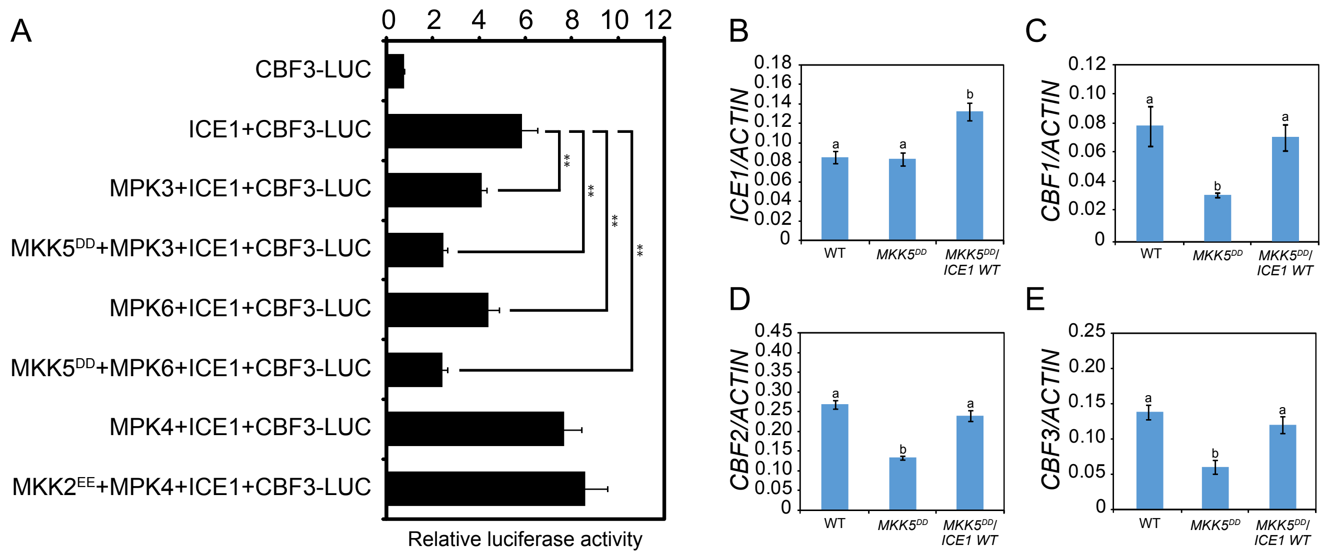

Figure S1. MKK5-MPK3/6 cascade negatively regulates *CBF* gene expression. Related to Figure 2 and 3.

(A) *CBF3-LUC* alone or together with other plasmids was transfected to wild-type protoplasts. The luciferase activity was examined 16 h after transfection. For each transfection, *ZmUBQ::GUS* was also co-transfected, and the activity of GUS was assessed and used as the internal control. Asterisks indicate statistically significant differences (**P < 0.01, Student's *t*-test).

(B-E) The seedlings of wild-type, *MKK5^{DD}*, *MKK5^{DD}/ICE1 pro::ICE1-YFP* were pretreated with Dex for 9 h and then subjected to low temperature treatment for 3 h. Transcript accumulation of *ICE1* and *CBF* genes was assessed by qRT-PCR, and *ACTIN8* was used as the internal control.

Data are means ± SD (n=3). Different letters represent statistically significant differences (P < 0.05, one-way ANOVA).

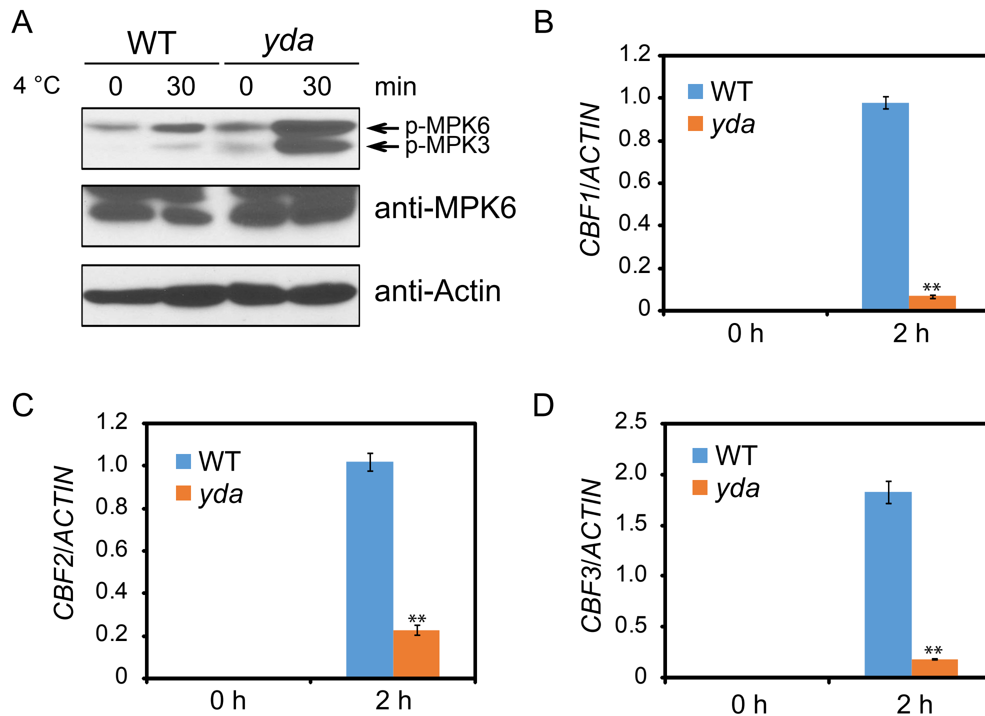

Figure S2. YDA is not required for the cold-induced activation of MPK3 and MPK6. Related to Figure 1.

(A) The seedlings of wild-type and *yda* mutants were treated with low temperature for 0, 15, and 30 min. The immunoblotting assays were performed using anti-pTEpY, anti-MPK6, and anti-Actin.

(B-D) The 12-day-old seedlings of wild-type and *yda* were treated with 4°C for 2 h. Transcript accumulation of *CBF* genes was assessed by qRT-PCR, and *ACTIN8* was used as the internal control.

Data are means ± SD (n=3). Asterisks indicate statistically significant differences (**P < 0.01, Student's *t*-test).

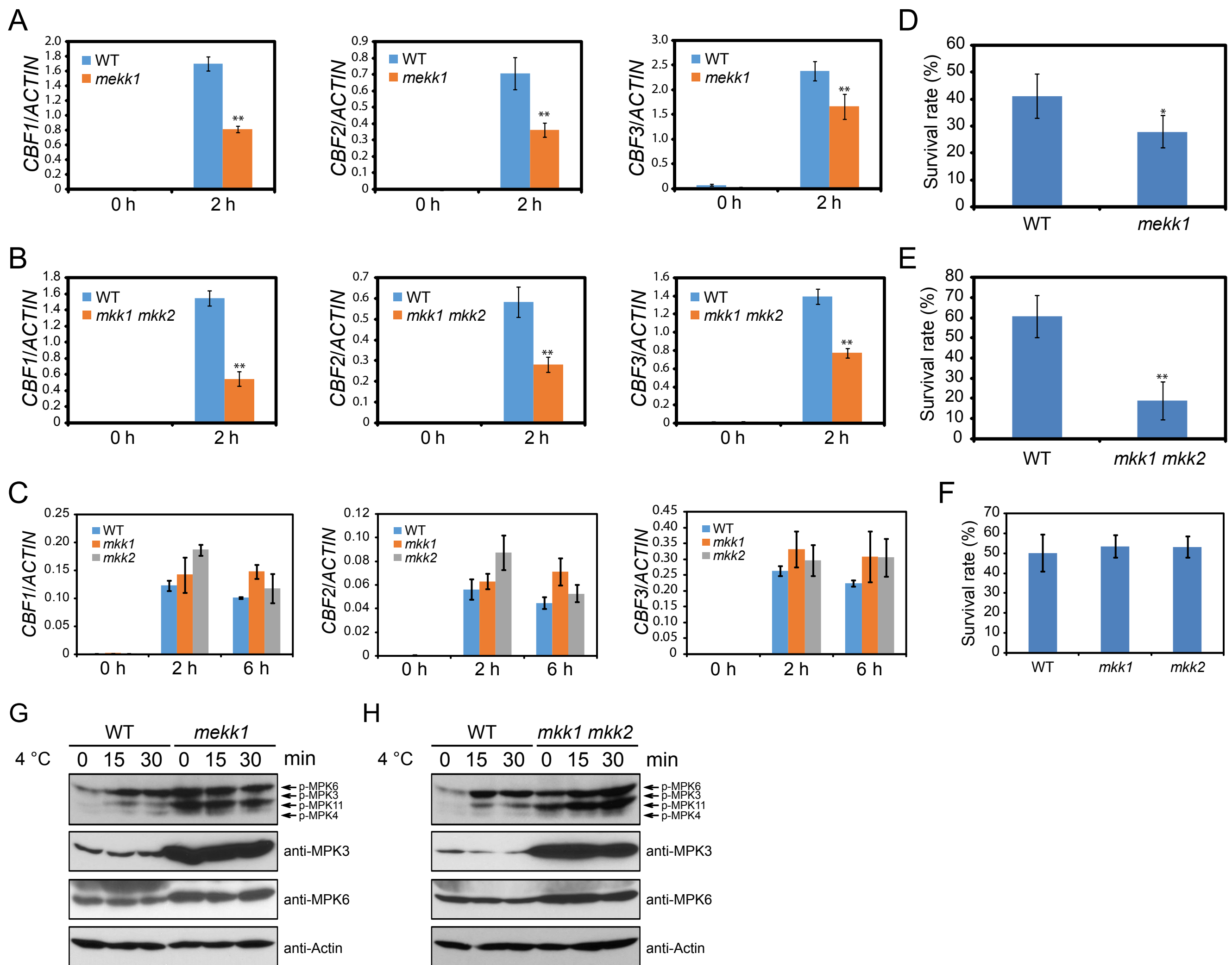

Figure S3. *mekk1* single mutant and *mkk1 mkk2* double mutant are hypersensitive to freezing. Related to Figure 4.

(A-C) The seedlings of *mekk1* (A), *mkk1 mkk2* (B), *mkk1*, and *mkk2* (C) were treated with low temperature (4°C). The transcript accumulation of *CBF* genes was assessed by qRT-PCR, and *ACTIN8* was used as the internal control.

(D-F) The survival rates of freezing-treated *mekk1* (D), *mkk1 mkk2* (E), *mkk1*, and *mkk2* (F) seedlings after 5 days of recovery at 23°C.

(G-H) The 12-day-old seedlings of wild-type and *mekk1* (G) or *mkk1 mkk2* (H) mutants were treated with low temperature for 0, 15, and 30 min. The immunoblotting assays were performed using anti-pTEpY, anti-MPK3, anti-MPK6, and anti-Actin.

Data in (A)-(F) are means ± SD (n=3). Asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01, Student's *t*-test).

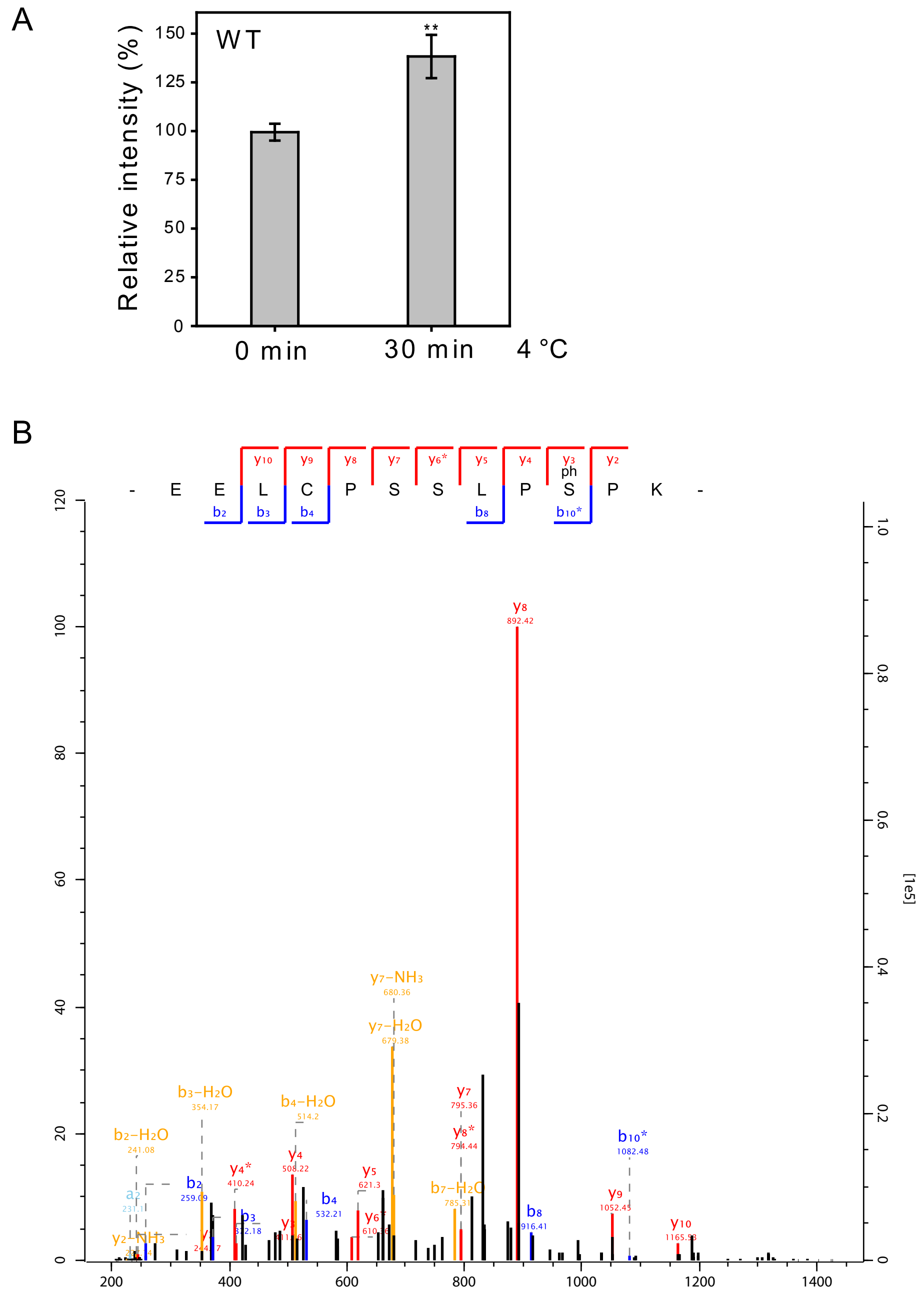

Figure S4. The phosphorylation of ICE1 *in vivo*. Related to Figure 5.

(A) The wild-type seedlings were treated with or without low temperature (4°C) for 30 min. The total proteins were extracted and applied for quantitative phosphoproteomics analysis. The relative phosphorylation level of ICE1 was assessed.

(B) The phosphorylation of the Ser403 residue of ICE1. Phosphoproteomics was performed for wild-type seedlings and the phosphorylation residues of the ICE1 protein were analyzed.

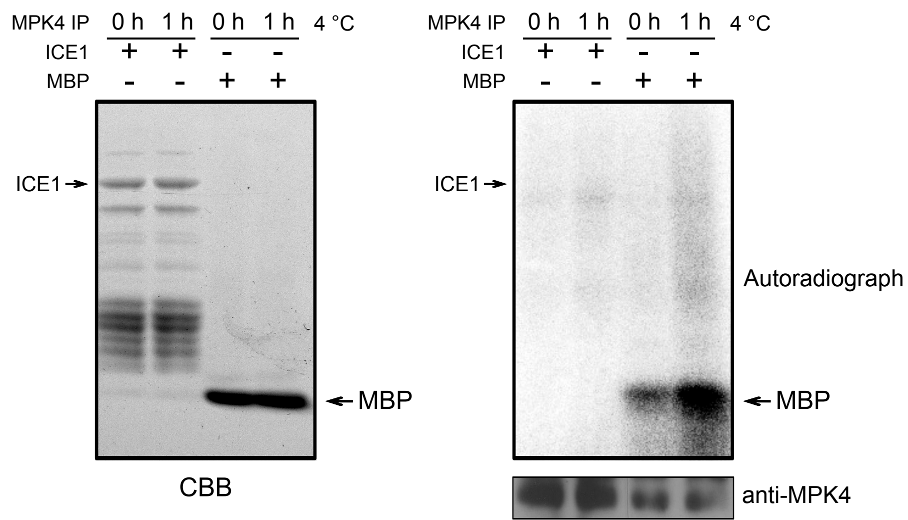

Figure S5. MPK4 is not able to phosphorylate ICE1. Related to Figure 5.

MPK4 was immunoprecipitated from wild-type seedlings incubated at 23°C or at 4°C for 1 h. The purified MPK4 was used for immunoprecipitation-kinase assay using ICE1 or MBP as a substrate.

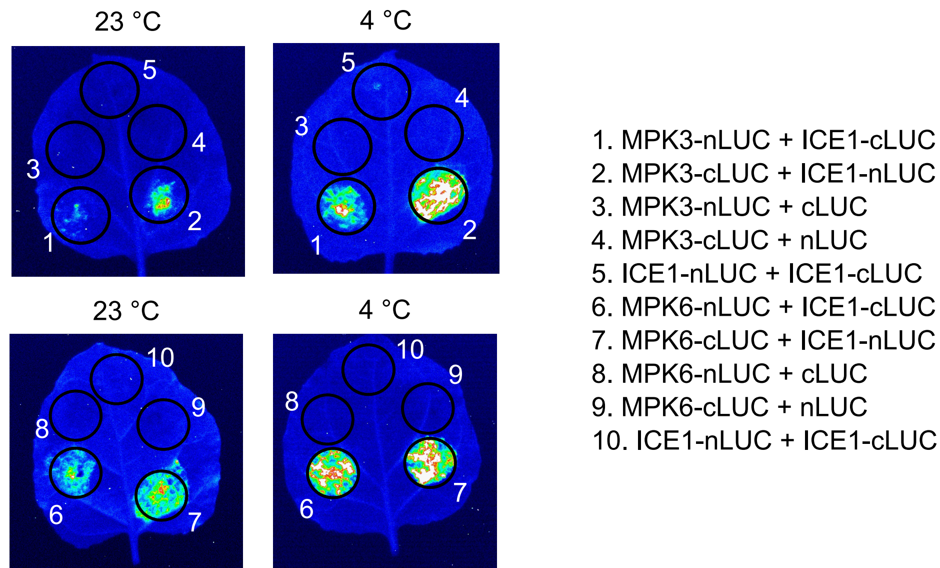

Figure S6. MPK3 and MPK6 interact with ICE1. Related to Figure 5 and 6.
The split luciferase complementation assay was applied to test the interaction between MPK3/6 and ICE1. The indicated constructs were transiently expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*-mediated method. Before taking images, the plants were treated with or without low temperature (4°C) for 1 h. Three biological replicates were conducted, and similar results were obtained.

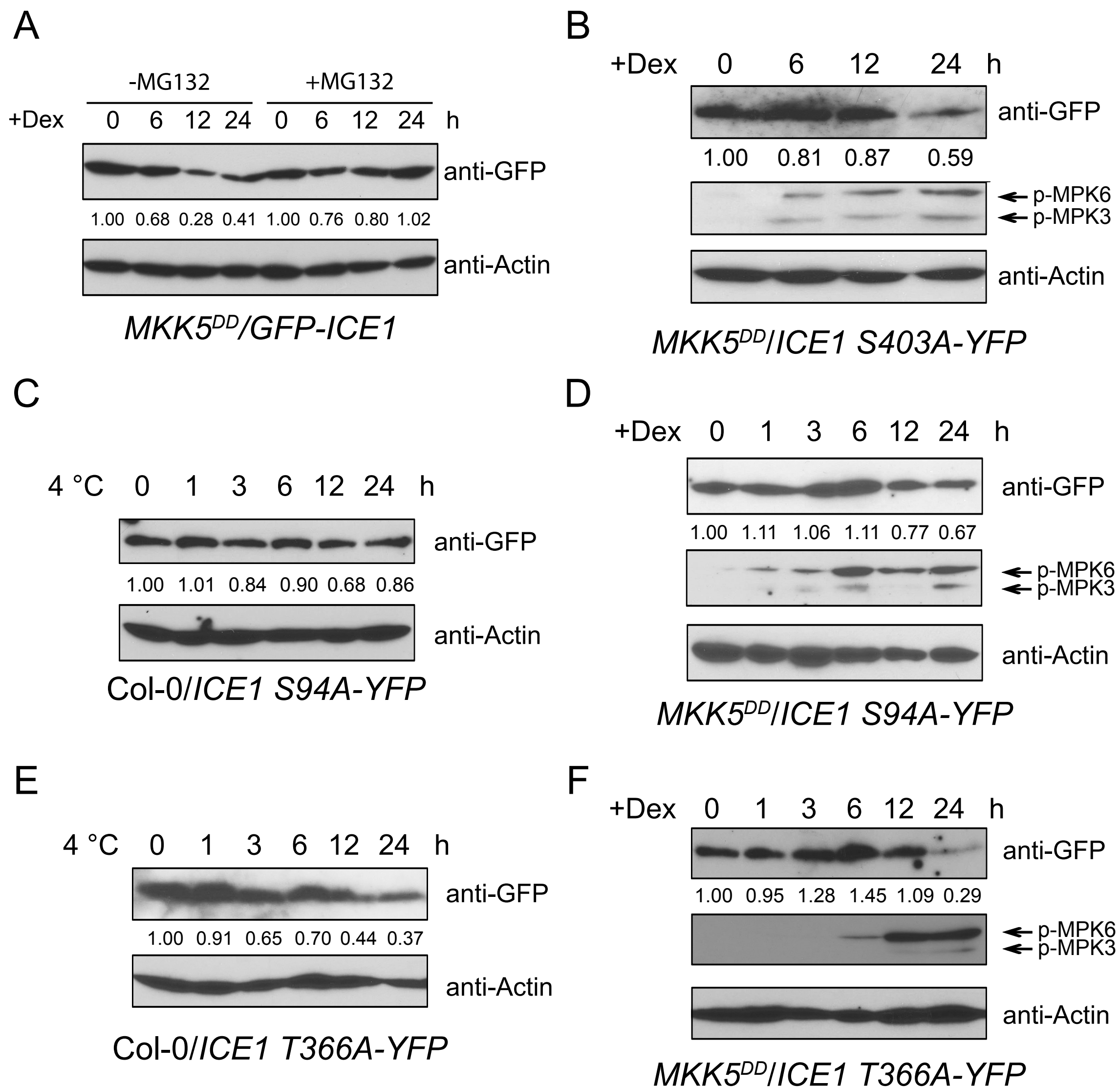

Figure S7. The phosphorylation sites of Ser94, Thr366, and Ser403 are required for the degradation of ICE1. Related to Figure 6.

(A) The seedlings of *MKK5^{DD}/35S::GFP-ICE1* were treated with or without MG132 before they were incubated with Dex. The total proteins were extracted and immunoblotting assays were performed using anti-GFP and anti-Actin.

(B-F) The seedlings of each transgenic plant were treated with Dex or low temperature (4°C). The total proteins were extracted and immunoblotting assays were performed using anti-GFP, anti-pTEpY, and anti-Actin. The band intensity was evaluated using ImageJ software. All experiments were repeated three times with similar results.

Table S1. Comparison of the phosphorylation levels of the selected MAPK pathway components before and after cold treatment. Related to Figure 1.

Type	Gene ID	Gene name	Phosphorylation site	Cold/Con*	p-value
MAPKKK	AT4G08500	MEKK1	S62	1.59	6.09E-03
	AT1G63700	YDA	S720	1.35	1.07E-01
	AT1G63700	YDA	S794	1.92	1.01E-02
MAPKK	AT4G29810	MKK2	S65	1.78	2.61E-03
MAPK	AT3G45640	MPK3	Y198	2.35	7.27E-03
	AT4G01370	MPK4	T201	2.41	2.47E-03
	AT4G01370	MPK4	Y203	2.50	2.71E-03
	AT2G43790	MPK6	T221	2.75	3.12E-03
	AT2G43790	MPK6	Y223	2.75	3.22E-03
	AT1G18150	MPK8	S539	0.46	2.58E-03
	AT3G18040	MPK9	Y187	1.23	5.28E-01
	AT1G73670	MPK15	S511	0.58	5.16E-03
	AT1G73670	MPK15	Y254	0.58	2.43E-02
	AT5G19010	MPK16	Y189	0.73	5.94E-02
	AT2G01450	MPK17	S397	1.04	5.97E-01
	AT1G53510	MPK18	Y189	1.00	9.95E-01
	AT3G14720	MPK19	Y189	1.33	3.14E-01
	AT2G42880	MPK20	Y189	0.81	1.95E-01

*The proteins from the control and cold-treated wild-type seedlings were extracted for quantitative phosphoproteomics analysis. The phosphosites that were detected in all three biological replicates of samples were selected for quantitative analysis. The peak intensity of each identified phosphosite was calculated. The fold change between cold-treated and mock-treated samples was determined by dividing the average values of three biological replicates between the two samples.

Table S2. Primers used in this study. Related to STAR methods.

Primer name	Sequence	Usage
CBF1 RT-LP	GGAGACAATGTTTGGGATGC	Quantitative real-time PCR
CBF1 RT-RP	TTAGTAACTCCAAAGCGACACG	Quantitative real-time PCR
CBF2 RT-LP	TGACGTGTCCTTATGGAGCTA	Quantitative real-time PCR
CBF2 RT-RP	CTGCACTCAAAAACATTTGCA	Quantitative real-time PCR
CBF3 RT-LP	GATGACGACGTATCGTTATGGA	Quantitative real-time PCR
CBF3 RT-RP	TACACTCGTTTCTCAGTTTTACAAAC	Quantitative real-time PCR
ICE1 RT-LP	TGCCTGCTAAGAATCTGATGGC	Quantitative real-time PCR
ICE1 RT-RP	AGATCCAGGAGGAGTTGACTCA	Quantitative real-time PCR
Actin RT-LP	ATGACTCAGATCATGTTTGAGACC	Quantitative real-time PCR
Actin RT-RP	TCAGTAAGGTCACGACCAGCAA	Quantitative real-time PCR
CRLK1 RT-LP	GTAGCAGCTTTTGCTTACAAATGCA	semi-quantitative RT-PCR
CRLK1 RT-RP	TTCTGACTCCTCCACTATCGGA	semi-quantitative RT-PCR
CRLK2 RT-LP	GCATACCTAGATATAACTACAAGG	semi-quantitative RT-PCR
CRLK2 RT-RP	CTGTCAAGTTCACAAGATTCC	semi-quantitative RT-PCR
LB1.3	ATTTTGCCGATTTCGGAAC	Genotyping
LB1 (SAIL)	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	Genotyping
SALK_052557 LP	AATCGGAACCTCGAGATGAAG	Genotyping
SALK_052557 RP	TTCACTCAAATCCTGACCCTG	Genotyping
SALK_151594 LP	ATTTTTGTCAACAATGGCCTG	Genotyping
SALK_151594 RP	TCTGCCTTTTCACGGAATATG	Genotyping
SALK_127507 LP	CTCTGGCTCATCGCTTATGTC	Genotyping
SALK_127507 RP	ATCTATGTTGGCGTTTGCAAC	Genotyping
SALK_027645 LP	CCACCACTAGCAGAGAAACATAAC	Genotyping
SALK_027645 RP	AACATGCTATCTGCCATCTGC	Genotyping
SAIL_511_H01 LP	TTCTTTTCCCAAATGGATTCC	Genotyping
SAIL_511_H01 RP	GTTAAAGCCATCCCTGACTCC	Genotyping
SALK_056245 LP	TTGCTCTGAATACACAGCAGC	Genotyping
SALK_056245 RP	GTCTTAGAGATCAGCGGGGAC	Genotyping
SALK_016240 LP	TACAGGCGAAAAACATGAACC	Genotyping
SALK_016240 RP	ATTGGGCCTTGAATTCAATTC	Genotyping
SALK_103505 LP	TTTATGGACGAGGTCCTCATG	Genotyping
SALK_103505 RP	CCTTGTCCTAGAACGGTTGTG	Genotyping
SALK_105078 LP	GAGCAGCTGTAGGACGATTTG	Genotyping
SALK_105078 RP	TCTCGCAGGAAATTTCAGTTC	Genotyping
ICE1 g LP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCGATTAGA GTAAATCCGAGT	Plasmid construction Transgenic plants
ICE1 g RP	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATCATACCA GCATACCCTGC	Plasmid construction Transgenic plants
ICE1 proto LP	CGCGGATCCATGAACAGCGACGGTGTTTGGCT	Plasmid construction Protoplast
ICE1 proto RP	TTGCGGCCGCTCAAACCAAACCAGCGTAACCTG	Plasmid construction Protoplast
ICE1 GST LP	CCGGAATTCATGAACAGCGACGGTGTTTGGCT	Plasmid construction *In vitro* kinase assay
ICE1 GST RP	TTGCGGCCGCTCAAACCAAACCAGCGTAACCTG	Plasmid construction *In vitro* kinase assay
ICE1 S94A LP	CTATTGATTCTTCTTCTTCTTGTGCTCCTTCTCAAGCTTTTAG TCTT	Point mutation
ICE1 S94A RP	AAGACTAAAAGCTTGAGAAGGAGCACAAGAAGAAGAAGAA TCAATAG	Point mutation
ICE1 S203A LP	TCCGTTGGAGTTGGAAGGTTTTGGTGCTCCTGCTAATGGT	Point mutation
ICE1 S203A RP	ACCATTAGCAGGAGCACCAAAACCTTCCAACTCCAACGGA	Point mutation
ICE1 T366A LP	CACAATGAACTTGAGTCAGCTCCTCCTGGATCTTTGC	Point mutation
ICE1 T366A RP	GCAAAGATCCAGGAGGAGCTGACTCAAGTTCATTGTG	Point mutation
ICE1 T382A LP	AAGCTTCCATCCGTTGGCACCTACACCGCAAAC	Point mutation

ICE1 T382A RP	GTTTGCGGTGTAGGTGCCAACGGATGGAAGCTT	Point mutation
ICE1 T384A LP	CCATCCGTTGACACCTGCACCGCAAACTCTTTC	Point mutation
ICE1 T384A RP	GAAAGAGTTTGCGGTGCAGGTGTCAACGGATGG	Point mutation
ICE1 S403A LP	GTTGTGTCCCTCTTCTTTACCAGCTCCTAAAGGCCAGCAA	Point mutation
ICE1 S403A RP	TTGCTGGCCTTTAGGAGCTGGTAAAGAAGAGGGACACAAC	Point mutation
MPK3 LP	CGCGGATCCATGAACACCGGCGGTGGCCAA	Plasmid construction Kinase and protoplast assay
MPK3 RP	TTGCGGCCGCCTAACCGTATGTTGGATTGAGT	Plasmid construction Kinase and protoplast assay
MPK6 LP	CGCGGATCCATGGACGGTGGTTCAGGTCAAC	Plasmid construction Kinase and protoplast assay
MPK6 RP	TTGCGGCCGCCTATTGCTGATATTCTGGATTG	Plasmid construction Kinase and protoplast assay
MPK4 LP	CGCGGATCCATGTCGGCGGAGAGTTGTTTCG	Plasmid construction Protoplast assay
MPK4 RP	TTGCGGCCGCTCACACTGAGTCTTGAGGATTG	Plasmid construction Protoplast assay
MPK3-LUC LP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGA GATAGAACCATGAACACCGGCGGTGGCCAAT	Plasmid construction Split-LUC assay
MPK3-LUC RP	GGGGACCACTTTGTACAAGAAAGCTGGGTCACCGTATG TTGGATTGAGTGCT	Plasmid construction Split-LUC assay
MPK6-LUC LP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAG ATAGAACCATGGACGGTGGTTCAGGTCAAC	Plasmid construction Split-LUC assay
MPK6-LUC RP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGCTGATA TTCTGGATTGAAAGC	Plasmid construction Split-LUC assay
ICE1-LUC LP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAG ATAGAACCATGGGTCTTGACGGAAACAATG	Plasmid construction Split-LUC assay
ICE1-LUC RP	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATCATACC AGCATACCCTGCT	Plasmid construction Split-LUC assay