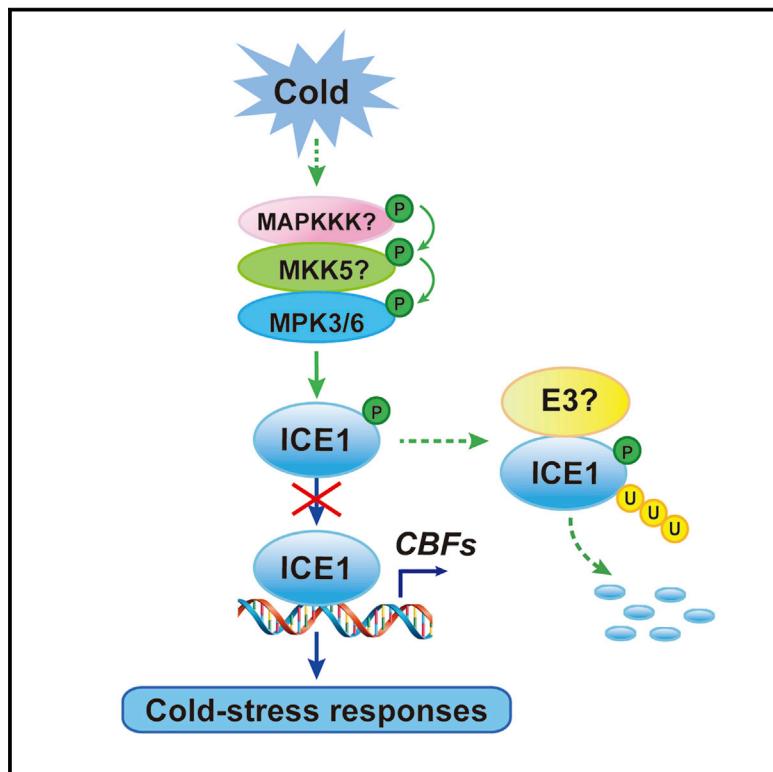


# Developmental Cell

## MPK3- and MPK6-Mediated ICE1 Phosphorylation Negatively Regulates ICE1 Stability and Freezing Tolerance in *Arabidopsis*

### Graphical Abstract



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### In Brief

ICE1 is a key regulator of the cold-activated CBF transcription factors in plants. Li et al. show that cold-activated mitogen-activated protein kinases MPK3 and MPK6 phosphorylate the ICE1 protein to reduce its stability and transcriptional activity, which consequently negatively regulates CBF expression and freezing tolerance in plants.

### Highlights

- Cold activates mitogen-activated protein kinases MPK3 and MPK6
- MPK3/MPK6 phosphorylate and destabilize the ICE1 protein
- MPK3/MPK6 activation attenuates plant freezing tolerance

# MPK3- and MPK6-Mediated ICE1 Phosphorylation Negatively Regulates ICE1 Stability and Freezing Tolerance in *Arabidopsis*

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## SUMMARY

Low temperatures affect plant growth, development, productivity, and ecological distribution. Expression of the C-repeat-binding factor (CBF) transcription factors is induced by cold stress, which in turn activates downstream cold-responsive (COR) genes that are required for the acquisition of freezing tolerance. Inducer of CBF expression 1 (ICE1) is a master regulator of CBFs, and ICE1 stability is crucial for its function. However, the regulation of ICE1 is not well understood. Here, we report that mitogen-activated protein kinase 3 (MPK3) and MPK6 interact with and phosphorylate ICE1, which reduces its stability and transcriptional activity. Consistently, the *mpk3* and *mpk6* single mutants and the *mpk3 mpk6* double mutants show enhanced freezing tolerance, whereas MPK3/MPK6 activation attenuates freezing tolerance. Phosphor-inactive mutations of ICE1 complement freezing sensitivity in the *ice1-2* mutant. These combined results indicate that MPK3/MPK6 phosphorylate and destabilize ICE1, which negatively regulates CBF expression and freezing tolerance in plants.

## INTRODUCTION

Low temperatures substantially attenuate plant growth, development, and geographical distribution, and adversely affect crop quality and productivity. Temperate plants can acquire cold tolerance after exposure to low but nonfreezing temperatures (called cold acclimation), which enhances their survival under freezing stress. Cold acclimation is a complex process that involves many physiological and biochemical pathways (Thomashow, 1999). The C-repeat (CRT) binding factor (CBF)/dehydration-responsive element binding factor 1 transcription factors belong to the Apetala2/ethylene-responsive factor subfamily,

and have crucial roles in plant cold acclimation (Liu et al., 1998; Stockinger et al., 1997). CBF proteins bind to the cold- and dehydration-responsive DNA regulatory element, also called the CRT element, on the promoters of cold-responsive (COR) genes, and activate COR gene expression under cold stress, thereby conferring plant freezing tolerance (Gilmour et al., 1998; Jia et al., 2016; Liu et al., 1998; Stockinger et al., 1997; Zhao et al., 2016).

Several transcription factors regulate CBF expression, including Inducer of CBF expression 1 (ICE1), phytochrome-interacting factor 4/7 (PIF4/7), MYB15, ethylene insensitive 3, calmodulin-binding transcription activator 3, brassinazole resistant 1 (BZR1), and CESTA (Agarwal et al., 2006; Chinnusamy et al., 2003; Doherty et al., 2009; Eremina et al., 2016; Lee and Thomashow, 2012; Li et al., 2017; Shi et al., 2012). ICE1 is considered as a master regulator of CBF genes (Chinnusamy et al., 2003). ICE1 encodes a MYC-like basic-helix-loop-helix transcription factor that binds to canonical MYC cis-elements (CANNTG) in CBF promoters and activates their expression (Chinnusamy et al., 2003; Ding et al., 2015). Emerging evidence indicates that ICE1 is regulated posttranslationally by several factors, including high expression of osmotically responsive gene 1 (HOS1), SAP and Miz (SIZ1), and open stomata 1 (OST1) (Ding et al., 2015; Dong et al., 2006; Miura et al., 2007). HOS1 acts as a RING-type ubiquitin E3 ligase that ubiquitinates and mediates the cold-induced degradation of ICE1, which in turn negatively regulates CBF expression at low temperatures (Dong et al., 2006). SIZ1-mediated ICE1 sumoylation stabilizes ICE1 and controls CBF expression (Miura et al., 2007). Our recent study showed that OST1/SnRK2.6, a Ser/Thr protein kinase, originally identified in ABA signaling, is activated by cold stress, subsequently interacts with and phosphorylates ICE1, which suppresses HOS1-mediated ICE1 degradation and positively regulates CBF expression and freezing tolerance (Ding et al., 2015). Previous studies showed that MYB15 and jasmonate ZIM-domain 1/4 also physically interact with ICE1 to repress CBF expression and negatively regulate freezing tolerance (Agarwal et al., 2006; Hu et al., 2013). As posttranslational regulation of ICE1 is essential for its function, it is important to identify other modulators that regulate ICE1.

Other protein kinase families have been implicated in plant responses to low temperature, including cold-responsive protein kinase 1, calcium/calmodulin-regulated receptor-like kinase 1 (CRLK1), mitogen-activated protein kinases (MAPKs), calcineurin-B-like interacting protein kinases, and  $\text{Ca}^{2+}$ -dependent protein kinases (Furuya et al., 2013; Huang et al., 2011; Kim et al., 2003; Liu et al., 2017; Rodriguez et al., 2010; Teige et al., 2004; Yang et al., 2010a, 2010b). MAPK cascades have evolved to transduce environmental and developmental cues into intracellular responses using three classes of protein kinases: MAP kinase kinase kinases (MAP3Ks; also called MAPKKKs or MEKKs), MAP kinase kinases (MAP2Ks; also called MKKs or MEKs), and MAPKs (Rodriguez et al., 2010). In plants, MAP3Ks are activated by stimulated plasma membrane receptors. Activated MAP3Ks phosphorylate and activate MAP2Ks, which in turn phosphorylate and activate MAPKs (Rodriguez et al., 2010). Several lines of evidence suggest that the MAPK pathway mediated by MEKK1-MKK2-MPK4/6 has a positive role in plant responses to cold stress (Teige et al., 2004; Yang et al., 2010b). MPK4 and MPK6 are activated by cold stress in plants (Ichimura et al., 2000; Teige et al., 2004). MKK2 is also activated by cold stress in plants, and consequently activates MPK4 and MPK6 (Teige et al., 2004). CRLK1 interacts with MEKK1, leading to MAPK activation and freezing tolerance (Yang et al., 2010b). These results suggest that the MAPK cascade participates in plant cold-stress responses. However, the underlying mechanisms of MAPK activity in cold signaling have not been elucidated.

Here, we show that MPK3 and MPK6 interact with and phosphorylate ICE1 in *Arabidopsis thaliana*. MPK3/MPK6 phosphorylation of ICE1 inhibits its transcriptional activity and facilitates ubiquitination-mediated ICE1 degradation under cold stress, thereby negatively regulating plant freezing tolerance and *CBF* expression. Our results identify a mechanism in which MPK3/MPK6 negatively regulate plant freezing tolerance by phosphorylating and destabilizing ICE1.

## RESULTS

### ICE1 Interacts with MPK3 and MPK6

ICE1 is regulated by multiple posttranslational modifications, which are crucial for its stability and transcriptional activity (Ding et al., 2015; Dong et al., 2006; Miura et al., 2007). To further explore ICE1 regulation, we performed a yeast two-hybrid assay to identify interacting proteins. The full-length ICE1 protein had strong self-activation activity, whereas the C-terminal region of ICE1 (from 358 to 494 amino acids, required for protein-protein interaction) abolished its activity (Agarwal et al., 2006). So we chose this C-terminal region cloned into the pGKBT7 vector as the bait in our assay (Figure S1A). This experiment identified MPK3 and MPK6 as interacting proteins, and they were selected for further study.

We performed yeast two-hybrid to confirm the interaction of full-length MPK3/MPK6 with full-length ICE1 (Ding et al., 2015) (Figure 1A). Then, we examined the ICE1 functional domains required for interaction with MPK3/MPK6 in yeast. ICE1 contains a serine-rich region at the N terminus, and a MYC-like basic-helix-loop-helix domain and possible zipper region at the C terminus (Figure 1B). The ICE1 C-terminal region strongly interacted

with MPK3 and MPK6, whereas deletion of the C-terminal region reduced these interactions, indicating that the ICE1 C-terminal region is required for interaction with MPK3/MPK6 in yeast (Figures 1B and S1B).

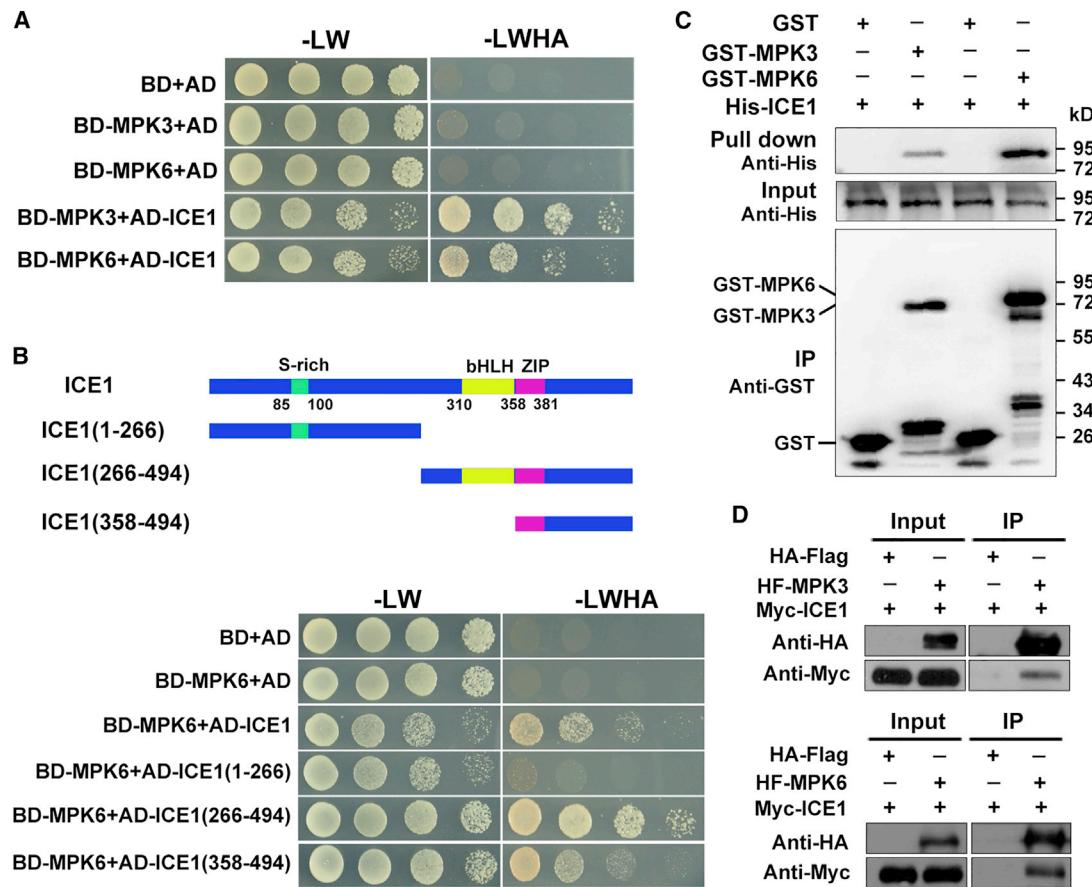
Next, we performed a glutathione S-transferase (GST) pull-down assay to confirm the interactions between MPK3/MPK6 and ICE1 *in vitro*. We tagged ICE1 with 6×His and incubated the tagged protein with GST-MPK3, GST-MPK6, or GST alone. The results showed that His-ICE1 was pulled down by GST-MPK3 and GST-MPK6, but not by GST alone (Figure 1C), indicating that ICE1 directly binds to MPK3/MPK6 *in vitro*.

We performed a coimmunoprecipitation (coIP) assay using *Nicotiana benthamiana* leaves transiently co-expressing 35S:HA-Flag-MPK3/MPK6 (35S:HF-MPK3/MPK6) and 35S:Myc-ICE1 constructs. Protein extracts were immunoprecipitated with anti-hemagglutinin (HA) agarose, and the precipitated proteins were analyzed by immunoblotting with anti-Myc antibody. A band with the expected mobility of Myc-ICE1 was successfully detected in the anti-HA immunoprecipitates of leaves expressing HF-MPK3 and HF-MPK6 (Figure 1D). By contrast, no Myc-ICE1 was detected in the anti-HA immunoprecipitates of leaves expressing HF-tagged empty vector. These combined results indicate that MPK3 and MPK6 interact with ICE1 *in vitro* and *in vivo*.

### MPK3 and MPK6 Negatively Regulate Freezing Tolerance

We hypothesized that the ICE1 interacting proteins MPK3 and MPK6 might be involved in plant cold-stress responses. Therefore, we performed freezing tolerance assays using the following *Arabidopsis* knockout mutants: *mpk3-1* (Zhao et al., 2014), *mpk3-2*, *mpk6-3*, and *mpk6-4* (Xu et al., 2008). The *mpk3* and *mpk6* mutants displayed substantially increased freezing tolerance (reflected by survival rate) under non-acclimated (NA) and cold-acclimated (CA) conditions compared with the wild-type Columbia (Col) (Figures 2A–2D). Next, we examined ion leakage, which is an indicator of stress-induced plasma membrane damage, in the *mpk3* and *mpk6* mutants. Ion leakage in the *mpk3* and *mpk6* mutants under NA and CA conditions was consistently lower than that in the wild type (Figures 2E and 2F). These results suggest that MPK3 and MPK6 are negative regulators of basal and acquired freezing tolerance in *Arabidopsis*.

To further study the role of MPK3 and MPK6 in the freezing stress response, we examined freezing tolerance in the conditional loss-of-function *mpk3 mpk6* double mutant, named MPK3SR (*mpk3 mpk6 pMPK3:MPK3<sup>TG</sup>*). The *mpk3 mpk6* loss-of-function mutant is lethal, but can be rescued with MPK3<sup>TG</sup>, which is a 4-amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3, 4-d]pyrimidine (NA-PP1)-sensitized version of MPK3. MPK3<sup>TG</sup> loses its function after the addition of NA-PP1 (Su et al., 2017; Xu et al., 2014). The NA-PP1-treated MPK3SR plants (line nos. 28 and 64) displayed increased freezing tolerance and reduced ion leakage compared with the wild-type, with or without cold acclimation (Figures 2G–2I and S2A–S2C). Similar freezing tolerance and ion-leakage responses were observed in another conditional *mpk3 mpk6* double mutant, named MPK6SR (*mpk3 mpk6 pMPK6:MPK6<sup>YG</sup>*) (Su et al., 2017; Xu et al., 2014) after NA-PP1 treatment (Figures S2D–S2F). NA-PP1-treated MPK3SR plants



**Figure 1. Interactions between ICE1 and MPK3/MPK6**

(A) ICE1 interacts with MPK3 and MPK6 in yeast. Yeast were grown on SD/-Leu/-Trp (-LW) or SD/-Leu/-Trp/-His/-Ade (-LWHA) medium. The empty pGADT7 prey vector was used as a negative control.

(B) The ICE1 C terminus interacts with MPK6 in yeast. Full-length and truncated ICE1 constructs with specific deletions (top). Interaction between full-length and truncated ICE1 constructs and MPK6 in yeast (bottom).

(C) *In vitro* pull-down assay shows the interactions of ICE1 with MPK3 and MPK6. GST, GST-MPK3, or GST-MPK6 was immobilized on glutathione Sepharose beads, incubated with His-ICE1 protein, and subjected to immunoblot analysis with anti-His antibody.

(D) ICE1 associates with MPK3 and MPK6 in *N. benthamiana* leaves. Total proteins were extracted from *N. benthamiana* leaves co-transfected with Myc-ICE1 and HA-Flag-MPK3/MPK6 or HA-Flag empty vector control, and immunoprecipitated using anti-HA agarose beads. Coimmunoprecipitated Myc-ICE1 was detected with anti-Myc antibody.

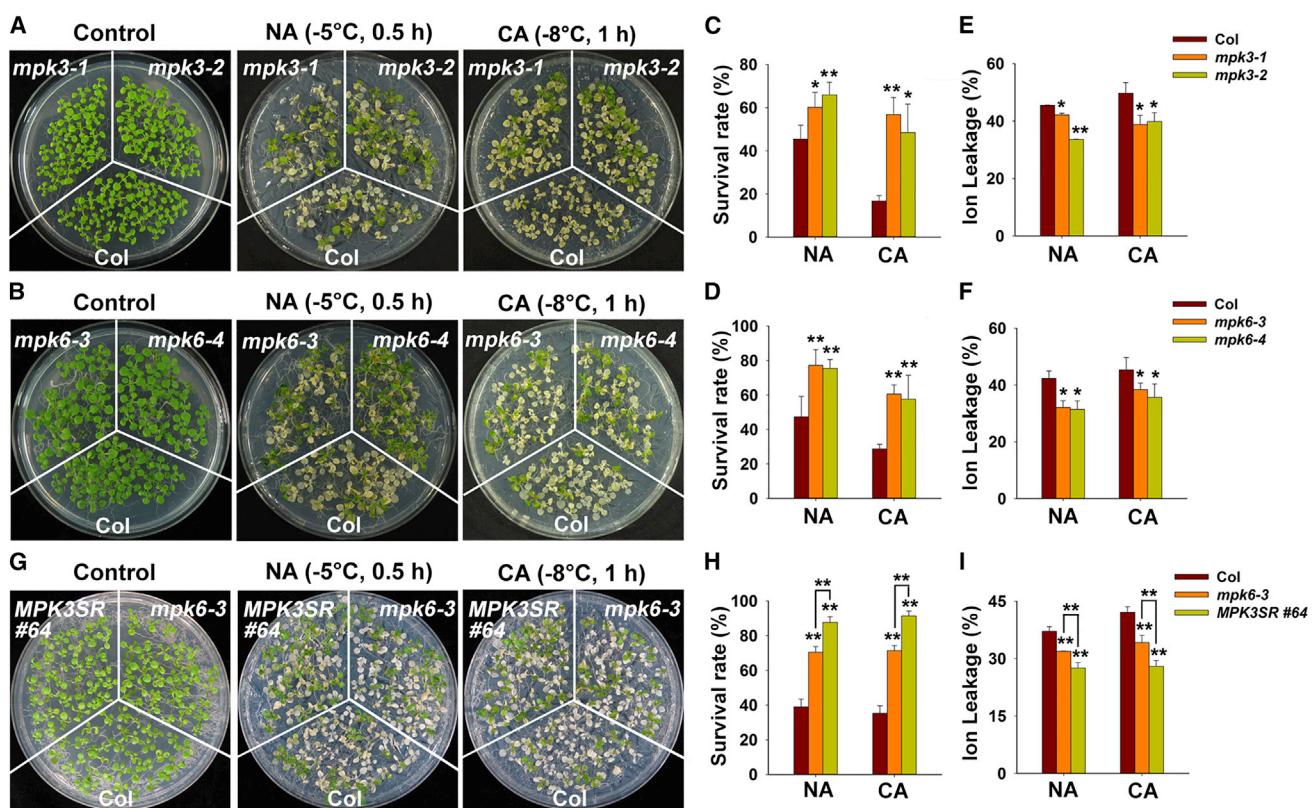
See also Figure S1.

(line no. 64) also displayed greater freezing tolerance than *mpk6-3* mutants (Figures 2G–2I). These results further demonstrate that MPK3 and MPK6 negatively regulate plant freezing tolerance.

MAPK activation requires dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues in the TXY motif by activated MAPKKs (Cobb and Goldsmith, 1995). Previous studies reported that MPK3 and MPK6 can be activated by the expression of MKK5<sup>DD</sup> (T215D/S221D), a constitutively active form of MKK5 (Liu and Zhang, 2004; Ren et al., 2002). We examined steroid-inducible gain-of-function MKK5<sup>DD</sup> transgenic *Arabidopsis* plants, and found that MPK3/MPK6 activation in dexamethasone-treated MKK5<sup>DD</sup> reduced freezing tolerance and increased ion leakage compared with that of control plants with or without cold acclimation (Figures S2G–S2I). These combined results suggest that the constitutively activated MKK5<sup>DD</sup> negatively regulates plant freezing tolerance.

### MPK3 and MPK6 Acts Upstream of ICE1 in the CBF-Dependent Pathway

Having shown that MPK3 and MPK6 are involved in cold-stress responses and interact with the CBF master regulator ICE1, we next evaluated whether MPK3/MPK6 mediate plant freezing tolerance via the CBF-dependent pathway by analyzing the expression of CBFs and CBF target genes in *mpk3-2* and *mpk6-3*. Cold-induced expression of CBF genes and targets, such as *COR15A*, *KIN1*, and *RD29A*, was significantly higher in *mpk3-2* and *mpk6-3* mutants than in wild-type plants (Figures 3A–3D). Consistently, the expression of CBFs and CBF target genes in NA-PP1-treated *MPK3SR* plants was also higher than that in the wild-type plants under cold treatment (Figures S3A and S3B). By contrast, MPK3/MPK6 activation in MKK5<sup>DD</sup> dramatically reduced cold induction of CBFs and CBF target gene expression compared with that in wild-type plants (Figures S3C and S3D). These results



**Figure 2. Freezing Phenotypes of *mpk3*, *mpk6*, and *mpk3 mpk6* Mutants**

(A–F) Freezing phenotypes (A and B), survival rates (C and D), and ion leakage (E and F) of the *mpk3* and *mpk6* mutants under non-acclimated (NA) and cold-acclimated (CA) conditions. Wild-type (*Columbia*), *mpk3*, and *mpk6* plants were grown on half-strength Murashige and Skoog (MS) plates at 22°C for 14 days before being subjected to freezing treatment. For the NA treatment, freezing began at 0°C, the temperature dropped by 1°C per hr until reaching -5°C, and then the plants were held at -5°C for 0.5 hr. For the CA plants (pretreated at 4°C for 3 days), freezing began at 0°C, the temperature dropped by 1°C per hr until reaching -8°C, and then the plants were held at -8°C for 1 hr. After treatment, the plants were incubated at 4°C under dark conditions for 12 hr and then shifted at 22°C under a 16-hr light/8-hr dark photoperiod. Representative images were taken after recovery for 3 days, and the survival rates and ion leakage were measured.

(G–I) Freezing phenotypes (G), survival rates (H), and ion leakage (I) of *mpk6-3* and *MPK3SR* (line no. 64). Five-day-old seedlings of wild-type, *mpk6-3*, and *mpk3 mpk6* plants were grown on half-strength MS plates, transferred to half-strength MS plates containing 0.5 μM NA-PP1 inhibitor, and then held for an additional 9 days before subjecting plants to NA or CA freezing treatments as described in (A)–(D) above.

In (C)–(F), (H), and (I), data are the mean values of three technical replicates ± SD. \*p < 0.05, \*\*p < 0.01 (t test) indicates a significant difference between wild-type plants and mutants. At least three independent experiments were performed with similar results.

See also Figure S2.

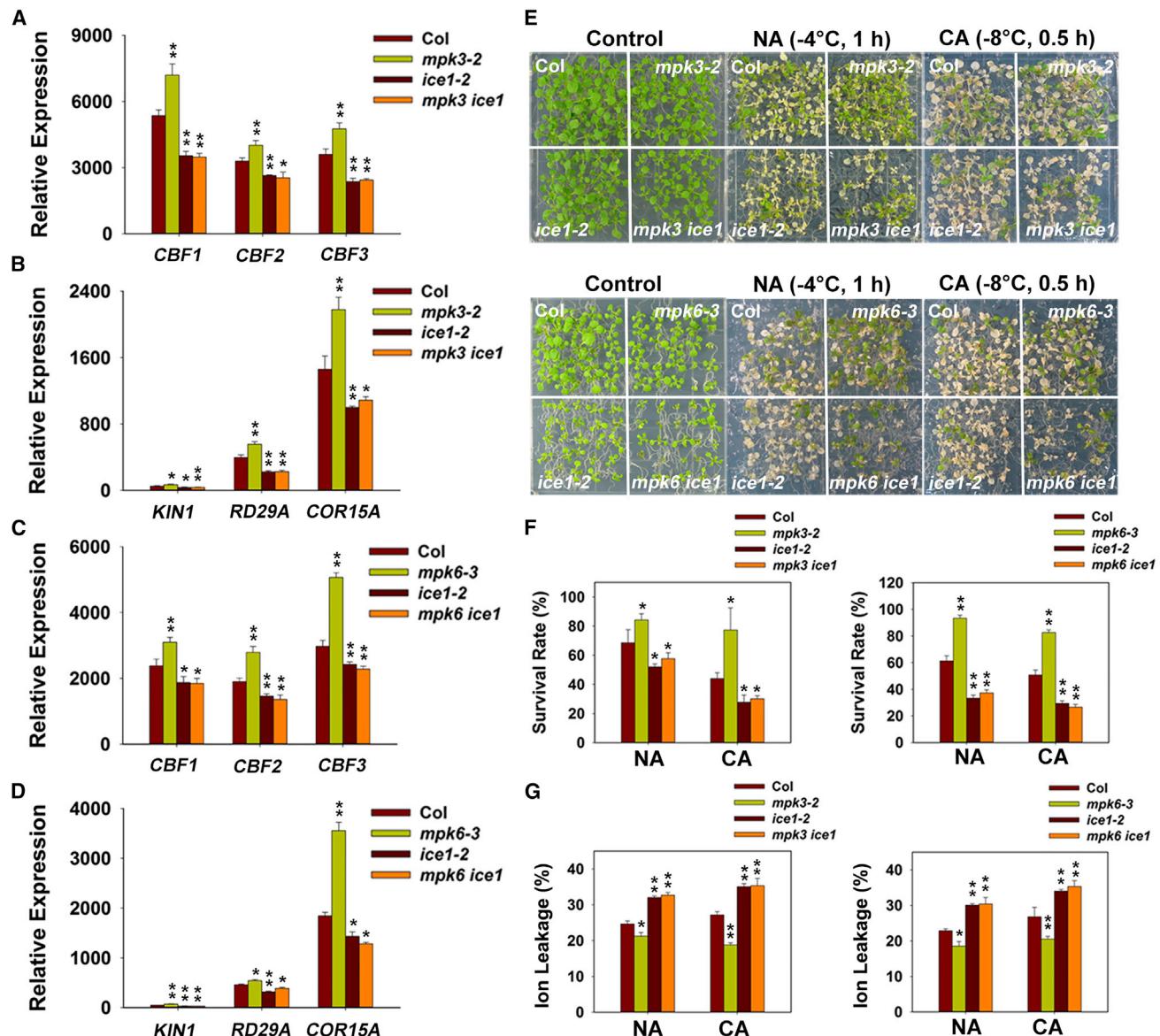
suggest that MPK3 and MPK6 negatively regulate *CBF* gene expression.

To explore the genetic interactions between MPK3/MPK6 and ICE1, we generated *mpk3-2 ice1-2* and *mpk6-3 ice1-2* double mutants. Consistent with our previous study (Ding et al., 2015), the *ice1-2* mutant was hypersensitive to freezing stress. The *mpk3-2 ice1-2* and *mpk6-3 ice1-2* double mutants resembled the *ice1-2* mutant in terms of freezing sensitivity, ion leakage, and cold-induced expression of *CBFs* and their target genes (Figure 3). These results suggest that MPK3 and MPK6 act upstream of ICE1 to negatively regulate *CBF* gene expression.

#### MPK3 and MPK6 Phosphorylate ICE1 In Vitro and In Vivo

Previous studies reported that MPK3 and MPK6 physically interact with and phosphorylate their substrates to modulate plant physiological and biochemical processes (Mao et al., 2011; Meng et al., 2013; Zhang et al., 2015). As MPK3 and

MPK6 interact with ICE1, we hypothesized that ICE1 might be a substrate of MPK3 and MPK6. Therefore, we purified recombinant His-tagged ICE1 and performed *in vitro* phosphorylation assays. Recombinant His-MPK3 and His-MPK6 strongly phosphorylated ICE1 after activation by constitutively active MKK5<sup>DD</sup> (Figure 4A). By contrast, neither MPK3 nor MPK6 phosphorylated ICE1 in the absence of constitutively active MKK5<sup>DD</sup> (Figure 4A). We analyzed the ICE1 amino acid sequence and found six potential MAPK phosphorylation sites (Ser94, Ser203, Thr366, Thr382, Thr384, and Ser403) (Enslen and Davis, 2001; Sharrocks et al., 2000) (Figure 4B). After mutating all six Ser/Thr residues to Ala (ICE1<sup>6A</sup>), ICE1 was no longer phosphorylated by MPK3/MPK6 (Figure 4C). Next, we tested whether native MPK3/MPK6 phosphorylated ICE1 by performing an in-gel kinase assay using total proteins extracted from *mpk3*, *mpk6*, and wild-type Col under cold stress. Cold-induced MPK3 or MPK6 kinase activity was observed when recombinant



**Figure 3. MPK3 and MPK6 Act Upstream of ICE1 in the CBF-Dependent Pathway**

(A and B) Expression of CBF genes (A) and their target genes (B) in *mpk3-2* and *mpk3-2 ice1-2* mutants under cold stress. Plants were grown for 14 days on half-strength MS plates at 22°C, and treated at 4°C for 3 or 24 hr to analyze CBF or CBF target gene expression. Total RNAs were extracted and subjected to qRT-PCR analysis. Gene expression levels in wild-type control plants at 22°C were set to 1.0.

(C and D) Expression of CBF genes (C) and their target genes (D) in *mpk6-3* and *mpk6-3 ice1-2* mutants under cold stress. Plants were grown for 14 days on half-strength MS plates at 22°C, and treated at 4°C for 3 or 24 hr to analyze CBF or CBF target gene expression. Gene expression levels in wild-type control plants at 22°C were set to 1.0.

(E–G) Freezing phenotypes (E), survival rates (F), and ion leakage (G) of *mpk3-2 ice1-2* and *mpk6-3 ice1-2* double mutants. Plants were grown for 14 days on half-strength MS plates at 22°C, and treated at -4°C for 1 hr for NA plants and -8°C for 0.5 hr for CA plants.

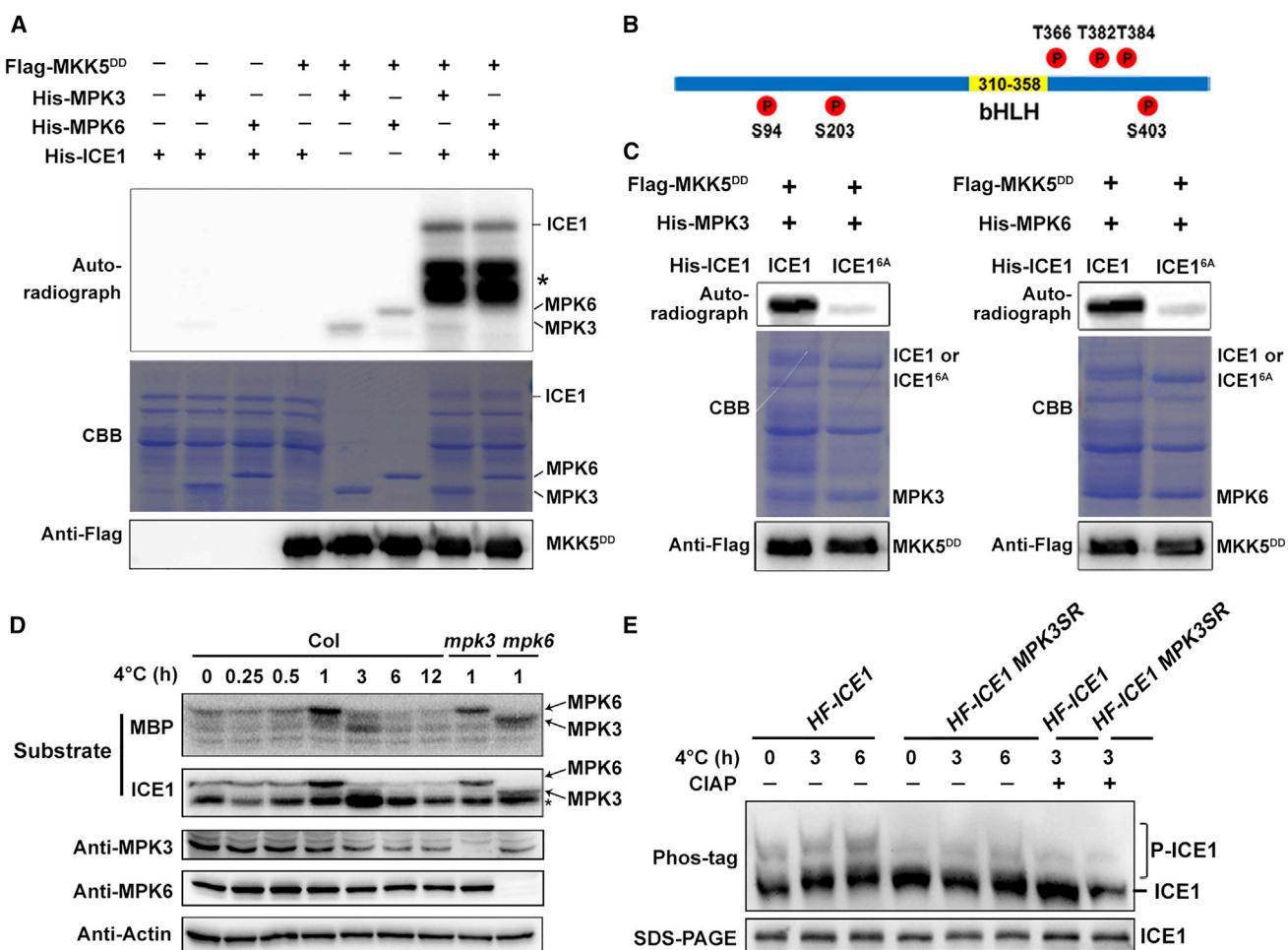
In (A)–(D), (F), and (G), data are the mean values of three technical replicates  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$  (t test) indicate significant differences between wild-type plants and mutants. Similar results were obtained from three independent experiments.

See also Figure S3.

His-ICE1 and MBP proteins were used as substrates; however, this cold-induced MPK3 or MPK6 activity was not detected in *mpk3* and *mpk6* mutants (Figure 4D). These results suggest that ICE1 is a substrate of MPK3/MPK6.

To examine whether MPK3/MPK6 phosphorylate ICE1 *in vivo*, we performed Phos-tag mobility shift assays (Bethke et al., 2009).

A transgenic plant expressing 35S:HF-ICE1 (Ding et al., 2015) was crossed with a conditional loss-of-function *mpk3 mpk6* double mutant (*MPK3SR* no. 64) to generate 35S:HF-ICE1 *MPK3SR* plants, which were subjected to cold treatment at 4°C. Protein extracts were separated in a Phos-tag gel and were detected by immunoblot analysis. Under normal temperatures, both



**Figure 4. MPK3/MPK6-Mediated ICE1 Phosphorylation In Vitro and In Vivo**

(A) ICE1 phosphorylation mediated by activated MPK3 and MPK6 *in vitro*. Reactions lacking the specified components (—) were used as controls. MPK3 and MPK6 were activated with recombinant Flag-MKK5<sup>DD</sup> protein. Recombinant proteins were separated by 10% SDS-PAGE after incubation in protein kinase buffer containing [ $\gamma$ -<sup>32</sup>P]ATP. Phosphorylated ICE1 was detected by autoradiography after gel electrophoresis (top panel). The intense bands denoted by asterisk in the right two panels represent the degradation products of ICE1. Recombinant MPK3, MPK6, and ICE1 were detected by Coomassie brilliant blue (CBB) staining (middle panel). Flag-MKK5<sup>DD</sup> protein was detected by anti-Flag antibody (bottom panel).

(B) Illustration of putative MAPK phosphorylation sites in ICE1.

(C) Phosphorylation assays of wild-type and mutated ICE1 by activated MPK3/MPK6 *in vitro*. Recombinant His-ICE1 and His-ICE1<sup>6A</sup> proteins were incubated with activated MPK3 and MPK6. Phosphorylated ICE1 was detected by autoradiography after gel electrophoresis (top panel). Recombinant MPK3, MPK6, and ICE1 were detected by CBB staining (middle panel). Flag-MKK5<sup>DD</sup> protein was detected by anti-Flag antibody (bottom panel).

(D) In-gel kinase assay of MPK3/MPK6 after cold treatment. Total proteins were extracted from 2-week-old plants treated at 4°C for the indicated times, and separated in SDS-PAGE gels containing 0.3 mg/mL MBP or recombinant His-ICE1 protein as a substrate. The mpk3 and mpk6 mutants treated at 4°C for 1 hr were used as controls. MPK3/MPK6 kinase activity was detected with autoradiography. MPK3 and MPK6 proteins were detected with anti-MPK3 and anti-MPK6 antibodies, and actin was used as a control. The star represents phosphorylation of ICE1 by other unknown protein kinase(s).

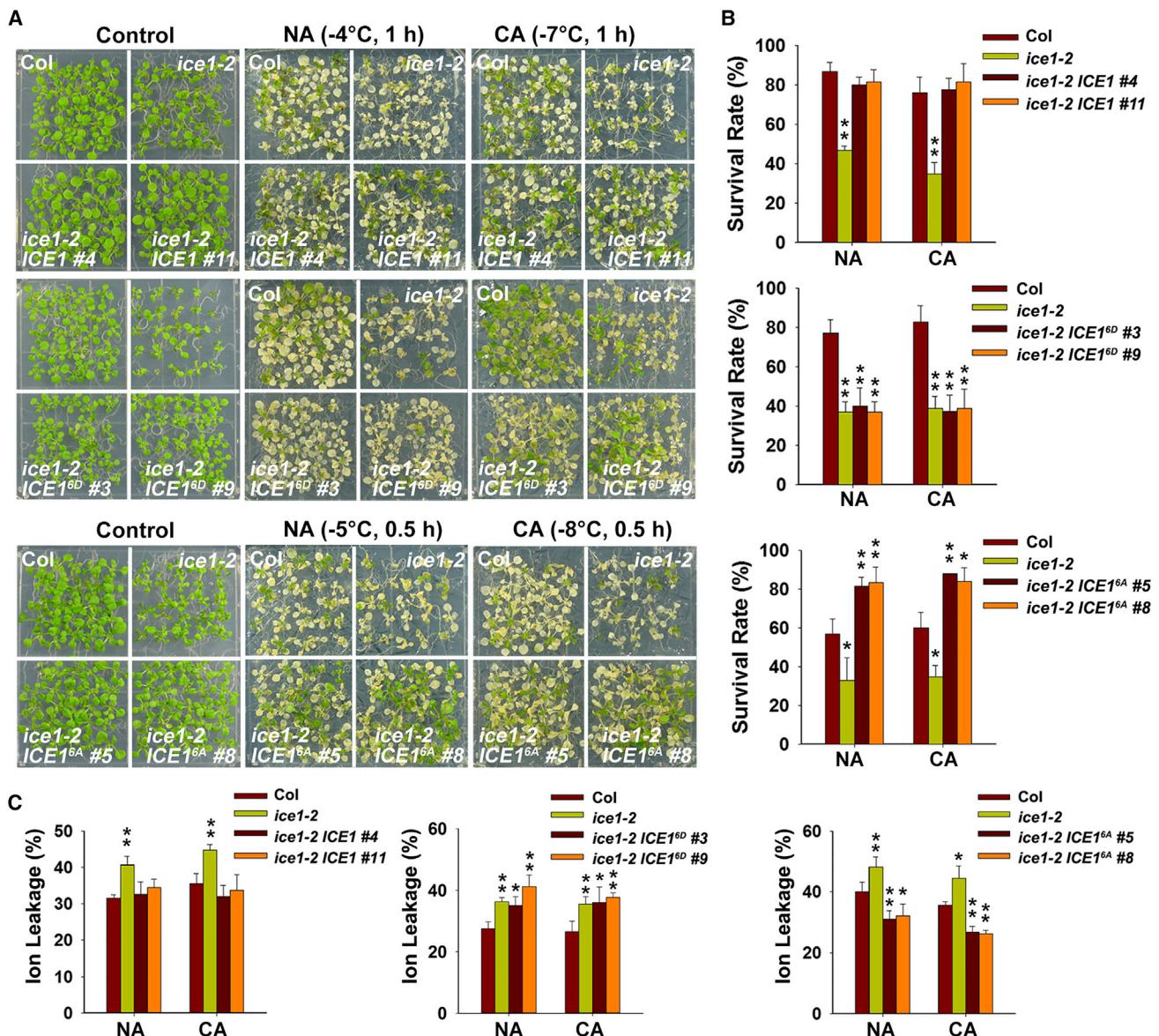
(E) ICE1 phosphorylation under cold stress *in vivo*. 35S:HF-ICE1 transgenic plants in wild-type (Col) or MPK3SR (line no. 64) backgrounds were treated at 4°C for 0, 3, and 6 hr. Protein extracts from the transgenic plants were separated in a Phos-tag gel, and HF-ICE1 was then detected with anti-HA antibody (upper panel). Total HF-ICE1 proteins in the wild type (Col) and MPK3SR (line no. 64) were determined by immunoblotting with anti-HA antibody as controls (lower panel).

unphosphorylated and phosphorylated ICE1 protein bands were detected. Under cold treatment, the lower ICE1 bands were shifted to higher molecular weight, which was nearly abolished by the addition of calf intestinal alkaline phosphatase(CIAP) (Figure 4E). This observed upshift of ICE1 bands was suppressed in the MPK3SR mutant with or without cold treatment (Figure 4E). These combined results suggest that MPK3/MPK6 modulate ICE1 phosphorylation under cold stress. Given that cold-activated OST1 phosphorylates ICE1 (Ding et al., 2015), the phos-

phorylated bands observed in the MPK3SR mutant should be at least partially mediated by OST1 under cold stress. Moreover, other unknown protein kinase(s) may be involved in the phosphorylation of ICE1 under warm temperatures.

#### MPK3/MPK6-Mediated ICE1 Phosphorylation Compromises ICE1 Function in Freezing Tolerance

To determine the biological significance of MPK3/MPK6-mediated ICE1 phosphorylation, we generated transgenic plants



**Figure 5. MPK3/MPK6-Mediated ICE1 Phosphorylation Attenuates ICE1 Function in the Cold-Signaling Pathway**

(A) Freezing phenotypes of *ICE1:ICE1-GFP*, *ICE1:ICE1<sup>6D</sup>-GFP*, and *ICE1:ICE1<sup>6A</sup>-GFP* transgenic plants. Wild-type, *ice1-2*, and transgenic plants were grown on half-strength MS plates at 22°C for 14 days, and then treated at -4°C for 1 hr or -5°C for 0.5 hr (for NA plants), or at -7°C for 1 hr or -8°C for 0.5 hr (for CA plants).

(B) Survival rates of *ICE1:ICE1-GFP*, *ICE1:ICE1<sup>6D</sup>-GFP*, and *ICE1:ICE1<sup>6A</sup>-GFP* transgenic plants described in (A).

(C) Ion leakage assays of *ICE1:ICE1-GFP*, *ICE1:ICE1<sup>6D</sup>-GFP*, and *ICE1:ICE1<sup>6A</sup>-GFP* transgenic plants described in (A).

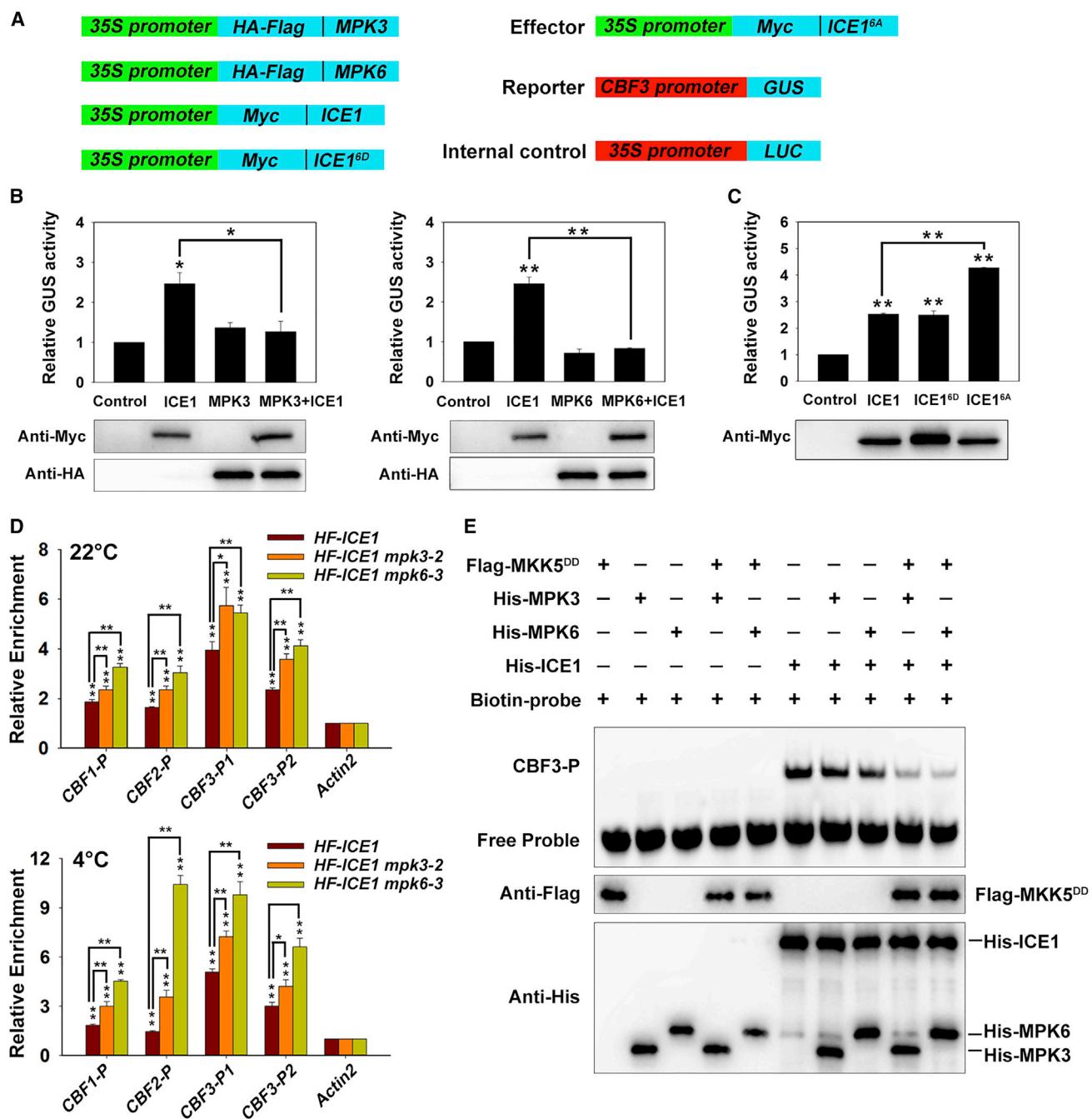
In (B) and (C), data are means of three technical replicates  $\pm$  SD. \*p < 0.05, \*\*p < 0.01 (t test) indicate significant differences between mutants and wild-type plants. Similar results were obtained from three independent experiments.

See also Figure S4.

expressing wild-type and mutated ICE1 tagged with GFP under the control of its native promoter, *ICE1:ICE1-GFP*, *ICE1:ICE1<sup>6A</sup>-GFP* (phosphor-inactive mutant), and *ICE1:ICE1<sup>6D</sup>-GFP* (phosphor-mimic mutant) in the *ice1-2* mutant background. Two independent lines with similar *ICE1* expression levels for each genotype were selected for further analysis (Figure S4A). The reduced freezing tolerance of *ice1-2* was fully rescued by *ICE1-GFP* and *ICE1<sup>6A</sup>-GFP*, but was not rescued by *ICE1<sup>6D</sup>-GFP* (Figures 5A and 5B). Consistently, *ICE1-GFP* and *ICE1<sup>6A</sup>-GFP*

fully complemented the increased ion leakage of *ice1-2*, but *ICE1<sup>6D</sup>-GFP* failed to do so (Figure 5C). These results suggest that MPK3/MPK6-mediated ICE1 phosphorylation attenuates ICE1 function in cold signaling.

Next, we examined whether MPK3/MPK6-mediated ICE1 phosphorylation affects the interaction between MPK3/MPK6 and ICE1. The results of a yeast two-hybrid assay indicated that both *ICE1<sup>6D</sup>* and *ICE1<sup>6A</sup>* interact with MPK6 in yeast (Figure S4B). Consistently, *ICE1<sup>6A</sup>* also interacted with MPK6 in a



**Figure 6. MPK3/MPK6-Mediated ICE1 Phosphorylation Suppresses ICE1 Transcriptional Activity**

(A) Schematics of all constructs used for GUS transactivation assays in *N. benthamiana* leaves. The CBF3 promoter was fused to the GUS reporter gene. The 35S promoter was fused to the LUC gene as an internal control. The effector constructs were 35S:HF-MPK3, 35S:HF-MPK6, 35S:MYC-ICE1, 35S:MYC-ICE1<sup>6D</sup>, and 35S:MYC-ICE1<sup>6A</sup>.

(B) ICE1-mediated activation of CBF3 expression was attenuated by MPK3 and MPK6 activity. CBF3:GUS was co-transformed with other constructs into *N. benthamiana* leaves, and leaves transfected with CBF3:GUS were used as a control. All transfections included the internal control (35S:LUC). After transfection, the *N. benthamiana* leaves were incubated at 22°C for 36 hr. Relative GUS activity (GUS/LUC) indicates the CBF3 expression level. ICE1 binding activity (top). Myc-ICE1 was detected with anti-Myc antibody (middle). HF-MPK3 or HF-MPK6 was detected with anti-HA antibody (bottom).

(C) The effect of phosphorylation on ICE1 transcriptional activity. Relative GUS activity (GUS/LUC) indicates the CBF3 expression level. ICE1 binding activity (top). ICE1 proteins were detected with anti-Myc antibody (bottom).

(D) ChIP analysis of ICE1 binding to the promoters of CBF genes. 35S:HF-ICE1 mpk3-2 and 35S:HF-ICE1 mpk6-3 plants (14 days old) were treated at 4°C. Chromatin was purified from the transgenic seedlings and immunoprecipitated with anti-HA antibody or without antibody. The amounts of indicated DNA in the immune complex were determined by qRT-PCR. An Actin2/8 fragment was amplified as a control. Relative enrichment was calculated as Input% of indicated DNA/Input% of control.

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coIP assay in *N. benthamiana* leaves expressing 35S:HF-MPK6 and 35S:Myc-ICE1<sup>6A</sup> (Figure S4C). These results suggest that MPK3/MPK6-mediated ICE1 phosphorylation does not affect the interaction of ICE1 with MPK3/MPK6.

### MPK3 and MPK6 Attenuate ICE1 Transcriptional Activity

To explore how MPK3/MPK6 affect ICE1 function and regulate *CBF* expression, we performed transient transactivation assays using the *CBF3* promoter fused to *GUS* (*CBF3:GUS*) (Ding et al., 2015) (Figure 6A). ICE1 and MPK3/MPK6 effector constructs were expressed under control of the 35S promoter, and were co-transfected with the *CBF3:GUS* reporter construct and the internal control (35S:LUC) into *N. benthamiana* leaves. Consistent with a previous study, ICE1 activated *CBF3* expression (Ding et al., 2015), whereas co-expression of MPK3/MPK6 obviously reduced ICE1-activated *CBF3* expression (Figure 6B). ICE1<sup>6A</sup> and wild-type ICE1 displayed similar transcriptional activities, whereas ICE1<sup>6A</sup> displayed enhanced transcriptional activity (Figure 6C). These results suggest that phosphorylation negatively regulates ICE1 transcriptional activity.

Next, we performed chromatin immunoprecipitation (ChIP) analyses to determine the effect of MPK3/MPK6 on ICE1 binding to *CBF* promoters. Transgenic plants expressing 35S:HF-ICE1 (Ding et al., 2015) were crossed with *mpk3-2* and *mpk6-3* mutants to generate HF-ICE1 *mpk3-2* and HF-ICE1 *mpk6-3* plants. ChIP assays showed that HF-ICE1 did bind to the promoters of *CBF* genes, and this binding was higher in the *mpk3-2* and *mpk6-3* mutant backgrounds before or after cold treatment (Figure 6D). Then, we performed electrophoresis mobility shift assays to analyze the effect of MPK3/MPK6 on ICE1 binding to *CBF* promoters *in vitro*. Recombinant His-ICE1 did bind to the *CBF3* promoter, which is consistent with the results of previous studies (Chinnusamy et al., 2003; Ding et al., 2015) (Figures 6E and S5). By contrast, MPK3/MPK6-mediated ICE1 phosphorylation clearly reduced its binding to the *CBF3* promoter (Figure 6E). These combined results suggest that MPK3 and MPK6 regulate cold signaling by affecting the transcriptional activity of ICE1.

### MPK3 and MPK6 Facilitate ICE1 Degradation under Cold Treatment

ICE1 is degraded under cold treatment (Dong et al., 2006). Therefore, we evaluated whether ICE1 stability was affected by MPK3/MPK6 *in vivo*. ICE1 protein levels were almost unchanged in HF-ICE1 *mpk3-2* and significantly increased in HF-ICE1 *mpk6-3* and HF-ICE1 MPK6SR before cold treatment (Figure 7A). Consistent with previous results (Ding et al., 2015; Dong et al., 2006), ICE1 protein was degraded in HF-ICE1 transgenic plants under cold treatment (Figure 7A). By contrast, ICE1 degradation was significantly reduced in HF-ICE1 *mpk3-2*, HF-ICE1 *mpk6-3*,

and HF-ICE1 MPK6SR transgenic plants after cold treatment (Figure 7A).

Next, we examined ICE1 degradation in *mpk3-2* and *mpk6-3* mutants using a cell-free protein degradation assay. Recombinant His-ICE1 protein was incubated with total protein extracts (equal concentration) from *mpk3-2* and *mpk6-3*, the wild-type Col plants. Then we monitored the amount of His-ICE1 remaining in the reactions by immunoblotting after the addition of ATP. The results showed that increasing incubation time with ATP gradually increased His-ICE1 degradation in wild-type plants; by contrast, His-ICE1 degradation occurred more slowly in *mpk3-2* and *mpk6-3* plants (Figure 7B). These results suggest that MPK3 and MPK6 mediate ICE1 degradation.

We examined whether MPK3 and MPK6 promote ICE1 degradation by phosphorylating ICE1. We measured the degradation of inactive ICE1<sup>6A</sup> using *ice1-2* 35S:HF-ICE1 (*ice1-2 HF-ICE1*) and *ice1-2 HF-ICE1<sup>6A</sup>* transgenic plants, which showed comparable transcript and protein expression levels of their respective transgenes (Figure S6). In the presence of the protein synthesis inhibitor cycloheximide, the level of wild-type HF-ICE1 protein was greatly reduced in *ice1-2 HF-ICE1* transgenic plants, but was only moderately changed in *ice1-2 HF-ICE1<sup>6A</sup>* transgenic plants (Figure 7C). These results indicate that MPK3/MPK6-mediated ICE1 phosphorylation affects ICE1 protein stability (Zhao et al., 2017 [this issue of *Developmental Cell*]).

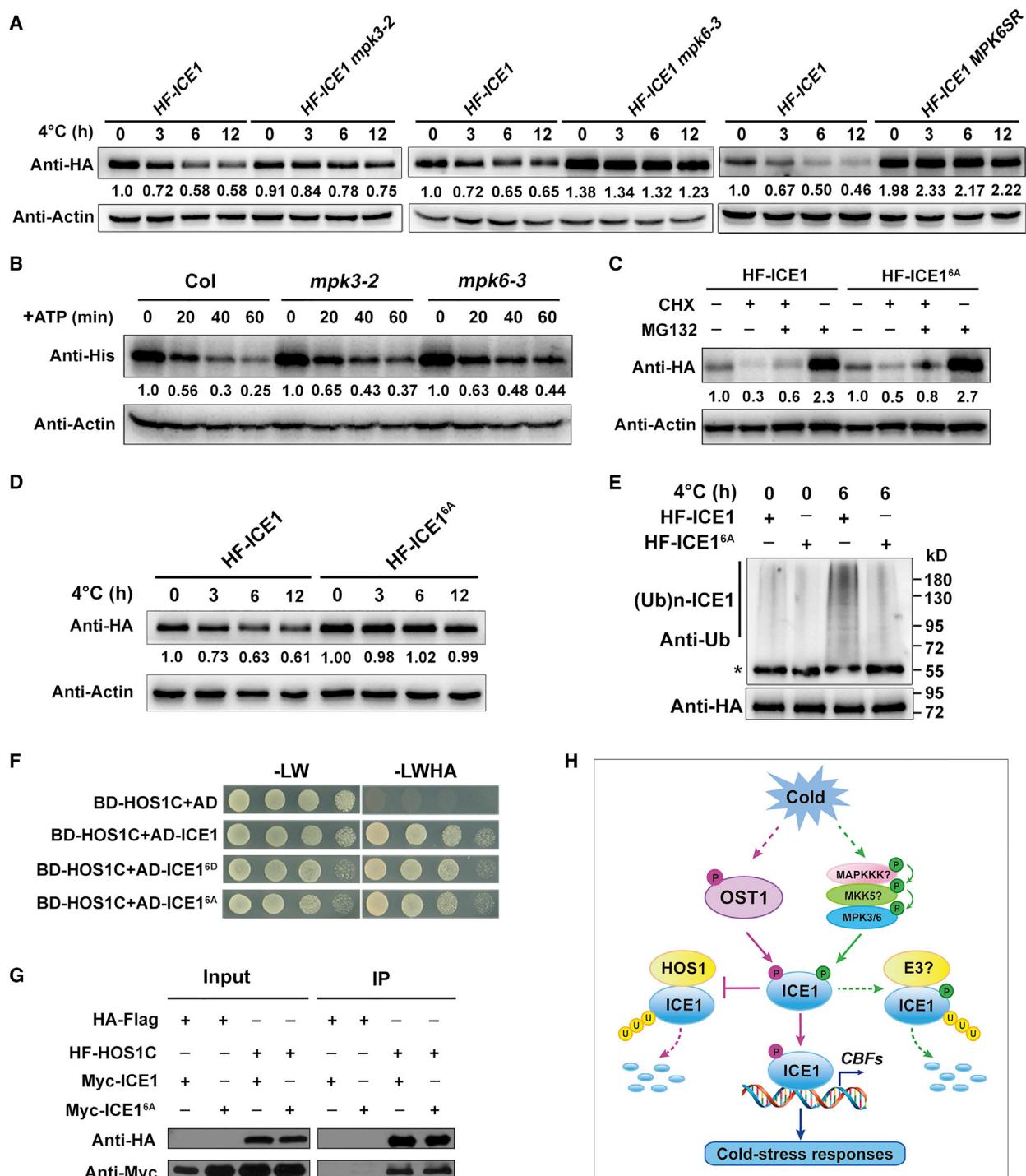
We determined the effect of MPK3/MPK6-mediated ICE1 phosphorylation on ICE1 stability under cold stress using *ice1-2 HF-ICE1* and *ice1-2 HF-ICE1<sup>6A</sup>* transgenic plants. The wild-type HF-ICE1 protein was substantially reduced after cold treatment compared with the levels before treatment, whereas the HF-ICE1<sup>6A</sup> protein levels were stably maintained after cold treatment (Figure 7D). Next, we examined the ubiquitination of ICE1 and ICE1<sup>6A</sup> in *ice1-2 HF-ICE1* and *ice1-2 HF-ICE1<sup>6A</sup>* transgenic plants untreated and treated at 4°C for 6 hr. After immunoprecipitation with anti-HA antibody, more high-molecular-weight polypeptide bands corresponding to polyubiquitinated forms of HF-ICE1 were detected in *ice1-2 HF-ICE1* transgenic plants after cold treatment than those before cold treatment using an anti-ubiquitin antibody, which is consistent with previous studies (Ding et al., 2015; Dong et al., 2006). By contrast, ubiquitination of HF-ICE1<sup>6A</sup> was significantly attenuated in *ice1-2 HF-ICE1<sup>6A</sup>* transgenic plants after cold treatment compared with that in *ice1-2 HF-ICE1* plants (Figure 7E). These combined results suggest that MPK3/MPK6-mediated ICE1 phosphorylation facilitates ICE1 ubiquitination, which enhances ICE1 proteolysis under cold stress.

A previous study reported that ICE1 interacts with HOS1 (Dong et al., 2006). Next, we tested whether MPK3/MPK6-mediated ICE1 phosphorylation affects the interaction between HOS1

(E) Electrophoresis mobility shift assay showing the ICE1 binding capacity. Reactions lacking the specified components (—) were used as controls. Recombinant Flag-MKK5<sup>DD</sup> protein was used to activate MPK3 and MPK6. His-ICE1 was incubated with activated MPK3 and MPK6 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. ICE1 binding capacity (top). Flag-MKK5<sup>DD</sup> was detected with anti-Flag antibody (middle). His-MPK3 and His-MPK6 were detected with anti-His antibody (bottom).

In (B)–(D), data are the mean values of three technical replicates ± SD. \*p < 0.05, \*\*p < 0.01 (t test) indicate significant differences between mutants and wild-type plants. At least three independent experiments were performed with similar results.

See also Figure S5.



**Figure 7. MPK3 and MPK6 Promote ICE1 Degradation under Cold Stress**

(A) ICE1 stability was reduced by MPK3 and MPK6 activation under cold stress. 35S:HF-ICE1, 35S:HF-ICE1 mpk3-2, and 35S:HF-ICE1 mpk6-3 plants (14 days old) were treated at 4°C for 0, 3, 6, and 12 hr. Five-day-old seedlings of 35S:HF-ICE1 and 35S:HF-ICE1 MPK6SR plants were grown on half-strength MS plates, transferred to half-strength MS plates containing 0.5 μM NA-PP1 inhibitor, and held for an additional 9 days, and then treated at 4°C for 0, 3, 6, and 12 hr. ICE1 was detected with anti-HA antibody. Actin was used as a control.

(B) MPK3 and MPK6 promote ICE1 degradation in a cell-free degradation assay. Equal amounts of total proteins extracted from 14-day-old wild-type (Col), mpk3-2, and mpk6-3 plants were incubated with recombinant His-ICE1 protein in the presence of ATP. ICE1 was detected with anti-His antibody. Actin was used as a control.

(C) Stability of ICE1 and ICE1<sup>6A</sup> proteins *in vivo*. The 14-day-old *ice1-2* 35S:HF-ICE1 and *ice1-2* 35S:HF-ICE1<sup>6A</sup> plants were treated with 100 μM cycloheximide (CHX), 50 μM MG132, or 100 μM CHX for 1 hr. ICE1 was detected with anti-HA antibody. Actin was used as a control.

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with ICE1. ICE1<sup>6D</sup> and ICE1<sup>6A</sup> interacted with the C terminus of HOS1 (HOS1C) in yeast (Figure 7F). The interaction of ICE1<sup>6A</sup> with HOS1C also was detected in a coIP assay in *N. benthamiana* leaves expressing 35S:HF-HOS1C and 35S:Myc-ICE1<sup>6A</sup> (Figure 7G). These results suggest that MPK3/MPK6-mediated ICE1 phosphorylation does not affect the HOS1-ICE1 interaction, and that the degradation of phosphorylated ICE1 might be executed by presently unidentified E3 ligases.

## DISCUSSION

MPK3 and MPK6 function in several cellular signaling pathways by phosphorylating downstream partner proteins in response to biotic and abiotic stresses (Rodriguez et al., 2010). Here, we show that MPK3 and MPK6 negatively regulate the ICE1-CBF-COR cascade in response to cold stress in plants (Figure 7H). Several lines of biochemical and genetic evidence strongly support our hypothesis. (1) ICE1 protein is phosphorylated by MPK3 and MPK6. (2) ICE1 phosphorylation by MPK3/MPK6 inhibits its transcriptional activity and promotes its degradation under cold stress. (3) The *mpk3*, *mpk6*, and *mpk3 mpk6* mutants exhibit enhanced freezing tolerance and increased induction of *CBF* expression in response to cold. Conversely, MPK3/MPK6 activation attenuates plant freezing tolerance and induction of *CBF* expression in response to cold. (4) The *ice1-2* mutant is epistatic to the *mpk3* and *mpk6* mutants in freezing tolerance.

It has been suggested that the *Arabidopsis* MAPK pathway mediated by MEKK1-MKK2-MPK4/6 is involved in plant responses to cold stress (Furuya et al., 2013; Ichimura et al., 2000; Rodriguez et al., 2010; Teige et al., 2004; Yang et al., 2010b). The *mkk2* mutant displays enhanced sensitivity to freezing, and MKK2 activity is enhanced by cold stress to upregulate MPK4 and MPK6 activity (Teige et al., 2004). A recent study reported that MPK6 phosphorylates MYB15, thereby regulating freezing tolerance (Kim et al., 2017).

Despite the progress in this field, the exact functions of MPK3 and MPK6 in cold-stress responses were not clear. Here, we showed that *mpk3*, *mpk6*, and *mpk3 mpk6* mutants exhibit enhanced freezing tolerance. MPK3 and MPK6 interact and phosphorylate ICE1. Phosphorylated ICE1 is unstable and is rapidly degraded, in a manner that resembles the phosphorylation-mediated degradation of other key regulators such as PIF4, NPR1 (nonexpressor of *PR* genes 1), BZR1, and MYC2 (Bernardo-Garcia et al., 2014; He et al., 2002; Spoel et al., 2009; Zhai et al., 2013). Therefore, MPK3 and MPK6 phosphorylation destabilizes ICE1, thereby inhibiting *CBF* expression and negatively regulating freezing tolerance.

Previous studies reported that MPK3, MPK6, and their upstream MAPKs (MKK4 and MKK5) are key regulators of stomatal development (Gudesblat et al., 2007; Lampard et al., 2009; Wang et al., 2007). Loss-of-function *MPK3/MPK6* mutations result in stomatal clustering (Gudesblat et al., 2007; Wang et al., 2007). ICE1 and its closely related paralog ICE2 drive three key transitional steps in stomatal differentiation (Kanaoka et al., 2008), and the *ice1 ice2* double mutant develops an epidermis that lacks stomata (Kanaoka et al., 2008). In the present study, we also found that *MPK3/MPK6* is upstream of *ICE1* in regulating stomatal differentiation (Figure S7A). Phosphor-inactive and mimic analyses indicate that ICE1 activity in stomatal differentiation is enhanced when ICE1 is mutated to ICE1<sup>6A</sup> (Figures S7B–S7D). These results suggest that MPK3/MPK6-mediated ICE1 phosphorylation has dual roles in regulating freezing tolerance and stomatal differentiation, which might be an effective strategy to integrate environmental cues and developmental processes in plants.

The RING-type ubiquitin E3 ligase HOS1 polyubiquitinates ICE1, which promotes its degradation under cold stress (Dong et al., 2006). In the present study, we found that MPK3/MPK6-mediated ICE1 phosphorylation does not affect the HOS1-ICE1 interaction. However, the phosphor-inactive ICE1 mutation inhibits polyubiquitination and degradation of ICE1 under cold stress. Consistent with our results, a previous study indicated that the ICE1<sup>S403A</sup> substitution inhibited ICE1 polyubiquitination, but this was not the principal ubiquitination site for HOS1 (Miura et al., 2011). Given that Ser403 is one of the sites of MPK3/MPK6-mediated ICE1 phosphorylation, we speculate that other ubiquitin E3 ligases may be involved in mediating ICE1 degradation under cold stress.

OST1-mediated ICE1 phosphorylation at Ser278 enhances ICE1 stability and positively regulates *CBF* expression and freezing tolerance (Ding et al., 2015). Nevertheless, MPK3/MPK6 promotes the ICE1 degradation by phosphorylating ICE1 at completely different phosphorylation sites, thereby reducing *CBF* expression and freezing tolerance. Therefore, multiple residues of ICE1 are phosphorylated by different kinases (OST1 and MPK3/MPK6), both of which are activated by low temperatures, thereby conferring opposing effects on ICE1 stability (Figure 7H). Crucial factors such as NPR1 and MYC2 are phosphorylated at different residues, which differentially affects their function (Saleh et al., 2015; Sethi et al., 2014; Zhai et al., 2013). Although we have not yet clarified the detailed dynamic changes induced by OST1- and MPK3/MPK6-mediated ICE1 phosphorylation, the available evidence suggests the following model: OST1 is activated during the early stages of

(D) Stability of ICE1 and ICE1<sup>6A</sup> under cold stress. The 14-day-old *ice1-2* 35S:HF-ICE1 and *ice1-2* 35S:HF-ICE1<sup>6A</sup> plants were treated at 4°C for 0, 3, 6, and 12 hr. ICE1 was detected with anti-HA antibody. Actin was used as a control.

(E) Polyubiquitination of ICE1 and ICE1<sup>6A</sup> *in vivo* under cold stress. The 14-day-old *ice1-2* 35S:HF-ICE1 and *ice1-2* 35S:HF-ICE1<sup>6A</sup> plants were treated at 4°C for 0 and 6 hr. Total protein extract was immunoprecipitated with anti-HA agarose beads, and precipitated proteins were subjected to immunoblot analysis with anti-ubiquitin antibody (upper panel). Immunoblot analysis of the total protein extract using anti-HA antibody as a loading control (lower panel). The star represents non-specific bands derived from anti-HA agarose beads.

(F) Interaction of HOS1 with ICE1, ICE1<sup>6D</sup> and ICE1<sup>6A</sup> in yeast.

(G) Interaction of HOS1 with ICE1, and ICE1<sup>6A</sup> in *N. benthamiana* leaves.

(H) Model of the role of MPK3/MPK6 in plant responses to cold stress.

See also Figure S6.

cold stress and phosphorylates ICE1 to inhibit HOS1-mediated degradation, which results in the cold induction of *CBF* genes. When ICE1 levels subsequently accumulate, ICE1 is phosphorylated by MPK3/MPK6 and consequently degraded through the 26S proteasome pathway, which inhibits CBF-dependent cold signaling. Consistent with this model, previous studies showed that, although ICE1 is a positive regulator of cold signaling, ICE1 is degraded in response to long-term cold treatment (Ding et al., 2015; Dong et al., 2006). The regulation of ICE1 stability by two kinases may fine-tune the turnover of ICE1 and the duration of its transcriptional activity in response to cold stress. Future studies will elucidate how multiple phosphorylation pathways are coordinated to regulate ICE1 function under cold stress in plants.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <https://doi.org/10.1016/j.devcel.2017.09.025>.

## AUTHOR CONTRIBUTIONS

S.Y. directed the project. H.L. and S.Y. designed the experiments. H.L., Y.D., and Y.S. performed the experiments. All authors discussed and interpreted the results. H.L. and Y.S. wrote the manuscript.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Mouse monoclonal anti-Myc	Sigma-Aldrich	Cat#M4439; RRID: AB_439694
Mouse monoclonal anti-HA	Sigma-Aldrich	Cat#H3663; RRID: AB_262051
Mouse monoclonal anti-GST	Beijing Protein Innovation	Cat#AbM59001-2H5-PU
Mouse monoclonal anti-His	Beijing Protein Innovation	Cat#AbM59012-18-PU
Rabbit polyclonal anti-Actin	Easybio	Cat#BE0027; RRID: AB_1107572
Rabbit polyclonal anti-MPK3	Sigma-Aldrich	Cat#M8318; RRID: AB_477247
Rabbit polyclonal anti-MPK6	Sigma-Aldrich	Cat#A7104; RRID: AB_476760
Mouse monoclonal anti-Flag	Sigma-Aldrich	Cat#F3165; RRID: AB_259529
Mouse monoclonal anti-Ubiquitin	Santa Cruz Biotechnology	Cat#SC-8017; RRID: AB_628423
<b>Bacterial and Virus Strains</b>		
BL21(DE3)pLysS	TIANGEN	Cat#CB106-02
AH109	Towfly	Cat#6453471VEC
BL21-CodonPlus (DE3)-RIPL	WEIDI	Cat#EC1007
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Anti-c-HA agarose beads (affinity gel)	Sigma-Aldrich	Cat#A7470; RRID: AB_10109522
Glutathione Sephadex 4 Fast Flow	GE Healthcare	Cat#17-5132-02
Ni Sepharose 6 Fast Flow	GE Healthcare	Cat#17-5318-01
MG132	Sigma-Aldrich	Cat#C2211
Anti-Flag M2 Magnetic Beads	Sigma-Aldrich	Cat#M8823; RRID: AB_2637089
Cocktail	Roche	Cat#04693132001
Flag peptides	Beijing Protein Innovation	N/A
Phos-tag Acrylamide AAL-107	Nard Institute, Ltd	Cat#AAL-107
Myelin Basic Protein	Life Technologies	Cat#13228-010
<b>Critical Commercial Assays</b>		
Cycloheximide	Sigma-Aldrich	Cat#R750107
NA-PP1	Toronto Research Chemicals	Cat#A603004
Dexamethasone	Sigma-Aldrich	Cat#D1756
ATP	Sigma-Aldrich	Cat#A6559
4-Methylumbelliferyl-β-D-glucuronide hydrate	Sigma-Aldrich	Cat#M9130
Luciferase Assay Reagent	Promega	Cat#E1483
<b>Experimental Models: Organisms/Strains</b>		
<i>Arabidopsis: mpk3-1</i> (SALK_151594)	Zhao et al., 2014	N/A
<i>Arabidopsis: mpk3-2</i> (SALK_100651)	Xu et al., 2008	N/A
<i>Arabidopsis: mpk6-3</i> (SALK_127507)	Xu et al., 2008	N/A
<i>Arabidopsis: mpk6-4</i> (SALK_062471)	Xu et al., 2008	N/A
<i>Arabidopsis: MPK3SR #28</i>	Xu et al., 2014; Su et al., 2017	N/A
<i>Arabidopsis: MPK3SR #64</i>	Xu et al., 2014; Su et al., 2017	N/A
<i>Arabidopsis: MPK6SR #58</i>	Xu et al., 2014; Su et al., 2017	N/A
<i>Arabidopsis: ice1-2</i> (SALK_003155)	Ding et al., 2015	N/A
<i>Arabidopsis: mpk3-2 ice1-2</i>	this paper	N/A
<i>Arabidopsis: mpk6-3 ice1-2</i>	this paper	N/A
<i>Arabidopsis: 35S:HA-Flag-ICE1 MPK3SR #64</i>	this paper	N/A
<i>Arabidopsis: 35S:HA-Flag-ICE1 MPK6SR #58</i>	this paper	N/A
<i>Arabidopsis: 35S:HA-Flag-ICE1</i>	Ding et al., 2015	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Arabidopsis</i> : <i>ice1-2 ICE1:ICE1-GFP</i>	this paper	N/A
<i>Arabidopsis</i> : <i>ice1-2 ICE1:ICE1<sup>6D</sup>-GFP</i>	this paper	N/A
<i>Arabidopsis</i> : <i>ice1-2 ICE1:ICE1<sup>6A</sup>-GFP</i>	this paper	N/A
<i>Arabidopsis</i> : <i>35S:HA-Flag-ICE1 mpk3-2</i>	this paper	N/A
<i>Arabidopsis</i> : <i>35S:HA-Flag-ICE1 mpk6-3</i>	this paper	N/A
<i>Arabidopsis</i> : <i>Flag-MKK5<sup>DD</sup></i>	<a href="#">Ren et al., 2002</a>	N/A
<i>Arabidopsis</i> : <i>35S:HA-Flag-ICE1<sup>6A</sup></i>	this paper	N/A
<i>Arabidopsis</i> : <i>ice1-2 35S:HA-Flag-ICE1</i>	this paper	N/A
<i>Arabidopsis</i> : <i>ice1-2 35S:HA-Flag-ICE1<sup>6A</sup></i>	this paper	N/A
Oligonucleotides		
yeast two-hybrid primers <i>EcoR I-MPK3-F</i> : CGGAATTCTATGAAACACCGGGCGGTG	Beijing Genomics Institute	Custom order
yeast two-hybrid primers <i>BamHI-MPK3-R</i> : CGGGATCCCTAACCGTATGTTGGA	Beijing Genomics Institute	Custom order
other primers see <a href="#">Table S1</a>	Beijing Genomics Institute	Custom order
Recombinant DNA		
<i>GST-MPK3</i>	this paper	N/A
<i>GST-MPK6</i>	this paper	N/A
<i>HA-Flag-MPK3</i>	this paper	N/A
<i>HA-Flag-MPK6</i>	this paper	N/A
<i>His-ICE1</i>	this paper	N/A
<i>Myc-ICE1</i>	<a href="#">Hu et al., 2013</a>	N/A
<i>Myc-ICE1<sup>6A</sup></i>	this paper	N/A
<i>Flag-MKK5<sup>DD</sup></i>	<a href="#">Xu et al., 2008</a>	N/A
<i>His-MPK3</i>	<a href="#">Xu et al., 2008</a>	N/A
<i>His-MPK6</i>	<a href="#">Xu et al., 2008</a>	N/A
<i>ICE1-GFP</i>	this paper	N/A
<i>ICE1<sup>6D</sup>-GFP</i>	this paper	N/A
<i>ICE1<sup>6A</sup>-GFP</i>	this paper	N/A
<i>HA-Flag-ICE1</i>	this paper	N/A
<i>HA-Flag-ICE1<sup>6A</sup></i>	this paper	N/A
<i>HA-Flag-HOS1C</i>	<a href="#">Ding et al., 2015</a>	N/A
Software and Algorithms		
Image J	National Institutes of Health	1.48u

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Shuhua Yang ([yangshuhua@cau.edu.cn](mailto:yangshuhua@cau.edu.cn)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

*Arabidopsis thaliana* accession Col-0 was used in this study. Plants were grown on ½ Murashige-Skoog (MS) medium (Phytotechlab) containing 0.8% agar at 22°C under a 16-h light/8-h dark photoperiod.

**METHOD DETAILS**

**Plasmid Construction and Plant Transformation**

To generate the native promoter-driven *ICE1-GFP* (*ICE1:ICE1-GFP*) construct, *ICE1* genomic sequence containing 1.5-kb native promoter was PCR amplified and cloned into pBluescript SK+ with *Pst* I and *Bam*H I. The full-length CDS of *ICE1* was amplified from *Arabidopsis* cDNA and clone into pBluescript SK+ vector with *Bam*H I and *Kpn* I to generate pBlue-*ICE1*. The *Pst* I/*Bam*H I fragment

of *ICE1* promoter and *BamH I/Kpn I* coding sequence (CDS) fragment of *ICE1* were cloned into *BamH I/Kpn I* site of pCAMBIA1300-GFP. The phosphor-inactive *ICE1* (*ICE1<sup>6A</sup>*) and phosphor-mimic *ICE1* mutants (*ICE1<sup>6D</sup>*) forms were generated by site-directed mutagenesis PCR using pBlue-*ICE1* as template, and cloned into pCAMBIA1300-GFP.

The vectors were transformed into *ice1-2* mutant by *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998), and the transformants were identified by screening hygromycin resistance. Independent lines with expression of tagged *ICE1* were identified based on qRT-PCR and/or immunoblotting analyses, and homozygous transgenic plants were used in this study.

To generate the 35S:HF-*ICE1* and 35S:HF-*ICE1<sup>6A</sup>* constructs, the CDS fragments of wild-type *ICE1* and *ICE1<sup>6A</sup>* were amplified and cloned into the pCM1307 vector. To generate the His-*ICE1* and His-*ICE1<sup>6A</sup>* constructs, the CDS fragments of wild-type *ICE1* and *ICE1<sup>6A</sup>* were amplified and cloned into the pET32a vector.

To generate the 35S:Myc-*ICE1*, 35S:Myc-*ICE1<sup>6D</sup>* and 35S:Myc-*ICE1<sup>6A</sup>* constructs, the CDS fragments of wild-type *ICE1*, *ICE1<sup>6D</sup>* and *ICE1<sup>6A</sup>* were amplified and cloned into the pFGC-YFP<sup>C</sup> vector.

To generate the tagged MPK3 and MPK6 constructs, the CDS fragments of *MPK3* and *MPK6* were amplified by PCR and fused with HA-Flag or GST in pCM1307 and pGEX4T-1 vectors to generate the 35S:HF-*MPK3*, 35S:HF-*MPK6*, GST-*MPK3* and GST-*MPK6* constructs. All constructs were confirmed by DNA sequencing, and primers used for different constructs are listed in Table S1.

### Physiological Analyses

The freezing tolerance and ion leakage assays were performed as described previously (Shi et al., 2012). For the freezing tolerance assays, *Arabidopsis* seedlings were grown on ½ MS medium containing 0.8% agar at 22°C for two weeks and subjected to freezing treatment. For the NA treatment, freezing began at 0°C, the temperature dropped by 1°C per hour until reaching the desired temperature, and then the plants were held at this temperature for 0.5 h or 1 h. For the CA plants (pretreated at 4°C for 3 d), freezing began at 0°C, the temperature dropped by 1°C per hour until reaching the desired temperature, and then the plants were held at this temperature for 0.5 h or 1 h. After treatment, the plants were incubated at 4°C under dark conditions for 12 h and then shifted to light at 22°C. Representative images were taken after recovery for 3 days, and the survival rates and ion leakage were measured.

For ion leakage assays, the above freezing-treated seedlings were placed in 15 mL tubes containing 5 mL deionized water (S0), shaken for 15 min and detected S1. The samples were boiled at 100°C water for 15 min, shaken at 22°C for 1 h and then detected S2. The value (S1-S0)/(S2-S0) was calculated as ion leakage.

For NA-PP1 (TRC) experiments, 5-d-old seedlings grown on ½ MS medium were transferred to plates containing 0.5 μM NA-PP1 and grown for 9 d, followed by freezing treatment. For DEX (Sigma-Aldrich) experiments, 13-d-old seedlings grown on ½ MS medium were treated with 0.5 μM DEX for 24 h, followed by freezing treatment.

### RNA Extraction and qPCR Analysis

Total RNAs were extracted from 14-d-old *Arabidopsis* seedlings grown on ½ MS medium using TRIzol reagent (Invitrogen). After DNase (Takara) treatment, total RNA was used for reverse transcription. qPCR analyses were performed on a 7500 real time PCR system (Life Technologies) using a SYBR Green reagent (Takara) with gene-specific primers (Table S1). The relative expression levels were calculated as described previously (Shi et al., 2012).

### Protein Extraction and Immunoblot Analysis

Total proteins were extracted from 2-week-old seedlings overexpressing *ICE1* or *ICE1<sup>6A</sup>* with or without 4°C treatment with protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150mM NaCl, 5 mM DTT, 1% Triton X-100, 2% NP40 and 1 × protease inhibitor cocktail), and separated by SDS-PAGE. ICE1 protein was detected with anti-HA antibody (Sigma-Aldrich).

To detect ubiquitinated ICE1, total proteins were extracted from 2-week-old seedlings overexpressing ICE1 or ICE1<sup>6A</sup> at 4°C treatment for 24 h, then immunoprecipitated using anti-HA agarose beads (Sigma-Aldrich) for 2 h at 4°C. The immunoprecipitated proteins were detected by immunoblotting with anti-ubiquitin antibody (Sigma-Aldrich).

### Protein Interaction Assays

#### Yeast Two-Hybrid Assay

To screen the cDNA library, the C-terminal coding fragment of *ICE1* (from 358-494 bp) was cloned into the pGKKT7 bait vector and then transformed into the yeast strain AH109 (Clontech), followed by transformation with the *Arabidopsis* cDNA library. The yeast transformants were screened on the selective medium SD/-Leu/-Trp/-His (-LWH). To confirm protein-protein interactions, the full-length CDS fragments of *MPK3* and *MPK6* were cloned into pGKKT7 and the full length CDS fragment of *ICE1* and truncated *ICE1* were cloned into pGADT7. The yeast transformants were screened on the selective medium SD/-Leu/-Trp/-His/-Ade (-LWHA).

#### In Vitro Pull-Down Assay

The pull-down assay was performed as described previously (Wang et al., 2011). Briefly, Recombinant GST-*MPK3* and GST-*MPK6* proteins were purified using glutathione beads (GE Healthcare), and His-*ICE1* was purified using Ni-NTA agarose (Qiagen). Glutathione beads containing 5 μg GST, GST-*MPK3* or GST-*MPK6* were incubated with 5 μg His-*ICE1* at 4°C for 2 h. After the proteins were eluted from the beads, the proteins were detected by immunoblotting with anti-His antibody (Beijing Protein Innovation).

### Co-IP Assay

Co-IP assay was performed as described previously (Ding et al., 2015). The full-length CDSs of *MPK3* and *MPK6* were cloned into the HA-Flag tag and *ICE1* was cloned into (Hu et al., 2013) with the Myc tag to generate 35S:HF-*MPK3*, 35S:HF-*MPK6* and 35S:Myc-*ICE1*. These resulting constructs were infiltrated into *N. benthamiana* via *Agrobacterium*-mediated method. After 36 h, total proteins were extracted with IP buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM dithiothreitol, 1% Triton X-100, 2% NP40 and 1 × protease inhibitor cocktail, followed by incubated with an anti-HA agarose beads for 2 h. Beads were washed five times with washing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 2% NP40), and resuspended with IP buffer. The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with anti-Myc antibody (Sigma-Aldrich).

### Phosphorylation Assays

#### In Vitro Phosphorylation Assay

The *in vitro* phosphorylation assay was performed as described previously (Mao et al., 2011). Recombinant His-tagged MPK3 and MPK6 (0.2 µg) were activated by incubation with recombinant MKK5<sup>DD</sup> (0.05 µg) in the reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 50 µM ATP and 1 mM DTT) at 28°C for 0.5 h. Activated MPK3 and MPK6 was then used to phosphorylate recombinant ICE1 proteins (1:10 enzyme substrate ratio) in the reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 25 µM ATP 1 µCi [γ-<sup>32</sup>P] ATP and 1 mM DTT) at 28°C for 30 min. The reactions were stopped by the addition of 5 × SDS sample buffer. The phosphorylated ICE1 was visualized by autoradiography after separation in a 10% SDS-PAGE gel. The levels of phosphorylation were quantitated using a Typhoon 9410 Variable Mode Imager (GE Healthcare).

#### In Vivo Phosphorylation Assay

ICE1 protein phosphorylated *in vivo* was detected by a mobility shift assay using the Phos-tag reagent (NARD Institute) as described previously (Mao et al., 2011). Briefly, proteins were extracted from wild-type Col and *MPK3SR* #64 plants expressing HF-ICE1 treated with or without 4°C, and separated in a 6% SDS-PAGE gel containing 50 µM Phos-tag and 200 µM MnCl<sub>2</sub>. After electrophoresis, the gel was incubated in the transfer buffer containing 10 mM EDTA three times, and washed in transfer buffer (50 mM Tris, 40 mM Glycine) for 10 min, and then transferred to a nitrocellulose membrane, HF-ICE1 was detected with the anti-HA antibody (Sigma-Aldrich).

### Cell-Free Protein Degradation Assay

The cell-free protein degradation assay of ICE1 was performed as described previously (Ding et al., 2015) with minor modifications. Briefly, total proteins were extracted from 2-week-old seedlings in degradation buffer (50 mM Tris-MES (pH=8.0), 500 mM Sucrose, 1 mM MgCl<sub>2</sub>, 10 mM EDTA (pH=8.0), 5 mM DTT). Equal amounts of proteins from *mpk3-2*, *mpk6-3* and wild-type Col plants were incubated with recombinant His-ICE1 protein for different time periods. ICE1 protein was detected by immunoblotting with anti-His antibody.

### Transcriptional Activity Assay

Transcriptional activity assay was performed as described previously (Ding et al., 2015). *N. benthamiana* leaves were cotransfected with *CBF3:GUS* combined with 35S:Myc-*ICE1*, 35S:Myc-*ICE1*<sup>6D</sup>, 35S:Myc-*ICE1*<sup>6A</sup>, 35S:HF-*MPK3*, or 35S:HF-*MPK6* for 36 h. 35S:LUC was included for all the samples as an internal transfection efficiency control. The luciferase (Luc) activity was detected using luciferase as a substrate (Promega). The GUS activity was analyzed using methyl umbelliferyl glucuronide (Sigma-Aldrich). The GUS/Luc ratio was used to determine *CBF3* promoter activity.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of proteins was achieved by measuring the intensity of relevant protein bands from western blot using ImageJ. At least three independent replicates were performed for each experiment. Representative results from one of the independent repeats were shown, and the data indicate mean of three technical replicates ± SD. Student's *t* test was used to determine the difference between two groups of data at a specific time point (\* *P* < 0.05, \*\* *P* < 0.01).

## DATA AND SOFTWARE AVAILABILITY

Images and other data are available at Mendeley (<https://doi.org/10.17632/jydknn3gmr.1>).