CPK28-NLP7 module integrates cold-induced Ca\(^{2+}\) signal and transcriptional reprogramming in Arabidopsis

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Exposure to cold triggers a spike in cytosolic calcium (Ca\(^{2+}\)) that often leads to transcriptional reprogramming in plants. However, how this Ca\(^{2+}\) signal is perceived and relayed to the downstream cold signaling pathway remains unknown. Here, we show that the CALCIUM-DEPENDENT PROTEIN KINASE 28 (CPK28) initiates a phosphorylation cascade to specify transcriptional reprogramming downstream of cold-induced Ca\(^{2+}\) signal. Plasma membrane (PM)–localized CPK28 is activated rapidly upon cold shock within 10 seconds in a Ca\(^{2+}\)-dependent manner. CPK28 then phosphorylates and promotes the nuclear translocation of NIN-LIKE PROTEIN 7 (NLP7), a transcription factor that specifies the transcriptional reprogramming of cold-responsive gene sets in response to Ca\(^{2+}\), thereby positively regulating plant response to cold stress. This study elucidates a previously unidentified mechanism by which the CPK28-NLP7 regulatory module integrates cold-evoked Ca\(^{2+}\) signal and transcriptome and thus uncovers a key strategy for the rapid perception and transduction of cold signals from the PM to the nucleus.

INTRODUCTION

With climate change, temperature extremes are becoming more frequent, which have severe negative consequences on plant physiology and biochemistry. To survive, plants have evolved exquisite thermosensory systems to perceive and react to both low and high temperatures, thereby adapting their metabolism, growth, and architecture to their immediate environmental conditions.

Calcium (Ca\(^{2+}\)) is a ubiquitous and evolutionarily conserved second messenger that plays a critical role in cold responses of plants and animals (1, 2). Cold induces the transient elevation of Ca\(^{2+}\) levels in the cytosol ([Ca\(^{2+}\)\(_{\text{cyt}}\)] (referred to as the Ca\(^{2+}\) signature hereafter), a well-established phenomenon that is considered to be one of the earliest signaling events in response to cold (1, 2). The channel TRANSIENT RECEPTOR POTENTIAL POTENTIAL (TRP) MELASTATIN 8 (TRPM8) mediates the perception of cold and triggers a cold-induced Ca\(^{2+}\) signature in mouse (Mus musculus) (3, 4). In plants, CHILLING TOLERANCE DIVERGENCE 1 (COLD1), a membrane-localized protein identified from rice (Oryza sativa), works together with G protein α subunit 1 (RGA1) to elicit the cold-induced Ca\(^{2+}\) influx and confers cold sensing, although the underlying mechanism is unknown (5). Several Ca\(^{2+}\) transporters and channels, including annexin 1 (AtANN1), MID1-COMPLEMENTING ACTIVITY 1 (MCA1), MCA2, and CYCLIC NUCLEOTIDE-GATED CHANNEL 9 (OsCNGC9), were shown to involve cold-evoked Ca\(^{2+}\) influx in Arabidopsis (Arabidopsis thaliana) and rice (6–8).

Pharmacological, genetic, and genomic evidence indicate that cold-induced Ca\(^{2+}\) signatures are likely perceived by putative Ca\(^{2+}\) sensors and decoded by downstream targets of the Ca\(^{2+}\) signal within cold regulatory networks, thereby resulting in transcriptional reprogramming in Arabidopsis (9). Members of the C-REPEAT BINDING FACTOR/DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1 (CBF/DREB1) family are key transcription factors that act upstream of COLD-RESPONSIVE (COR) genes to induce their expression and promote cold tolerance (10, 11). Arabidopsis CBF genes (CBF1–CBF3) are rapidly induced by cold stress (12), which is at least partially dependent on Ca\(^{2+}\) (7). Previous studies showed that the expression of CBF genes is indirectly modulated by Ca\(^{2+}\)/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE 1/2 (CRLK1/2) (13, 14) and directly regulated by the members of the CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR (CAMTA) family of transcription factors (15, 16). While the involvement of CRLK1/2 and CAMTAs in plant cold responses has been established, it is unclear whether they confer transcriptional reprogramming triggered by cold-induced Ca\(^{2+}\) signals.

Plants have three major families of calcium sensors: CALMODULIN (CaM), CALCINEURIN B-LIKE (CBL), and CALCIUM-DEPENDENT PROTEIN KINASE/CALCIUM-SENSOR PROTEIN KINASES (CDPKs/CPKs) (17). Unlike CaM and CBL that must relay Ca\(^{2+}\) by diffusing to their immediate environmental conditions.

In this study, we report that cold shock rapidly activates the Ca\(^{2+}\)-dependent activity of CPK28 in plants. CPK28 phosphorylates the transcription factor NIN-LIKE PROTEIN 7 (NLP7), leading to its translocation from the cytosol to the nucleus in a Ca\(^{2+}\)-dependent

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manner upon cold stress. In the nucleus, NL7 plays an important role in plant responses to cold stress and COR gene expression (1); however, it is unclear how Ca$^{2+}$ signaling is relayed into cold signaling. CPKs have both Ca$^{2+}$ sensing and responding function (23). To examine the possibility that CPKs might transmit the cold-induced Ca$^{2+}$ signal, we conducted quantitative polymerase chain reaction (qPCR) to analyze the expression of CPK genes in a rapid response to cold stress. We observed that the two CPK genes CPK28 and CPK32 were induced by cold after cold treatment for 1 hour, with the cold induction of CPK28 being much more pronounced than that of CPK32 (Fig. 1A). Therefore, we determined the freezing phenotypes of cpk28 mutants (24). Both cpk28-1 and cpk28-3 knockout mutants displayed decreased freezing tolerance compared with the wild type under both nonacclimated (NA) and cold-acclimated (CA) conditions (Fig. 1B and C, and fig. S1A). The freezing-sensitive phenotype of the cpk28-1 mutant was fully rescued by the introduction of a genomic copy of CPK28 cloned in-frame with the coding sequence of green fluorescent protein (CPK28pro:CPK28-GFP) (Fig. 1B and C, and fig. S1B). To further explore the function of CPK28 in regulating freezing tolerance, we generated transgenic plants overexpressing CPK28 with a Myc tag driven by the Super promoter (25) (Super:CPK28-Myc, CPK28-OE); these CPK28-OE plants exhibited enhanced freezing tolerance compared with the wild type (Fig. 1D, E, and F, and fig. S1, C to E). These results thus demonstrate that CPK28 positively regulates plant tolerance to cold stress.

**RESULTS**

**CPK28 positively regulates plant freezing tolerance**

Ca$^{2+}$ plays an important role in plant responses to cold stress and COR gene expression (1); however, it is unclear how Ca$^{2+}$ signaling is relayed into cold signaling. CPKs have both Ca$^{2+}$ sensing and responding function (23). To examine the possibility that CPKs might transmit the cold-induced Ca$^{2+}$ signal, we conducted quantitative polymerase chain reaction (qPCR) to analyze the expression of CPK genes in a rapid response to cold stress. We observed that the two CPK genes CPK28 and CPK32 were induced by cold after cold treatment for 1 hour, with the cold induction of CPK28 being much more pronounced than that of CPK32 (Fig. 1A). Therefore, we determined the freezing phenotypes of cpk28 mutants (24). Both cpk28-1 and cpk28-3 knockout mutants displayed decreased freezing tolerance compared with the wild type under both nonacclimated (NA) and cold-acclimated (CA) conditions (Fig. 1B and C, and fig. S1A). The freezing-sensitive phenotype of the cpk28-1 mutant was fully rescued by the introduction of a genomic copy of CPK28 cloned in-frame with the coding sequence of green fluorescent protein (CPK28pro:CPK28-GFP) (Fig. 1B and C, and fig. S1B). To further explore the function of CPK28 in regulating freezing tolerance, we generated transgenic plants overexpressing CPK28 with a Myc tag driven by the Super promoter (25) (Super:CPK28-Myc, CPK28-OE); these CPK28-OE plants exhibited enhanced freezing tolerance compared with the wild type (Fig. 1D, E, and F, and fig. S1, C to E). These results thus demonstrate that CPK28 positively regulates plant tolerance to cold stress.

Cold rapidly activates CPK28 in a Ca$^{2+}$-dependent manner

Because CPK28 harbors four conserved EF-hand motifs, we performed microscale thermophoresis (MST) assays, and found that CPK28 did bind to Ca$^{2+}$ (Fig. 2A), which is consistent with the previous finding (26). As CPK28 contains four EF-hand motifs at its C-terminal domain, we determined whether these EF-hands of CPK28 are important for its Ca$^{2+}$ binding by mutating Asp to Ala at each EF-hand to obtain CPK28<sub>EFm</sub> (D378A, D415A, D457A, and D487A) according to the previous study (27). CPK28<sub>EFm</sub> showed Ca$^{2+}$-binding activity sixfold lower than CPK28 (Fig. 2A). We also examined the Ca$^{2+}$-binding activity of truncated CPK28 (∆CPK28) lacking four EF-hands and found that it failed to bind Ca$^{2+}$ (fig. S2A). These results indicate that CPK28 is a Ca$^{2+}$ sensor in an EF-hand motif-dependent manner. Next, we investigated whether the kinase activity of CPK28 is regulated by cold using plant extracts incubated with a recombinant fusion protein between glutathione S-transferase and the kinase-dead version of BOTRYTIS-INDUCED KINASE 1 (GST-BIK1<sub>K105E</sub>) as a substrate (28) in an in-gel kinase assay. Compared to 22°C, cold shock (4°C) rapidly and strongly activated CPK28 kinase activity within 10 s of exposure in wild-type plants but not in the cpk28-1 mutant (Fig. 2B and C, and fig. S2B). This cold activation of CPK28 was blocked by the application of EGTA (Fig. 2B). To further confirm this result, we performed an in-gel kinase assay using immunoprecipitated CPK28 proteins extracted from Super:CPK28-Myc plants that were treated at 4°C for 10 s without EGTA or for 10 s after pretreatment with 25 mM EGTA for 4 hours. The result showed that CPK28 was apparently activated by cold treatment, and this activation was inhibited by EGTA (Fig. 2D).

In addition, neither the subcellular localization of CPK28 at the plasma membrane (PM) nor its protein stability was obviously affected by cold treatment (fig. S3, A and B). These data suggest that cold stress activates the Ca$^{2+}$-dependent activity of CPK28.

To dissect whether the Ca$^{2+}$-binding and kinase activities of CPK28 are required for its regulation of freezing tolerance, we transformed the cpk28-1 mutant with genomic fragments for CPK28<sub>EFm</sub> (lacking strong Ca$^{2+}$ binding) and CPK28<sub>D188A</sub> (the kinase-dead form of CPK28) (28) driven by the CPK28 promoter. Neither CPK28<sub>EFm</sub> nor CPK28<sub>D188A</sub> complemented the freezing sensitivity of cpk28-1 under both NA and CA conditions (Fig. 2E to H, and fig. S3, C to E). Therefore, the Ca$^{2+}$-binding and kinase activities of CPK28 are essential for its positive regulation of plant tolerance to freezing stress. These results suggest that CPK28 may act downstream of the cold-induced Ca$^{2+}$ signal to sense and transduce Ca$^{2+}$ signaling.

**NLP7 is a partner of CPK28 to positively regulate plant cold responses**

Considering that CPK28 localizes to the PM (fig. S3A), we hypothesized that CPK28 may target a component in the PM or the cytosol to decode the Ca$^{2+}$ signature in response to cold stress. To this end, we performed liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis using Super:CPK28-GFP transgenic plants treated at 4°C for 0.5 hours. We identified several previously reported CPK28 targets, such as METHIONINE ADENOSYLTRANSFERASE 1 (MAT1) and MAT3 (24), and 14-3-3 proteins (table S1) (29). Intriguingly, NLP7, a key transcription factor involved in nitrate response (20, 30, 31), was also identified with a high score, suggesting that it might form a complex with CPK28 (table S1).

To determine whether NLP7 physically interacts with CPK28, we performed in vitro pull-down assays. We established that GST-tagged CPK28 can bind to His-tagged NLP7, but not to its homologous protein NLP8 tagged with His (Fig. 3A). We extended these observations in planta with coimmunoprecipitation (co-IP) assays by transient infiltration of constructs encoding HA-FLAG (HF)–tagged CPK28 and NLP7-Myc or HF-CPK28 and NLP8-Myc in Nicotiana benthamiana leaves. We observed a clear interaction between NLP7-Myc and HF-CPK28, but not between NLP8-Myc and HF-CPK28 (Fig. 3B). We confirmed this result with a bimolecular fluorescence complementation (BiFC) assay in N. benthamiana leaves, which also revealed that CPK28 interacts with NLP7 around the PM (Fig. 3C). CPK3 or NLP8 was not found to interact with NLP7 and CPK28, respectively (Fig. 3C). Hence, CPK28 interacts with NLP7 in vitro and in vivo.

Since NLP7 forms a protein complex with CPK28 in vivo, we asked whether NLP7 is involved in modulating the cold response in plants. Freezing tolerance assays showed that nlp7-1 (32) and nlp7-4 (33) mutants are much more sensitive to freezing tolerance compared with the wild type under NA and CA conditions (Fig. 3, D and E, and fig. S4, A to C). Furthermore, the introduction of an NLP7 genomic fragment cloned in-frame with GFP and under the control of the NLP7 promoter (NLP7pro:NLP7-GFP) fully rescued the freezing sensitivity of the nlp7-1 mutant (Fig. 3, D and E, and fig. S4A), demonstrating that the impaired freezing tolerance seen in nlp7 mutants is indeed caused by the loss of NLP7 function. By contrast,
overexpressing NLP7 with a Myc tag enhanced the freezing tolerance of the transgenic seedlings compared with the wild type (Fig. 3, F to H, and fig. S4, D to F). Thus, NLP7 may work together with CPK28 to regulate plant responses to cold stress.

Cold induces the relocation of NLP7 to the nucleus in a Ca\(^{2+}\)-dependent manner

To further investigate the role of NLP7 in plant tolerance to cold stress, we examined the NLP7 expression and NLP7 protein stability before or after cold treatment. The transcript and protein levels of NLP7 did not appear to obviously change at early stages of cold exposure (5, 15, 30, and 45 min) (fig. S5, A and B). Previous studies have demonstrated that nitrate triggers the nuclear retention of NLP7 (20, 34). NLP7-GFP mainly localized in the cytosol under nitrogen starvation conditions, while nitrate triggers the nuclear retention of NLP7-GFP (20, 34). These findings prompted us to determine whether the subcellular localization of NLP7 was affected by low temperature in root cells of stable NLP7pro:NLP7-GFP transgenic plants. When seedlings were continually grown on half-strength Murashige and Skoog (MS) medium at 22°C, we detected NLP7-GFP fluorescence in both the cytoplasm and the nuclei of root cells, with the cytosolic signal being predominant in most root cells (Fig. 4A). However, we observed strong fluorescence signals for NLP7-GFP mainly in the nuclei after cold treatment at 4°C for 15 min (Fig. 4A). EGTA treatment markedly suppressed the cold-induced accumulation of NLP7 in the nucleus (Fig. 4A). These results suggest that cold induces the translocation of NLP7 from the cytoplasm to the nucleus in a Ca\(^{2+}\)-dependent manner.

To further validate this result, we monitored the nuclei-cytoplasmic partitioning of NLP7 in NLP7pro:NLP7-GFP transgenic seedlings using cell fractionation assays. Immunoblot analysis showed that ~75% of total NLP7 protein abundance was associated with the cytoplasm, with ~20% residing in the nucleus under normal conditions at 22°C (Fig. 4, B and C). However, treatment with cold (4°C) for 15 min led
to the predominant nuclear accumulation of NLP7 (representing ~75% of total NLP7), accompanied by its reduction in the cytosolic fraction (only ~25%) (Fig. 4, B and C). Further immunoblot analysis showed that the nuclear accumulation of NLP7 takes place within 5 min of cold treatment (Fig. 4, D and E), indicating that cold rapidly promotes NLP7 nuclear shuttling. Moreover, application of EGTA largely blocked the cold-induced nuclear accumulation of NLP7 (Fig. 4, D and E). The cold-triggered accumulation of NLP7 in the nucleus was not affected by treatment with cycloheximide (CHX; a protein synthesis inhibitor) (fig. S5, C and D), indicating that cold...
promotes the movement of preexisting cytosolic-localized NLP7 to the nucleus and that NLP7 nuclear trafficking is a direct response to cold stress. Together, these results demonstrate that nucleo-cytoplasmic partitioning of endogenous NLP7 is rapidly and dynamically regulated by low temperature in a Ca²⁺-dependent manner.

**CPK28 and Ca²⁺ are required for the nuclear shuttling of NLP7 upon cold stress**

As our results above indicated that NLP7 interacts with a kinase (Fig. 3, A to C), we asked whether NLP7 is a substrate for phosphorylation by CPK28. Accordingly, we performed in vitro phosphorylation assays using recombinant purified proteins and found that NLP7 is strongly phosphorylated by CPK28 (Fig. 5A). Moreover, we identified five potential residues (Ser⁷⁸³, Ser⁷⁹³, Ser⁸⁰⁷, Ser⁸⁰⁸, and Thr⁸¹⁷) adjacent to the Phox and Bem1 (PB1) domain [functions as a protein-protein interaction domain (31)] that might be phosphorylated by CPK28 based on LC-MS/MS assays (fig. S6, A to F). We then repeated the in vitro phosphorylation assays using truncated NLP7 (∆NLP7; comprising amino acids 778 to 853) containing Ala substitutions in place of these Ser or Thr residues. Compared to ∆NLP7,

Fig. 3. NLP7 is a partner of CPK28 to positively regulate freezing tolerance. (A) GST pull-down assay showing the interaction between His-NLP7 and GST-CPK28. Recombinant His-NLP7, His-NLP8, and GST-CPK28 were detected with anti-His and anti-GST antibodies, respectively. NLP8 was used as a control. (B) Co-IP assay showing the NLP7-CPK28 interaction in N. benthamiana leaves. The immunoprecipitated products were detected with anti-HA and anti-Myc antibodies, respectively. NLP8 was used as a control. (C) Interaction between NLP7 and CPK28 by BIFC assay. The construct combinations were cotransfected in N. benthamiana leaves. The interaction signal was detected by confocal microscopy after 18 hours of incubation. NLP8 and CPK3 were used as negative controls. Scale bars, 40 μm. (D) and (E) Freezing phenotypes (D) and survival rates (E) of wild-type, nlp7-1, and nlp7-1 NLP7pro:NLP7-GFP (nlp7 NLP7) seedlings. (F and G) Freezing phenotypes (F) and survival rates (G) of wild-type and Super:NLP7-Myc-overexpressing (NLP7-OE #2) plants. (H) NLP7 protein levels in seedlings of wild-type, nlp7-1, and NLP7-overexpressing lines (NLP7-OE #2 and #11). NLP7 was detected with anti-Myc antibody. Actin2 was used as a control. In (E) and (G), data are means ± SEM of three independent experiments, each with three technical replicates (*P < 0.05 and **P < 0.01, Student’s t test).
\[ \Delta NLP7^{S783A} \text{ single-mutant and } \Delta NLP7^{S807,808A} \text{ double-mutant versions of } \Delta NLP7 \text{ showed slightly decreased phosphorylation intensity, whereas other single mutants including } \Delta NLP7^{S793A} \text{ and } \Delta NLP7^{T817A} \text{ did not markedly affect their phosphorylation profile (Fig. 5B). However, when } \Delta NLP7^{S783,793,807,808A} (\Delta NLP7^{4A}), \text{ and } \Delta NLP7^{S783,793,807,808A,T817A} (\Delta NLP7^{5A}) \text{ were used as substrates, phosphorylation intensity drastically decreased in order, with the phosphorylation signal of } \Delta NLP7^{5A} \text{ nearly abolished (Fig. 5B). These data suggest that these five residues in NLP7 are phosphorylated by CPK28 in vitro. Further LC-MS/MS assay identified three of the above five residues (Ser793, Ser807, and Ser808) as being phosphorylated in NLP7pro:NLP7-GFP transgenic seedlings treated at 4°C for 30 min (fig. S6, G and H). We failed to detect phosphorylation sites of Ser783 and Thr817 in vivo, possibly because of their weak phosphorylation status in plants. Furthermore, we tested whether Ca\(^{2+}\) could enhance the kinase activity of CPK28 with modified 

Fig. 4. Cold promotes NLP7 nuclear shuttling dependently on Ca\(^{2+}\). (A to E) Effect of cold and EGTA on the localization of NLP7-GFP in root cells of NLP7pro:NLP7-GFP seedlings. Twelve-day-old seedlings were treated at 4°C for 15 min after pretreatment with or without 25 mM EGTA for 4 hours. Seedlings at 22°C were used as controls. (A) Representative images of NLP7-GFP localization at 22°C (left), after 4°C for 15 min (middle), and after EGTA and 4°C for 15 min (right). Scale bars, 50 μm. (B and D) Immunoblots of NLP7-GFP abundance. (C and E) Quantification of immunoblot signals shown in (B) and (D). NLP7-GFP was detected with anti-GFP antibody. Phosphoenolpyruvate carboxylase (PEPC) and histone H3 (H3) were used as loading controls for cytosolic and nuclear fractions, respectively. In (C), the amount of NLP7 (B) in total, soluble, and nuclear fractions divided by that of the relevant marker equaled \( R_T, R_S, \) and \( R_N \) respectively, and then \( R_T/R_T \) (the ratio was 1.0), \( R_S/R_T \), and \( R_N/R_T \) (relative protein level) were calculated. Total (T), soluble (S), and nuclear (N). In (E), the ratio of band intensity for NLP7 relative to H3 in untreated seedlings was set to 1.0. In (C) and (E), data are means ± SEM of three independent experiments (* \( P < 0.05 \) and ** \( P < 0.01 \), Student’s t test).
NLP7-GFP displayed a stable and low phosphorylation state before or after cold treatment in the *cpk28-1* mutant that was comparable to the phosphorylation state of NLP7 5A-GFP in seedlings expressing NLP7pro:NLP7 5A-GFP (Fig. 5D). Furthermore, the phosphorylation intensity of NLP7-GFP markedly decreased after the application of EGTA to NLP7pro:NLP7-GFP seedlings (Fig. 5F), indicating that the cold-induced NLP7 phosphorylation is Ca\(^{2+}\)-dependent. Therefore, NLP7 is phosphorylated by CPK28 in planta under cold stress in a Ca\(^{2+}\)-dependent manner.

Second, we examined NLP7-GFP fluorescence in NLP7pro:NLP7-GFP transgenic seedlings in the wild-type and *cpk28-1* mutant backgrounds to determine whether phosphorylated NLP7 by CPK28 rapidly shuttles from the cytosol to the nucleus in response to cold stress. Notably, we observed strong NLP7-GFP fluorescence in the nuclei of root cells of NLP7pro:NLP7-GFP seedlings after cold treatment, whereas most of the NLP7-GFP fluorescence remained in the cytoplasm of *cpk28-1* NLP7pro:NLP7-GFP seedlings (Fig. 6A), indicating that the cold-induced NLP7 phosphorylation is Ca\(^{2+}\)-dependent. Therefore, NLP7 is phosphorylated by CPK28 in planta under cold stress in a Ca\(^{2+}\)-dependent manner.

Last, we evaluated the function of NLP7 phosphorylation in regulating freezing tolerance. The freezing-sensitive phenotypes of the cold-induced nuclear import of NLP7 5A-GFP (Fig. 6, A to C). These results collectively support the notion that CPK28 phosphorylates NLP7 around the PM, which is quickly followed by their shuttling to the nucleus. However, it remains unknown how NLP7 proteins shuttle from the cytoplasm to the nucleus upon cold stress. As NLP7 has a possible NLS motif (Fig. S6A), we asked whether cold-triggered NLP7 nuclear relocation is associated with the NLS motif. To this end, we generated a construct harboring NLP7pro:NLP7(\(\Delta\)NLS)-GFP, in which the NLS domain was deleted, and expressed it in *N. benthamiana* leaves. We observed that NLP7(\(\Delta\)NLS)-GFP was still localized to the nucleus after cold treatment (fig. S7A), suggesting that cold-triggered NLP7 nuclear relocation may not be related to NLS motif. It is worthy to note that five phosphorylated residues are close to the PB1 domain (fig. S6A), which was reported to function as a protein-protein interaction domain (31). Therefore, it is possible that phosphorylation of NLP7 at these five residues may change NLP7 structure and disrupt the function of PB1 domain and thus inhibit the interaction of NLP7 and its nuclear transport proteins (e.g., importins) or the proteins that bind NLP7 to the cytoplasm. This hypothesis needs further investigation.
mutant were fully rescued by overexpressing wild-type NLP7 (nlp7 NLP7-OE lines #1 and #6), but not when NLP7S205A was overexpressed (nlp7 NLP 5A -OE #20 and #22) (Fig. 6D and fig. S7, B to D). These data suggest that phosphorylation of NLP7 is essential for regulating freezing tolerance in plants.

CPK10, CPK30, and CPK32 were previously shown to phosphorylate Ser205 of NLP7 in the nucleus in response to nitrate (20). We thus explored whether the phosphorylation of NLP7 mediated by CPK10, CPK30, and CPK32 might also participate in cold responses. To this end, we mutated Ser205 to Ala in NLP7 and introduced the resulting encoding construct into the nlp7-1 mutant, under the control of the Super promoter (fig. S8A). Overexpressing NLP7S205A in the nlp7-1 mutant failed to rescue the sensitivity of nlp7-1 to nitrate (fig. S8B) but fully restored the freezing sensitivity of nlp7-1 (fig. S8, C and D), suggesting that the phosphorylation of NLP7 by CPK10/30/32 is not required for its regulation of cold responses. The nitrate phenotype of nlp7-1 was fully rescued by overexpressing NLP7S2A (fig. S8B), indicating that the CPK28-mediated phosphorylation of NLP7 is not required for its function in nitrate responses. These results suggest that phosphorylation of NLP7 at different sites under different conditions (here, cold and nitrate) plays distinct roles, and the freezing sensitivity phenotype of nlp7-1 is not due to the nitrate deficiency.

**CPK28 is required for NLP7-mediated regulation of freezing tolerance**

To dissect the genetic hierarchy between CPK28 and NLP7, we crossed cpk28-1 with nlp7-1 to generate the cpk28 nlp7 double mutant. Freezing tolerance assays showed that the freezing sensitivity of cpk28 nlp7 is comparable to that of the nlp7-1 single mutant (Fig. 7, A and B). Next, we overexpressed NLP7-Myc in cpk28-1, which failed to rescue the freezing sensitivity of cpk28-1 (Fig. 7, F to H). Similarly, overexpression of CPK28-Myc in nlp7-1 did not suppress the freezing sensitivity of the nlp7-1 mutant (Fig. 7, C to E). These results demonstrate that CPK28 is required for NLP7 for the regulation of freezing tolerance.

**NLP7 is a key transcription factor that regulates transcriptional reprogramming downstream of the cold-induced Ca^{2+} signature**

To dissect how the transcription factor NLP7 regulates plant responses to cold stress after it is imported into the nucleus, we performed...
transcriptome deep sequencing [RNA sequencing (RNA-seq)] of 14-day-old wild-type Col-0 and nlp7-1 mutant seedlings exposed to 4°C for 0, 3, or 24 hours. In total, 6686 genes were regulated by cold stress in the wild type (absolute fold change ≥ 2; \( P < 0.05 \)), which we termed COR genes (Fig. 7A and dataset S1). NLP7 appeared to regulate the expression of 2182 genes (4°C, 3 and 24 hours), of which 1172 were NLP7-regulated COR genes (Fig. 8A, fig. S9A, and datasets S2 and S3). Gene Ontology (GO) term analysis of these NLP7-regulated COR genes revealed a marked enrichment for genes involved in processes such as phytohormone responses, metabolic processes, and abiotic stimuli (fig. S9B). Moreover, hierarchical clustering analysis showed that some genes are involved in cold tolerance, lipid metabolism, osmotic protectants, and phytohormone regulators (Fig. 8D). The early COR genes CBF1 and CBF3, which belong to the ETHYLENE RESPONSIVE FACTOR-APETALA 2 (ERF-AP2) superfamily of transcription factors, were down-regulated by NLP7 (Fig. 8D). Other genes encoding basic helix-loop-helix (bHLH)-type and B-box (BBX)-type transcription factors, zinc finger proteins, and RNA binding proteins involved in regulating gene expression and stress responses were also regulated by NLP7 in response to cold stress (Fig. 8D). Some of these genes have been previously reported to play important roles in plant tolerance to cold stress, including DWARF AND DELAYED FLOWERING 1 (DDF1) (35), bHLH70/MYC70 (36), and COLD AND CIRCADIAN REGULATED 2 [CCR2; also named GLYCINE-RICH RNA-BINDING PROTEIN 7 (GRF7)] (37). Plant hormones such as gibberellic acid (GA) and auxin have also been implicated in plant cold responses (38). Genes involved in phytohormone metabolism and responses, such as GA20 oxidase 3 (GA20ox3), GA3ox1, GA2ox1, SMALL AUXIN UP-REGULATED RNAs (SAURs), and INDOLE-3-ACETIC AND INDUCIBLE (IAA), were also regulated by NLP7 (Fig. 8D), suggesting that NLP7 may act as a link between phytohormones and cold responses. Moreover, NLP7 regulated late COR genes encoding protectant proteins such as LATE-EMBRYOGENESIS-ABUNDANT (LEA) and EARLY DEHYDRATION-INDUCIBLE (ERD) (39). Lipids are key components of cell membranes and are essential modulators of membrane fluidity and cold tolerance (40). Some genes encoding regulators participate in lipid biosynthesis and freezing tolerance, including SPHINGOLD LCB DESATURASE 1 (SLD1) (41), DIACYLGlycerol ACYLTRANSFERASE 1 (DGAT1) (42), and ERD7 (Fig. 8D) (43). In addition to lipids, soluble sugars, proline, and lignin are also important for cold tolerance (39). The genes SUCROSE SYNTHASE 1 (SUS1), FUMARASE 2 (FUM2), and CINNAMOYL COA REDUCTASE 1 (CCR1) involved in regulating the biosynthesis of these substances were regulated by NLP7 (Fig. 8D). Thus, NLP7 is a key transcription factor that orchestrates the cold-responsive transcriptome,
Fig. 8. NLP7 controls the cold-responsive transcriptome. (A) Venn diagram showing the number of genes regulated by cold, NLP7, and Ca^{2+} (absolute fold change ≥ 2; P < 0.05). (B) Hierarchical clustering of 687 NLP7- and Ca^{2+} common–regulated COR genes as detected by RNA-seq of nlp7-1 and EGTA treatment compared to the wild type. Pink indicates increased expression, and blue indicates decreased expression. (C) Venn diagram showing the overlap between NLP7-regulated COR genes, as detected by RNA-seq (absolute fold change ≥ 1.5; P < 0.05), and NLP7 direct target genes, as determined by ChIP-seq. (D) Hierarchical clustering analysis showing representative COR genes regulated by NLP7. Genes bound and directly regulated by NLP7 are highlighted in red. The Z score scale represents transcripts per kilobase million (TPM). In (A) to (D), data were obtained from three independent experiments. (E) Distribution of NLP7-binding sites in the CBF1–CBF3 promoters. Two independent repeats are shown. (F) The occupancy of NLP7 in different regions of CBFs (CBF1 to CBF3) was assessed by ChIP-qPCR in NLP7pro:NLP7-Myc plants. IgG was used as negative controls. (G) Relative transcript levels for CBF genes in wild-type, nlp7-1, cpk28-1, and complementation lines (cpk28-1 CPK28 and nlp7-1 NLP7) seedlings treated or not with cold treatments. In (F) and (G), data are means ± SD (**P < 0.01, Student’s t test).
possibly by integrating many internal signals to regulate plant freezing tolerance.

Next, we dissected whether NLP7-regulated COR transcriptome is associated with cold-evoked Ca\textsuperscript{2+} by comparing with COR genes modulated by EGTA. First, we found that Ca\textsuperscript{2+} modulates the expression of a large set of COR genes (1653 genes) (absolute fold change ≥ 2; \( P \leq 0.05 \)) (Fig. 8A, fig. S9C, and datasets S4 to S6), indicating that Ca\textsuperscript{2+} plays a critical role in controlling the cold-responsive transcriptome. Second, we overlapped 1172 NLP7-regulated CORs with 1653 Ca\textsuperscript{2+}-regulated CORs and found that 400 of these 1172 NLP7-regulated COR genes (about 34%) were also dependent on Ca\textsuperscript{2+} (Fig. 8A and dataset S7). Hierarchical clustering analysis clearly separated NLP7-dependent COR genes as a function of their sensitivity to EGTA (Fig. 8B). Therefore, we conclude that NLP7 at least partially modulates the expression of COR gene sets downstream of Ca\textsuperscript{2+}.

To further identify direct targets of NLP7 at the genome-wide level, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) using 14-day-old NLP7pro:NLP7-GFP transgenic seedlings treated at 4°C for 0.5 hours. We identified 1389 and 1844 peaks bound by NLP7 from two independent experiments, respectively, of which more than 20% were located within promoter regions (fig. S10A and datasets S8 and S9). However, we unexpectedly found that more than 40% of peaks were located within the first exon region (fig. S10A and datasets S8 and S9). Moreover, the binding region peaks of the NLP7 are deviated from the transcription start site (fig. S10B). One possibility for this phenomenon might be due to the tight binding of NLP7 to RNA polymerase II under cold stress. Alternatively, NLP7 has other functions beyond transcriptional regulation in cold stress responses, as transcription factors have been reported to function in the regulation of pre-mRNA splicing via recruitment splicing factors (44). These peaks from two biological replicates correspond to 1149 and 1565 genes bound by NLP7, respectively, and 764 overlapped (fig. S10C and datasets S10 and S11). These genes were selected for further study (fig. S10C). Among these NLP7-bound genes, 183 genes were NLP7-regulated COR genes (absolute fold change ≥ 1.5; \( P \leq 0.05 \)) (Fig. 8C and datasets S12 to S15).

By analyzing NLP7-bound and NLP7-regulated COR genes, we found that CBF1 and CBF3 are bound and regulated by NLP7 (Fig. 8, D and E). CBF2 transcript levels in the nlp7-1 mutant were about 60% of wild-type levels as seen by RNA-seq, and its promoter was also bound by NLP7 (Fig. 8E). On the basis of the binding region of NLP7 on CBF1, CBF2, and CBF3 (Fig. 8E), we performed ChIP-qPCR to test whether NLP7 binds CBF1–CBF3 in planta. NLP7 could bind the promoter and exon regions of CBF1 to CBF3 (Fig. 7F). Next, we determined whether NLP7 regulates the expression of CBF1–CBF3 genes under cold conditions by qPCR. We observed that cold markedly induced the expression of these genes; however, their cold induction was much lower in cpk28-1 and nlp7-1 mutants than in the wild type (Fig. 8G), which was consistent with the RNA-seq data (Fig. 8D). Moreover, the impaired cold induction in these two mutants was fully rescued by their corresponding complement lines (Fig. 8G). Notably, cold-induced expression of CBF1–CBF3 was also inhibited in wild-type seedlings pretreated with EGTA (fig. S10D). In addition to CBF1–CBF3, genes involved in cold responses including SLD1, DDF1, and DREB1D/CBF4 were bound and directly regulated by NLP7 (Fig. 8D).

NLP7 was shown to bind to the nitrate-responsive cis-element (NRE) in the promoters of its target genes (30). Using our collective results, we performed a de novo discovery for enriched motifs in NLP7-binding sites within genic regions, which identified the element 5’-xxAA(G/A, A/G)GxCAx-3’ as the top-scoring motif (61%, \( P = 1.8 \times 10^{-99} \)), which was named NRE-like hereafter (fig. S10E). Electrophoretic mobility shift assay (EMSA) confirmed that NLP7 bound to this NRE-like motif in the CBFI1 promoter (fig. S10F).

NL7-bound regions in the CBFI (CBFI-P1) and CBF2 (CBF2-P1) detected in the ChIP-qPCR (Fig. 8F) also contained the NRE-like motif. Together, these results demonstrate that the key transcription factor NLP7 acts downstream of CPK28 to decode the cold-induced Ca\textsuperscript{2+} signature by regulating a large set of COR genes, thereby positively regulating plant responses to cold stress.

**DISCUSSION**

Ca\textsuperscript{2+} is a ubiquitous second messenger that triggers plant responses to cold stress in plants (1). There are some Ca\textsuperscript{2+} sensor or Ca\textsuperscript{2+}-related proteins such as CaMs, CBLs, CDPKs/CPKs, CRLKs, and CAMTAs in plants (23). CRLK1 has been reported to regulate plant freezing tolerance, and its activation is enhanced by CaM (13, 14). In addition, the transcription activators CAMTAs contained calmodulin-binding motifs that positively regulate the expression of CBFs (15, 45), which is dependent on the binding of CAMTAs with CaM (45). Moreover, CAMTA3 and CAMTA5 regulate CBF1 only in a rapid (but not to gradual) reduction in temperature (46). However, it is unclear whether they confer cold-induced transcriptional reprogramming associated with Ca\textsuperscript{2+}. Therefore, it remains unclear which intracellular Ca\textsuperscript{2+} sensor is responsible for cold-evoked Ca\textsuperscript{2+} sensing and decoding or how they act. In this study, we demonstrated that the kinase CPK28 is rapidly activated by cold in a Ca\textsuperscript{2+}-dependent manner and mediates the nuclear shuttling of the transcription factor NLP7 by direct phosphorylation, which, in turn, specifies transcriptional reprogramming (Fig. 9). NLP7 regulates many COR genes downstream of the Ca\textsuperscript{2+}, and its nuclear relocation is also dependent on the Ca\textsuperscript{2+} under cold stress. Moreover, mutation of NLP7 causes a severe defect in plant freezing tolerance, indicating that NLP7 acts as a key factor in plant cold responses. Therefore, this study uncovers a previously unrecognized function of the Ca\textsuperscript{2+} sensor CPK28 as a master regulator in orchestrating cold-activated signaling through the Ca\textsuperscript{2+}-CPK28-NLP7 cascade that allows plants to respond and adapt to cold stress.

Cold shock can trigger Ca\textsuperscript{2+} influx into the cytosol within seconds through Ca\textsuperscript{2+} channels and transporters in plants. For example, OsNCNC9 were reported to mediate cold-evoked Ca\textsuperscript{2+} influx in rice (8). We previously showed that Ca\textsuperscript{2+} transporter AtANN1 plays a critical role in the regulation of Ca\textsuperscript{2+} influx in cold stress response in Arabidopsis (7). The Ca\textsuperscript{2+} transport activity of AtANN1 is enhanced by the protein kinase OST1. These findings suggest a critical link between OST1-AtANN1 and cold-induced Ca\textsuperscript{2+} generation. In this study, we demonstrated that CPK28 is rapidly activated within 10 s after cold shock and phosphorylates NLP7 to specify transcriptional reprogramming under cold stress. It remains unclear whether there is a link between CPK28-NLP7 module and OST1-AtANN1 module. This awaits further investigation.

The PM is one of the initial sites of cold perception (47). Changes in cell membrane fluidity are critical to modulating COR gene expression (48, 49). However, little is known about how these changes are sensed and then rapidly transduced to the nucleus in plants. In the bacterium Bacillus subtilis, physiological, transcriptional, and crystal structure data support the notion that the histidine kinase DesK senses membrane fluidity via its membrane-spanning helices.
that CPK28 is rapidly activated by cold within 10 s in a Ca²⁺-dependent manner (52). This mechanism therefore relies on a switch-off mode of action. Activated CPK28 phosphorylates the transcription factor NLP7, thereby preventing its nuclear import and phosphorylated NLP7 (stabilized by CPK28) is rapidly activated and, in turn, phosphorylates NLP7. This action triggers the nuclear translocation of NLP7 to shuttle from the cytosol to the nucleus, thereby specifying transcriptional reprogramming and consequently promoting freezing tolerance in Arabidopsis.

Fig. 9. Working model for CPK28-NLP7 in cold-induced Ca²⁺ signature sensing and transduction. Under normal conditions, the CPK28 protein kinase is in an inactive state so that the transcription factor NLP7 mostly localizes in the cytosol. Upon cold stress, a potential calcium channel opens, leading to a rapid cold-induced influx of Ca²⁺ in the cytosol (Ca²⁺ signature), which is sensed by CPK28. After binding to Ca²⁺, CPK28 is rapidly activated and, in turn, phosphorylates NLP7. This action triggers the nuclear translocation of NLP7 to shuttle from the cytosol to the nucleus, thereby specifying transcriptional reprogramming and consequently promoting freezing tolerance in Arabidopsis.

(50, 51). With a drop in temperature, DesK functions as a kinase to transfer a phosphoryl group to the transcription factor DesR, which then activates the expression of des, encoding a lipid desaturase, thereby increasing lipid disorder and helping restore the appropriate membrane fluidity (50). In Arabidopsis, we previously showed that the PM-localized COLD-RESPONSIVE PROTEIN KINASE 1 (CRPK1) is activated after 1 to 3 hours of cold treatment and attenuates the cold response by phosphorylating and facilitating the import of 14-3-3 proteins into the nucleus to indirectly promote CBF protein degradation (52). This mechanism therefore relies on a switch-off mode of action to avoid an excessive cold response. In this study, we found that CPK28 is rapidly activated by cold within 10 s in a Ca²⁺-dependent manner. Activated CPK28 phosphorylates the transcription factor NLP7 and triggers its nuclear translocation within 5 min or less, thereby initiating a rapid cold response by reshaping the cold-responsive transcriptome. Considering that the cold-induced reduction in membrane fluidity is a primary cold sensing event that may be sensed by PM-localized proteins in plants (47), the CPK28-NLP7 phosphorylation cascade may form a sensing-transducing module downstream of the changes in membrane fluidity that rapidly and directly transmits the cold signal from the PM to the nucleus to switch on the cold response.

NLP7 has been implicated as a central hub of primary nitrate responses (20, 30, 31). CPK10, CPK30, and CPK32 were shown to be imported into the nucleus in response to nitrate and enhance the nuclear retention of NLP7 by phosphorylation (20). In this study, we demonstrated that NLP7 acts as a key factor in plant responses to cold stress by specifying transcriptional reprogramming. Cold stimulated the nuclear translocation of NLP7 after phosphorylation in the cytoplasm by PM-localized CPK28, which is different from the regulatory mechanism imposed onto NLP7 by CPK10, CPK30, and CPK32, whereby NLP7 relocates to the nucleus before being phosphorylated in response to nitrate, leading to its nuclear retention (20).

Considering that NLP7 regulates nitrate and cold responses, we asked whether there is a cross-talk of different responses mediated by NLP7 and how NLP7 distinguishes them. We found that over-expression of NLP7S205A (harporing mutations of CPK28 phosphorylation sites) failed to rescue the freezing sensitivity of nlp7-1 but fully rescued nitrate sensitivity. By contrast, the overexpression of NLP7A2005S (carrying a mutation of CPK10/30/32 phosphorylation site) showed the opposite behavior, as it failed to restore the nitrate sensitivity of nlp7-1 (20) but fully restored freezing tolerance. Given that CPK28 is not obviously responsive to nitrate (20), it is possible that the CPK28-NLP7 module may not be associated with nitrate signaling. Ser305 is located at a nitrate signal–responsive domain in the N-terminal region (31), and C-terminal region containing Ser783, Ser93, Ser807, Ser808, and Thr417 is responsible for cold stress response. Therefore, this phosphocode-based regulation of NLP7 by different CPKs is important for its function in responses to diverse signals.

In addition, we also compared our RNA-seq data and data from a previous study (53) with two published NLP7 RNA-seq data in nitrate responses (20, 34) and found that, using different sets of RNA-seq data, more than half of nitrate-responsive (NR) genes are COR genes (fig. S11A). By comparing NR genes and COR genes regulated by NLP7, we found that 75 and 116 genes were overlapped, accounting for 15.9 and 10.8% of NLP7-regulated NR genes, respectively (fig. S11B) (20, 34). Moreover, we compared our ChIP-seq data with the published NLP7 ChIP-seq data involved in nitrate responses (34). The results showed that 12 genes are both COR and NR genes directly bound and regulated by NLP7, accounting for 13.2% of NLP7-targeted NR genes (fig. S11C), implicating the possible involvement of NR genes in NLP7-mediated cold stress responses. Together, these data suggest that although the target genes of nitrate-activated and cold stress–activated NLP7 are quite different, there are some common target genes of NLP7 in responses to nitrate and cold stress, which implies a possible link between nitrogen and cold signaling. The underlying mechanism awaits further investigation in the future study.

CPK28 has been reported to be a negative regulator in immune signaling (28, 54). In this study, we found that CPK28 positively regulates plant freezing tolerance. An outstanding question is how CPK28 distinguishes different upstream signals. As CPK28 is rapidly activated by cold and phosphorylated by calcium (50), the specific Ca²⁺ signature is likely one determinant for CPK28 to recognize distinct signals. An interesting finding demonstrated that CPK28 achieves generative growth through the tissue-specific balance of jasmonic acid (JA) and GA, without affecting JA-mediated defense responses (55). Therefore, it is also possible that CPK28 confers different signals in a tissue-specific manner. Moreover, an interesting paper showed that a single phosphorylation site in CPK28 can differentially direct its function in immune signaling (56). Therefore, different phosphorylation sites in CPK28 may be required for distinct pathways. Furthermore, other upstream regulatory factors may also be important for the activation of CPK28 in response to environmental cues.
this study are shown as follows: cpk28-1 (GK_523B08) (24), cpk28-3 (Wiscdslox_264d03) (24), nlp7-1 (Salk_26134C) (32), and nlp7-4 that harbors a point mutation (C to T) and thus results in a premature termination of NLP7 at the 62 residue (33). The transgenic Arabidopsis plants including Super:CPK28-Myc (CPK28-Myc), Super:NLP7-GFP (NLP7-GFP), Super:NLP7-Myc (NLP7-Myc), NLP7pro:CPK28-Myc, NLP7pro:NLP7-GFP, NLP7pro:NLP7-Myc, NLP7pro:NLP7-GFP, and NLP7pro:NLP7-Myc cpk28-1, NLP7-Myc cpk28-1, CPK28pro:CPK28-GFP [19], CPK28pro:CPK28-GFP cpk28-1, CPK28pro:CPK28-Myc cpk28-1, CPK28pro:NLP7-GFP nlp7-1, NLP7pro:NLP7-GFP nlp7-1, NLP7pro:NLP7-GFP nlp7-1, NLP7pro:Myc nlp7-1, and NLP7pro:Myc nlp7-1 were generated by transformation with Agrobacterium tumefaciens (GV3101) using the floral-dip method (57). All transgenic plants were selected by hygromycin B, and T3 or T4 homozygous transgenic plants were used in this study. The transgenic plants with the cpk28-1 and nlp7-1 background were genotyped by PCR to identify homozygous cpk28-1 and nlp7-1 mutants, respectively. Proteins in the transgenic plants were detected by immunoblots with anti-GFP (TransGen) or anti-Myc (Sigma-Aldrich) antibody. The cpk28 nlp7 double mutant was generated by crossing cpk28-1 with nlp7-1. PCR genotyping was used to identify homozygous cpk28-1 and nlp7-1 in the cpk28 nlp7 double mutant. Seeds were stratified at 4°C for 3 days and planted on half-strength MS medium containing 0.8% agar and 2% sucrose under a 16-hour light and 8-hour dark cycle at 22°C. Fourteen-day-old seedlings grown on molecular genetics research laboratory (MGRL) (University of Tokyo) growth medium (58) under a 16-hour light and 8-hour dark cycle at 22°C were used to analyze nitrate responses. N. benthamiana plants were selected by hygromycin B, and T3 or T4 homozygous transgenic plants were used in this study. The details of ChIP-qPCR were described in a previous study (49). The method for obtaining NLP7 point mutations is the same as that for CPK28 point mutations. Primers used for vector construction are shown in table S2.

**Protein expression and purification from Escherichia coli**

Plasmids were transformed to strain BL21 (codon plus). A single clone of each transformant was selected and incubated in 5 ml of LB liquid medium supplemented with corresponding antibiotics at 37°C for 12 hours and then transferred to 500 ml of LB liquid medium with corresponding antibiotics at 37°C for 2 to 4 hours until the OD_{600} (optical density at 600 nm) reached to 0.4 to 0.6. Unless otherwise indicated, recombinant proteins were expressed in E. coli with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 hours at 16°C. Recombinant proteins were purified according to the manufacturer’s instructions.

**Quantitative PCR**

Total RNA was extracted from fresh leaf tissues with TRIzol reagent (Sigma-Aldrich). cDNA was synthesized using the HiScript II Reverse Transcriptase (Promega). qPCR was performed with the SYBR Premix Ex Taq Kit (Takara) using a 7500 Fast Real-Time PCR System (Applied Biosystems). Genespecific primers used for qPCR are listed in table S2.

**ChIP-qPCR assay**

The details of ChIP-qPCR were described in a previous study (60) with some modifications. Briefly, 2.5 g of 14-day-old NLP7pro:NLP7-Myc seedlings was formaldehyde fixed, and the nuclear proteins were extracted and sonicated. The protein-DNA complexes were precipitated with anti-Myc agarose beads (Sigma-Aldrich) or control immunoglobulin G (IgG) serum after the samples were preclayed by protein A agarose beads. The protein-DNA complexes were heated to reverse the cross-linking. The DNA was extracted and amplified by qPCR. Gene-specific primers used for ChIP-qPCR assay are listed in table S2.

**Freezing treatment**

Unless otherwise indicated, 2-week-old seedlings grown on half-strength MS plates at a 22°C chamber were treated at 4°C for 4 days (CA) before freezing treatment or directly subjected to freezing treatment (NA). The plants were placed in a freezing incubator (RuMED) with the following program: The initial temperature was set at 0°C, and the temperature was dropped 1°C every 1 hour until reaching the indicated temperatures. After treatment, the seedlings were kept at 4°C for 12 hours in the dark and then were transferred to a 22°C growth chamber for recovery. The survival rates were counted after 3 days, and the seedlings that can grow new leaves are considered survival ones. Three independent experiments were performed for each genotype or treatment at each temperature point.

**EGTA treatment**

For freezing tolerance assays, 14-day-old seedlings were treated with 25 mM EGTA for 4 hours at 22°C (NA) or treated with 25 mM EGTA at 4°C for 4 days (CA) and then subjected to freezing tolerance assay. For gene expression, confocal imaging, and cell fractionation assays, 12-day-old seedlings were treated with 25 mM EGTA for 4 hours at 22°C and then put to a 4°C chamber for the time indicated.
Plant protein extraction and IP

Total proteins were extracted from *N. benthamiana* leaves or transgenic *Arabidopsis* with extraction buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Triton X-100, 0.1% NP-40, 5 mM dithiothreitol (DTT), and 1× cocktail (EDTA-free protease inhibitor, Roche)]. The lysates were cleared by centrifugation at 13,000g twice, 15 min each. For IP assays, total protein extracts were incubated with affinity tag conjugated to agarose beads at 4°C for 2 hours, including anti-HA agarose beads (Sigma-Aldrich, #A2095) and GFP-trap agarose beads (Chromotek). After IP, beads were washed five times with extraction buffer at 4°C, 5 min each, and immunoprecipitated products were eluted by 5× SDS loading buffer and then heated at 99°C for 5 min. Next, the proteins were separated on SDS–polyacrylamide gel electrophoresis (PAGE) gels [10% (w/v) or 12% (w/v)] and transferred to nitrocellulose membranes. Immunoblots were probed with related antibodies as shown in the figure legends. Equal loading was confirmed by probing with anti-Actin2 antibody (EASYBIO, #BE0027).

Cell fractionation

Cytoplasmic and nuclear protein isolation was performed as previously described (61). Tissues were homogenized in lysis buffer [300 mM sucrose, 20 mM tris-HCl (pH 8.0), 5 mM MgCl₂, 5 mM KCl, 0.3% Triton X-100, 5 mM NP-40, 5 mM β-mercaptoethanol, and 35% glycerol] supplemented with 5 mM DTT and 1× cocktail, and 5% of the extracts was used as total proteins. The lysate was then filtered through Miracloth (Calbiochem) and centrifuged at 5000g for 10 min (4°C), and the supernatant was collected (soluble fraction). The pellet was washed five times with lysis buffer. After the last wash, the pellet was resuspended with nuclear protein extraction buffer [50 mM tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, 5 mM DTT, and 1× cocktail]. Boiled samples with 5× SDS loading buffer were separated on the 12% (w/v) SDS-PAGE gel and detected with antibodies against GFP (TransGen, HT801-01), cytoplasmic marker phosphoenolpyruvate carboxylase (PEPC) (Agrisera, #AS09 458; RRID: AB_1312), and nuclear marker histone H3 (Millipore, #05-499; RRID: AB_2787688).

Microscale thermophoresis

MST assay was performed as previously described (7). Buffers in the recombinant GST, GST-CPK28, GST-CPK28Em, and GST-ΔCPK28 proteins were replaced with phosphate buffered saline with 0.005% Tween-20 (PBST) buffer (pH 7.4) using column A (NanoTemper Technologies). Next, 10 mM GST, GST-CPK28, GST-CPK28Em, and GST-ΔCPK28 proteins were labeled with excess NHS NT-647 dye at a molar ratio of 1:5 for 30 min at a 22°C chamber in the dark. Free unlabeled dye was removed by column B, which was reequilibrated with PBST buffer. CaCl₂ (1 mM) was serially diluted with Hepes buffer [20 mM Hepes (pH 7.4) and 150 mM KCl] and kept constant with the dilution ratio being 0.5, which was mixed with the same amount of labeled protein. Last, the mixtures were loaded into capillaries (NanoTemper Technologies) and analyzed by NanoTemper Monolith Instrument (NT.115) (NanoTemper Technologies) with 20% LED power and 20% MST power. Dissociation constant (Kd) was calculated using Signal Thermophoresis and T-Jump Data.

In vitro pull-down assay

In vitro pull-down assay was carried out as previously described (62). In brief, 0.5 µg of His-NLP7 proteins or His-NLP8 was incubated with 5 µg of GST-CPK28 and immunoprecipitated by GST beads (Thermo Fisher Scientific, catalog no. 17-5132-02) at 4°C for 2 hours. The mixture was gathered by centrifugation at 500g for 5 min, followed by washing with PBS buffer five times. Proteins were separated on 10% (w/v) SDS-PAGE and detected with anti-GST (Beijing Protein Innovation, catalog no. AbM59001-2H5-PU) and anti-His (Beijing Protein Innovation, catalog no. AbM59012-18-PU) antibodies.

Co-IP assay

The resulting plasmids were transformed into *N. benthamiana* leaves and expressed for about 48 hours. Then, *N. benthamiana* leaves were lysed by IP buffer [2 mM EDTA (pH 8.0), 50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, and 0.1% Triton X-100]. The lysates were cleared by centrifugation at 13,000g twice, 15 min each. The remaining supernatant was incubated with the indicated affinity tag agarose beads at 4°C for 2 hours. After five times of washes, the products were analyzed and detected by immunoblots with anti-HA (Sigma-Aldrich, #H3663) and anti-Myc antibodies (Sigma-Aldrich, #M4439).

Bimolecular fluorescence complementation

The constructs (CPK28-YNE + NLP7-YCE; CPK28-YNE + NLP8-YCE; and CPK3-YNE + NLP7-YCE) were transfected into *N. benthamiana* leaves. After incubation for 18 hours, the yellow fluorescent protein (YFP) fluorescence signal was detected using a confocal laser scanning microscope (Zeiss LSM880 Airyscan).

Mass spectrometry

For identification of interacting proteins of CPK28 and phosphorylation sites of NLP7 in planta, total proteins were prepared from *SuperCPK28-GFP* and *NLP7pro:NLP7-GFP* stable transgenic plants treated at 4°C for 0.5 hours using IP buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 0.1% NP-40, 20% glycerol, 5 mM DTT, and 1× cocktail] and immunoprecipitated by GFP-trap agarose beads (Chromotek) at 4°C for 2 hours. The immunoprecipitated proteins were washed with IP buffer five times (500g, 5 min each) and then eluted with 0.1 M glycine solution (pH 2.8) and mixed with 1 M tris solution immediately to sustain proteins at suitable pH (pH 7.5). Proteins were digested by trypsin and subjected to a Thermo Q-Exactive high-resolution mass spectrometer (Thermo Fisher Scientific) for LC-MS/MS analysis.

For analysis of phosphorylation sites of NLP7 in vitro, 0.1 µg of recombinant GST-CPK28 or GST-CPK28Em proteins was incubated with 1 µg of MBP-His-NLP7 in the kinase reaction buffer [20 mM MgCl₂, 20 mM Heps-KOH (pH 7.5), 5 mM DTT, and 100 µM ATP] at 30°C for 30 min. Next, proteins were digested with trypsin and subjected to a Thermo Q-Exactive high-resolution mass spectrometer (Thermo Fisher Scientific) for LC-MS/MS analysis.

Phosphorylated protein detection

Proteins were extracted by nuclear extraction buffer [50 mM tris-HCl (pH 8.0), 1% SDS, 5 mM DTT, and 1× cocktail] from *NLP7pro:NLP7-GFP*, *NLP7pro:NLP7-GFP* cpk28-1, and *NLP7pro:NLP7-GFP* transgenic plants treated at 4°C for the indicated time. Proteins were separated on 6% phos-tag-PAGE gel containing 50 µM phos-tag AAL solution (Boppard-China), 100 µM MnCl₂ at cooling conditions (90 V, 4 hours), and 12% (w/v) SDS-PAGE gel (150 V, 1.5 hours), respectively. After washing with buffer [38.64 mM glycine, 47.88 mM tris, 20% (v/v) methanol, and 10 mM EDTA (pH 8.0)], proteins on the phos-tag-PAGE gel were transferred to nitrocellulose.
membranes (200 mA, 3 hours). Proteins on SDS-PAGE gel were directly transferred to nitrocellulose membranes (200 mA, 2 hours). Anti-GFP antibody (TransGen, HT801-01) was used to detect phosphorylated and unphosphorylated NLP7. Anti-H3 antibody (Millipore, #05-499; RRID: AB_2787688) was used to detect H3 proteins.

**In vitro and in vivo phosphorylation**

For in vitro kinase assay, 1 μg of purified MBP-His-NLP7 proteins or His-

\[ \Delta NLP7 \] variants was incubated with 0.1 μg of purified GST-

CPK28 in 20 μl of kinase reaction buffer [20 mM MgCl₂, 20 mM tris-

HCl (pH 7.5), 1 mM DTT, 1 mM CaCl₂, 50 μM ATP, and 1 μCi of \([\gamma-32P]ATP\). The mixture was kept at 30°C for 30 min, and the reaction was stopped by adding 5× SDS loading buffer. Proteins were boiled at 99°C for 5 min and then separated on 10% (w/v) SDS-PAGE. Proteins were stained by Coomassie brilliant blue (CBB). After 12 to 24 hours, the phosphorylated proteins were visualized by autoradiography (Typhoon 9410 imager).

The procedure of the modified in vitro kinase assay was the same as that of the in vitro kinase assay except that its reaction system did not contain \([\gamma-32P]ATP\). The His-

\[ \Delta NLP7 \] proteins were detected with anti-His antibody, and related types of CPK28 including GST-

CPK28, GST-CPK28 

\[ \Delta \] Fm , and GST-CPK28 

\[ \Delta \] 188A were stained by CBB. λPP was used to process phosphorylated proteins.

For in-gel kinase assay, five steps were processed. In the first step, total proteins were extracted from 14-day-old wild-type, 

\[ \Delta \] CPK28, and 

\[ \Delta \] Super:CPK28-Myc seedlings treated at 4°C for the time indicated with the plant extraction buffer [25 mM NaF, 1 mM Na₃VO₄, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 50 mM Hepes-

KOH (pH 7.5)] supplemented with 5 μM DTT and 1× cocktail. Proteins were then separated on 8% (w/v) SDS-PAGE containing GST-BIK1 

\[ K105F \] (0.1 mg/ml) as a substrate (150 V, 1.5 hours) (28). In the second step, the gel was washed with buffer containing 1 mM DTT, 5 mM NaF, 0.1 mM Na₃VO₄, bovine serum albumin (0.5 mg/ml), 0.1% Triton X-100, and 25 mM tris-

HCl (pH 7.5) for three times, 20 min each. This step is important for subsequent protein renaturation. In the third step, the gel was incubated with renatured buffer [2 mM DTT, 5 mM NaF, 0.1 mM Na₃VO₄, and 25 mM tris-

HCl (pH 7.5)] at 4°C for 1, 12 to 13, and 1 hours, respectively. In the fourth step, the gel was incubated with kinase reaction buffer [12 mM MgCl₂, 1 mM CaCl₂, 1 mM DTT, 0.1 mM Na₃VO₄, and 25 mM Hepes-

KOH (pH 7.5)] at 22°C for 30 min, followed by adding 60 μCi of \([\gamma-32P]ATP\) and 9 μl of cold ATP (1 mM) and kept at 22°C for an additional 1.5 to 2 hours. In the last step, the gel was removed from the reaction buffer and then washed with the buffer (containing 5% trichloroacetic acid and 1% sodium pyrophosphate) for five times, 30 min each, followed by autoradiography (Typhoon 9410 imager).

**Electrophoretic mobility shift assay**

EMSA was performed using the biotin-labeled probes and the Lightshift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s instructions. Briefly, 2 μg of purified His-

\[ NLP7 \] (RWP-RK) was incubated with biotin-labeled CBFI promoter probes at 22°C for 30 min. Twenty-five– to 50-fold unlabeled probes to labeled probe were used.

**RNA sequencing**

To analyze NLP7-regulated COR genes, 2-week-old Arabidopsis seedslings were treated with or without cold at 4°C for 0, 3, and 24 hours, respectively. To analyze Ca²⁺-regulated COR genes, 2-week-old Arabidopsis seedslings presprayed with 25 mM EGTA or water (–EGTA) at 22°C for 4 hours were harvested (0 hour) or were treated with cold at 4°C for 3 and 24 hours, respectively. Three independent replicates were performed each experiment. Total RNAs were isolated, and then the libraries were sequenced on an Illumina Nova-seq-6000 platform. Paired-end reads [150 base pairs (bp)] were generated. Raw data of fastq format were first subjected to quality control using FastQC (v.0.11.9) (63). Then, reads were mapped to the Arabidopsis genome (TAIR10) using HISAT2 (v.2.2.0) (64) with default parameters. The read counts of each gene were obtained by featureCounts (v.2.0.1) (65), and transcripts per kilobase million (TPM) of each gene was calculated using an in-house R script. The differential expression analysis was performed using DESeq2 (v.1.30.0, R package) (66). P value <0.05 and log₂ fold change ≥1 or log₂ fold change ≤−1 were set as the threshold for significantly differential expression.

Two criteria were used to define NLP7-regulated COR genes: (i) Genes had to be up- or down-regulated (fold changes of at least 2) after cold treatment for either 3 or 24 hours in wild-type plants, and (ii) genes were either up (NLP7-repressed)– or down-regulated (NLP7-activated) (fold changes of at least 2) in the nlp7-1 mutant compared with wild-type plants. In addition, NLP7-regulated genes (fold changes of at least 1.5) were selected for overlapping with ChiP-seq data.

**ChIP sequencing**

ChIP-seq analysis was performed as previously described (60) with some modifications. Two independent replicates were performed in this experiment. In brief, 2.5-g 2-week-old NLP7pro:N-

LP7-GFP or wild-type seedlings grown on half-strength MS treated at 4°C for 30 min were harvested, which were fixed within 36 ml of PBS buffer supplemented with 1 ml of 37% formaldehyde solution for 12 min, followed by treatment with 2 M glycine for 5 min under vacuum conditions. The seedlings were collected and ground in liquid nitrogen, and nuclear proteins were extracted according to the previous study (60). Next, the samples were sufficiently broken by ultrasound (Diagenode) so that the genome DNA is broken to about 250 bp in size. Then, the samples were diluted and immunoprecipitated by GFP-Trap agarose beads (Chromotek) overnight. After washing the beads with buffers (low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and TE buffer) (60) at 4°C for 5 min each, DNA was prepared and extracted by a DNA extraction kit. Extracted DNA was used in library preparation using the KAPA Hyper Prep kit (Illumina). Samples were barcoded and subjected to 150-bp paired-end deep sequencing on an Illumina Nova-seq-6000 platform. For all ChIP-seq datasets, raw fastq data were trimmed using the Trim Galore (https://github.com/FelixKrueger/TrimGalore) with Q20, and then the cleaned reads were aligned to the Arabidopsis genome (TAIR10) using Bowtie2 v2.2.9 with default parameters (67). Unique aligned reads were extracted by the SAMtools v1.4.1 program with a MAQ20 (map quality > 20) filtration (68). Peaks were identified by MACS2 v2.1.1 with default parameters (q < 10⁻⁵) (69). To search for conserved binding motifs in consistent NLP7-binding regions, the 500-bp sequence surrounding the peak summit of each consistent peak was extracted and submitted to the online version of MEME-ChIP under default settings (70).

**Quantification and statistical analysis**

Band intensity quantification of in-gel kinase assay results and protein levels was performed using the Image Lab. For survival rates,
data are represented as means ± SEM, and statistical significance was examined by Student’s t test (*P < 0.05 and **P < 0.01) under the same treatment. For relative kinase activity and relative intensity, data are represented as means ± SD, and statistical significance was examined by Student’s t test (*P < 0.05 and **P < 0.01). Survival rates in Fig. 6 were examined by one-way analysis of variance (ANOVA), and different letters represent significant difference at P < 0.05 under the same treatment temperature. Relative protein expression, data are represented as means ± SD, and statistical significance was divided by that of the relevant marker equaled RRT, RRS, and RRS, respectively. Second, RRT/RRT (the ratio was 1.0), RRS/RRS, and RRS/RR were calculated.

SUPPLEMENTAL MATERIALS

Supplemental material for this article is available at https://science.org/doi/10.1126/sciadv.abn7901.

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