICE1, which also contributes to stabilizing ICE1. Our study thus reveals that OST1 positively regulates cold signaling in the CBF-dependent pathway.

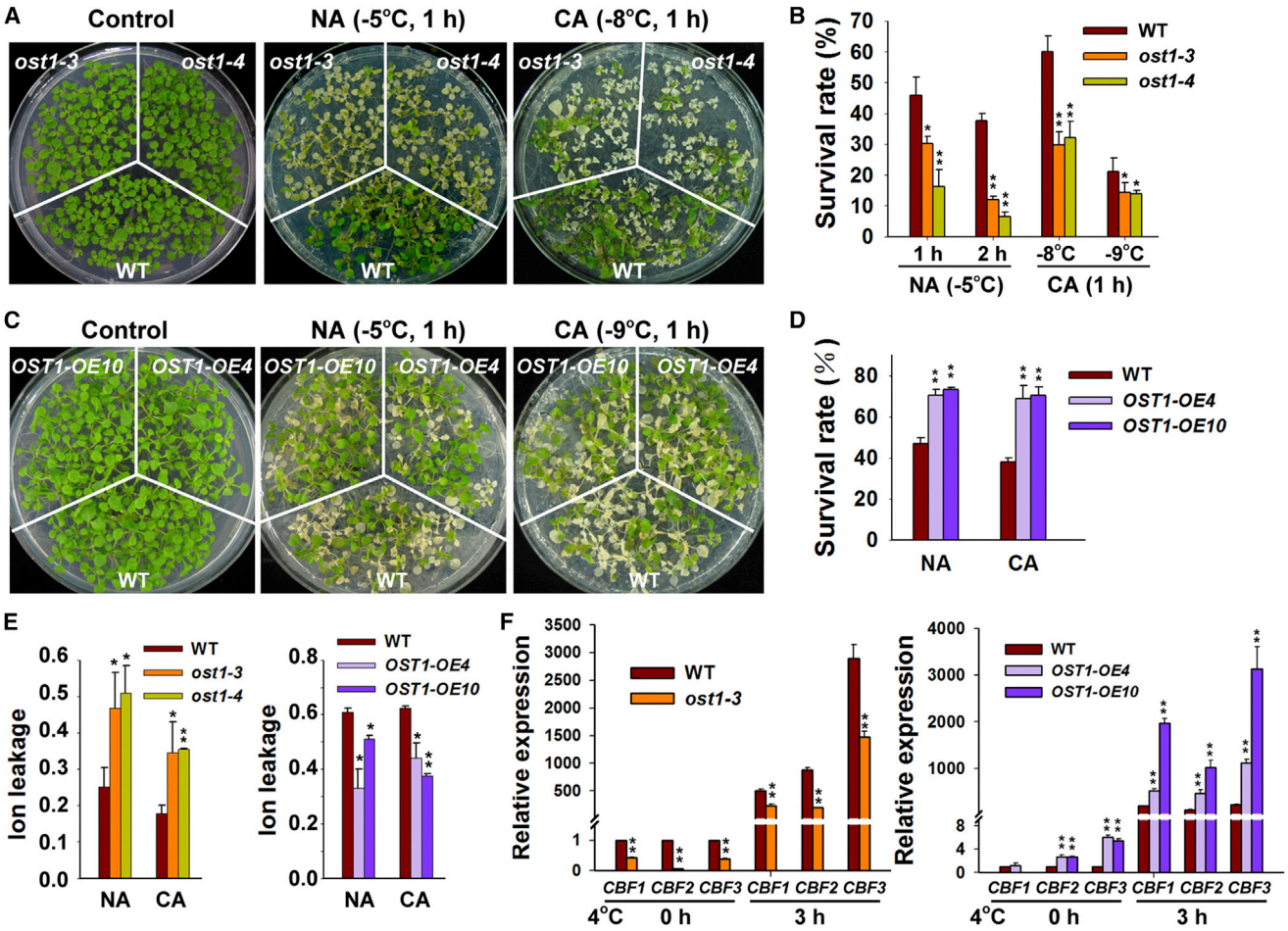

Figure 1. OST1 Mediates Plant Responses to Freezing Stress

(A and B) Freezing phenotypes (A) and survival rates (B) of the *ost1* mutants. Two-week-old plants grown on MS plates at 22°C were treated at −5°C for 1 hr or 2 hr for NA plants and at −8°C or −9°C for 1 hr for acclimated plants (CA; 4 days at 4°C).

(C and D) Freezing phenotypes (C) and survival rates (D) of 16-day-old *Super:OST1-Myc* plants.

(E) Ion leakage assay of the plants in (A) and (C).

(F) Expression of *CBFs* in 2-week-old seedlings under cold stress.

In (B, D, E and F), data are means of three replicates ± SD, and the asterisks indicate significant differences compared with the WT under the same treatment conditions (*$p < 0.05$, **$p < 0.01$, t test).

RESULTS

OST1 Is a Positive Regulator in Freezing Tolerance

To identify protein kinases involved in cold stress, we collected a set of T-DNA insertion mutants of genes that encode protein kinases from public resources and conducted a freezing tolerance assay. Two knockout mutants in *OST1/SnRK2.6/SnRK2E*, *ost1-3/snrk2.6/snrk2e*, and *ost1-4* (Figures S1A and S1B available online) showed defective phenotypes under freezing stress and were chosen for further study.

Under nonacclimated (NA) and cold-acclimated (CA) conditions, the *ost1-3* and *ost1-4* mutants displayed freezing-sensitive phenotypes compared with the WT Col (Figure 1A). The survival rates of the *ost1* mutants were much lower than the WT under both conditions (Figure 1B). Consistent with this, ion leakage, an indicator of plasma membrane injury caused by cold stress, was dramatically higher in *ost1* than in the WT after freezing treatment (Figure 1E). Furthermore, overexpression of *OST1* in *ost1-3* fully complemented the freezing phenotypes *ost1-3* (Figures S1C–S1E). These results indicate that OST1 is required for freezing tolerance in *Arabidopsis*.

To further assess the role of OST1 in freezing stress, we generated transgenic plants overexpressing *OST1-Myc* driven by a super promoter (*OST1-OE*) (Figure S1F). These transgenic plants showed enhanced freezing tolerance compared with the WT under both NA and CA conditions (Figures 1C and 1D). Ion leakage in these transgenic plants was consistently decreased compared with the WT (Figure 1E), suggesting that OST1 positively regulates freezing tolerance.

OST1 Is Activated by Cold Stress

We tested whether cold stress has effects on protein level and localization of OST1 in *OST1-OE* transgenic plants. The OST1 protein level and its localization in the nucleus and cytosol

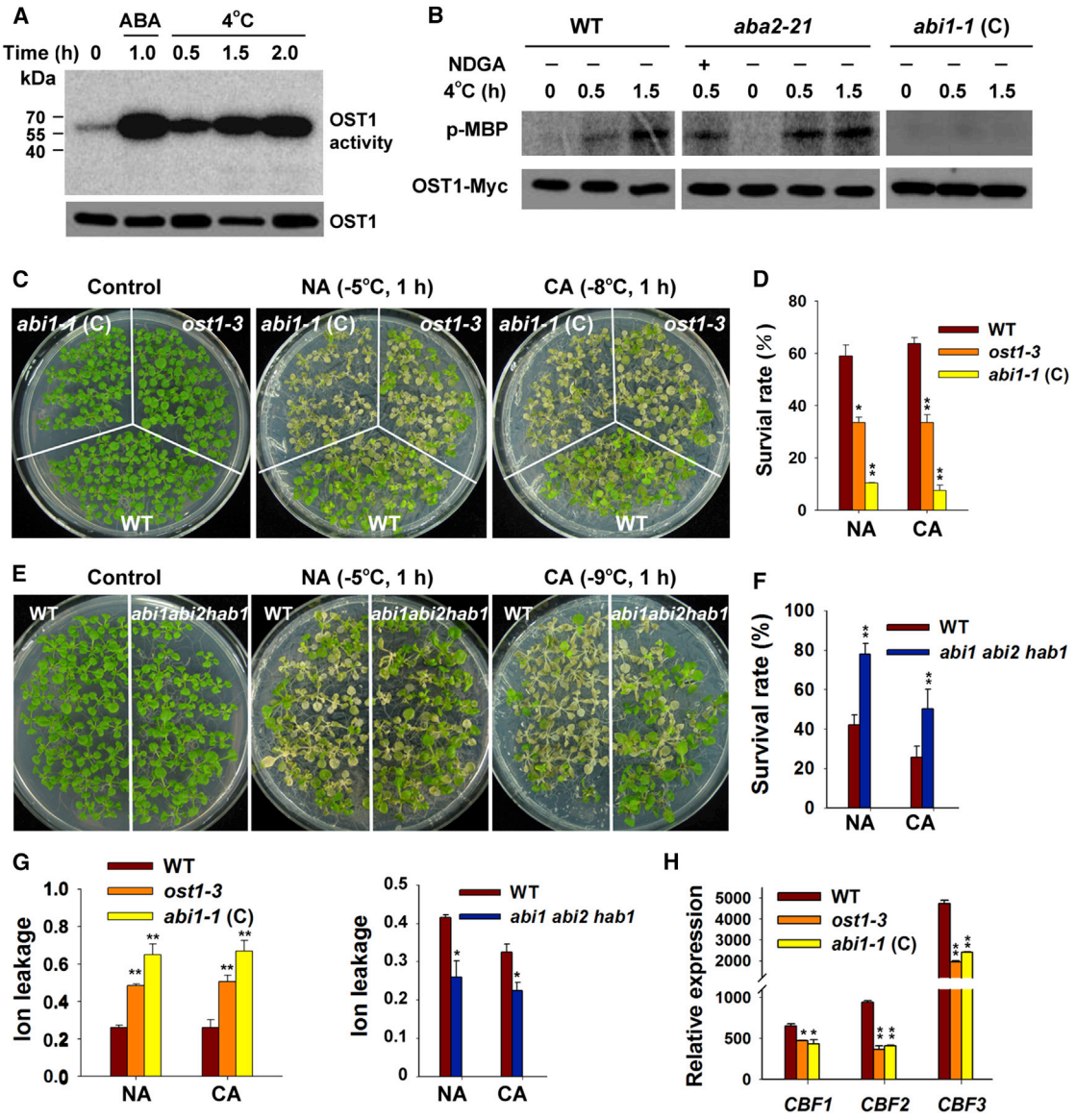

Figure 2. Cold Stress Activates OST1 Protein Kinase

(A) In-gel kinase assay of OST1 under cold stress. Total proteins prepared from 2-week-old *Super:OST1-Myc* plants treated at 4°C for the indicated times were incubated with anti-Myc agarose beads and separated by SDS-PAGE gel containing 0.3 mg/mL MBP substrate. Plants treated with 50 μM ABA were used as a control. Radioactivity is shown in the top panel. OST1-Myc detected with anti-Myc antibody is shown in the bottom.

(B) OST1 kinase activity in *aba2-21* and *abi1-1* (C) mutants under cold stress. Protoplasts prepared from *aba2-21* and *abi1-1* (C) expressing *OST1-Myc* were treated at 4°C for the indicated times. For NDGA treatment, 100 μM NDGA was added for 1 hr before cold treatment. One μCi of [γ-^{32}P] was used for the WT and *aba2-21*, and 4 μCi of [γ-^{32}P] was used for *abi1-1* (C). OST1 was immunoprecipitated with anti-Myc antibody and subjected to kinase assay using MBP as substrate or to immunoblot analysis with anti-Myc antibody. Radioactivity is shown in the top. OST1-Myc serves as a loading control. All these experiments were done at the same conditions with the same exposure time.

(C and D) Freezing phenotypes (C) and survival rates (D) of 2-week-old *ost1-3* and *abi1-1* (C) under freezing stress.

(E and F) Freezing phenotypes (E) and survival rates (F) of 2-week-old *abi1 abi2 hab1* mutant under freezing stress.

(G) Ion leakage of the plants in (C) and (E).

(H) Expression of *CBFs* in 2-week-old seedlings under cold stress. The expression of gene in untreated WT was set to 1.

In (D and F–H), data are means of three replicates ± SD, and the asterisks indicate significant differences compared with the WT under the same treatment conditions (*$p < 0.05$, **$p < 0.01$, t test).

were not obviously altered after cold treatment (Figures S2A and S2B). To examine whether OST1 kinase activity is affected by cold stress, we performed an in-gel kinase assay using *OST1-OE* plants. ABA was used to activate OST1 as a control (Mustilli et al., 2002) (Figure 2A). Intriguingly, we found that OST1 was activated rapidly after cold treatment in *OST1-OE* plants (Figure 2A). Previous studies indicated that ABA-deficient mutants show defect in freezing tolerance and expression of *COR* genes

(Mantyla et al., 1995; Xiong et al., 2001), suggesting that ABA is involved in cold responses. In order to see whether ABA plays a role in cold-activated OST1, we compared the OST1 activity between the WT and ABA-deficient mutant *aba2-21* (Mang et al., 2012) under cold stress. The similar cold-induced OST1 activities were observed in both the WT and *aba2-21* protoplasts expressed *OST1-Myc* (Figure 2B). *aba2-21* mutant contains <10% ABA of WT (Mang et al., 2012) (Figure S2C). The cold activation of OST1 in *aba2-21* was not further compromised by the ABA inhibitor nordihydroguaiaretic acid (NDGA) (Mang et al., 2012) (Figure 2B). In addition, ABA contents in both WT and *aba2-21* mutants were moderately decreased after cold treatment (Figure S2C). Taken together, these results suggest that activation of OST1 by cold and ABA may be through two distinct pathways.

In ABA signaling, the PP2C protein ABI1 interacts with OST1 and negatively regulates OST1 activity (Umezawa et al., 2009; Vlad et al., 2009; Yoshida et al., 2006). We next addressed whether ABI1 affects cold-induced OST1 activity by examining OST1 activity in the gain-of-function mutant *abi1-1* in Columbia-0 accession (here as *abi1-1* (C)) (Luo et al., 2014), which carries the same mutation as *abi1-1* in L*er* (Leung et al., 1994; Meyer et al., 1994). OST1 activity induced by cold was abolished in *abi1-1* (C) (Figure 2B). Consistently, *abi1-1* (C) mutant and *ABI1*-overexpressing (*ABI1-OE*) plants exhibited reduced freezing tolerance and increased ion leakage compared with the WT (Figures 2C, 2D, 2G, and S2D–S2G). In contrast, the *abi1 abi2 hab1* loss-of-function mutant showed higher survival rate and lower ion leakage than the WT after freezing treatment (Figures 2E–2G). These results suggest that ABI1 represses cold-induced OST1 activity, which in turn negatively controls freezing tolerance.

OST1 Positively Regulates the Expression of *CBF* Genes

Next, we examined whether OST1-mediated plant responses to cold stress is dependent on the CBF pathway. Compared with the WT, the basal expression of *CBF1-CBF3* genes was downregulated in *ots1-3*, whereas it was upregulated in *OST1-OE* plants without cold treatment (Figure 1F). Furthermore, expression of *CBF* genes was rapidly induced by cold in both WT and *ost1-3*; however, the cold induction of these genes in *ost1-3* was consistently decreased compared with the WT (Figure 1F). Cold induction of CBF regulons, including *COR15A* and *KIN1*, was also significantly attenuated in *ost1-3* compared with the WT (Figure S1G). In contrast, cold induction of *CBFs* and their regulons was dramatically increased in *OST1-OE* plants compared with the WT (Figures 1F and S1H). These results suggest that OST1 acts upstream of *CBFs* to regulate CBF-dependent gene expression.

Similar to *ost1-3*, cold-induced expression of *CBFs* in *abi1-1* (C) and *ABI1-OE* plants was decreased compared with the WT (Figures 2H and S2H). These results further demonstrate that OST1-mediated cold signaling is suppressed by ABI1.

OST1 Interacts with ICE1

Because OST1 positively regulates the expression of *CBFs*, we next tested whether OST1 interacts with ICE1, the transcription factor upstream of *CBFs*. A yeast two-hybrid assay showed that ICE1 interacted with OST1 and its homolog SnRK2.3 but not SnRK2.4 (Figures 3A and S3). We further identified the functional domains required for their interaction in yeast. ICE1 protein contains a putative acidic domain, serine-rich region in the N terminus, and MYC-like bHLH domain and possible zipper region at the C terminus (Chinnusamy et al., 2003). Deletion of the N-terminal regions of ICE1 did not obviously affect the interaction between OST1 and ICE1, whereas deletion of the C terminus abolished their interaction (Figures 3A and 3B), indicating that C terminus of ICE1 is required for its binding to OST1. To determine the functional domain of OST1 interacting with ICE1, we generated two truncated forms of OST1 (Figure 3C) and found that Domain II of OST1 is necessary for the OST1-ICE1interaction (Figure 3D).

The OST1-ICE1 interaction was verified by an in vitro pull-down assay. The His-ICE1 protein, but not the MBP-His protein, was able to pull down the GST-OST1 protein (Figure 3E). In addition, a coimmunoprecipitation (coIP) assay was performed in *Nicotiana benthamiana* leaves expressing *OST1-Myc* and *HA-FLAG-ICE1* (*HF-ICE1*), and OST1 was pulled down by ICE1 (Figure 3F). Further, a bimolecular fluorescence complementation (BiFC) assay revealed that the OST1-ICE1 association occurred in the nuclei of tobacco epidermal cells (Figure 3G). These results collectively indicate that OST1 interacts with ICE1.

OST1 Phosphorylates ICE1 and Enhances Its Stability under Cold Stress

We next examined whether OST1 can phosphorylate ICE1 in vitro. His-OST1 showed strong autophosphorylation activity. When incubated with OST1, ICE1 was phosphorylated (Figure 4A), suggesting that ICE1 is a substrate of OST1 in vitro.

To determine whether ICE1 is phosphorylated in an OST1-dependent manner *in planta*, ICE1 proteins were immunoprecipitated from *HF-ICE1* transgenic plants and treated with calf intestinal alkaline phosphatase (CIAP). At 22°C, the position of ICE1 band derived from *HF-ICE1* plants was not obviously altered with CIAP treatment (Figure 4B). However, the ICE1 protein extracted from 4°C-treated plants migrated more slowly compared with that at 22°C without CIAP treatment. Moreover, this migration of ICE1 protein at 4°C was dramatically inhibited after CIAP treatment (Figure 4B), indicating that ICE1 is phosphorylated *in planta* especially under cold conditions. In contrast, no very obvious migration of ICE1 protein was detected in *ost1-3* plants expressing *HF-ICE1* at 4°C before and after CIAP treatment (Figure 4B), suggesting that the phosphorylation of ICE1 is predominantly mediated by OST1 under cold conditions.

We next tested whether ICE1 stability is influenced by OST1 in vivo. ICE1 is shown to be degraded by HOS1 under cold stress (Dong et al., 2006). Consistent with this, the ICE1 protein was degraded in *HF-ICE1* transgenic plants upon cold treatment (Figure 4C). However, the degradation of ICE1 was significantly reduced in *HF-ICE1/OST1-Myc* transgenic plants under cold stress (Figure 4C). This phenomenon was also consistently observed in *Arabidopsis* protoplasts expressing *HF-ICE1/OST1-Myc*. Cold-induced ICE1 degradation was blocked by expression of OST1 and addition of MG132, a 26S proteosome inhibitor (Figures S4A and S4B).

To further dissect the role of OST1 in ICE1 stability, we examined the ubiquitination of ICE1 in *HF-ICE1* and *HF-ICE1/OST1-Myc* transgenic plants. ICE1 protein was ubiquitinated by cold stress in *HF-ICE1* transgenic plants, which is consistent with a previous study (Dong et al., 2006). However, the ubiquitination of ICE1 was evidently inhibited in *HF-ICE1/OST1-Myc*

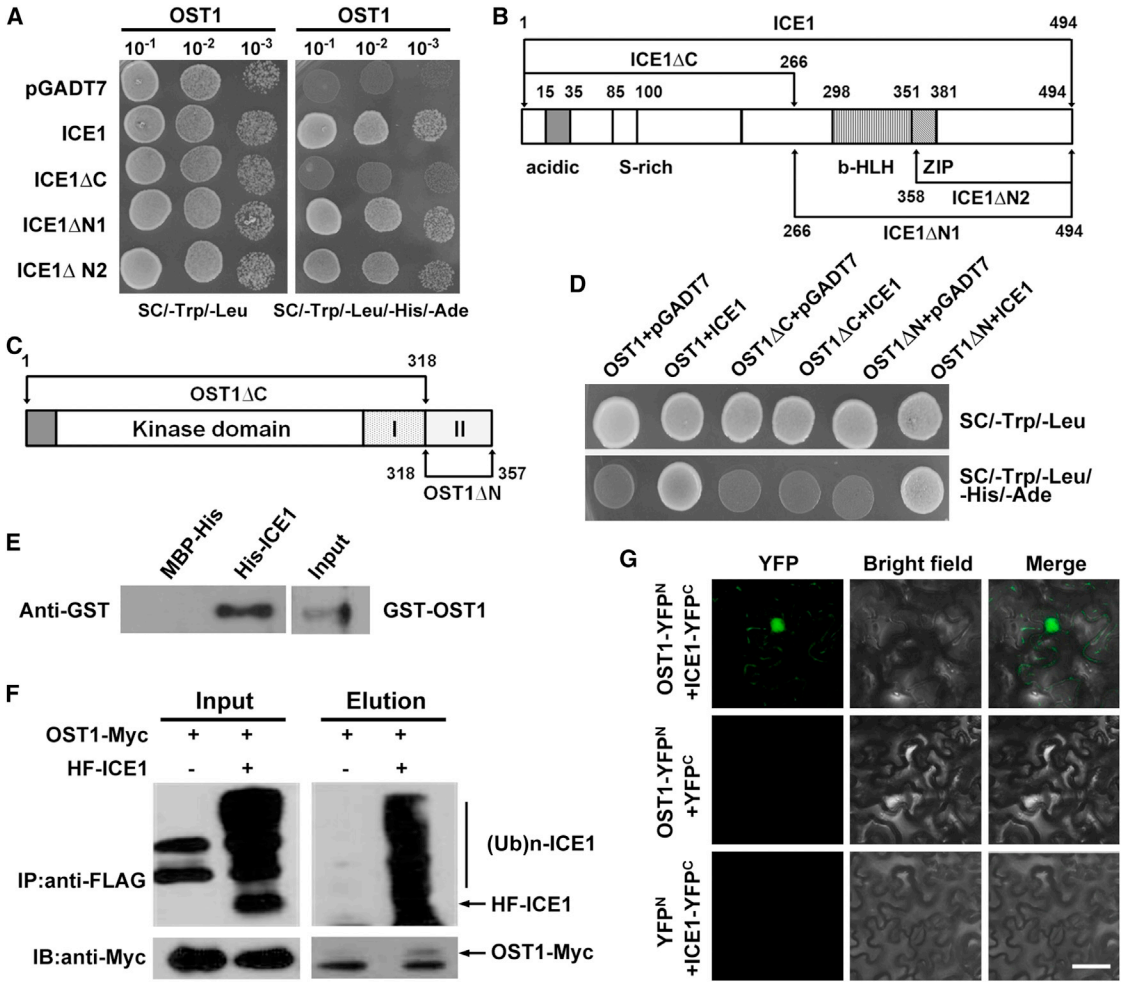

Figure 3. OST1 Interacts with ICE1

(A) The interaction of OST1 and ICE1 in yeast.

(B and C) Diagram of ICE1-truncated proteins (B) and OST1-truncated proteins (C) used for the yeast two-hybrid assays described in (A) and (D).

(D) Interaction of ICE1 and OST1 in yeast.

(E) Interaction of OST1 and ICE1 proteins in vitro. The GST-OST1 proteins were incubated with immobilized His-ICE1 or MBP-His, and the proteins immunoprecipitated with His-beads were detected using anti-GST1 antibody.

(F) Interaction of OST1 and ICE1 proteins in vivo. The total protein extracts from *N. benthamiana* leaves transfected with *35S:HA-FLAG-ICE1* (*35S:HF-ICE1*)/*Super:OST1-myc* or *Super:OST1-Myc* alone were immunoprecipitated with anti-FLAG Sepharose beads. The proteins from crude lysates (left, input) and immunoprecipitated proteins (right) were detected with anti-Myc antibody.

(G) BiFC analysis of the interaction between OST1 and ICE1 in *N. benthamiana*. Scale bars represent 20 μm.

transgenic plants (Figure 4D). These results suggest that the OST1-mediated ICE1 phosphorylation stabilizes ICE1 by compromising its ubiquitination under cold conditions.

Next, we examined ICE1 degradation in the WT, *ost1-3*, and *OST1-OE* plants using a cell-free protein degradation assay. ICE1 protein was extracted from *Arabidopsis* protoplasts that expressed *HF-ICE1* and incubated with total proteins prepared from the WT, *ost1-3*, and *OST1-OE* plants in the presence of ATP. ICE1 was degraded faster in *ost1-3* than the WT under cold stress. However, the degradation of ICE1 was largely suppressed in *OST1-OE* plants (Figure 4E). These data provide further evidence that ICE1 stability is positively regulated by OST1 *in planta*.

We next searched for possible phosphorylation sites in the ICE1 protein according to the OST1 phosphoproteomics data (Wang et al., 2013a). Strikingly, we found that S278 of ICE1 might be a putative phosphorylation site by OST1. To dissect the possible biological function of this potential phosphorylation site of ICE1, WT ICE1 and the phosphorylated active form of ICE1 carrying a serine to aspartate mutation ($ICE1^{S278D}$) were transformed into the WT plants and protoplasts, and ICE1 stability was examined. The ICE1 protein levels were decreased in both stable transgenic plants and *Arabidopsis* protoplasts expressing *HF-ICE1* after 3 hr of cold treatment (Figures 4F and S4C). However, the stability of $ICE1^{S278D}$ was dramatically enhanced in transgenic plants and protoplasts expressing $HF\text{-}ICE1^{S278D}$ compared with WT ICE1 (Figures 4F and S4C), suggesting that phosphorylation at S278 of ICE1 is required for ICE1 stability. We also generated construct expressing

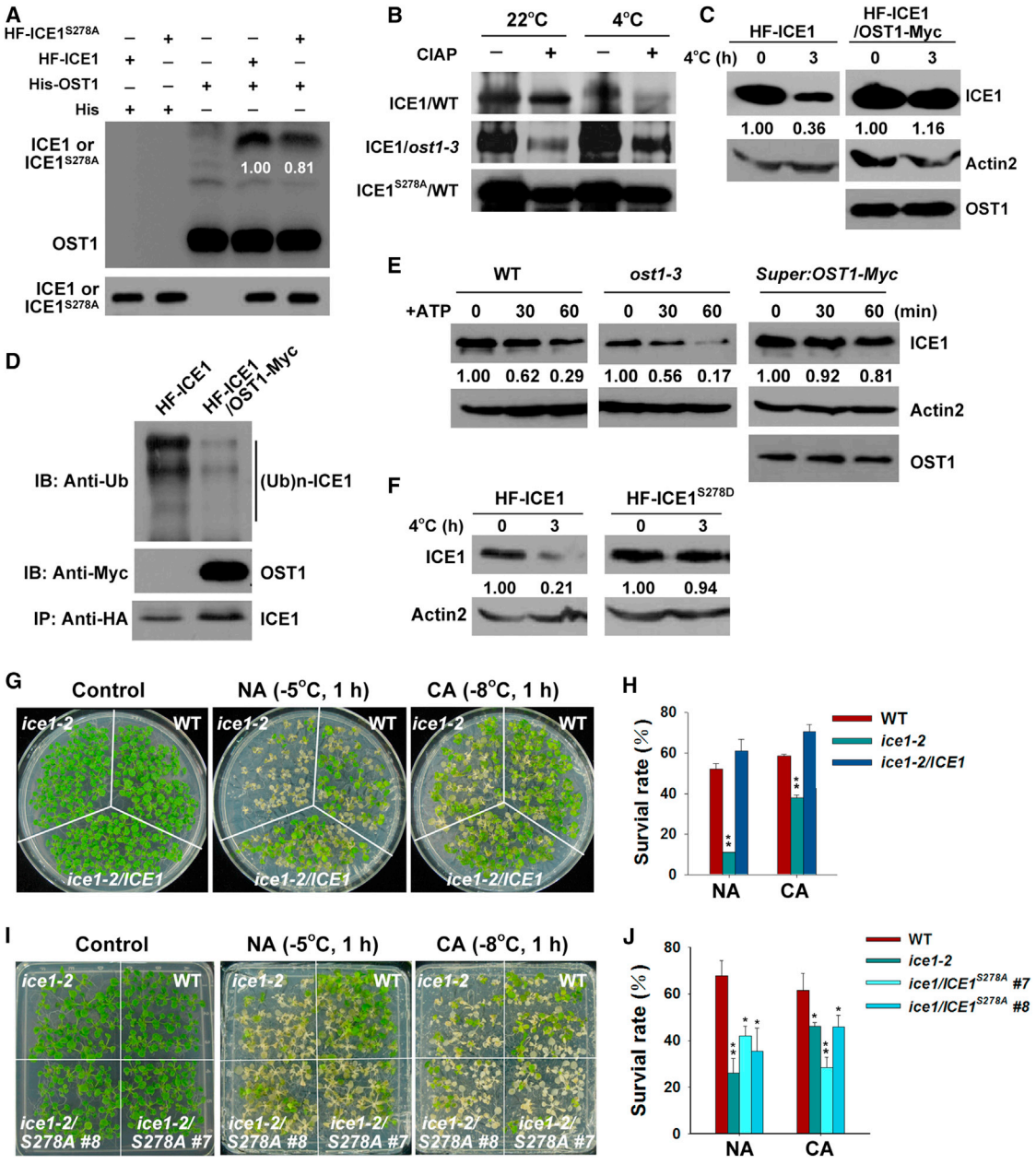

Figure 4. OST1 Phosphorylates ICE1 under Cold Stress

(A) Phosphorylation of ICE1 and ICE1^{S278A} by OST1 in vitro. The purified proteins were separated by 10% SDS-PAGE after incubation in protein kinase assay buffer containing [γ-^{32}P]ATP. Radioactivity is shown in the top, and ICE1 and ICE1^{S278A} proteins detected with anti-HA antibody are shown in the bottom.

(B) Phosphorylation of ICE1 under cold stress in vivo. Two-week-old *35S:HF-ICE1, 35S:HF-ICE1/ost1-3* and *35S:HF-ICE1*^{S278A} plants were treated at 4°C for 1 hr. ICE1 proteins immunoprecipitated with anti-HA antibody were treated with calf intestinal alkaline phosphatase (CIAP) at 37°C for 30 min and then subjected to immunoblot analysis using anti-HA antibody.

(C) ICE1 stability is enhanced by OST1 in vivo under cold stress. Two-week-old *35S:HF-ICE1* or *35S:HF-ICE1/Super:OST1-Myc* transgenic plants were treated at 4°C for 0 and 3 hr. ICE1 was detected with anti-HA antibody; OST1 was detected with anti-Myc antibody, and Actin2 served as a control.

(D) Ubiquitination of ICE1 is suppressed by OST1 in vivo. Two-week-old *35S:HF-ICE1* or *35S:HF-ICE1/Super:OST1-Myc* seedlings were treated at 4°C for 6 hr, and the proteins were subjected to immunoblot analysis using anti-HA or anti-Ubiquitin (Ub) antibody for ICE1 and anti-Myc antibody for OST1.

(E) OST1 suppresses ICE1 degradation. In vitro cell-free degradation assays were performed. ICE1 was detected with anti-HA antibody, and OST1 was detected with anti-Myc antibody.

(F) The stability of ICE1 and ICE1^{S278D} under cold stress. Stable transgenic plants expressing ICE1 and ICE1^{S278D} were treated at 4°C for 0 and 3 hr. ICE1 proteins were detected with anti-HA antibody. Actin2 served as a loading control.

(G and I) Freezing phenotypes of 12-day-old *ice1-2* plants overexpressing ICE1 (G) and ICE1^{S278A} (I).

(H and J) Survival rates of 12-day-old *ice1-2* plants overexpressing WT ICE1 (H) and ICE1^{S278A} (J). Data are means of three replicates ± SD, and asterisks indicate significant differences compared to the WT under the same treatment conditions (*$p < 0.05$, **$p < 0.01$, t test).

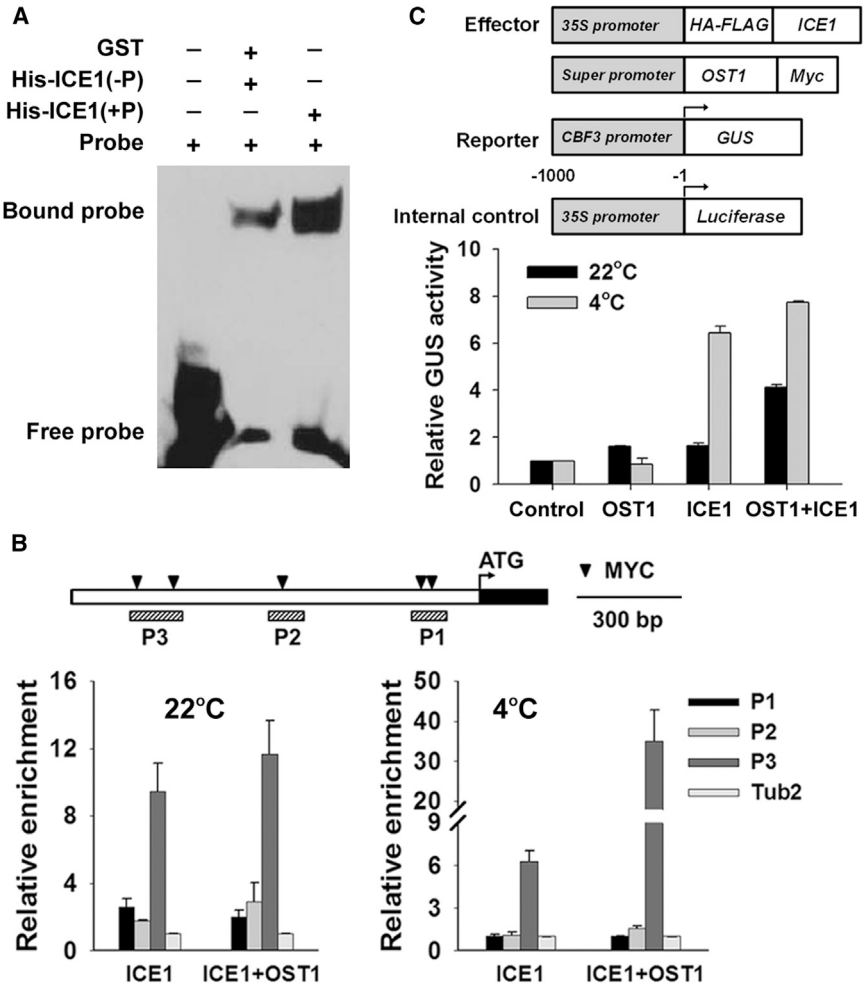

Figure 5. OST1 Promotes the Transcriptional Activity of ICE1 under Cold Stress

(A) EMAS assay to examine ICE1 binding to the *CBF3* promoter. His-ICE1 was immunoprecipitated with His agarose and incubated with purified GST-OST1 or GST with kinase reaction buffer at 30°C for 30 min. A biotin-labeled *CBF3* DNA fragment was then incubated with His-ICE1 or phosphorylated His-ICE1 protein.

(B) ChIP analysis of ICE1 binding to the MYC motifs in the *CBF3* gene. The upstream region and part of an exon of *CBF3* are shown with a white box and black box, respectively. The arrowheads in the top indicate the sites containing MYCs in the *CBF3* promoter. Hatched boxes represent the DNA fragments amplified in the ChIP assay. Protoplasts expressing *35S:HF-ICE1* alone or *35S:HF-ICE1/Super: OST1-Myc* were treated at 22°C or 4°C and subjected to ChIP assays.

(C) Activation of *CBF3* expression by ICE1 is promoted by OST1 in *N. benthamiana*. The various constructs used are shown in the top. *CBF3:GUS* was cotransformed with other constructs into *N. benthamiana* leaves, and leaves transfected with *CBF3:GUS* were only used as a control. After transfection, the plants were incubated at 22°C for 36 hr and 4°C for an additional 3 hr. Relative GUS activity (GUS/Luc) indicating the level of *CBF3* expression activated by ICE1 is shown in the bottom. In (B) and (C), data are means of three replicates ± SD.

nonphosphorylation mutated form of ICE1 carrying a serine 278 to alanine mutation ($ICE1^{S278A}$) for in vitro kinase assay. $ICE1^{S278A}$ could also be phosphorylated by OST1, but the signal was slightly weaker than ICE1 (Figure 4A), implying the existence of other OST1 phosphorylation sites in ICE1. When $ICE1^{S278A}$ was expressed in *Arabidopsis*, the migration of ICE1 was drastically suppressed in $ICE1^{S278A}$ transgenic plants under cols stress (Figure 4B), indicating that S278 of ICE1 is the major phosphorylation site of OST1 in vivo under cold conditions.

We next determined whether phosphorylation of ICE1 is required for its function by introducing $ICE1^{S278A}$ into *ice1-2* (Figure S4D). Compared with the WT, the *ice1-2* mutant showed increased freezing sensitivity and ion leakage, which could be fully rescued by *ICE1* (Figures 4G, 4H, and S4E), but could not be rescued by $ICE1^{S278A}$ (Figures 4I, 4J, and S4F). These data indicate that phosphorylation of ICE1 at S278 is required for its function in cold signaling.

OST1 Enhances the Binding Ability of ICE1 to the *CBF3* Promoter

To investigate how OST1 affects ICE1 in regulation of *CBF* gene expression, we analyzed the effect of OST1 on ICE1 binding activity by electrophoresis mobility shift assay (EMSA). ICE1 protein was able to bind to the *CBF3* promoter (Figure 5A), which is consistent with a previous study (Chinnusamy et al., 2003). When ICE1 was phosphorylated by OST1 in vitro, the binding affinity of phosphorylated ICE1 to the *CBF3* promoter was dramatically enhanced (Figure 5A).

The effect of OST1 on the ability of ICE1 to bind the *CBF3* promoter was further examined by chromatin immunoprecipitation (ChIP) assays. *Arabidopsis* protoplasts expressing *HF-ICE1* alone or *HF-ICE1/OST1-Myc* were treated at 22°C or 4°C. ICE1 specifically bound to the *CBF3* promoter, and this binding activity was enhanced when OST1 was overexpressed at 22°C (Figure 5B). Furthermore, the OST1-mediated ICE1 binding to the *CBF3* promoter was further enhanced by cold stress (Figure 5B). These results suggest that OST1 promotes ICE1 binding to the *CBF3* promoter in vivo.

To further test the effect of OST1 on ICE1 transcriptional activity, we performed transient transactivation assays using the *CBF3* promoter fused to the *GUS* gene as a reporter. ICE1 and OST1 effector constructs were expressed under the control of the *35S* or super promoter and transfected together with the reporter construct into leaves of *N. benthamiana*. The expression of ICE1 protein activated *CBF3* expression (Figure 5C), whereas coexpression of OST1 dramatically increased ICE1-activated *CBF3* expression. In addition, cold treatment enhanced the OST1-induced promotion of ICE1-induced *CBF3* expression (Figure 5C). These results strongly

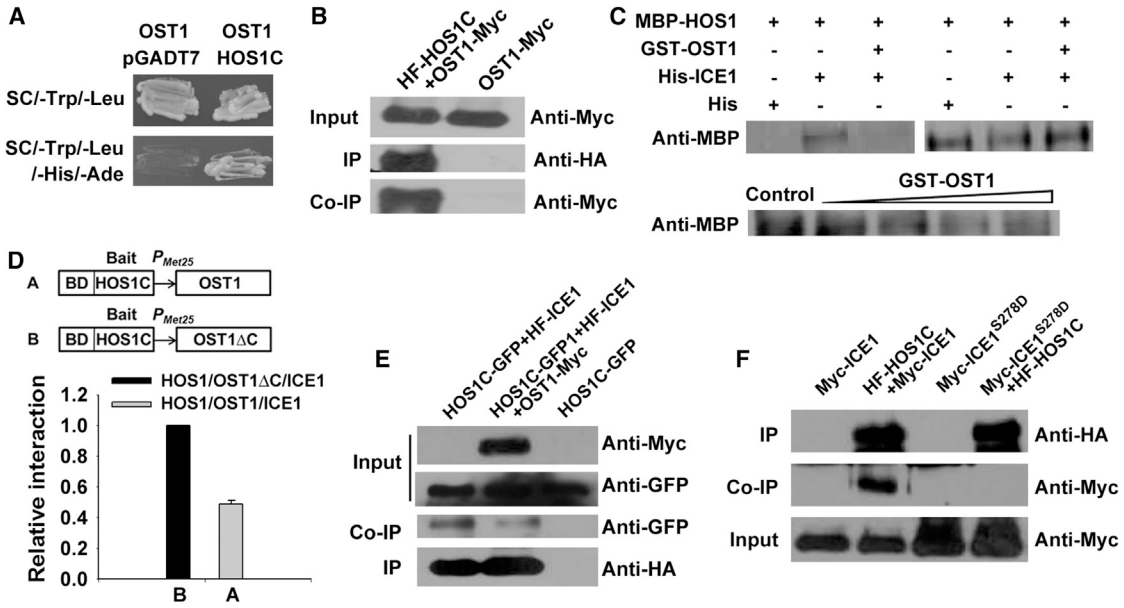

Figure 6. OST1 Competes with ICE1 for Interaction with HOS1

(A) The interaction of OST1 and the C terminus of HOS1 (HOS1C) in yeast.

(B) Coimmunoprecipitation of OST1 and HOS1 proteins in vivo. *N. benthamiana* leaves were transfected with *Super:OST1-Myc* alone or *Super:OST1-myc/35S:HF-HOS1C*. The total protein extracts were immunoprecipitated with anti-HA Sepharose beads, and the proteins from crude lysates (input), and immunoprecipitated proteins were detected with anti-Myc antibody.

(C) The in vitro interaction between ICE1 and HOS1 is weakened by OST1. GST-OST1 protein combined with His-ICE1 or His-MBP was incubated with immobilized MBP-HOS1. MBP-HOS1 input is shown in the right. Immunoprecipitated proteins were detected with anti-MBP antibody. The gradient in the bottom indicates an increasing amount of GST-OST1.

(D) Yeast three-hybrid assays showing the effects of OST1 on the HOS1-ICE1 interaction. Yeast cells were transformed with pB-HOS1C-OST1 or pB-HOS1C-OST1ΔC with pA-ICE1 and cultured on SC/-Trp/-Leu/-Met medium. The β-galactosidase assay was performed. The interaction value for HOS1-ICE1-OST1ΔC was set to 1, and the relative interaction for HOS1-ICE1-OST1 is shown in the bottom. The data are mean values of three replicates ± SD.

(E) Interactions of OST1, HOS1, and ICE1 proteins in vivo. *Arabidopsis* protoplasts were transfected with *HOS1C-GFP/HF-ICE1* (lane 1), *HOS1C-GFP/HF-ICE1/OST1-Myc* (lane 2), or *HOS1C-GFP* alone (lane 3). Proteins from crude lysates (input) were detected with anti-GFP and anti-Myc antibodies. Total protein extracts were immunoprecipitated with anti-HA Sepharose beads. Immunoprecipitated proteins were detected with anti-GFP antibody.

(F) The effect of ICE1 phosphorylation on HOS1-ICE1 interaction. *N. benthamiana* leaves were transfected with *HF-HOS1C* and *Myc-ICE1* or *Myc-ICE1*^{S278D}. Proteins from crude lysates (input) were detected with anti-Myc antibody. The total protein extracts were immunoprecipitated with anti-HA Sepharose beads. Immunoprecipitated proteins were detected with anti-Myc antibody.

support that OST1 modulates cold signaling by controlling the transcriptional ability of ICE1.

The OST1-HOS1 Interaction Interferes with the HOS1-ICE1 Interaction

Because ICE1 interacts with HOS1 (Dong et al., 2006) and OST1 (Figure 3), we next tested whether OST1 is also associated with HOS1. In yeast, OST1 was able to interact with the C terminus of HOS1 (Figure 6A), which is also the interacting domain with ICE1 (Dong et al., 2006). Moreover, a coIP assay confirmed the interaction of HOS1 and OST1 in *N. benthamiana* leaves (Figure 6B).

To examine whether OST1 affects the HOS1-ICE1 interaction in vitro, MBP-HOS1, GST-OST1, and His-ICE1 proteins expressed in *E. coli* were used for a pull-down assay. HOS1 interacted with ICE1, whereas this interaction was greatly reduced by the addition of OST1 (Figure 6C). Moreover, the attenuation of this interaction was dependent on the amount of OST1 (Figure 6C). These data demonstrate that OST1 and HOS1 compete for binding to ICE1 in vitro.

A yeast three-hybrid assay was performed to further explore the effect of OST1 on the HOS1-ICE1 interaction. We generated a prey construct expressing ICE1, two bait constructs expressing C-terminal HOS1 and a mock OST1 bridge (OST1ΔC) that did not interact with ICE1 (Figure 4D) or the full-length OST1 bridge protein. The HOS1-ICE1 interaction was dramatically decreased in yeast cells expressing OST1 but not in those expressing OST1ΔC (Figure 6D).

We then analyzed the effect of OST1 on the HOS1-ICE1 interaction *in planta*. *HOS1C-GFP*, *HF-ICE1*, and *OST1-Myc* were expressed in *Arabidopsis* protoplasts, and a coIP assay was performed. HOS1 could interact with ICE1, and the HOS1-ICE1 interaction was evidently weakened by coexpression of OST1 (Figure 6E). Moreover, the interaction of HOS1 and ICE1^{S278D} was examined in *N. benthamiana* expressing *HF-HOS1C* and *Myc-ICE1*^{S278D}. ICE1^{S278D} failed to interact with HOS1 (Figure 6F), suggesting that the phosphorylated ICE1 protein inhibits its interaction with HOS1. Furthermore, we determined the effect of OST1 on ICE1 stability in the presence of HOS1. *HF-ICE1*, *OST1-Myc*, and *HOS1-HA* were expressed in *Arabidopsis* protoplasts, respectively, and total proteins were extracted and mixed them with combinations under cold conditions for a semi-in vitro degradation assay.

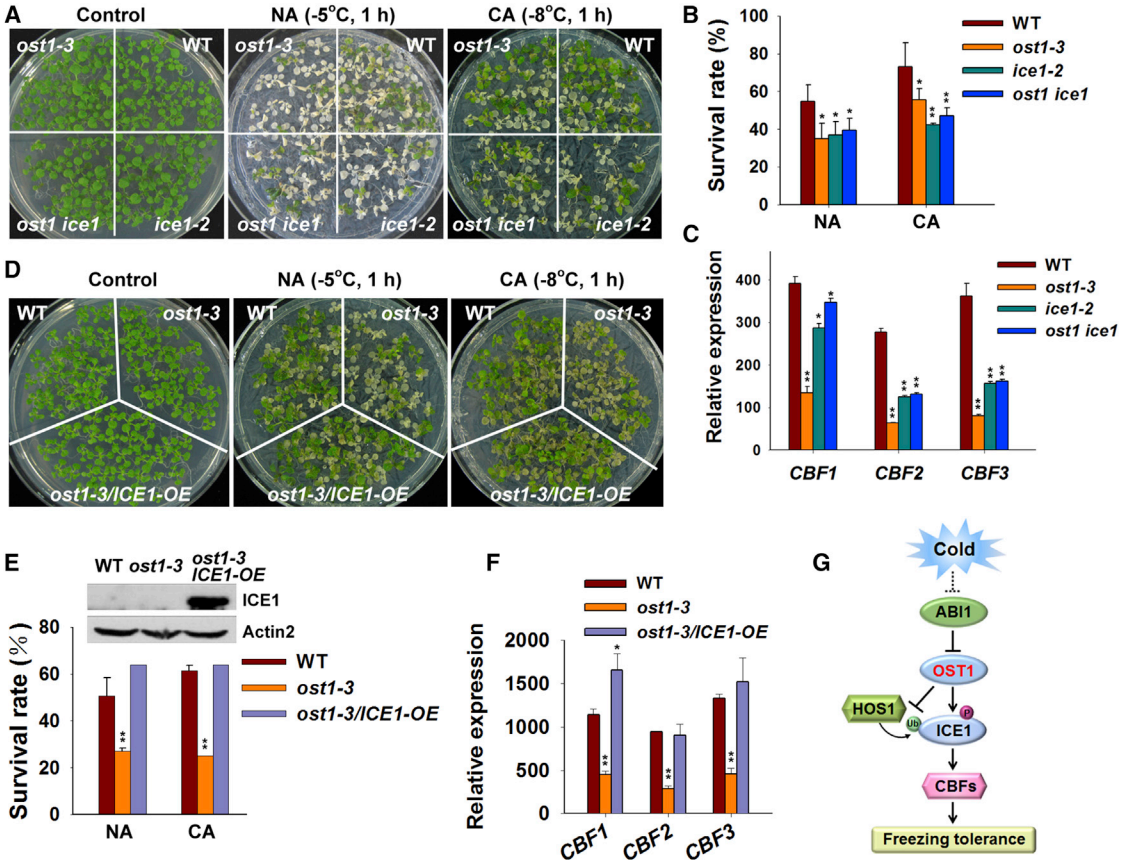

Figure 7. Genetic Interaction of *OST1* and *ICE1*

(A and B) Freezing phenotypes (A) and survival rates (B) of 2-week-old *ost1-3*, *ice1-2*, and *ost1-3 ice1-2* plants.

(C) Expression of *CBFs* in 2-week-old seedlings described in (A) after cold treatment at 4°C for 3 hr.

(D and E) Freezing phenotypes (D) and survival rates (E) of 2-week-old *ost1-3* plants overexpressing *ICE1*. The corresponding protein levels are shown at the top of (E).

(F) Expression of *CBFs* in 2-week-old seedlings described in (D) after cold treatment at 4°C for 3 hr. The expression of gene in untreated WT was set to 1.

In (B), (C), (E), and (F), data are means of three replicates ± SD, and asterisks indicate significant differences between the mutants and WT under the same treatment conditions (*$p < 0.05$, **$p < 0.01$, t test).

(G) The proposed model showing that OST1 modulates cold signaling by stabilizing ICE1.

Cold stress activates OST1 protein kinase, which is suppressed by ABI1. The cold-activated OST1 subsequently phosphorylates ICE1 and inhibits HOS1-mediated degradation of ICE1. In addition, OST1 interacts with HOS1 and facilitates the release of HOS1 to bind to ICE1. As a result, the stability and transcriptional activity of ICE1 are enhanced, thereby promoting the expression of *CBFs* and freezing tolerance.

ICE1 was degraded by HOS1, whereas overexpression of OST1 inhibited the degradation of ICE1 by HOS1 (Figure S4G). Taken together, these results suggest that OST1 phosphorylates ICE1, which in turn suppresses HOS1-mediated degradation of ICE1.

Genetic Interaction of *OST1* and *ICE1*

To explore the genetic interaction of *OST1* and *ICE1*, we generated *ice1-2 ost1-3* double mutant. Similar to *ice1-2* and *ost1-3*, the *ice1-2 ost1-3* double mutant displayed enhanced freezing sensitivity and decreased cold induction of *CBF* genes (Figures 7A–7C). When *ICE1* was overexpressed in *ost1-3*, the freezing hypersensitivity and decreased cold-induced *CBFs* expression of *ost1-3* were fully suppressed (Figures 7D–7F). These results further support that OST1 acts upstream of ICE1 in the CBF-dependent pathway.

DISCUSSION

Currently, the most thoroughly understood cold signaling pathway is ICE1-CBF-COR cascade; however, the protein kinases that act upstream in this cascade have remained elusive. In this study, we demonstrate that OST1, the positive regulator in the ABA signaling pathway, is a crucial protein kinase in the CBF-dependent pathway to regulate cold responses. Under cold stress, OST1 phosphorylates ICE1 and inhibits the degradation of ICE1 mediated by HOS1. Meanwhile, the OST1 protein also competes with HOS1 for binding to ICE1, thus liberating ICE1 from the HOS1-ICE1 complex. The dual role of OST1 contributes to the enhancement of ICE1 stability to increase *CBF* expression and freezing tolerance (Figure 7G).

OST1 is regarded as a central component in stomatal movement regulated by ABA (Fujita et al., 2009; Mustilli et al., 2002;

Yoshida et al., 2002). OST1 protein kinase activity was shown to be activated by ABA, NaCl, and low-humidity stress (Mustilli et al., 2002; Yoshida et al., 2006), but not cold stress (Yoshida et al., 2006). However, we show here that OST1 kinase is activated in plants under cold stress (Figure 2). This discrepancy is probably due to the different experiment systems used. Intriguingly, cold activates OST1 at the similar levels in the WT and ABA-deficient mutant *aba2-21* (Mang et al., 2012). Previous reports have shown that mild increases in endogenous ABA levels are observed after 6 hr of cold treatment in *Arabidopsis* (Lang et al., 1994) and after 12 hr of cold treatment in tomato (Daie and Campbell, 1981). In this study, we found that endogenous levels of ABA in both WT and *aba2-21* mutant are moderately reduced following 0.5 hr of cold treatment (Figure S2). This is not contradictory because 6–24 hr of cold treatment was used in the former studies (Daie and Campbell, 1981; Lang et al., 1994), whereas OST1 is activated after 0.5–1.5 hr of cold treatment in this study (Figure 2). Based on all these results, we postulate that ABA plays little role, if any, in the cold induction of OST1 activity at the early stage of cold response. Meanwhile, mutations of genes in ABA biosynthesis (*aba1* and *aba3*) result in impaired freezing tolerance (Mantyla et al., 1995; Xiong et al., 2001). In these ABA-deficient mutants, the reduced accumulation of ABA would decrease the expression of ABA responsive genes whose encoding proteins may function against different abiotic stresses, including cold and salt stress. It is likely that this process would mainly determine the basal resistance of the plants to cold stress.

Notably, cold activation of OST1 is abolished in the *abi1-1* dominant mutant. Moreover, *ost1*, *abi1-1* (C), and *ABI1-OE* plants show decreased cold induction of *CBF* genes and freezing sensitivity, whereas the *abi1 abi2 hab1* mutant displays the opposite freezing phenotypes (Figure 2). These data suggest that cold stress can activate OST1 perhaps by inactivating ABI1 somehow in an ABA-independent manner. Considering that ABA receptors PYR/PYLs have ABA-independent functions (Hao et al., 2011), one possibility is that cold stress may change the conformation of some PYR/PYL proteins in an unknown mechanism or affect other unknown proteins (such as proteins involved in cold sensing) that could directly interact with ABI1 and release its inhibition on OST1. A previous study has shown that phospholipase Dα1 (PLDα1)-derived phosphatidic acid (PA) in the plasma membrane can interact with ABI1 to mediate the ABA effects on stomata movement (Mishra et al., 2006). Cold stress can induce an increase in PA in the plasma membrane (Welti et al., 2002). Therefore, it is also possible that ABI1 might be tethered by PA under cold stress, which leads to removal of the ABI1 inhibition of OST1. Further study on how OST1 is activated by cold stress will shed more light on the understanding of cold perception in plants.

Under cold stress, ICE1 exists majorly as phosphorylated form in plants, and this phosphorylation is predominantly mediated by OST1 (Figure 4). The OST1-ICE1 interaction and phosphorylation of ICE1 at S278 by OST1 attenuates ICE1 proteolysis mediated by HOS1 (Figures 4 and 5). Indeed, ICE1 protein was found to remain stable within a 2 hr cold treatment, and thereafter it began to degrade (Figure S4H). It is noteworthy that the ICE1-interacting domain of OST1 (Domain II) is also the docking site for ABI1 (Yoshida et al., 2006). So it is likely that ABI1 could also compete with ICE1 for binding to OST1 and prevent OST1 from dephosphorylation by ABI1, which consequently results in feedback activation of OST1. A previous study showed that a mutated form of ICE1, ICE1^{S403A}, has increased protein stability and transactivation activity under cold stress (Miura et al., 2011). These findings suggest that the phosphorylation of ICE1 by different protein kinases execute distinct functions. At the early stages of cold stress, ICE1 phosphorylated by OST1 is a stable and active form that activates the expression of *CBFs* to enhance plant freezing tolerance; however, ICE1 that is phosphorylated at later stages by other unknown protein kinases may be more easily recognized by HOS1 and subsequently degraded via the 26S-proteasome pathway (Dong et al., 2006). Given that ICE1 is also regulated by ubiquitination and sumoylation (Dong et al., 2006; Miura et al., 2007), it will be interesting to investigate the homeostasis regulation of ICE1 through multiple protein modifications.

Stomatal movement plays important roles in plant responses to environmental stimuli, including CO_2 exchanges, water transpiration, and plant defense responses (Shimazaki et al., 2007). *Commelina communis* stomata close rapidly when plants are shifted from 27°C to 7°C, which might be a result of increased calcium uptake by guard cells (Wilkinson et al., 2001). ICE1 regulates plant stomatal development by interacting with an intrinsic positive regulator, SPEECHLESS, that directs entry into the stomatal cell lineage (Kanaoka et al., 2008). OST1 can directly phosphorylate and activate the ion channel SLAC1 to regulate stomatal movement (Geiger et al., 2009). The results suggest a potential link between the cold response and stomatal development/movement. Intriguingly, both *OST1* and *ICE1* are predominantly expressed in guard cells and vascular tissues (Kanaoka et al., 2008; Mustilli et al., 2002), which is consistent with our results showing their function in the same cold signaling pathway. However, the *CBFs* and their target genes are expressed in all leaf cells under cold stress (Novillo et al., 2007). The different expression patterns of *CBFs* and *ICE1*/*OST1* imply that the reduced expression of *CBF*s by cold stress in *ost1* and *ice1* mutants should mainly occur in the tissues where *ICE1* and *OST1* are expressed. Further investigation will decipher the interplay of stomatal development/movement and cold responses in plants.

EXPERIMENTAL PROCEDURES

Plants Materials and Growth Conditions

Arabidopsis ecotype Col plants were grown on MS medium at 22°C under a 16 hr light/8 hr dark photoperiod. The mutants *ost1-3/snrk2.6* (SALK_008068), *ost1-4* (GK-516B05), *abi1-1* (Col) (Luo et al., 2014), *aba2-21* (Mang et al., 2012), *ice1-2* (Kanaoka et al., 2008), and *abi1 abi2 hab1* (Hua et al., 2012) were used in this study. Details of transgenic plants and plasmid constructions were described in the Supplemental Information.

Physiological Analyses

The freezing tolerance and ion leakage assays were performed as described (Shi et al., 2012). The detailed procedures were described in Supplemental Information. ABA contents were determined as described previously (Fu et al., 2012).

In Vitro Pull-Down Assay

The pull-down assay was performed as described (Wang et al., 2011). Detailed procedures are provided in the Supplemental Information.

Yeast Two-Hybrid and Three-Hybrid Assays

The yeast two-hybrid and three-hybrid assays were performed as described (Lian et al., 2011; Wang et al., 2011). The yeast strain was transformed with pairs of plasmids (pB-HOS1C-OST1 and pA-ICE1 or pB-HOS1C-mOST1 and pA-ICE1) in yeast three hybrid assay.

Protein Kinase Assay

In-gel kinase assay was performed as described previously (Fujii et al., 2007). Detailed in vitro and in-gel kinase assays are provided in the Supplemental Information.

CoIP Assay

The total proteins were extracted from *N. benthamiana* leaves expressing *Super:OST1-Myc/35:HF-ICE1* or *Super:OST1-Myc* constructs and incubated with anti-FLAG agarose beads. Proteins bound to the beads were detected with anti-Myc antibody (Sigma-Aldrich).

For the interference assay, the total proteins were extracted from *Arabidopsis* protoplasts expressing *Super:OST1-Myc/35:HF-ICE1* or *Super:OST1-Myc/35:HF-ICE1/Super:HOS1C-GFP* or *Super:HOS1C-GFP*, incubated with anti-HA agarose beads and detected with anti-GFP antibody (Sigma-Aldrich).

For the HOS1-ICE1^{S278D} interaction assay, total proteins were prepared from *N. benthamiana* leaves expressing *35S:HF-HOS1C/35S:Myc-ICE1*^{S278D}, *35S:HF-HOS1C/35S:Myc-ICE1*, *35S:Myc-ICE1*^{S278D}, and *35S:Myc-ICE1* constructs and immunoprecipitated with HA agarose beads. The samples were detected by anti-Myc and anti-HA antibodies (Sigma-Aldrich).

ChIP Assay

ChIP assay was performed on *Arabidopsis* protoplasts expressing *35:HF-ICE1* alone or *35:HF-ICE1/Super:OST1-Myc* as described (Shi et al., 2012). The enriched DNA fragments were analyzed by qRT-PCR using the primers listed in the Supplemental Information.

EMSA Assay

His-ICE1 and GST-OST1 proteins were expressed in *E. coli* and purified using His and glutathione sepharose beads, respectively (Amersham Biosciences). His-ICE1 was incubated with GST-OST1 or GST with kinase reaction buffer at 30°C for 30 min. A biotin-labeled *CBF3* DNA fragment was then incubated with His-ICE1 or phosphorylated His-ICE1. The EMSA assay was described in the Supplemental Information.

Cell-free and Semi-In Vivo Protein Degradation Assays

Cell-free and semi-in vivo protein degradation assays were performed as described (Liu et al., 2010; Wang et al., 2013b). Equal amounts of proteins from the WT Col, *ost1-3*, and *Super:OST1-Myc* plants were incubated with HF-ICE1 protein expressed in *Arabidopsis* protoplasts at 22°C for 30 and 60 min, and they were detected by immunoblotting with an anti-HA antibody. The detailed semi-in vivo protein degradation assay was described in the Supplemental Information.

Additional Methods

Additional information on plasmid construction, gene expression analysis, and transient transactivation assay is provided in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.12.023.

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