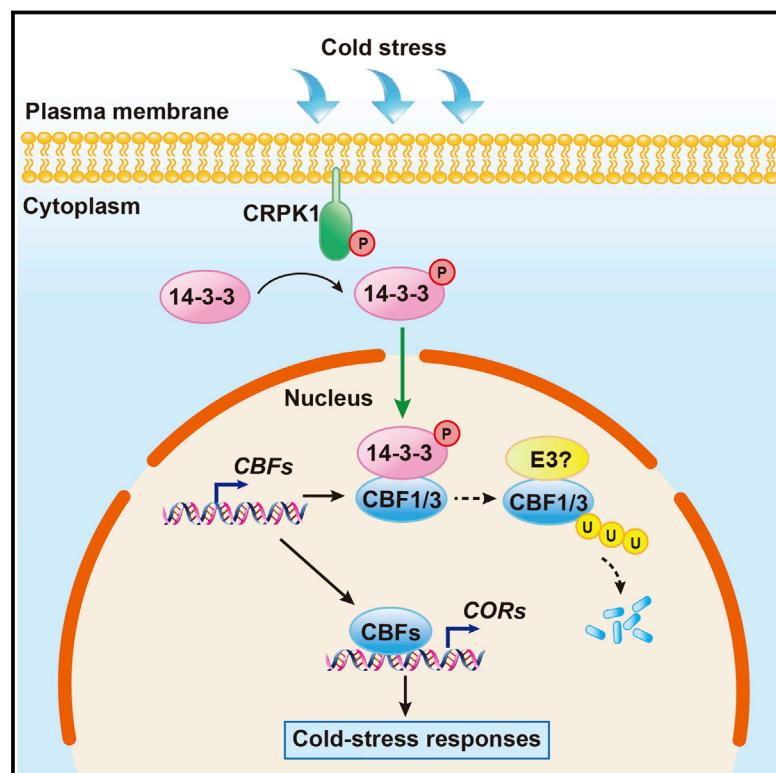


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Plasma Membrane CRPK1-Mediated Phosphorylation of 14-3-3 Proteins Induces Their Nuclear Import to Fine-Tune CBF Signaling during Cold Response

Graphical Abstract



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In Brief

How the plasma membrane senses and transduces cold signals remains unknown. Liu et al. demonstrate that the cold-activated plasma membrane CRPK1 phosphorylates 14-3-3 proteins, which are imported from the cytosol to the nucleus and interact with CBF proteins to promote their destabilization, thus fine-tuning CBF-dependent cold signaling.

Highlights

- Cold activates the plasma membrane-localized protein kinase CRPK1
- CRPK1 interacts with and phosphorylates 14-3-3 proteins
- Phosphorylated 14-3-3 proteins translocate from the cytosol to the nucleus
- In the nucleus, 14-3-3 proteins interact with CBFs and promote their degradation

Plasma Membrane CRPK1-Mediated Phosphorylation of 14-3-3 Proteins Induces Their Nuclear Import to Fine-Tune CBF Signaling during Cold Response

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SUMMARY

In plant cells, changes in fluidity of the plasma membrane may serve as the primary sensor of cold stress; however, the precise mechanism and how the cell transduces and fine-tunes cold signals remain elusive. Here we show that the cold-activated plasma membrane protein cold-responsive protein kinase 1 (CRPK1) phosphorylates 14-3-3 proteins. The phosphorylated 14-3-3 proteins shuttle from the cytosol to the nucleus, where they interact with and destabilize the key cold-responsive C-repeat-binding factor (CBF) proteins. Consistent with this, the *crpk1* and *14-3-3 $\kappa\lambda$* mutants show enhanced freezing tolerance, and transgenic plants overexpressing *14-3-3 λ* show reduced freezing tolerance. Further study shows that CRPK1 is essential for the nuclear translocation of 14-3-3 proteins and for 14-3-3 function in freezing tolerance. Thus, our study reveals that the CRPK1-14-3-3 module transduces the cold signal from the plasma membrane to the nucleus to modulate CBF stability, which ensures a faithfully adjusted response to cold stress of plants.

INTRODUCTION

Low temperature adversely affects the survival, growth, and development of plants. During evolution, plants have acquired sophisticated mechanisms to respond to low temperatures. For example, in many temperate plants, cold acclimation increases freezing tolerance after exposure to nonfreezing low temperatures (Thomashow, 1999). Emerging studies in *Arabidopsis* have explored the transcriptional network of cold acclimation pathways. Three C-repeat (CRT)-binding factors (CBFs), also known as dehydration-responsive element (DRE)-binding proteins (DREBs), play pivotal roles in cold acclimation (Jia et al., 2016; Liu et al., 1998; Stockinger et al., 1997; Zhao et al., 2016). These transcription factors are rapidly induced by cold stress and bind to CRT/DRE DNA regulatory elements in the promoters of a subset of cold-regulated (*COR*) genes;

expression of these *COR* genes renders plants able to tolerate freezing stress (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998; Thomashow, 1999). Because of their key role in cold acclimation, the transcriptional regulation of CBFs has been extensively studied (Shi et al., 2015). Inducer of CBF expression 1 (ICE1), calmodulin-binding transcription activator 3 (CAMTA3), and brassinazole-resistant 1 (BZR1) positively regulate CBF expression (Chinnusamy et al., 2003; Doherty et al., 2009; Li et al., 2017), whereas MYB15 and ethylene-insensitive 3 (EIN3) negatively regulate CBF expression (Agarwal et al., 2006; Shi et al., 2012).

Although transcriptional regulation of CBF has been well studied, little is known about the translational and posttranslational regulation of CBF proteins. Protein kinase-mediated phosphorylation has been found to modulate cold response of plants. A recent study identified one protein kinase, open stomata 1 (OST1), that positively regulates freezing tolerance. Under cold stress, OST1 is activated and phosphorylates ICE1, which inhibits ICE1 degradation mediated by the E3 ligase high expression of osmotically responsive gene 1 (HOS1) (Agarwal et al., 2006), thus positively regulating CBF expression and freezing tolerance (Ding et al., 2015). Other protein kinases, including the mitogen-activated protein kinase kinase 2 (MKK2) and CBL-interacting protein kinase 3 (AtCIPK3), have been implicated in plant cold responses (Kim et al., 2003; Teige et al., 2004). However, how these protein kinases function in cold signaling remains unclear.

The 14-3-3 proteins are phosphopeptide-binding proteins and are highly conserved in all eukaryotic cells. The 14-3-3 proteins are involved in a wide range of biological processes (Denison et al., 2011), and they usually interact with phosphorylated proteins to mediate signal transduction by altering their activity, stability, conformation, subcellular localization, or affinity to other proteins (Paul et al., 2012). *Arabidopsis* 14-3-3 proteins regulate the activities of plasma membrane H⁺-ATPase (Fuglsang et al., 2007) and salt overly sensitive 2 (SOS2) kinase (Zhou et al., 2014), the stability of aminocyclopropane-1-carboxylic acid synthase isoforms (Catalá et al., 2014; Yoon and Kieber, 2013), and the localization of the transcription factor BZR1/brassinosteroid-insensitive 1-EMS suppressor 1 (BES1) (Gampala et al., 2007). In addition, phosphorylation of 14-3-3 proteins is important for their functions. In animals, phosphorylation of 14-3-3 proteins causes a decrease in binding to Raf-1 protein kinase (Van Der Hoeven

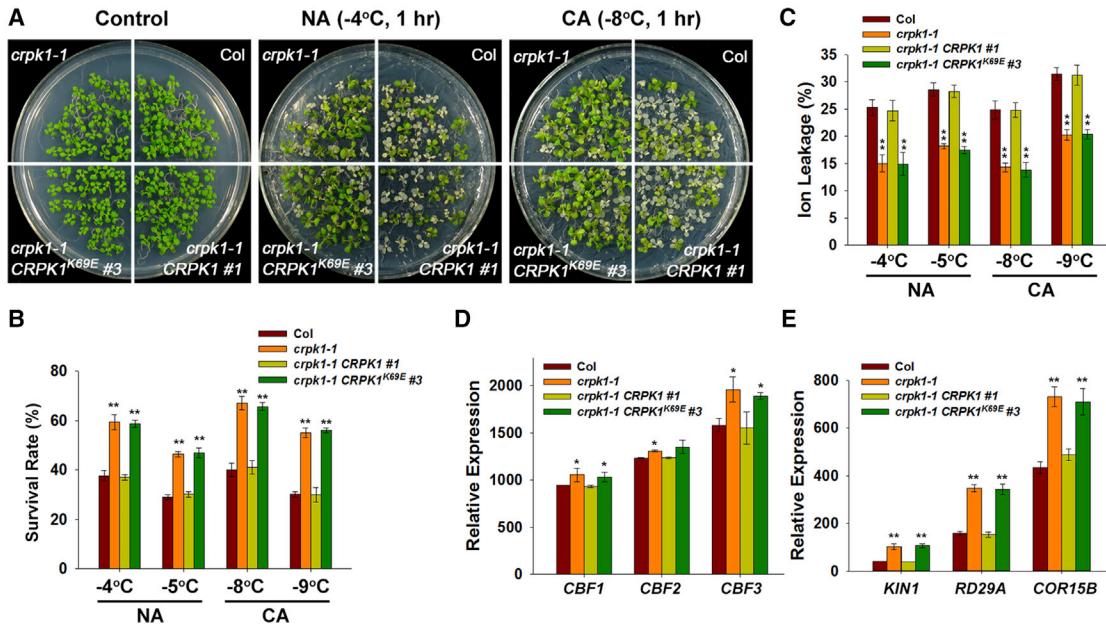


Figure 1. CRPK1 Negatively Modulates Freezing Tolerance in *Arabidopsis*

(A–C) Freezing tolerance of the *crpk1-1* mutant and complemented lines with wild-type *CRPK1* and the kinase-dead form *CRPK1^{K69E}* driven by its native promoter (*crpk1-1 CRPK1* and *crpk1-1 CRPK1^{K69E}*) under non-acclimated (NA) or cold-acclimated (CA) conditions. The 13-day-old plants grown on half-strength Murashige–Skoog (1/2 MS) plates at 22°C were treated at –4°C or –5°C for 1 hr (NA) or were pretreated at 4°C for 3 days and then treated at –8°C or –9°C for 1 hr. After recovery at 22°C for 3 days, representative pictures of plants are shown (A), survival rates were counted (B), and ion leakage was measured (C). (D and E) Expression of *CBFs* (D) and target genes (E) in *crpk1-1*, *crpk1-1 CRPK1*, and *crpk1-1 CRPK1^{K69E}* plants under cold stress. Total RNA was extracted from 12-day-old seedlings treated at 4°C for 3 hr for *CBFs* and 24 hr for *CBF* targets and then subjected to qRT-PCR. *Actin2/8* was used as a control. The expression of these genes in untreated wild-type was set to 1.

The data in (B)–(E) are shown as means of three replicates ± SD, and the asterisks indicate significant differences by Student's t test (*p < 0.05 and **p < 0.01) when compared to the wild-type. The above experiments were repeated three times with similar results.

See also Table S1 and Figure S1.

et al., 2000), and phosphorylation of 14-3-3 ζ leads to the disruption of its dimeric structure (Powell et al., 2003). In *Arabidopsis*, 14-3-3 κ and χ are phosphorylated by SNF1-related protein kinase 2.8 (SnRK2.8) (Shin et al., 2007). Nevertheless, our understanding of the molecular mechanism that regulates 14-3-3 proteins in plants remains very limited.

Although CBF-dependent cold-signaling pathways have been extensively studied, the mechanisms by which plants sense and transduce the cold signal remains unclear. One widely proposed hypothesis is that plasma membrane fluidity provides the primary signal for cold perception in plants (Orvar et al., 2000). In this study, we found that the plasma membrane-localized kinase cold-responsive protein kinase 1 (CRPK1) is activated by cold stress and phosphorylates 14-3-3 proteins, which in turn translocate from the cytoplasm into the nucleus to promote the degradation of CBFs via the 26S proteasome pathway. Our study shows that the 14-3-3 proteins act as integrators to transduce the cold signal from the plasma membrane to the nucleus to regulate the plant response to cold stress.

RESULTS

CRPK1 Negatively Regulates Freezing Tolerance

To identify protein kinases involved in freezing tolerance, we performed a genetic screen for protein kinase mutants (Ding et al.,

2015), and we isolated several candidate receptor-like protein kinase mutants with altered freezing tolerance. In this study, we further characterized a mutant (SALK_004253C) that contains a T-DNA insertion in *At1g16670*, and we named this allele *cold-responsive protein kinase 1* (*crpk1-1*) (Figures S1A and S1B). The *crpk1-1* null mutant showed significantly enhanced freezing tolerance compared to the wild-type under both non-acclimated (NA) and cold-acclimated (CA, 4°C for 3 days) conditions (Figures 1A and 1B). Ion leakage indicates injury of the plasma membrane caused by cold stress. Accordingly, the *crpk1-1* mutant showed less electrolyte leakage than the wild-type with or without cold acclimation (Figure 1C). The second allele, *crpk1-2* (SALK_054771), was isolated (Figures S1A and S1B), and it showed enhanced freezing tolerance similar to *crpk1-1* (Figures S1C and S1D). Two independent transgenic lines expressing *CRPK1* genomic fragment (*CRPK1:CRPK1*) fully complemented the freezing tolerance of *crpk1-1* (Figures 1A–C and S1F–S1H), indicating that the freezing tolerance of *crpk1-1* is caused by the mutation of *CRPK1*.

To test whether CRPK1 mediates cold signaling via a CBF-dependent pathway, we examined the expression of *CBF1–CBF3* in the *crpk1-1* mutant under cold stress. Cold induction of three *CBF* genes in *crpk1-1* mutants was slightly higher than in the wild-type (Figures 1D and S1J); however, the expression of CBF targets, including *RD29A*, *COR15B*, and *KIN1*,

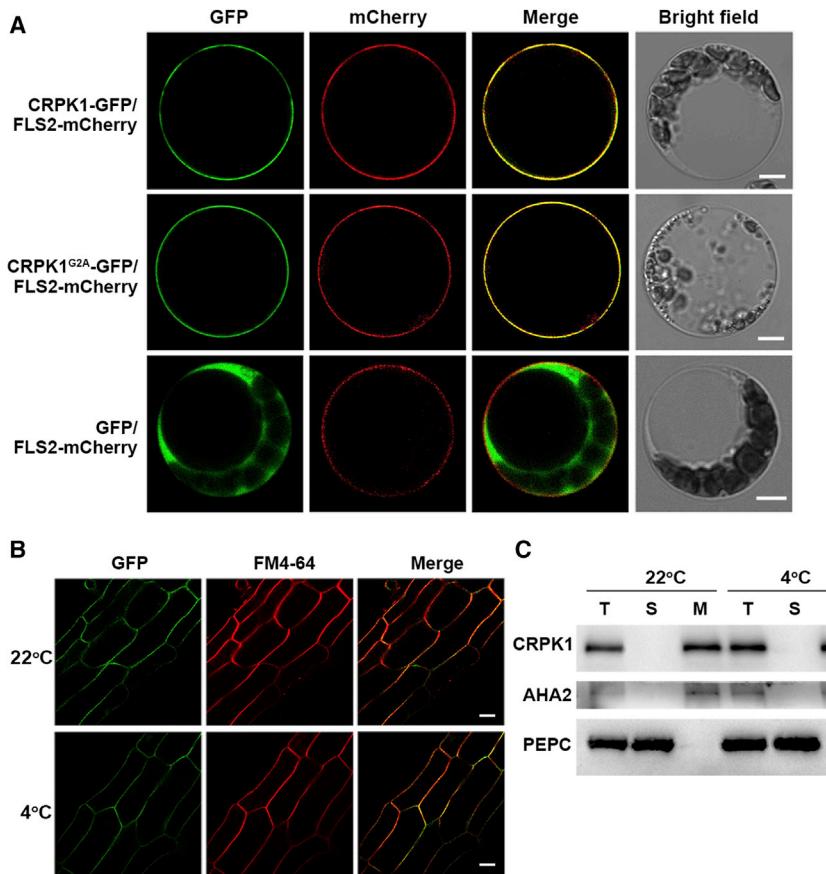


Figure 2. Characterization of CRPK1 Protein

(A) Localization of CRPK1 in *Arabidopsis* protoplasts. *CRPK1-GFP* and *FLS2-mCherry* (marker for plasma membrane) or *CRPK1^{G2A}-GFP* (carrying a G2A mutation in a putative myristylation site of CRPK1) and *FLS2-mCherry* plasmids were co-transformed into *Arabidopsis* mesophyll protoplasts. The GFP and mCherry signals were visualized under confocal microscopy after 16-hr incubation at 22°C. GFP was used as a control. Scale bar, 10 μm.

(B) Localization of CRPK1 in transgenic plants expressing *CRPK1-GFP* driven by its native promoter (*CRPK1:CRPK1-GFP*). The 7-day-old *CRPK1:CRPK1-GFP* stable transgenic plants were treated with or without 4°C for 0 and 3 hr, followed by staining with FM4-64 (a plasma membrane stain) for 5 min. The signals in root tips were immediately visualized under confocal microscopy. Scale bar, 25 μm.

(C) Subcellular fractionation analysis of CRPK1. Total (T), soluble (S), and membrane (M) proteins were prepared from 7-day-old *CRPK1:CRPK1-GFP* transgenic plants treated at 4°C for 0 and 3 hr. The immunoblots were probed with anti-GFP antibody, and specific antibodies were used to detect plasma membrane ATPase (AHA2) as a membrane marker and phosphoenolpyruvate carboxylase (PEPC) as a soluble marker. See also Table S1.

significantly increased in *crpk1-1* compared to the wild-type (Figures 1E and S1K). These results suggest that CRPK1 negatively impacts on the CBF pathway.

CRPK1 Protein Localizes to the Plasma Membrane

CRPK1 encodes an unknown protein kinase that belongs to the receptor-like cytoplasmic kinase (RLCK) family (Shiu and Bleeker, 2001). To determine the subcellular localization of CRPK1, we generated the Super:*CRPK1-GFP* construct harboring Super promoter-driven CRPK1 fused to GFP and 35S:*FLS2-mCherry* as a plasma membrane marker (Robatzek et al., 2006), and we co-transformed them into wild-type Col protoplasts. Under microscopy, the CRPK1-GFP was co-localized with FLS2-mCherry at the plasma membrane (Figure 2A). When we mutated the potential myristylation site of CRPK1 (Gly2) to Ala, the signal of CRPK1^{G2A}-GFP was still observed at the plasma membrane (Figure 2A), suggesting that the plasma membrane localization was not due to the myristylation of G2 at the N terminus of CRPK1. We also generated stable transgenic plants expressing *CRPK1:CRPK1-GFP*, and we observed the CRPK1-GFP signal at the plasma membrane before or after cold treatment, which was overlapped with the signal dyed with FM4-64, a plasma membrane stain (Figure 2B). Immunoblot analysis using *CRPK1:CRPK1-GFP* transgenic plants detected CRPK1 protein only in the membrane fraction at both 22°C and 4°C (Figure 2C). These results suggest that CRPK1 is localized

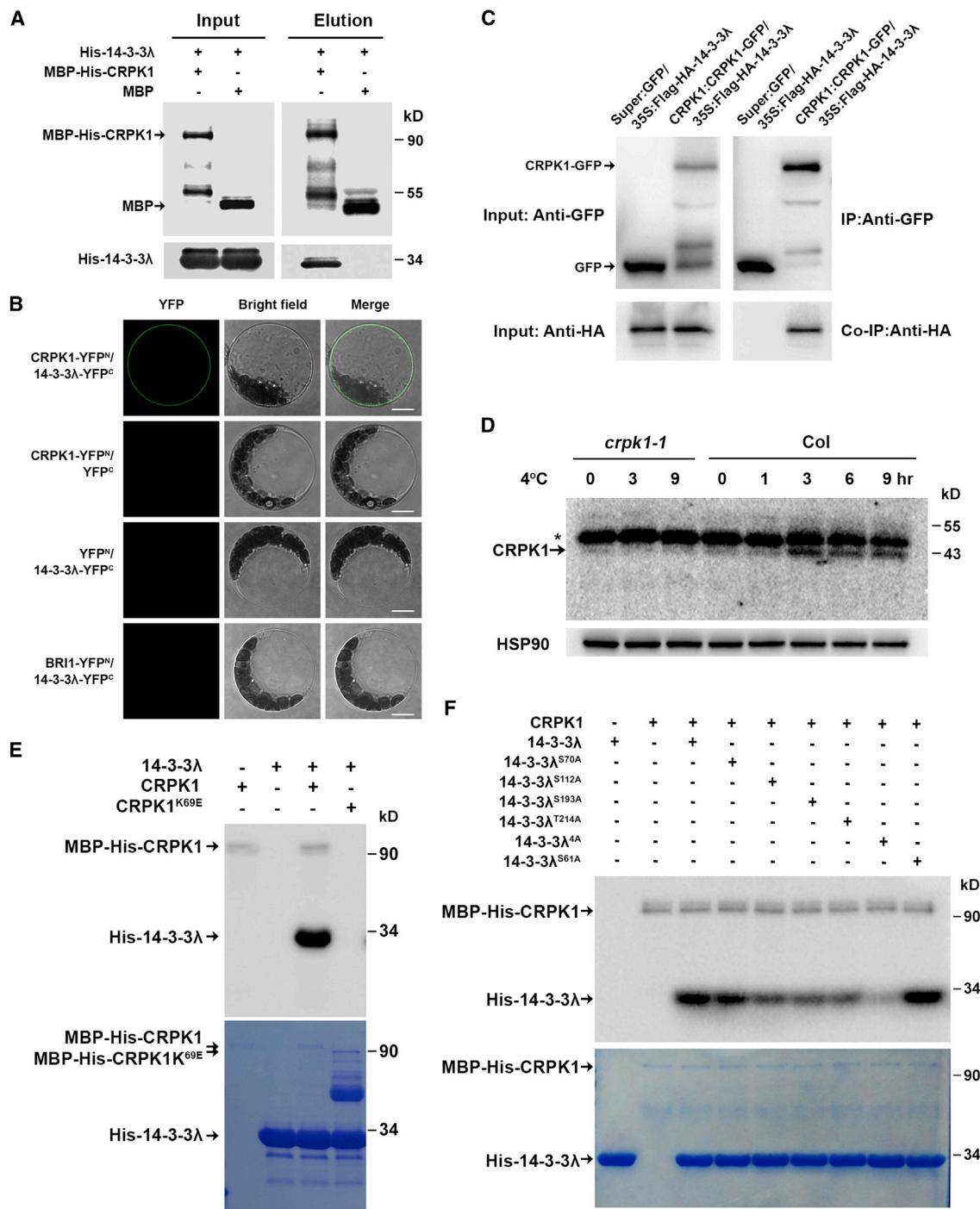
at the plasma membrane and the localization is not affected by cold stress.

CRPK1 Interacts with 14-3-3 Proteins

To explore how CRPK1 functions in the freezing tolerance of plants, we performed a yeast two-hybrid screening to identify its interacting proteins. Of 78 identified clones, 21 clones encode members of the 14-3-3 protein family, namely, 14-3-3 λ , 14-3-3 κ , 14-3-3 ϵ , and 14-3-3 ω (Figure S2A). In vitro pull-down assay confirmed the interaction between CRPK1 and 14-3-3 λ (Figure 3A). To examine their interaction in vivo, we performed bimolecular fluorescence complementation (BiFC) assays, and we observed the yellow fluorescent protein (YFP) fluorescence signal only at the plasma membrane of *Arabidopsis* protoplasts with co-expression of 35S:*CRPK1-YFP^N* and 35S:14-3-3 λ -YFP^C (Figure 3B). Furthermore, co-immunoprecipitation (coIP) assays using transgenic plants expressing *CRPK1:CRPK1-GFP* and 35S:FLAG-HA-14-3-3 λ verified the in vivo interaction of CRPK1 and 14-3-3 λ (Figure 3C). These results indicate that CRPK1 interacts with 14-3-3 λ at the plasma membrane in vivo.

Cold-Activated CRPK1 Phosphorylates 14-3-3 λ In Vivo

As CRPK1 interacts with 14-3-3 λ , we then tested whether CRPK1 could phosphorylate 14-3-3 λ by an in-gel kinase assay using total proteins extracted from wild-type Col and *crpk1-1*

**Figure 3. CRPK1 Interacts with and Phosphorylates 14-3-3 Proteins**

(A) CRPK1 interacts with 14-3-3 λ in vitro. The purified MBP-His-CRPK1 or MBP protein was incubated with His-14-3-3 λ protein. After being immunoprecipitated with MBP-beads, the proteins were detected with an anti-His antibody.

(B) BiFC showing the interaction of CRPK1 and 14-3-3 λ in *Arabidopsis* protoplasts. Partial YFP constructs were fused with CRPK1 or 14-3-3 λ , and the fusions were co-expressed transiently in *Arabidopsis* protoplasts. The YFP signal was visualized by confocal microscopy. Scale bars, 20 μ m.

(C) CoIP of CRPK1 with 14-3-3 λ in planta. Total proteins were extracted from 2-week-old transgenic plants expressing Super:GFP 35S:FLAG-HA-14-3-3 λ and CRPK1:CRPK1-GFP 35S:FLAG-HA-14-3-3 λ , and they were immunoprecipitated with anti-GFP antibody. Input and immunoprecipitated proteins were analyzed by immunoblotting with anti-GFP and anti-HA antibodies, respectively.

(legend continued on next page)

mutant with or without cold treatment. By using 14-3-3 λ as substrate, the assay detected low activity at ~43 kDa, which is consistent with the predicted size of CRPK1 (with 390 amino acids). Intriguingly, this activity on 14-3-3 λ was significantly activated after a cold treatment of 3 hr (Figure 3D). As expected, this cold-induced activity of CRPK1 was not detected in the *crpk1-1* mutant (Figure 3D). CRPK1-Myc was also found to phosphorylate 14-3-3 λ in an in-gel kinase assay with immunoprecipitated CRPK1-Myc proteins extracted from *Super:CRPK1-Myc* plants, especially after treated at 4°C (Figure S2B). In addition, we used Phos-tag mobility shift assays to examine 14-3-3 λ phosphorylation by CRPK1. Total proteins were extracted from protoplasts of wild-type Col and *crpk1-1* expressing FLAG-HA-14-3-3 λ , with or without 4°C treatment, and separated in a Phos-tag gel. We observed an upshift of 14-3-3 λ protein in Col, and the upshifted band became much stronger after cold treatment but was nearly abolished after treatment with calf intestinal alkaline phosphatase (CIAP) (Figure S2C). These results strongly demonstrate that cold-activated CRPK1 phosphorylates 14-3-3 λ in vivo.

We next tested whether CRPK1 directly phosphorylates 14-3-3 λ in vitro. His-14-3-3 λ and MBP-His-CRPK1 recombinant proteins were expressed in *E. coli* and purified. In vitro phosphorylation assay showed both autophosphorylation of CRPK1 and phosphorylation of 14-3-3 λ by CRPK1 (Figure 3E).

To determine whether the kinase activity of CRPK1 is important for its biological function, we mutated the conserved Lys69, which is required for the activities of other kinases (Li and Nam, 2002), to Glu to generate a kinase-dead form of CRPK1 (CRPK1^{K69E}). The CRPK1^{K69E} failed to show either autophosphorylation or phosphorylation of 14-3-3 λ (Figure 3E). We then introduced the *CRPK1:CRPK1^{K69E}* construct into *crpk1-1*. As expected, the mutated transgene failed to rescue freezing tolerance of *crpk1-1* (Figures 1A–1C and S2D–S2F) or cold induction of CBF target genes (Figures 1D, 1E, S1J, and S1K). These results suggest that the kinase activity of CRPK1 is essential for its function in regulating freezing tolerance.

To investigate the potential phosphorylation sites of 14-3-3 λ by CRPK1, we performed mass spectrometry and found that Thr214 of 14-3-3 λ could be phosphorylated by CRPK1 (Figure S2G). Ser70 and Ser193 are conserved in 14-3-3 proteins in plants and animals (de Boer et al., 2013), and Ser112 was previously identified as a phosphorylation site of 14-3-3 in plant by mass spectrometry (de Boer et al., 2013). Therefore, we replaced these Ser and Thr residues of 14-3-3 λ with Ala (A) residue, producing 14-3-3 λ ^{S70A}, 14-3-3 λ ^{S112A}, 14-3-3 λ ^{S193A}, 14-3-3 λ ^{T214A}, and 14-3-3 λ ^{S70AS112AS193AT214A} (14-3-3 λ ^{4A}), to mimic non-phosphorylation. In vitro phosphorylation assays showed that CRPK1-mediated phosphorylation of 14-3-3 λ was reduced to

varying degrees when containing one point mutation. When these four amino acids were mutated to Ala, the phosphorylation was largely abolished (Figure 3F). As a control, the S61A mutation did not obviously affect 14-3-3 λ phosphorylation (Figure 3F). These results suggest that Ser70, Ser112, Ser193, and Thr214 of 14-3-3 λ are the major phosphorylated residues recognized by CRPK1.

Translocation of 14-3-3 Proteins to the Nucleus Requires Cold-Mediated CRPK1 Phosphorylation

Considering that CRPK1 is a plasma membrane protein and phosphorylates 14-3-3 proteins under cold stress, we next asked whether 14-3-3 proteins play a role in transducing the cold signal to the nucleus. We first examined whether cold stress altered the subcellular localization of 14-3-3 proteins. To this end, we generated transgenic plants expressing *Super:14-3-3 λ -GFP* in wild-type Col, and then we crossed *Super:14-3-3 λ -GFP* plant with *crpk1-1* mutant to generate *Super:14-3-3 λ -GFP crpk1-1* plants. We observed the 14-3-3 λ -GFP signals in both the cytoplasm and nucleus but predominantly in the cytoplasm of *Super:14-3-3 λ -GFP* root cells at 22°C. Surprisingly, strong GFP signals were observed in the nuclei of root cells after 4°C treatment (Figure 4A). In contrast, 14-3-3 λ -GFP signals were mainly detected in the cytoplasm of root cells at both 22°C and 4°C in the *crpk1-1* mutant (Figure 4B). These results suggest that cold stress induces translocation of the 14-3-3 λ protein in the nucleus, which requires CRPK1.

We also generated transgenic plants expressing *Super:14-3-3 λ ^{4A}-GFP* (non-phosphorylatable mutated form) and *Super:14-3-3 λ ^{4D}-GFP* (phosphomimic mutated form) transgenic plants. The 14-3-3 λ ^{4A}-GFP and 14-3-3 λ ^{4D}-GFP proteins were also majorly localized in the cytoplasm at 22°C, which was not obviously affected by cold treatment (Figures S3A and S3B), indicating that cold-induced nuclear import of 14-3-3 λ is disrupted when mutated at its CRPK1-mediated phosphorylation sites, whereas the 14-3-3 λ ^{4D} mutation may not mimic the corresponding phosphorylated form of 14-3-3 λ . Consistent with these results, cell fractionation assays showed that cold stress did not affect the total amount of 14-3-3 proteins in wild-type Col and *crpk1-1* mutant (Figures 4C and 4D). However, the nuclear portion of 14-3-3 proteins or 14-3-3 λ was dramatically increased in cold-treated wild-type compared with the untreated one (Figures 4C and S3C). In contrast, *crpk1-1* mutant showed a similar nucleo-cytoplasmic distribution of 14-3-3 proteins and 14-3-3 λ before and after cold treatment (Figures 4D and S3D). These data further demonstrate that CRPK1 phosphorylates 14-3-3 proteins to facilitate their nuclear translocation under cold stress.

(D) In-gel kinase assay of CRPK1 under cold stress. The 2-week-old wild-type Col and *crpk1-1* were treated at 4°C for the indicated time, and total protein extracts were separated on an SDS-PAGE gel containing 0.3 mg/mL 14-3-3 λ as a substrate. The autoradiograph of the gel is shown in the top panel, and HSP90 is shown as an equal loading control. The arrow shows intensity of CRPK1 activity and the asterisk shows nonspecific band.

(E) CRPK1 phosphorylates 14-3-3 λ in vitro. The purified recombinant MBP-His-CRPK1 or MBP-His-CRPK1^{K69E} and His-14-3-3 λ were incubated in protein kinase buffer with [γ -³²P]ATP and separated on 10% SDS-PAGE. The autoradiograph of the gel is shown in the upper panel, and the Coomassie brilliant blue-stained proteins are shown in the lower panel.

(F) Identification of 14-3-3 λ phosphorylation sites by CRPK1. Recombinant mutated His-14-3-3 λ proteins were incubated with MBP-His-CRPK1 as described in (E). Phosphorylated 14-3-3 λ proteins were visualized by autoradiography after gel electrophoresis.

See also Table S1 and Figure S2.

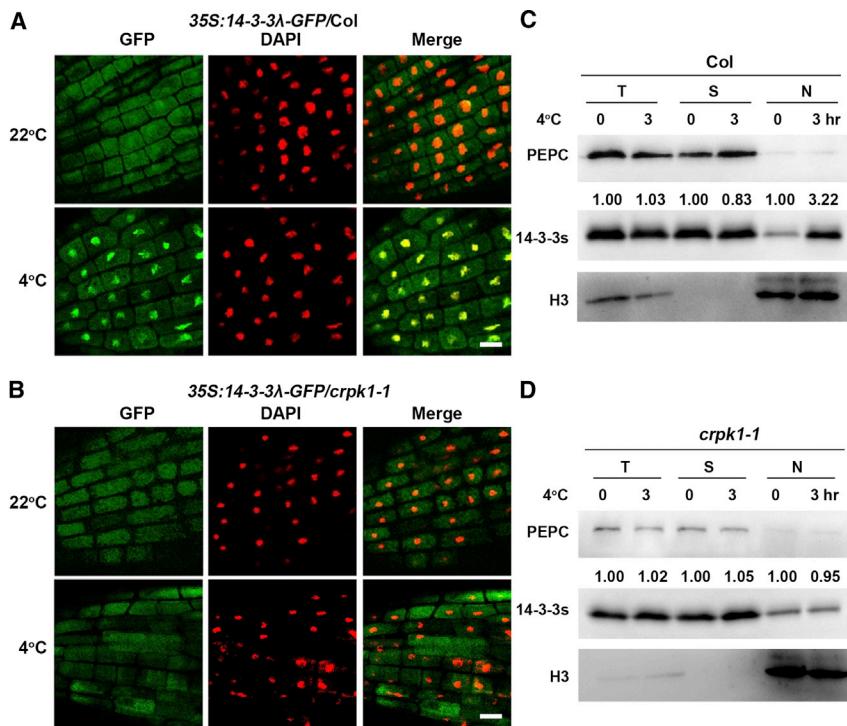


Figure 4. Cold Stress Induces 14-3-3 Translocation from the Cytosol into the Nucleus

(A and B) Subcellular localization of 14-3-3 λ in Col (A) and *crpk1-1* mutant (B) under cold stress. The 6-day-old transgenic plants expressing *Super:14-3-3λ-GFP* in Col and *crpk1-1* mutant were treated at 4°C for 0 and 3 hr. GFP signals in roots were visualized under confocal microscopy. DAPI was used to stain nuclei (pseudo-color, red). Scale bar, 20 μ m.

(C and D) Immunoblot analysis of 14-3-3 protein localization in Col (C) and *crpk1-1* mutant (D) under cold stress. Total (T) proteins were excreted from 12-day-old wild-type Col and the *crpk1-1* mutant. Nuclear (N) and soluble (S) proteins were separated by CellLytic PN Isolation/Extraction kit, and proteins were detected by immunoblotting with an anti-14-3-3 antibody. Anti-PEPC and anti-H3 antibodies were used as cytosolic and nuclear markers, respectively. The relative intensity of nuclear 14-3-3 proteins to H3 or total (soluble) 14-3-3 proteins to PEPC without cold treatment was set to 1.00.

See also Table S1 and Figure S3.

14-3-3 Proteins Negatively Regulate Plant Freezing Tolerance

Given that CRPK1 modulates freezing tolerance and cold-activated CRPK1 phosphorylates 14-3-3 λ to facilitate its nuclear import, we hypothesized that 14-3-3 proteins might function in regulating freezing tolerance. To test this hypothesis, we examined the freezing tolerance of single and double mutants of 14-3-3 λ and 14-3-3 κ , two close homologs in the 14-3-3 gene family. There was no significant difference in freezing tolerance or ion leakage between wild-type and 14-3-3 λ or 14-3-3 κ single mutants (Figures S4A–S4C). However, the 14-3-3 $\kappa\lambda$ double mutant showed enhanced freezing tolerance and decreased ion leakage compared with wild-type (Figures 5A–5C and S4A–S4C). *CBF3*, one of three *CBF* genes, was mildly upregulated, while all *COR* genes tested were dramatically upregulated in the 14-3-3 $\kappa\lambda$ double mutant compared to the wild-type plants (Figure 5D). A wild-type genomic fragment of 14-3-3 λ (14-3-3 λ :14-3-3 λ) fully complemented the freezing-tolerant phenotypes of the 14-3-3 $\kappa\lambda$ double mutant (Figures 5A–5C and S4A–S4D).

Two independent transgenic lines overexpressing 14-3-3 λ (OX1 and OX2) (Zhou et al., 2014) were also used for freezing tolerance assay. Both lines showed increased freezing sensitivity and decreased cold induction of *CBF3* and CBF-regulated *COR* genes (Figures 5E–5H and S5), indicating that the 14-3-3 proteins negatively regulate freezing tolerance.

CRPK1 Is Required for the Function of 14-3-3 Proteins in Freezing Tolerance

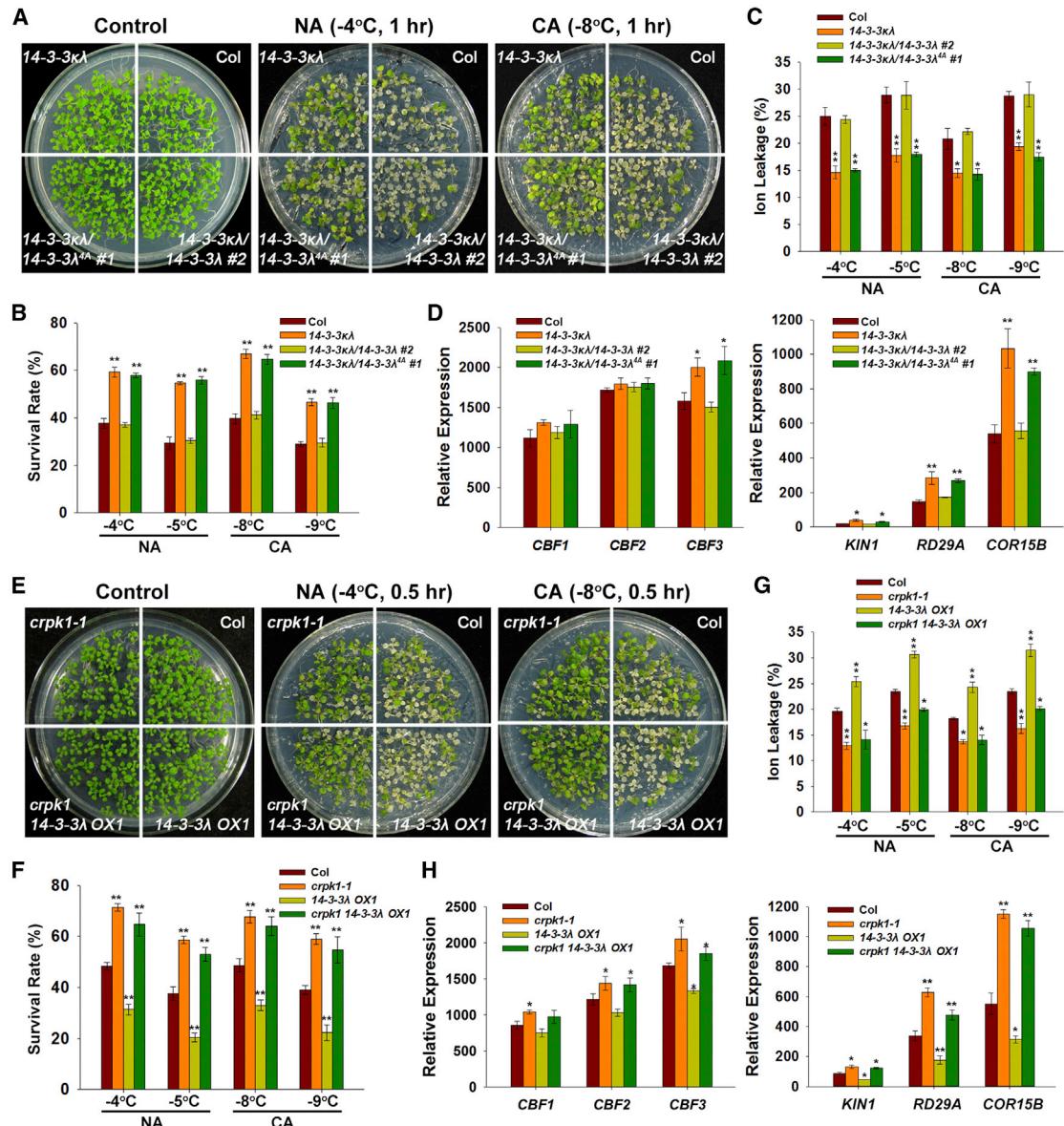
To test whether 14-3-3 phosphorylation is required for their function in freezing tolerance, we introduced 14-3-3 λ :14-3-3 λ^{4A} mutated form into 14-3-3 $\kappa\lambda$ double mutant, and we found that

14-3-3 λ :14-3-3 λ^{4A} could not rescue the freezing-tolerant phenotypes or expression of CBF regulons of the 14-3-3 $\kappa\lambda$ mutant (Figures 5A–5D and S4D–S4G). In addition, the freezing tolerance of the 14-3-3 $\kappa\lambda$ mutant was not rescued by 14-3-3 λ^{4D} (Figures S4D–S4G), supporting the notion that the 14-3-3 λ^{4D} mutation cannot mimic the 14-3-3 λ phosphorylated form.

To further dissect the requirement for CRPK1 for the function of 14-3-3 proteins in plant freezing tolerance, we generated *crpk1* 14-3-3 λ OX1 plants by crossing *crpk1-1* and 14-3-3 λ OX1 plants. The *crpk1-1* plants were freezing tolerant, whereas the 14-3-3 λ OX1 plants behaved like *crpk1-1* in terms of freezing tolerance and expression of CBF target genes (Figures 5E–5H). Collectively, these data further demonstrate that the function of 14-3-3 λ in regulating plant freezing tolerance requires CRPK1.

14-3-3 Proteins Interact with CBF Proteins

We next explored the biological relevance of the translocation of 14-3-3 proteins from the cytosol to the nucleus under cold stress. As cold induction of CBF target genes was affected in 14-3-3 $\kappa\lambda$ double mutant, this prompted us to speculate that 14-3-3 proteins may function via an association with CBF proteins. In addition, in immunoprecipitation/mass spectrometry assays using cold-treated *Super:CBF1-Myc* transgenic plants, we identified several 14-3-3 proteins, including 14-3-3 ϵ , 14-3-3 μ , 14-3-3 ν , 14-3-3 χ , 14-3-3 λ , 14-3-3 ν , and 14-3-3 ψ , as CBF1-interacting proteins (Table S2). Therefore, we tested the possibility of interaction between CBF proteins and 14-3-3 proteins. As a result, we found that 14-3-3 λ could pull down CBF3, and the mutations of 14-3-3 λ^{4A} and 14-3-3 λ^{4D} did not affect their direct interaction *in vitro* (Figure 6A).

**Figure 5. 14-3-3 Proteins Negatively Regulate Plant Freezing Tolerance**

(A–C) Freezing tolerance assays on the 14-3-3 $\kappa\lambda$ double mutant and complemented lines with wild-type 14-3-3 λ and 14-3-3 λ^{4A} driven by the native 14-3-3 κ promoter (14-3-3 $\kappa\lambda/\lambda$ and 14-3-3 $\kappa\lambda/\lambda^{4A}$), under NA and CA conditions. Representative pictures are shown (A), the survival rates were counted (B), and ion leakage was measured (C).

(D) Expression of CBFs and their target genes in the 14-3-3 $\kappa\lambda$ double mutants and the 14-3-3 $\kappa\lambda/\lambda^{4A}$ and 14-3-3 $\kappa\lambda/\lambda^{4A}$ complemented lines under cold stress.

(E–G) Freezing phenotype (E), survival rate (F), and ion leakage (G) of the crpk1-1, 35S:FLAG-HA-14-3-3 λ (14-3-3 λ OX1), and crpk1 14-3-3 λ OX1 plants under NA and CA conditions.

(H) Expression of CBFs and their target genes in the crpk1-1, 14-3-3 λ OX1, and crpk1 14-3-3 λ OX1 plants under cold stress.

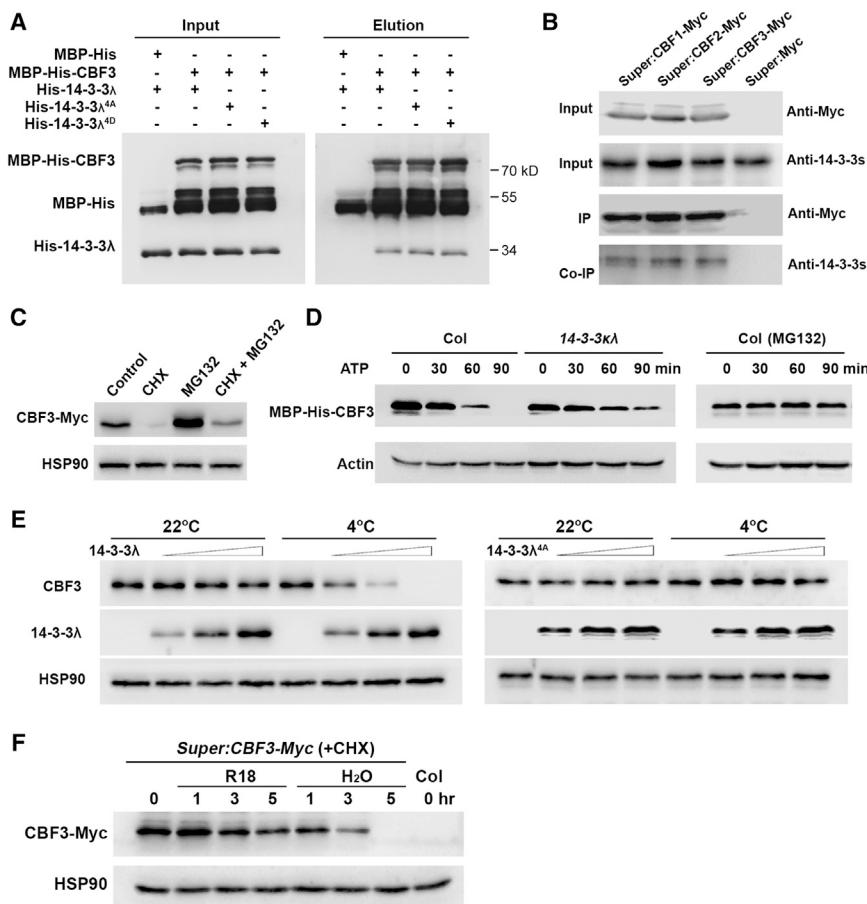
The data in (B)–(D) and (F)–(H) are shown as means of three replicates \pm SD, and the asterisks indicate significant differences by Student's t test (*p < 0.05 and **p < 0.01) when compared to the wild-type.

See also Table S1 and Figures S4 and S5.

Yeast two-hybrid assays verified the interaction of CBF1 and CBF3 with 14-3-3 proteins (Figure S6A). CoIP assays using transgenic plants expressing Super:CBFs-Myc (Figures 6B and S6B) confirmed the interaction of CBF proteins and 14-3-3 proteins in vivo.

14-3-3 Proteins Facilitate the Ubiquitin-Mediated Degradation of CBF1 and CBF3

We further assessed the significance of interaction between 14-3-3 proteins and CBF proteins. When characterizing CBF proteins, we found that CBF proteins were subjected to



(F) The effect of R18 on CBF3 degradation in planta. The 12-day-old seedlings of *Super:CBF3-Myc* were treated with 300 μM CHX and 20 μg/ml R18 or water for the indicated time, and total proteins were extracted and subjected to immunoblotting with an anti-Myc antibody. HSP90 was used as a control. See also Tables S1 and S2 and Figure S6.

degradation by the 26S proteasome pathway. CBFs-Myc levels in *Super:CBFs-Myc* transgenic seedlings decreased after 300-μM cycloheximide (CHX, an inhibitor of protein synthesis) treatment and increased after 50-μM MG132 (a proteasome inhibitor) treatment, and the effect of CHX was partially suppressed by MG132 (Figures 6C and S6C). Furthermore, cell-free degradation assays showed that CBF proteins were degraded in the presence of ATP, and this degradation was inhibited by MG132. Intriguingly, the degradation of CBF1 and CBF3, but not CBF2, was dramatically suppressed in the 14-3-3 $\kappa\lambda$ double mutant (Figures 6D and S6D), suggesting that the destabilization of CBF1 and CBF3 may be mediated by 14-3-3 κ and 14-3-3 λ .

We next examined whether 14-3-3 proteins affect the stability of CBF proteins in plant. To this end, we transformed increasing amounts of 35S:FLAG-HA-14-3-3 λ plasmid into *Arabidopsis* protoplasts isolated from *Super:CBFs-Myc* transgenic plants. With increasing expression of 14-3-3 λ , the levels of CBF1 and CBF3 gradually decreased under cold treatment. However, when we expressed increasing amounts of 14-3-3 λ ^{AA} in *Arabidopsis* protoplasts, the protein levels of CBF1 and CBF3 remained unchanged (Figures 6E and S6E). In contrast, CBF2

protein level was not affected by 14-3-3 λ (Figure S7E). R18 peptide has been reported to disrupt functional 14-3-3 interactions (Wang et al., 1999). When treated with R18, the degradation of CBF1 and CBF3, but not CBF2, significantly decreased in *Super:CBFs-Myc* transgenic plants (Figures 6F and S6F), further validating the role of 14-3-3 proteins in the degradation of CBF1 and CBF3 proteins.

The stability of CBF3 in wild-type and *crpk1-1* mutant was also examined. When the same amount of *Super:CBF3-Myc* and increasing amounts of *Super:14-3-3λ-GFP* were co-expressed into the wild-type Col protoplasts, CBF3 protein levels were decreased under cold stress. In contrast, CBF3 protein levels were unchanged in the *crpk1-1* protoplasts (Figure S6G). Together, these results suggest that 14-3-3 proteins negatively regulate the stability of CBF1 and CBF3, and phosphorylation of 14-3-3 λ by CRPK1 is required to promote the destabilization of CBF1 and CBF3.

CRPK1 and 14-3-3 Proteins Act Upstream of CBF Proteins to Regulate Cold Signaling

To examine the effect of 14-3-3 proteins on CBF protein levels in planta, we generated *CBF1:CBF1-Myc 14-3-3κλ* and

Figure 6. 14-3-3 Proteins Facilitate the Ubiquitin-Mediated Degradation of CBF3

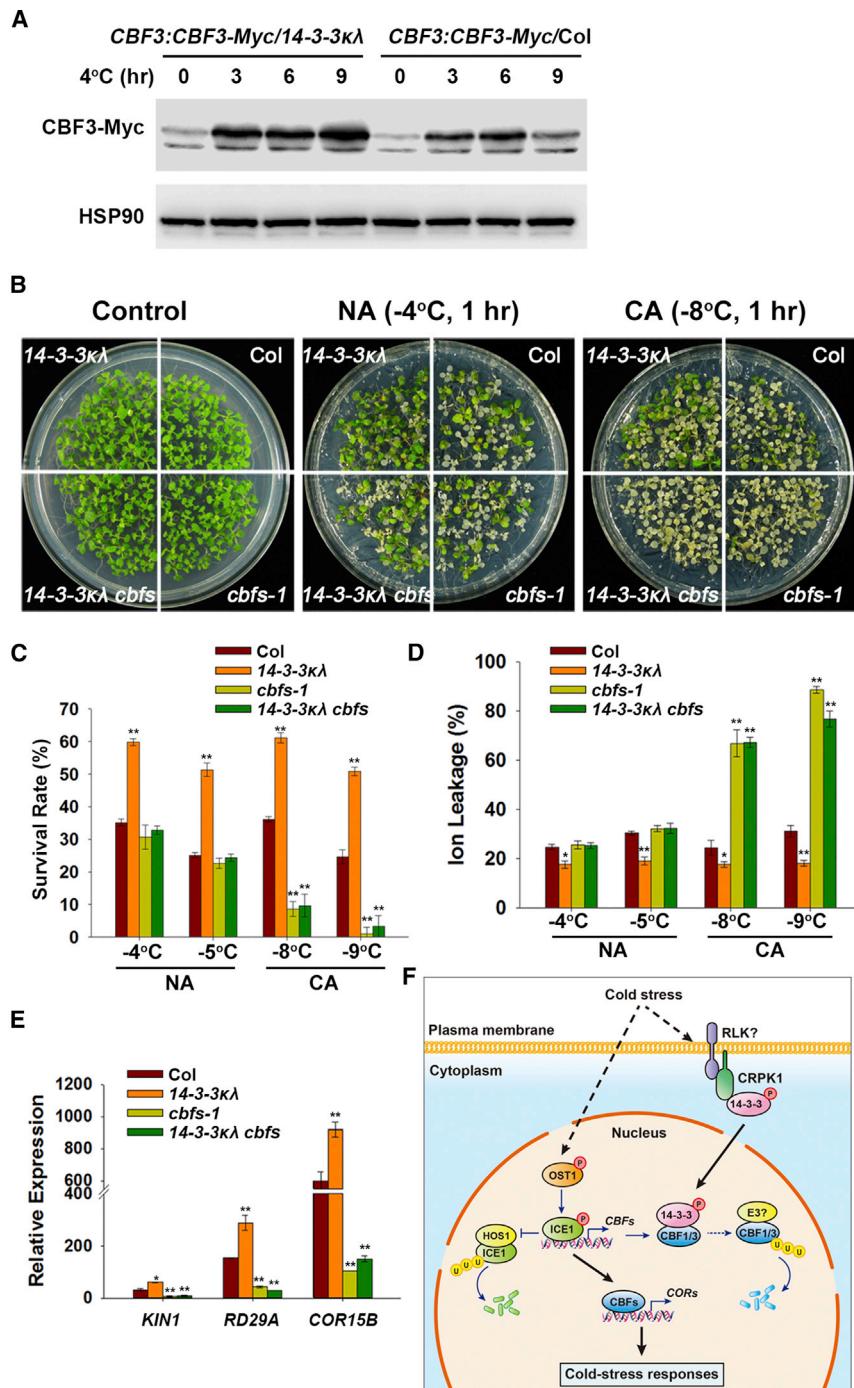
(A) In vitro pull-down assay showed the interaction of 14-3-3 λ with CBF3. The purified MBP-His-CBF3 or MBP-His protein was incubated with His-14-3-3 λ variant proteins. After being immunoprecipitated with MBP-beads, the proteins were detected with an anti-His antibody.

(B) CoIP assay showing the interaction of CBF proteins with 14-3-3 proteins in planta. Total proteins were extracted from 2-week-old *Super:CBF-Myc* seedlings, immunoprecipitated with an anti-Myc antibody, and detected with an anti-14-3-3s antibody.

(C) Immunoblot analysis of CBF3 protein in 2-week-old *Super:CBF3-Myc* seedlings treated with 300 μM CHX and/or 50 μM MG132 for 3 hr. CBF3 was detected with an anti-Myc antibody. HSP90 was used as a loading control.

(D) In vitro cell-free degradation assays. Recombinant purified MBP-His-CBF3 was incubated with total protein extracted from wild-type Col (treated with or without 50 μM MG132 for 3 hr) and the 14-3-3 $\kappa\lambda$ double mutant in the presence of ATP. CBF3 was detected with an anti-His antibody. Actin was used as a control.

(E) CBF3 protein is destabilized by 14-3-3 λ . Increasing amounts (0, 10, 20, and 30 μg, respectively) of 35S:Flag-HA-14-3-3 λ or 35S:Flag-HA-14-3-3 λ ^{AA} were transformed into protoplasts derived from the transgenic plants expressing *Super:CBF3-Myc* and incubated for 12 hr, followed by 22°C or 4°C for an additional 2 hr. CBF3 was detected with an anti-HA antibody and 14-3-3 λ with an anti-HA antibody. HSP90 was used as a control.



CBF3:CBF3-Myc 14-3-3 κ L plants by crossing *CBF1:CBF1-Myc* or *CBF3:CBF3-Myc* with the *14-3-3 κ L* mutant. The basal level of CBF1 was higher in *14-3-3 κ L* mutant than in the wild-type Col, which accounts for the increased basal freezing tolerance of *14-3-3 κ L* mutants. Cold induction of CBF3 protein was much higher in *14-3-3 κ L* mutants than in wild-type. Moreover, CBF3 protein levels in wild-type decreased at 9 hr of cold treatment, but they remained at high levels in the *14-3-3 κ L* mutant (Figure 7A). These data are consistent

Figure 7. Genetic Interaction of 14-3-3 and CBF Genes

(A) CBF3 accumulation in wild-type Col and the *14-3-3 κ L* double mutant under cold stress. The 13-day-old seedlings of *CBF3:CBF3-Myc 14-3-3 κ L* were treated at 4°C for the indicated time, and total proteins were extracted and subjected to immunoblotting with an anti-Myc antibody. HSP90 was used as a control.

(B–D) Freezing phenotype (B), survival rate (C), and ion leakage (D) of the *14-3-3 κ L cbfs* quintuple mutant.

(E) Expression of CBF target genes in the *crpk1 cbfs* and *14-3-3 κ L cbfs* mutants under cold stress.

(F) Model for CRPK1 and 14-3-3 proteins in regulating the CBF-signaling pathway. The unknown RLK might be involved in cold signal perception and phosphorylates CRPK1 under cold stress. The cold-activated CRPK1 phosphorylates 14-3-3 proteins, which translocate from the cytosol to the nucleus to promote proteasome-mediated degradation of CBF1 and CBF3, thus negatively regulating COR expression and freezing tolerance.

The data in (C)–(E) are shown as means of three replicates \pm SD, and the asterisks indicate significant differences by Student's t test (*p < 0.05 and **p < 0.01) when compared to the wild-type. See also Figure S7.

with the cold upregulation of CBF target COR genes in the *14-3-3 κ L* mutant (Figure 5D).

To dissect the genetic interaction of CRPK1, 14-3-3s, and CBFs, we generated *crpk1 cbfs* and *14-3-3 κ L cbfs* mutants by crossing *crpk1-1* and *14-3-3 κ L* with the *cbf1 cbf2 cbf3* (*cbfs-1*) triple mutant (Jia et al., 2016). The *crpk1-1* and *14-3-3 κ L* mutants are freezing tolerant, and cold-acclimated *cbfs-1* mutant is hypersensitive to freezing stress (Jia et al., 2016) (Figures 7B, 7C, and S7). The *crpk1 cbfs* and *14-3-3 κ L cbfs* mutants displayed increased freezing sensitivity and ion leakage but decreased expression of CBF target genes compared with the wild-type, as did the *cbfs-1* mutant (Figures 7B–7E and S7). These results reveal that

CRPK1 and 14-3-3 proteins act upstream of CBF proteins to negatively regulate plant freezing tolerance.

DISCUSSION

CBF genes are critical transcription factors for plant cold acclimation, and their transcriptional regulation has been extensively studied (Agarwal et al., 2006; Chinnusamy et al., 2003; Doherty et al., 2009; Shi et al., 2012); however, how the cold signal

transduces from the plasma membrane to the nuclear CBF proteins and how CBF proteins are regulated are largely unknown. Here we have shown that the CBF proteins are regulated at the posttranslational level via signal transduction from the plasma membrane. Cold stress activates the plasma membrane-localized protein kinase CRPK1, which phosphorylates 14-3-3 proteins in the cytoplasm, thereby triggering 14-3-3 proteins to translocate into the nucleus. In the nucleus, phosphorylated 14-3-3 proteins form a protein complex with CBF proteins to promote the 26S proteasome-mediated degradation of CBF proteins, thus attenuating the CBF signaling (Figure 7F).

In-gel kinase assays showed that CRPK1 is activated by cold stress (Figure 3). An intriguing question is how CRPK1 is activated by cold stress, as this seems to be directly responsible for adjusting plant freezing tolerance. CRPK1 belongs to the CRLK family, and it is localized to the plasma membrane under normal and cold conditions (Figure 2). However, CRPK1 lacks a classic transmembrane domain, as the protein kinase BRI1 kinase inhibitor 1 (BKI1) does. BKI1 interacts with the BR receptor BRI1 and inhibits BRI1 autophosphorylation in the absence of BR. However, when active BR binds to BRI1, BRI1 auto- or *trans*-phosphorylates itself, and then it promotes BKI1 phosphorylation, thus enhancing BKI1 dissociation from the plasma membrane and BRI1 (Wang and Chory, 2006). This prompts us to propose that CRPK1 might form a complex with a cold-stimulated receptor-like kinase (RLK) to coordinate cold signal perception. When the plant senses low temperature by an unidentified RLK, the autophosphorylation of this RLK would be activated, and then it would phosphorylate downstream targets to result in activation of the cold response. Meanwhile, prolonged cold stress would lead to activation of a negative feedback loop via CRPK1 activation, as overexpression of *CBFs* has dramatic negative impact on plant growth (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). In this case, CRPK1 would ensure proper adjustment of the intensity and/or duration of the cold stress response that would need to match the intensity and/or duration of the initial cold stimulus. Further investigation of the activation mechanism of CRPK1 will help us to understand cold perception in plant.

In this study, we found that 14-3-3 proteins are important intermediators that perceive and transduce the phosphorylation signal from the plasma membrane to the nucleus. The 14-3-3 proteins play prominent roles in many aspects of plant growth and development, as well as biotic and abiotic responses (Catalá et al., 2014; Cotelle et al., 2000; de Boer et al., 2013; Gampala et al., 2007; Gardino and Yaffe, 2011; Wang et al., 2011). They have been considered to function by binding to their target proteins to alter their functions (de Boer et al., 2013). Moreover, 14-3-3 proteins are phosphorylated by some protein kinases, such as SnRK2.8 and WPK4 (an SNF1-related wheat protein kinase) in plant (Ikeda et al., 2000; Shin et al., 2007). Our findings show that cold-activated CRPK1 phosphorylates 14-3-3 λ in plant (Figure 3). Most of the identified phosphorylation sites in 14-3-3 proteins have been shown in the flexible C terminus after the last α helix (de Boer et al., 2013), which can act as an auto-inhibitor and determinant for binding to their target proteins (Shen et al., 2003; Sinnige et al., 2005). We found that Ser70, Ser112, Ser193, and Thr214 are the major phosphorylated residues of 14-3-3 λ by

CRPK1 (Figure 3), among which the Ser70 and Ser193 sites of 14-3-3 λ are conserved phosphorylation sites in human 14-3-3 proteins (de Boer et al., 2013), and Thr214 is in the C terminus, suggesting the importance of CRPK1 on 14-3-3 λ function. In agreement with this notion, cold-induced nuclear import of 14-3-3 λ is dependent on CRPK1 (Figure 4). Therefore, 14-3-3 proteins play important roles in connecting the CRPK1-mediated membrane signal to the nucleus.

Under cold stress, the phosphorylated 14-3-3 proteins translocate to the nucleus, interacting with CBF proteins, and they promote 26S proteasome-mediated degradation of CBF proteins (Figures 4 and 6). Consistently, cold induction of CBF target genes is upregulated in *crpk1* and 14-3-3 $\kappa\lambda$ mutants (Figures 1 and 5). As cold induction of CBF genes, especially *CBF3* gene, is also mildly upregulated in *crpk1* and 14-3-3 $\kappa\lambda$ mutants, it is possible that the CRPK1 pathway also has a minor impact on the CBF transcription regulation, which remains to be determined. The 14-3-3 proteins have three high-affinity binding motifs: RSXpS/TXP (mode 1), RXXXpS/TXP (mode 2), and pS/T-X(1-2)-COOH (mode 3) (Yaffe et al., 1997). Unfortunately, we did not find any canonical binding motifs in the CBF proteins. Emerging studies have shown that 14-3-3 proteins can also bind to atypical motifs of some proteins and even can bind a non-phosphorylated motif (de Boer et al., 2013). Two criteria are used to identify a potential interaction site for 14-3-3 partners: putative binding sites may coincide with phosphorylation sites or disordered regions (de Boer et al., 2013). In animal, the most 14-3-3 protein-binding motifs are within disordered regions. Disorder enhances molecular recognition and protein-protein interaction, thus promoting proteins to interact with diverse partners (Bustos, 2012). By using PONDR-FIT (<http://www.disprot.org/>), we detected the structure of CBF proteins and found that the N and C termini of CBFs are disordered, suggesting that 14-3-3 proteins might interact with CBF proteins through these regions. Nevertheless, we cannot exclude that CBF proteins are phosphorylated by cold stress and, in turn, interact with 14-3-3 proteins. It will be interesting to define the 14-3-3 interaction domain and phosphorylation sites of CBF proteins. Furthermore, identification of the protein kinase(s) and E3 ligase(s) that phosphorylate and degrade CBF proteins, respectively, will shed more light on the molecular mechanism of CBF protein modifications.

Cold-induced CBF protein accumulation is compromised by the plasma membrane CRPK1-mediated phosphorylation of 14-3-3 proteins, indicating a negative feedback function in cold signal transduction from the plasma membrane. This mechanism helps plants to optimize their growth and stress resistance to the respective environmental conditions. Indeed, many studies have shown that the negative feedback regulation mechanisms are important for plant adaptation. Abscisic acid promotes S-nitrosylation of SnRK2.6, which negatively feedback regulates abscisic acid signaling (Wang et al., 2015). Cold stress inhibits ethylene production, but it promotes the accumulation of EIN3, a positive transcription factor in the ethylene-signaling pathway, to repress the expression of *CBFs* during cold acclimation (Shi et al., 2012). In this study, CRPK1 and 14-3-3 proteins play negative roles to prevent excessive cold responses, which allows plants to fine-tune stress

responses and developmental processes during changing environmental conditions.

STAR★METHODS

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

- KEY RESOURCES TABLE
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 - Generation of Multiple Mutants
 - Physiological Assays
 - qRT-PCR Assay
 - GFP Fluorescence Assay
 - Biochemistry
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2017.02.016>.

AUTHOR CONTRIBUTIONS

Z.L. and S.Y. designed the study. Z.L., Y.J., Y.D., Y.S., and Z.L. performed the experiments. Z.L., Y.J., Y.D., Y.S., Y.G., Z.G., and S.Y. analyzed the data. Z.L., Y.D., and S.Y. wrote the paper.

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STAR★METHODS**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Myc	Sigma-Aldrich	Cat#M4439
Mouse monoclonal anti-HA	Sigma-Aldrich	Cat#H3663
Mouse monoclonal anti-GFP	Abmart	Cat#M20004M
Mouse monoclonal anti-MBP	Beijing Protein Innovation	Cat#AbM59007-3-PU
Mouse monoclonal anti-His	Beijing Protein Innovation	Cat#AbM59012-18-PU
Mouse monoclonal anti-HSP90	this paper	N/A
Rabbit polyclonal anti-Actin	Easybio	Cat#BE0027
Rabbit polyclonal anti-14-3-3	Santa Cruz	Cat#SC-33752; RRID: AB_J1508
Rabbit polyclonal anti-PEPC	Agrisera	Cat#AS09 458; RRID: AB_1312
Mouse monoclonal anti-Histone H3	Millipore	Cat#05-499; RRID: AB_2787688
Mouse polyclonal anti-AHA2	this paper	N/A
Bacterial and Virus Strains		
BL21(DE3)pLysS	TIANGEN	Cat#CB106-02
AH109	Towfly	Cat#6453471VEC
NMY51	Dualsystems Biotech	Cat#P01001
Chemicals, Peptides, and Recombinant Proteins		
R18 peptides	Sigma-Aldrich	Cat#SML0108
Anti-c-Myc agarose beads (affinity gel)	Sigma-Aldrich	Cat#A7470
GFP-Trap® A	ChromoTek	Cat#gta-20
Ni Sepharose 6 Fast Flow	GE Healthcare	Cat#17-5318-01
MG132	Sigma-Aldrich	Cat#C2211
Cocktail	Roche	Cat#04693132001
DAPI	Beyotime Biotechnology	Cat#C1002
Phos-tag Acrylamide AAL-107	Nard Institute, Ltd	Cat#AAL-107
Critical Commercial Assays		
CellLytic PN Isolation/Extraction kit	Sigma-Aldrich	Cat#CELLYTPN1
Titansphere Phos-TiO Kit	GL Sciences	Cat#5010-21309
DUAL membrane kit3	Dualsystems Biotech	Cat#P01001
pUC-SPYNE/pUC-SPYCE for BiFC	Walter et al., 2004	N/A
Experimental Models: Organisms/Strains		
<i>Arabidopsis: CRPK1:CRPK1</i>	this paper	N/A
<i>Arabidopsis: CRPK1:CRPK1^{K69E}</i>	this paper	N/A
<i>Arabidopsis: CRPK1:CRPK1-GFP</i>	this paper	N/A
<i>Arabidopsis: Super:14-3-3λ^{4A}-GFP</i>	this paper	N/A
<i>Arabidopsis: Super:14-3-3λ^{4D}-GFP</i>	this paper	N/A
<i>Arabidopsis: 14-3-3λ:14-3-3λ</i>	this paper	N/A
<i>Arabidopsis: 14-3-3λ:14-3-3λ^{4A}</i>	this paper	N/A
<i>Arabidopsis: 14-3-3λ:14-3-3λ^{4D}</i>	this paper	N/A
<i>Arabidopsis: 35S:Flag-HA-14-3-3λ</i>	this paper	N/A
<i>Arabidopsis: Super:14-3-3λ-GFP</i>	this paper	N/A
<i>Arabidopsis: Super:CBF1-Myc</i>	this paper	N/A
<i>Arabidopsis: Super:CBF2-Myc</i>	this paper	N/A
<i>Arabidopsis: Super:CBF3-Myc</i>	this paper	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Arabidopsis</i> : <i>CBF1:CBF1-Myc</i>	Jia et al. (2016)	N/A
<i>Arabidopsis</i> : <i>CBF3:CBF3-Myc</i>	Jia et al. (2016)	N/A
<i>Arabidopsis</i> : 14-3-3 λ	Zhou et al. (2014)	N/A
<i>Arabidopsis</i> : 14-3-3 λ OX1	Zhou et al. (2014)	N/A
<i>Arabidopsis</i> : 14-3-3 λ OX2	Zhou et al. (2014)	N/A
<i>Arabidopsis</i> : <i>crpk1-1</i> (SALK_004253C)	ABRC	N/A
<i>Arabidopsis</i> : <i>crpk1-2</i> (SALK_054771)	ABRC	N/A
<i>Arabidopsis</i> : <i>cbf1 cbf2 cbf3 (cbfs-1, cbfs)</i>	Jia et al. (2016)	N/A
<i>Arabidopsis</i> : 14-3-3 λ cbfs	this paper	N/A
<i>Arabidopsis</i> : <i>crpk1 14-3-3λ OX1</i>	this paper	N/A
Oligonucleotides		
real-time PCR primers <i>CRPK1-qF</i> : GCTAGAC TTATGCCACCTAAC	Beijing Genomics Institute	Custom order
real-time PCR primers <i>CRPK1-qR</i> : CCGCATA CTCTGGTGTCTAAA	Beijing Genomics Institute	Custom order
<i>CRPK1-PBT3-F</i> : GGCCATTACGGCCATGGG TTGCTCCTGGTTATC	Beijing Genomics Institute	Custom order
<i>CRPK1-PBT3-R</i> : GGCGGAGGCAGGCCTATGG TACTGCTGAATGTA	Beijing Genomics Institute	Custom order
14-3-3 λ - <i>PPR3-F</i> : GGCCATTACGGCCCGGAT GGCAGCGACATTAGG	Beijing Genomics Institute	Custom order
14-3-3 λ - <i>PPR3-R</i> : GGCGGAGGCAGGCCTTAC ATAGAGTAGTAAAC	Beijing Genomics Institute	Custom order
other primers see Table S1	Beijing Genomics Institute	Custom order
Recombinant DNA		
<i>CRPK1</i> ^{G2A} -GFP	this paper	N/A
<i>CRPK1</i> -GFP	this paper	N/A
<i>His-14-3-3λ</i>	Zhou et al. (2014)	N/A
<i>His-14-3-3λ^{4A}</i>	this paper	N/A
<i>His-14-3-3λ^{4D}</i>	this paper	N/A
<i>MBP-His-CBF</i>	Jia et al. (2016)	N/A
<i>MBP-His-CRPK1</i>	this paper	N/A
<i>MBP-His-CRPK1</i> ^{K69E}	this paper	N/A
Software and Algorithms		
TMHMM Server v. 2.0	http://www.cbs.dtu.dk/services/TMHMM/	
PONDR-FIT	http://www.disprot.org/	
ImageJ	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).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis thaliana ecotype Col-0 plants were used in this study. Plants were grown at 22 ± 1°C under a 16/8 hr light/dark photo-period at 100 μmol m⁻² s⁻¹, with 50%–70% relative humidity on half-strength Murashige-Skoog (1/2 MS) plastic plates containing 1.5% sucrose and 0.8% agar.

METHOD DETAILS

Plasmid Construction and Plant Transformation

CRPK1 cDNA was fused with GFP, Myc, His and MBP in the pSuper1300 (pCAMBIA1300 vector containing a Super promoter, which consists of three copies of the octopine synthase upstream-activating sequence in front of the manopine synthase promoter) (Ni et al., 1995; Yang et al., 2010), and pMalC2 vectors to generate the *Super:CRPK1-GFP*, *Super:CRPK1^{G2A}-GFP* (carrying a G2A mutation in CRPK1), *Super:CRPK1-Myc* and *MBP-His-CRPK1* constructs. A *CRPK1* genomic fragment including the sequence from 1580 bp upstream of the *CRPK1* translation start codon to 196 bp downstream of the stop codon was amplified and cloned into pCAMBIA1300 to generate *CRPK1:CRPK1*, *CRPK1:CRPK1^{K69E}* (carrying a K69E mutation of CRPK1), and *CRPK1:CRPK1-GFP* (fused with GFP in the C terminus of CRPK1).

A full-length 14-3-3 λ genomic fragment was PCR-amplified and fused with GFP or Flag-HA in the pSuper1300, pCM1307, pCAMBIA1300, and PET28a vectors to generate *Super:14-3-3 λ -GFP*, *Super:14-3-3 λ ^{4A}-GFP*, *Super:14-3-3 λ ^{4D}-GFP*, *14-3-3 λ :14-3-3 λ* , *14-3-3 λ :14-3-3 λ ^{4A}*, *14-3-3 λ :14-3-3 λ ^{4D}*, *35S:Flag-HA-14-3-3 λ* , and *His-14-3-3 λ* .

The cDNAs of *CBF* were fused with Myc or MBP-His in the pSuper1300, pCAMBIA1300 and pMalC2 vectors to generate *Super:CBF-Myc*, *CBF:CBF-Myc* and *MBP-His-CBF* constructs.

The resulting vectors were transformed into *Arabidopsis* plants by floral dip (Clough and Bent, 1998). *Super:CRPK1-Myc* was transformed to the wild-type Col. *CRPK1:CRPK1*, *CRPK1:CRPK1^{K69E}*, and *CRPK1:CRPK1-GFP* constructs were transformed into the *crpk1-1* mutant. *Super:14-3-3 λ -GFP*, *Super:14-3-3 λ ^{4A}-GFP*, and *Super:14-3-3 λ ^{4D}-GFP* constructs were transformed into the wild-type Col. *14-3-3 λ :14-3-3 λ* , *14-3-3 λ :14-3-3 λ ^{4A}*, and *14-3-3 λ :14-3-3 λ ^{4D}* constructs were transformed into the *14-3-3 $\kappa\lambda$* double mutant. T3 or T4 homozygous transgenic plants were used in this study.

Generation of Multiple Mutants

The *crpk1 cbfs*, *14-3-3 $\kappa\lambda$ cbfs*, and *crpk1 14-3-3 λ OX1* plants were generated by crossing, and homozygous lines were genotyped and used for further study.

Physiological Assays

Freezing Tolerance Assays

The freezing tolerance assays were performed as described (Shi et al., 2012). Briefly, the 13-d-old seedlings were treated with or without cold acclimation at 4°C for 3 d and then put into a freezing chamber that was programmed to drop 1°C per hour from 1°C to the desired temperature. Then the seedlings were kept in the dark at 4°C for 12 hr and grown for an additional 3 d at 22°C before counting the survival rates.

Ion Leakage Assays

The ion leakage assays were performed as described (Shi et al., 2012). *Arabidopsis* seedlings were treated with low temperatures described in the figure legends and put into 15 mL tubes containing 5 mL deionized water (sample S0), followed by shaking for 15 min and then detecting (S1). The samples were subsequently boiled at 100°C for 15 min, followed by shaking at 22°C for 1 hr, and then detecting (S2). The ratio of S1-S0 to S2-S0 was calculated as ion leakage.

qRT-PCR Assay

Total RNA was extracted from 12-d-old plants with TRIzol reagent (Invitrogen) and was reverse transcribed with M-MLV reverse transcriptase (Promega). SYBR Green PCR Master Mix kit (Takara) was used to perform the quantitative real-time PCR. The reaction system and relative expression levels were performed as described (Shi et al., 2012). Briefly, relative transcript abundance was calculated using the comparative C_T method. For a standard control, expression of *ACTIN2/8* was used. After calculation of ΔC_T ($C_{T,\text{gene of interest}} - C_{T,\text{actin2/8}}$), $\Delta\Delta C_T$ [$\Delta C_T - \Delta C_{T,\text{WT(0 h)}}$] was calculated as instructed. The relative expression level was calculated as $2^{-\Delta\Delta C_T}$. A $2^{-\Delta\Delta C_T}$ value for the wild-type without cold treatment (0 h) was normalized to 1 [$2^{-(\Delta\Delta C_T(\Delta C_{T,\text{WT(0 h)} - \Delta C_{T,\text{WT(0 h)})}))} = 2^0 = 1$].

GFP Fluorescence Assay

For localization of CRPK1 in protoplasts, *Super:CRPK1-GFP* and *35S:FLS2-mCherry* (marker for plasma membrane) or *Super:CRPK1^{G2A}-GFP* and *35S:FLS2-mCherry* plasmids were co-transformed into *Arabidopsis* mesophyll protoplasts as described (Yoo et al., 2007). *35S:GFP* was used as a control. The GFP signal was visualized under confocal microscope (Leica sp5, Germany) after 16 hr incubation at 22°C.

7-d-old stable transgenic plants expressing *CRPK1:CRPK1-GFP* were treated with or without 4°C for 3 hr, and GFP signal in root tips was visualized using a confocal microscope. FM4-64 was used to stain plasma membrane.

Super:14-3-3 λ -GFP stable transgenic plants in wild-type Col and *crpk1-1* mutant background, *Super:14-3-3 λ ^{4A}-GFP* and *Super:14-3-3 λ ^{4D}-GFP* transgenic plants were used to show the subcellular localization of 14-3-3 λ at 22°C and 4°C. 6-d-old plants were treated with or without 4°C for 3 hr, and the GFP signal in roots was visualized under confocal microscope.

Biochemistry**Immunoblot Analysis**

For the CRPK1-GFP immunoblots, 12-d-old *CRPK1:CRPK1-GFP* transgenic seedlings were treated at 4°C for 0, 3, 6, 9, or 12 hr. The CRPK1-GFP fusion proteins were visualized on immunoblots using an anti-GFP antibody (Abmart).

For the CBF immunoblots, 12-d-old or 14-d-old *Super:CBFs-Myc* plants were treated with 300 μM CHX and/or 50 μM MG132 for 3 hr in Figure 6C, or treated with 20 μg/ml R18 (Sigma-Aldrich) or H₂O in 1/2 liquid MS for different hours as described (Yoon and Kieber, 2013) in Figure 6F, or they were treated at 4°C for various hours as shown in Figure 7A. The CBFs-Myc fusion proteins were visualized on immunoblots using an anti-Myc antibody (Sigma-Aldrich).

Cell Fractionation Assays

The procedure for membrane fractionation assays was performed as described (Hua et al., 2001). Briefly, 12-d-old transgenic plants of *CRPK1:CRPK1-GFP* grown in 1/2 MS medium were extracted in 0.5 mL fractionation extraction buffer (50 mM Tris pH 8.0, 2 mM EDTA, 20% glycerol, 1 mM DTT, 0.1% Triton X-100, and 1 × inhibitor cocktail). Homogenate was filtered through Miracloth (Calbiochem) and 50 μL was saved as total protein, and then the remainder was centrifuged at 5000 g for 5 min to remove organelles and debris. Supernatants were centrifuged at 100,000 g for 1 hr to precipitate membranes. Supernatants were used for the soluble sample, and membrane pellets were re-suspended in 450 μL protein extraction buffer. For western blotting, total proteins (T) and soluble fractions (S) were 1 × concentrated compared with membrane protein extracts (M).

Preparation of nuclear and cytoplasmic fractions was performed as described (Bao et al., 2014). Nuclear fractions (N) were 10-fold more concentrated, compared with soluble fractions (S) and total proteins (T). 14-3-3 proteins were detected using anti-14-3-3 antibodies (Santa Cruz). Anti-PEPC (Agrisera) and anti-H3 (Millipore) antibodies (Agrisera) were used as cytosolic and nuclear markers, respectively. ImageJ software was used for quantification of bands in the immunoblot. The relative intensity of nuclear 14-3-3s to H3, or total (soluble) 14-3-3 s to PEPC without cold treatment was set to 1.00.

Yeast Two-Hybrid Assays

A DUAL membrane yeast two-hybrid system (Dualsystems Biotech) was used to determine the interaction between CRPK1 and 14-3-3 proteins. The full-length 14-3-3s were cloned into the pPR3 vector, and full length CRPK1 was cloned into the pBT3-STE vector, and the resulting vectors were transformed into the yeast strain NMY51. Yeast growth is shown on SC-/Trp-/Leu or SC-/Trp-/Leu-/His-/Ade medium. pAl-Alg5 were used as positive prey control and pDL2-Alg5 were used as negative prey control. The pCCW-Alg5 control bait was used to carry out a bait dependency test.

For the interaction of 14-3-3s and CBF1 or CBF3, full length 14-3-3s were cloned into pGADT7 vector and full length *CBF1* and *CBF3* was cloned into pGBKT7, and the resulting vectors or empty vectors were transformed into the yeast strain AH109. Interactions were shown on SC-/Trp-/Leu or SC-/Trp-/Leu-/His medium supplemented with 10 mM 3-AT.

In Vitro Pull-Down Assays

The pull-down assays were performed as described (Ding et al., 2015). In Figure 3A and 3 μg purified fusion protein His-14-3-3λ was incubated with immobilized 10 μg MBP and MBP-His-CRPK1 fusion proteins at 4°C for 2 hr and then detected with an anti-MBP or an anti-His antibody (Beijing Protein Innovation).

For the pull-down assay in Figure 5A and 3 μg of His-14-3-3λ or His-14-3-3λ^{4A} or 14-3-3λ^{4D}-His protein was incubated with 5 μg of immobilized MBP-CBF3 at 4°C for 2 hr. Proteins retained on the beads were analyzed by immunoblotting with an anti-MBP or anti-His antibody.

Bimolecular Fluorescence Complementation (BiFC) Assays

BiFC assays were performed using *Arabidopsis* protoplasts as described (Bao et al., 2014). The CRPK1 coding sequence was fused in frame to the C terminus of YFP^N in pUC-SPYNE (Walter et al., 2004), and 14-3-3λ CDS was fused in frame to the C terminus of YFP^C in the pUC-SPYCE vector. After 16 hr of co-transformation into *Arabidopsis* protoplasts, the YFP signal was visualized using a confocal microscope.

Co-Immunoprecipitation Assays

The co-immunoprecipitation assays were performed as described (Ding et al., 2015). Transgenic plants of *CRPK1:CRPK1-GFP/35S:Flag-HA-14-3-3λ* and *Super:GFP/35S:Flag-HA-14-3-3λ* were used to detect the interaction of CRPK1 and 14-3-3λ. The *Super:CBFs-Myc* transgenic plants were used for the interaction of 14-3-3λ while CBFs and the *Super:Myc* empty vector transgenic plants were used as the negative control. The total proteins extracted from stable transgenic plants were immunoprecipitated with anti-GFP agarose (ChromoTek) or anti-Myc agarose (Sigma-Aldrich). The immunoprecipitates were separated on a 10% SDS-PAGE gel and detected with corresponding antibodies. Signals were visualized with X-ray film or Fusion Solo (Vilber Lourmat).

Kinase Assays

The in vitro phosphorylation assay was performed as described (Ding et al., 2015). Briefly, 5 μg purified fusion 14-3-3λ-His protein or 14-3-3λ^{4A}-His or 2 μg MBP and 1 μg MBP-His-CRPK1 or MBP-His-CRPK1^{K69E} were incubated in protein kinase assay buffer (50 mM HEPES pH 7.5, 5 mM MgCl₂, 1 mM DTT, 1 μCi [γ -³²P]ATP and 10 mM cold ATP). The phosphorylated proteins were analyzed by 10% SDS-PAGE and detected by Typhoon 9410 imager. Coomassie brilliant blue was used as a loading control.

In-gel kinase assays were performed as described (Ding et al., 2015) with some modifications. Briefly, total proteins were extracted from Col and *crpk1-1* treated with or without 4°C in protein extraction buffer [50 mM HEPES-KOH, pH 7.5, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, 25 mM NaF, 1 mM Na₃VO₄, 20% glycerol, 0.1% Triton X-100, and 1 × protease inhibitor cocktail (Roche)], or total proteins of *CRPK1-Myc* transgenic plant with or without 4°C were immunoprecipitated with anti-Myc agarose beads (Sigma-Aldrich). The

proteins were separated by 8% SDS-PAGE gel containing 0.3 mg/mL 14-3-3 λ as substrate. The gel was washed three times with washing buffer (1 mM DTT, 5 mM NaF, 0.1 mM Na₃VO₄, 0.5 mg/mL BSA, 0.1% Triton X-100, and 25 mM Tris-HCl, pH 7.5) for 20 min each, and proteins were renatured three times with buffer (2 mM DTT, 5 mM NaF, 0.1 mM Na₃VO₄, and 25 mM Tris-HCl, pH 7.5) for 1 hr, 12 hr and 1 hr at 4°C. After being incubated with kinase reaction buffer (2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 0.1 mM Na₃VO₄, and 25 mM HEPES-KOH, pH 7.5) for 30 min, the gel was incubated with 30 mL kinase reaction buffer supplemented with 70 μ Ci [γ -³²P]ATP and 8 μ L cold ATP (1 mM) at room temperature for 2 hr and then washed with 5% TCA and 1% sodium pyrophosphate five times for 30 min each. Radioactivity was detected by Typhoon 9410 imager.

Phos-tag Assays

Phos-tag assays were performed as described (Mao et al., 2011). In brief, 35S:Flag-HA-14-3-3 λ plasmid was transformed into protoplasts of Col and crpk1-1, which were cultured at 22°C for 16 hr, treated at 4°C for 0 and 3 hr, and then treated with or without calf intestinal alkaline phosphatase (CIAP) at 37°C for 30 min. The proteins were electrophoresed on an 8% SDS-PAGE gel supplemented with 50 μ M Phos-tag and 200 μ M MnCl₂, the gel was then incubated in the transfer buffer containing 10 mM EDTA three times, and washed in transfer buffer for 10 min. After transferred onto PVDF membrane, Flag-HA-14-3-3 λ protein was detected with an anti-HA antibody (Sigma-Aldrich).

Cell-Free Protein Degradation Assays

Cell-free protein degradation assays were performed as described (Ding et al., 2015). Purified His-CBF recombinant proteins were incubated with total proteins extracted from the wild-type Col and 14-3-3 $\kappa\lambda$ plants in the presence of ATP at 25°C for different time courses, and His-CBF proteins were detected by immunoblotting with an anti-HA antibody.

Mass Spectrometry Assays

To prepare samples for identifying putative phosphorylation site of 14-3-3 λ by mass spectrometry, 1 μ g MBP-His-CRPK1 and 10 μ g His-14-3-3 λ purified protein were incubated in 20 μ L of protein kinase assay buffer (50 mM HEPES pH 7.5, 5 mM MgCl₂, 1 mM DTT, and 10 mM cold ATP) at 30°C for 25 min. The reaction mixture was reduced by DTT, alkylated by iodoacetamide (IAM) and digested by trypsin overnight at 37°C. Then phosphopeptides were enriched using the Titansphere Phos-TiO Kit (GL Sciences) according to manufacturer's protocol. The resulting phosphopeptides were analyzed by LC-MS/MS.

To prepare samples for identifying interacting proteins by mass spectrometry, 100 mg total proteins extracted from Super:CBF1-Myc stable transgenic plants were immunoprecipitated with anti-Myc agarose (Sigma-Aldrich), then CBF1-Myc proteins were eluted by elution buffer (0.1 M Glycine-HCl, pH 2.8) and neutralized by 1 M Tris buffer. The samples were enriched by ultrafiltration tube (Millipore). The resulting proteins were used for LC-MS/MS analysis.

LC-MS/MS analysis was performed as follows: 100 μ g proteins were dissolved in 30 μ L 50 mM NH₄HCO₃ solution, the proteins were reduced with DTT and alkylated with IAM. The resulted proteins were incubated with trypsin (pH 8.5) at 37°C overnight. The resulted peptide was diluted with 0.1% formic acid, and centrifuged at 12000 rpm for 20 min. The supernatant was collected for nanoLC-MS analysis. Nanospray ESI-MS was performed on a Thermo Q-Exactive high resolution mass spectrometer (Thermo Scientific). Raw data from the mass spectrometer were preprocessed with Mascot Distiller 2.4 and the peak list was searched against TAIR 10.0 database. The search parameters are: Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Decoy database: Yes, Enzyme: Trypsin, Max. missed cleavages: 2, MS mass tolerance: 10 ppm, MSMS mass tolerance: 0.02 Da.

QUANTIFICATION AND STATISTICAL ANALYSIS

Band intensity quantification of 14-3-3 proteins was performed using the ImageJ. Statistic significance of survival rates, ion leakage and gene expression was examined by Student's t test (*p < 0.05, **p < 0.01).

DATA AND SOFTWARE AVAILABILITY

The accession number for the CRPK1 reported in this paper is the *Arabidopsis* Genome Initiative or GenBank/EMBL databases: At1g16670. Images and other data are available at Mendeley (<http://dx.doi.org/10.17632/cz9xbwprny.1>).