CellPress
PARTNER JOURNAL

Molecular Plant
Research Article

Cold-Induced CBF–PIF3 Interaction Enhances Freezing Tolerance by Stabilizing the phyB Thermosensor in *Arabidopsis*

Bochen Jiang[1,4], Yiting Shi[1,4], Yue Peng[1], Yuxin Jia[1], Yan Yan[1], Xiaojing Dong[1], Hui Li[1], Jie Dong[2], Jigang Li[1], Zhizhong Gong[1], Michael F. Thomashow[3] and Shuhua Yang[1,*]

[1]State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China

[2]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520, USA

[3]MSU-DOE Plant Research Lab, Michigan State University, East Lansing, MI 48824, USA

[4]These authors contributed equally to this article.

***Correspondence: Shuhua Yang (yangshuhua@cau.edu.cn)**

https://doi.org/10.1016/j.molp.2020.04.006

ABSTRACT

Growth inhibition and cold-acclimation strategies help plants withstand cold stress, which adversely affects growth and survival. PHYTOCHROME B (phyB) regulates plant growth through perceiving both light and ambient temperature signals. However, the mechanism by which phyB mediates the plant response to cold stress remains elusive. Here, we show that the key transcription factors mediating cold acclimation, C-REPEAT BINDING FACTORs (CBFs), interact with PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) under cold stress, thus attenuating the mutually assured destruction of PIF3–phyB. Cold-stabilized phyB acts downstream of CBFs to positively regulate freezing tolerance by modulating the expression of stress-responsive and growth-related genes. Consistent with this, *phyB* mutants exhibited a freezing-sensitive phenotype, whereas *phyB*-overexpression transgenic plants displayed enhanced freezing tolerance. Further analysis showed that the PIF1, PIF4, and PIF5 proteins, all of which negatively regulate plant freezing tolerance, were destabilized by cold stress in a phytochrome-dependent manner. Collectively, our study reveals that CBFs–PIF3–phyB serves as an important regulatory module for modulating plant response to cold stress.

Key words: CBFs, PIFs, phyB, freezing tolerance, *Arabidopsis*

Jiang B., Shi Y., Peng Y., Jia Y., Yan Y., Dong X., Li H., Dong J., Li J., Gong Z., Thomashow M.F., and Yang S. (2020). Cold-Induced CBF–PIF3 Interaction Enhances Freezing Tolerance by Stabilizing the phyB Thermosensor in *Arabidopsis*. Mol. Plant. **13**, 894–906.

INTRODUCTION

Low temperature is a major environmental factor adversely affecting plant growth and development. Plants have evolved elaborate regulatory mechanisms that allow them to adapt to environmental temperature changes, one of which is cold acclimation, whereby plants from temperate regions exhibit increased freezing tolerance after exposure to low, non-freezing temperatures (Thomashow, 1999; Shi et al., 2018). The transcription factors C-REPEAT BINDING FACTORs/DROUGHT RESPONSE ELEMENT BINDING FACTOR 1s (CBFs/DREB1s) play crucial roles in cold acclimation (Thomashow, 1999; Jia et al., 2016; Zhao et al., 2016; Liu et al., 2018). Upon induction by cold stress, CBFs activate a set of *COLD-REGULATED* (*COR*) genes, some of which encode cryoprotective proteins or enzymes involved in osmolyte biosynthesis or reactive oxygen species scavenging, finally leading to increased freezing tolerance (Guy et al., 1987; Jaglo-Ottosen et al., 1998; Liu et al., 1998).

In addition to providing energy for photosynthesis, light also functions as a key environmental cue that regulates multiple facets of plant growth and development (Rockwell et al., 2006; Jiao et al., 2007). Phytochromes (phyA to phyE) are photoreceptors that perceive red and far-red wavelengths through the conversion of the biologically inactive Pr form to the active Pfr form (Rockwell et al., 2006). Photoactivated phytochromes are rapidly translocated from the cytoplasm to the nucleus, where they directly interact with the BASIC HELIX-LOOP-HELIX (bHLH)

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, CAS.

transcription factors PHYTOCHROME-INTERACTING FACTORs (PIFs) (Leivar and Quail, 2011). This interaction promotes rapid phosphorylation and 26S proteasome pathway-mediated degradation of PIFs, thereby modulating the expression of PIF target genes to initiate phytochrome-mediated light responses (Al-Sady et al., 2006; Shen et al., 2007, 2008; Bae and Choi, 2008; Lorrain et al., 2008). Interestingly, the interaction of photoactive Pfr-phyB and PIF3 also induces the concomitant and relatively slow degradation of the phyB photoreceptor in response to long-term light exposure (Al-Sady et al., 2008; Ni et al., 2013, 2014; Leivar and Monte, 2014). This PIF3–phyB feedback regulation loop likely prevents unfettered prolongation of elevated phyB activity, which would severely limit plant growth and development.

Emerging evidence indicates that light and temperature signaling are tightly linked (Casal and Questa, 2018; Lu et al., 2019; Ding et al., 2020). The key component of light signaling, PIF4, is a central regulator of plant thermomorphogenesis and regulates warm temperature-induced hypocotyl and petiole growth, leaf hyponasty, and flowering (Koini et al., 2009; Kumar et al., 2012; Lau et al., 2018). Interestingly, the photoreceptor phyB was proved to be a thermosensor in plants (Jung et al., 2016; Legris et al., 2016). Increases in ambient temperature promote the dark reversion of phyB from the Pfr to Pr form, resulting in PIF4 accumulation and consequent hypocotyl elongation (Jung et al., 2016; Legris et al., 2016). Furthermore, phyB directly associates with the promoters of key target genes in a temperature-dependent manner (Jung et al., 2016). A recent study showed that phyB also perceives daytime temperature through a transcriptional activator, HEMERA, which interacts with PIF4 to induce the expression of PIF4 target genes and PIF4 accumulation at warm temperatures (Qiu et al., 2019).

In addition to regulating thermomorphogenesis, several light-signaling components were shown to modulate freezing tolerance and help plants better adapt to harsh environmental conditions. Phytochromes are essential for the full development of cold acclimation in *Arabidopsis thaliana* (Crosatti et al., 1999; Kim et al., 2002; Soitamo et al., 2008). Furthermore, a low ratio of red to far-red (R/FR) light derepresses circadian-gated *CBF* transcription and increases *COR* expression at 16°C (Franklin and Whitelam, 2007), suggesting a negative role of phyB in modulating *CBF* expression. In addition, *CBF* gene expression is repressed by phyB, PIF4, and PIF7 under long-day (LD) conditions (Lee and Thomashow, 2012). PIF3 was recently shown to negatively regulate the *CBF* pathway by directly binding to the *CBF* promoters (Jiang et al., 2017). Although the roles of light-signaling components in regulating plant freezing tolerance have been extensively studied, it remains unclear how the light-signaling components are regulated by cold stress.

In this report, we demonstrate that cold-induced CBF proteins interact with PIF3, which inhibits the co-degradation of PIF3 and phyB under cold stress. Further study indicated that cold-stabilized phyB promotes the degradation of PIF1, PIF4, and PIF5, and consequently increases freezing tolerance. Together, these findings reveal that cold-induced CBFs stabilize the phyB thermosensor to enhance plant freezing tolerance.

RESULTS

CBFs Interact with and Stabilize PIF3 under Cold Stress

We previously showed that PIF3 negatively regulates freezing tolerance by repressing the expression of *CBF* genes (Jiang et al., 2017). Since cold stress induces the accumulation of CBFs and inhibits light-induced degradation of PIF3 (Jiang et al., 2017), we asked whether CBFs could stabilize PIF3 via a feedback mechanism under cold conditions. To test this hypothesis, we first performed an *in vitro* pull-down assay to examine whether CBFs could physically interact with PIF3. We found that His-tagged PIF3 interacted with maltose-binding protein (MBP)-His-tagged CBF1, CBF2, and CBF3, but not with MBP-His alone (Figure 1A; Supplemental Figure 1A and 1B), suggesting that CBF proteins physically interact with PIF3 *in vitro*.

Co-immunoprecipitation (Co-IP) assays were then performed using *Arabidopsis* protoplasts co-transformed with *Super:PIF3-Myc* and *35S:HA-FLAG-CBF1*, *35S:HA-FLAG-CBF2*, *35S:HA-FLAG-CBF3*, or *HA-FLAG*, and strong interactions between CBFs and PIF3 were detected in plants (Figure 1B; Supplemental Figure 1C and 1D). Luciferase complementation imaging (LCI) assays also showed that CBFs were associated with PIF3 *in planta* when PIF3-nLuc was co-expressed with CBF1-, CBF2-, or CBF3-cLuc in *Nicotiana benthamiana* leaves (Figure 1C and Supplemental Figure 1E). By contrast, no interactions between CBF1 and PIF1, PIF4, or PIF5 were observed in either pull-down or LCI assays (Supplemental Figure 1F and 1G). These data indicate that CBFs specifically interact with PIF3 *in vitro* and *in vivo*.

Previous studies showed that CBF protein abundance was dramatically induced within several hours after cold treatment (Liu et al., 2017; Ding et al., 2018). Therefore, we examined how the interaction of PIF3 and CBF1 was regulated by different temperatures using the *35S:PIF3-His-Myc CBF1:CBF1-Myc* transgenic plants. As expected, the CBF1–PIF3 interaction was barely detected when CBF1 levels were very low before cold treatment. After 6 h of cold treatment, when accumulation of CBF1 drastically increased, the interaction was strikingly enhanced (Figure 1D). Collectively, these results demonstrate that CBFs interact with PIF3 *in planta* under cold conditions.

To explore the biological relevance of the CBF–PIF3 interactions under cold stress, we first determined whether CBFs could affect the DNA-binding activity of PIF3 by performing a chromatin immunoprecipitation (ChIP)–PCR assay using *35S:PIF3-Myc* plants. Our data showed that the binding affinity of PIF3 to the *CBF* gene promoters was not altered before or after cold treatment (Supplemental Figure 2A–2C). We next examined PIF3 protein abundance in *PIF3-Myc* and *PIF3-Myc cbfs* (where *cbfs* refers to the *cbf1,2,3* triple mutant) plants grown under LD conditions followed by transfer to 4°C at zeitgeber time 3 (ZT3; i.e., 3 h after dawn) for different times. We found that PIF3 levels in *PIF3-Myc* transgenic plants remained stable at 4°C (Figure 1E), consistent with a previous report (Jiang et al., 2017). However, PIF3 abundance was markedly reduced in *PIF3-Myc cbfs* plants after cold treatment (Figure 1E). Combined with the observation that *PIF3* expression was not obviously affected by CBFs under both normal and cold

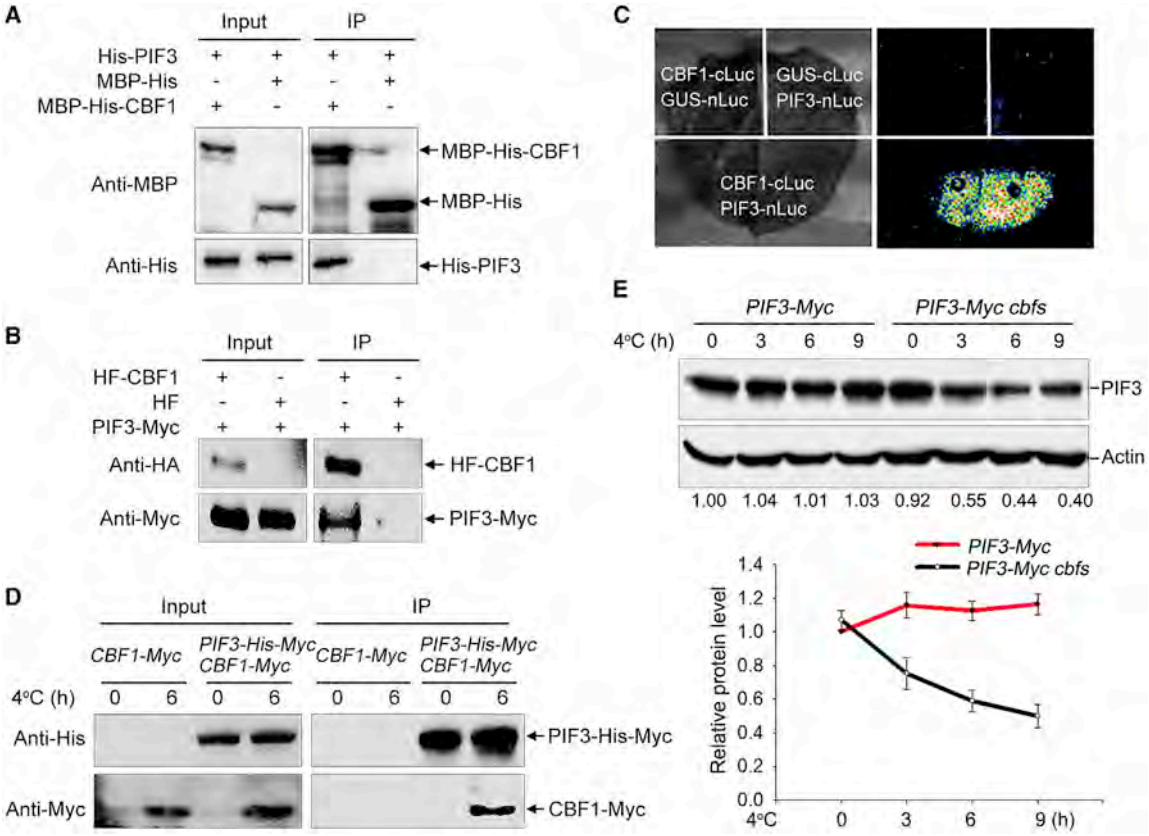

Figure 1. PIF3 Interacts with CBF1 *In Vitro* and *In Vivo*.

(A) Pull-down assay showing the interaction between His-PIF3 and MBP-His-CBF1 *in vitro*. Purified His-PIF3 fusion proteins were incubated with maltose-binding protein (MBP)-His-CBF1 or MBP-His alone, and pull-down assays were performed using MBP agarose beads, followed by immunoblot analysis with anti-MBP and anti-His antibodies.

(B) Co-IP assay showing the interaction between PIF3 and CBF1 proteins *in vivo*. *Arabidopsis* mesophyll protoplasts were co-transformed with *Super:PIF3-Myc* and *35S*: *HA-FLAG-CBF1* (*HF-CBF1*) or *35S:HA-FLAG* (*HF*). Protoplasts were incubated at 22°C for 14 h in darkness. Total protein extracts were immunoprecipitated with HA Sepharose beads. Crude lysate proteins (input) and immunoprecipitated proteins (right panels) were detected using anti-HA and anti-Myc antibodies.

(C) Luciferase complementation imaging (LCI) assay showing the interaction between PIF3 and CBF1 in *N. benthamiana* leaves. CBF1-cLuc/PIF3-nLuc, CBF1-cLuc/GUS-nLuc, and GUS-cLuc/PIF3-nLuc were co-expressed in *N. benthamiana* leaves for 48 h. GUS-nLuc and GUS-cLuc were used as negative controls.

(D) Co-IP assays showing that PIF3 and CBF1 interact under cold treatment. The *CBF1:CBF1-Myc* and *PIF3-His-Myc CBF1:CBF1-Myc* transgenic plants were grown for 12 days and then transferred to 4°C for 6 h. Total protein extracts were immunoprecipitated with His agarose beads. Crude lysate proteins (input) and immunoprecipitated proteins (right panels) were detected with anti-His and anti-Myc antibodies.

(E) The Stability of PIF3 protein in *35S:PIF3-His-Myc* and *35S:PIF3-His-Myc cbfs* plants under cold treatment. Twelve-day-old seedlings grown under LD conditions were transferred to 4°C at ZT3 (3 h after dawn) for the indicated period. Total proteins were extracted and subjected to immunoblot analysis with anti-Myc or anti-actin antibodies. The immunoblot results were quantified using ImageJ software. PIF3 protein levels were normalized to the actin loading control. The relative protein levels are shown below the blots, where relative protein level at 0 h was set to 1.0. Quantitative data are shown as the mean of three biological replicates ±SEM.

conditions (Supplemental Figure 2D), these results indicate that CBFs interact with PIF3 and enhance its stability under cold stress.

CBFs Suppress phyB Degradation under Cold Stress

It was previously shown that light-induced degradation of PIF3 is necessary for its co-degradation with phyB via feedback regulation (Ni et al., 2013, 2014). Therefore, we tested whether cold-stabilized PIF3 could affect phyB protein stability. Compared with that at 22°C under LD conditions, the stability of the phyB protein was increased after cold treatment under both white- and red-light conditions (Figure 2A and Supplemental Figure 3A). Moreover, cell-fractionation assays revealed substantial accumulation of phyB in the nucleus at 4°C (Figure 2B and Supplemental Figure 3B). We further observed that in *PIF3-Myc* overexpression transgenic plants under both white- and red-light LD conditions, light-induced degradation of phyB and PIF3 at 22°C was also suppressed at 4°C (Figure 2C and Supplemental Figure 3C). Along with the observation that *phyB* expression slightly decreased at 4°C (Supplemental Figure 2E), we conclude from these results that the co-degradation of PIF3 and phyB is inhibited by low temperature.

As CBFs interact with and stabilize PIF3 under cold conditions, we hypothesized that their interactions may also affect the

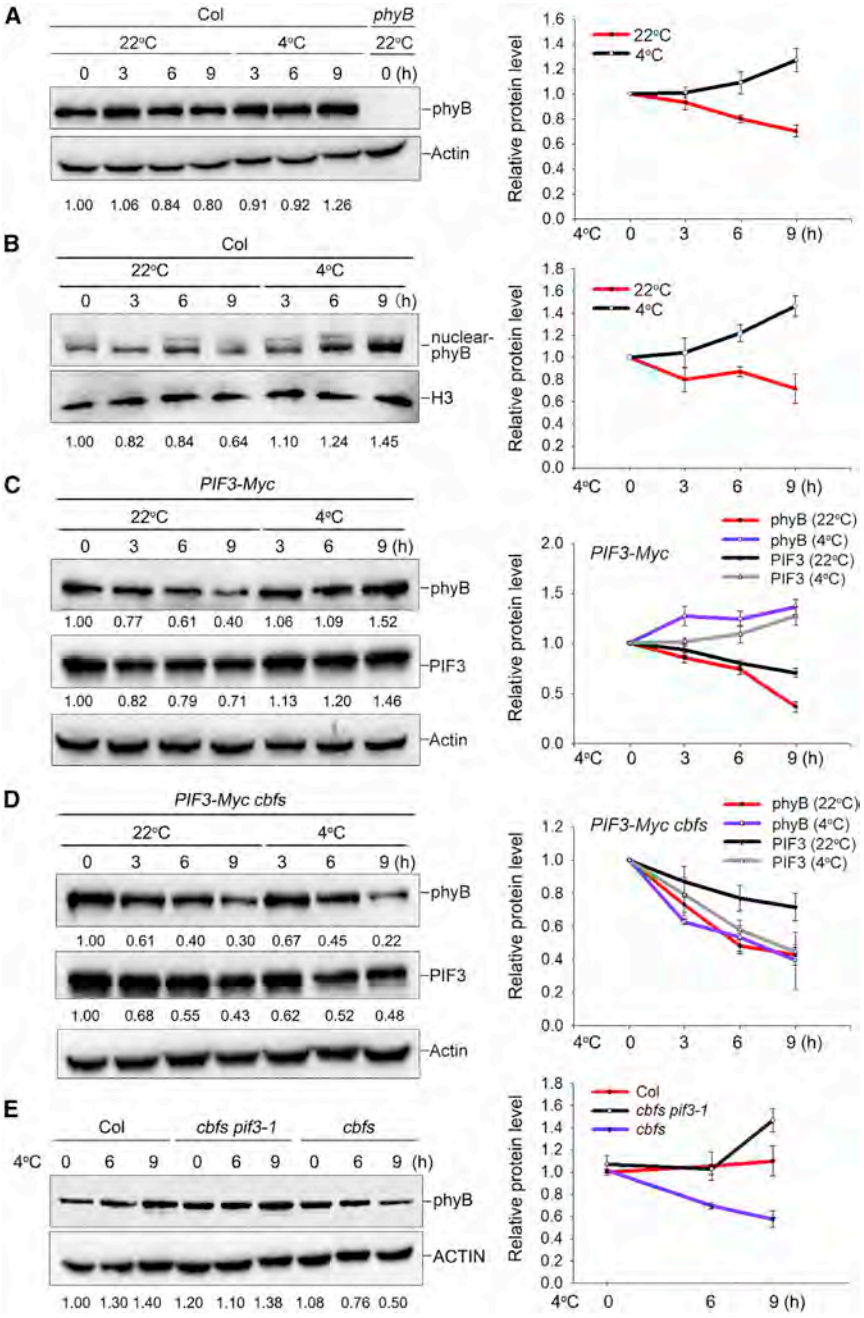

Figure 2. Cold-Induced CBFs Attenuate the PIF3-Mediated Feedback Regulation of phyB.

(A and B) Stability of phyB **(A)** and nuclear-localized phyB **(B)** proteins in wild-type plants under normal and cold conditions.

(C and D) Stability of phyB and PIF3 proteins in *PIF3-Myc* **(C)** and *PIF3-Myc cbfs* **(D)** plants under normal and cold conditions.

(E) Stability of phyB proteins in *cbfs* and *cbfs pif3-1* mutants under cold conditions.

Twelve-day-old seedlings grown under LD conditions were transferred to 4°C at ZT3 or maintained at 22°C for the indicated periods. Total proteins were extracted and subjected to immunoblot analysis with anti-Myc, anti-phyB, or anti-actin antibodies. The immunoblot results were quantified using ImageJ software. PIF3 and phyB protein levels were normalized to the actin loading control. The relative protein levels are shown below the blots, where relative protein level at 0 h was set to 1.0. Quantitative data are shown as means of three biological replicates ±SEM.

stability of phyB. To test this hypothesis, we examined the phyB protein levels in *PIF3-Myc cbfs* plants before and after cold treatment. Compared with the levels in *PIF3-Myc* plants (Figure 2C), the levels of phyB and PIF3 proteins in *PIF3-Myc cbfs* plants were dramatically reduced after cold treatment (Figure 2D). We then examined the phyB levels in the wild type (Columbia [Col]) and *cbfs* and *cbfs pif3-1* mutants under cold stress. We found that the phyB protein remained stable in Col but was notably degraded in the *cbfs* triple mutant after cold treatment. However, its degradation was largely abolished in the *cbfs pif3-1* mutant (Figure 2E). These results indicate that CBF-mediated stabilization of PIF3 compromises the degradation of phyB.

Previous studies showed that light-induced phosphorylation of PIF3 mediates the co-degradation of PIF3 and phyB through the recruitment of LRBs (Light-Response Bric-a-brack/Tramtrack/Broad) (Ni et al., 2013, 2014). Our data indicated that light-induced phosphorylation of PIF3 was reduced under cold stress (Supplemental Figure 3D) and that cold-induced CBFs had little, if any, effect on the interaction of PIF3 and phyB (Supplemental Figure 3E). Taken together, these data suggest that CBFs interact with PIF3 and stabilize phyB by attenuating PIF3–phyB co-degradation under cold stress.

phyB Positively Regulates Freezing Tolerance

As phyB was shown to be a thermosensor in *Arabidopsis* (Jung et al., 2016; Legris et al., 2016), we next sought to determine the role of phyB in regulating plant freezing tolerance. The *phyB* loss-of-function mutant exhibited impaired freezing tolerance with or without cold acclimation (Figure 3A–3C). The ion leakage of the *phyB* mutant was much higher than that of the wild type (Figure 3D and 3E), indicating that the plasma membrane damage caused by cold stress is more severe in the *phyB* mutant. Conversely, transgenic plants overexpressing *phyB* showed enhanced freezing tolerance compared with wild type under both non-acclimated and cold-acclimated conditions, accompanied by a higher survival rate and lower ion leakage (Figure 3A–3E). Given that phyB is a red-light photoreceptor, we then examined the freezing tolerance of the *phyB* mutant and overexpression plants supplied with only red light in LD conditions. Similar to the observations under white-light conditions, the *phyB* mutant exhibited decreased freezing

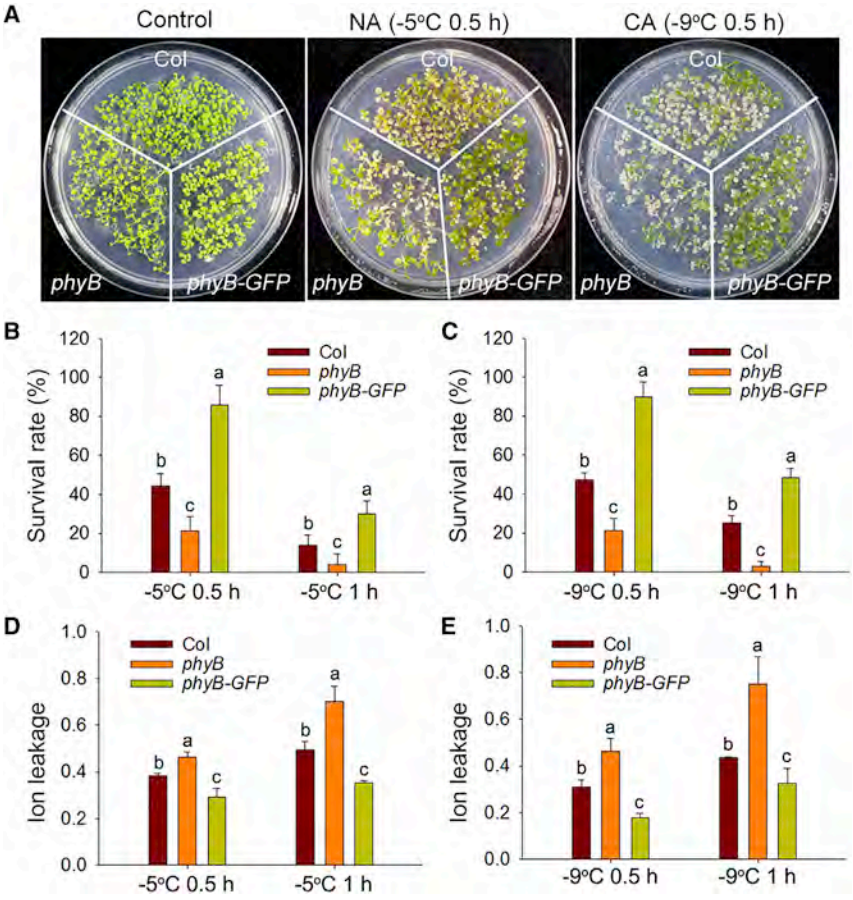

Figure 3. phyB Positively Regulates Plant Freezing Tolerance.

(A–E) Freezing phenotypes **(A)**, survival rates **(B and C)**, and ion leakage **(D and E)** of 12-day-old phyB mutants and *phyB*-overexpression plants. The seedlings were grown under a 16-h-white light/8-h-dark photoperiod (LD) at 22°C and either directly exposed to freezing treatment (non-acclimated, NA), or treated at 4°C for 3 days before freezing treatment (cold-acclimated, CA). Representative photographs were taken after a 3-day recovery period, survival rates were calculated, and the ion leakage was measured. Data are means of three biological replicates ±SEM. Lowercase letters indicate statistically significant differences based on one-way ANOVA with Tukey's HSD post hoc analysis.

tolerance, whereas *phyB*-overexpression plants showed enhanced freezing tolerance under red LD conditions with or without cold acclimation (Supplemental Figure 4A and 4B). These results suggest that phyB positively regulates basal and acquired freezing tolerance of *Arabidopsis* under both white- and red-light conditions.

To further assess the roles of phyB in the cold stress response, we performed an RNA-sequencing (RNA-seq) analysis using wild-type (Col) and *phyB* mutant plants before (0 h) and after cold treatment (3 h and 24 h) and then compared their transcriptomes. RNA-seq analysis revealed that 329 genes were upregulated (phyB-repressed genes) and 306 genes were downregulated (phyB-activated genes) in the *phyB* mutant (fold change ≥ 2; $P < 0.05$) (Supplemental Figure 5A and 5B; Supplemental Tables 1, 2, and 3). Gene ontology (GO) analysis showed that the 211 phyB-activated genes (0 h) and 169 phyB-activated *COR* genes (3 h and 24 h) were primarily involved in the responses to abiotic stresses, biotic stimulus, and flavonoid metabolic processes, whereas the 163 phyB-repressed genes and the 209 phyB-repressed *COR* genes were mostly enriched in the categories of responses to auxin, auxin-activated signaling pathway, developmental processes, signaling transduction, and regulation of growth (Supplemental Figure 5C and 5D). These data suggest that phyB positively regulates freezing tolerance possibly through modulating the expression of stress- and growth-related genes.

CBFs Positively Regulate Freezing Tolerance Partially through phyB

To further explore the genetic relationship between CBFs and phyB, we generated *phyB CBF1*:*CBF1-Myc* transgenic plants by genetic crossing of *phyB* mutant and *CBF1*:*CBF1-Myc* transgenic plants (Jia et al., 2016). The expression of *CBF1* and its target genes, including *COR47*, *RD29A*, and *KIN1*, was slightly upregulated in two independent *CBF1*:*CBF1-Myc* transgenic lines under cold treatment (Supplemental Figure 6A), suggesting that these transgenic plants represent weak *CBF1*-overexpression lines. Consistent with this, *CBF1*:*CBF1-Myc* transgenic plants showed a more freezing-tolerant phenotype with a higher survival rate and lower ion leakage than the wild type with or without cold acclimation (Figure 4A–4C). Non-acclimated *phyB CBF1*:*CBF1-Myc* plants largely phenocopied the *phyB* single mutants. Cold-acclimated *phyB CBF1*:*CBF1-Myc* plants were slightly but significantly more tolerant to freezing than *phyB*, but much more sensitive to freezing than *CBF1*:*CBF1-Myc* plants (Figure 4A–4C). These results suggest that phyB partially contributes to CBF1-mediated freezing tolerance in plants.

To define the role of CBFs in regulating the phyB signaling pathway, we asked how many CBF-regulated *COR* genes were also regulated by phyB. By comparing our list of phyB-regulated *COR* genes with those of CBF-regulated *COR* genes identified in a previous study (Zhao et al., 2016), we observed that about 20% of CBF-activated *COR* genes were downregulated whereas 30% of CBF-repressed *COR* genes were upregulated in *phyB* mutants after cold treatment (fold change ≥ 1.65) (Supplemental Figure 6B; Supplemental Tables 4 and 5). It appears that most of these misregulated genes are indirectly regulated by CBFs, because half of them lack CBF-recognized CRT/DRE *cis* elements in their promoters (Supplemental Tables 4 and 5).

We then performed quantitative PCR assays to further verify the expression of several selected CBF-induced *COR* genes

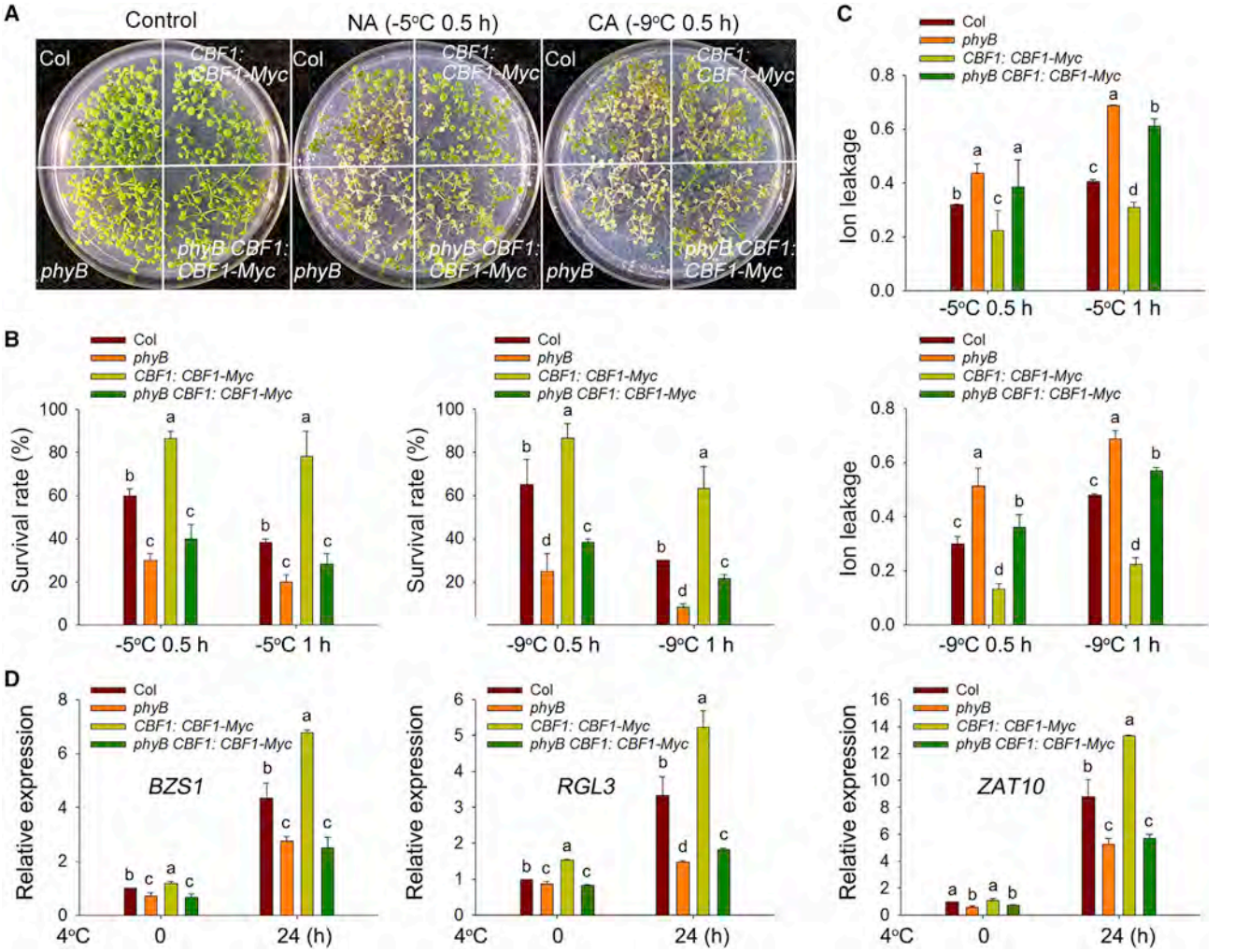

Figure 4. phyB Acts Downstream of CBF1 to Regulate the Expression of *COR* Genes and Plant Freezing Tolerance.

(A–C) Freezing phenotypes **(A)**, survival rates **(B)**, and ion leakage assays **(C)** of 12-day-old *phyB*, *CBF1*:*CBF1-Myc* (#3), and *phyB CBF1*:*CBF1-Myc* (#3) plants. Data are the means of three biological replicates ±SEM. Lowercase letters indicate statistically significant differences based on one-way ANOVA with Tukey's HSD post hoc analysis.

(D) *BZS1*, *RGL3*, and *ZAT10* expression in *phyB*, *CBF1*:*CBF1-Myc*, and *CBF1*:*CBF1-Myc phyB* plants. Twelve-day-old seedlings grown under LD conditions were transferred to 4°C for 24 h at ZT3 and subjected to quantitative PCR analysis. Actin was used as a normalization control. Gene expression in untreated wild-type Col was normalized to 1.0. Data are means of three biological replicates ±SD. Lowercase letters indicate statistical significance based on one-way ANOVA with Tukey's HSD post hoc analysis.

that were downregulated in the *phyB* mutant, *BZS1*, *RGL3*, and *ZAT10*, all of which are involved in phytohormone-modulated photomorphogenesis and freezing tolerance (Achard et al., 2008; Fan et al., 2012; Park et al., 2015; Zhao et al., 2016). Under normal temperatures, the basal transcript levels of *BZS1*, *RGL3*, and *ZAT10* were lower in the *phyB* mutant and higher in *CBF1*:*CBF1-Myc* plants than in the wild type. Moreover, cold induction of *BZS1*, *RGL3*, and *ZAT10* transcript levels was decreased in the *phyB* mutant but increased in *CBF1*:*CBF1-Myc* transgenic plants (Figure 4D). *phyB CBF1*:*CBF1-Myc* plants phenocopied the *phyB* mutant in terms of *BZS1*, *RGL3*, and *ZAT10* expression with or without cold treatment (Figure 4D). Together, these results indicate that CBF1 acts upstream of phyB in regulating freezing tolerance and the expression of a set of *COR* genes.

phyB Promotes the Degradation of PIF1, PIF4, and PIF5 under Cold Stress to Increase Plant Freezing Tolerance

It is well documented that phytochromes induce rapid phosphorylation and consequent degradation of the PIF1, PIF4, and PIF5 proteins in response to light at warm temperatures (Leivar and Quail, 2011). Moreover, PIF4 was shown to directly repress *CBF* genes under cold stress (Lee and Thomashow, 2012). Thus, the freezing hypersensitivity of the *phyB* mutant might result from an increase in PIF abundance. Next, we examined whether the levels of the PIF1, PIF4, and PIF5 proteins under cold stress are also regulated by phyB. Transgenic plants overexpressing *PIF1-Myc*, *PIF4-Myc*, and *PIF5-HA* grown under LD conditions were maintained at 22°C or transferred to 4°C at ZT3 for different times. Our immunoblot data showed that the PIF1, PIF4, and PIF5 proteins were stable at 22°C but were

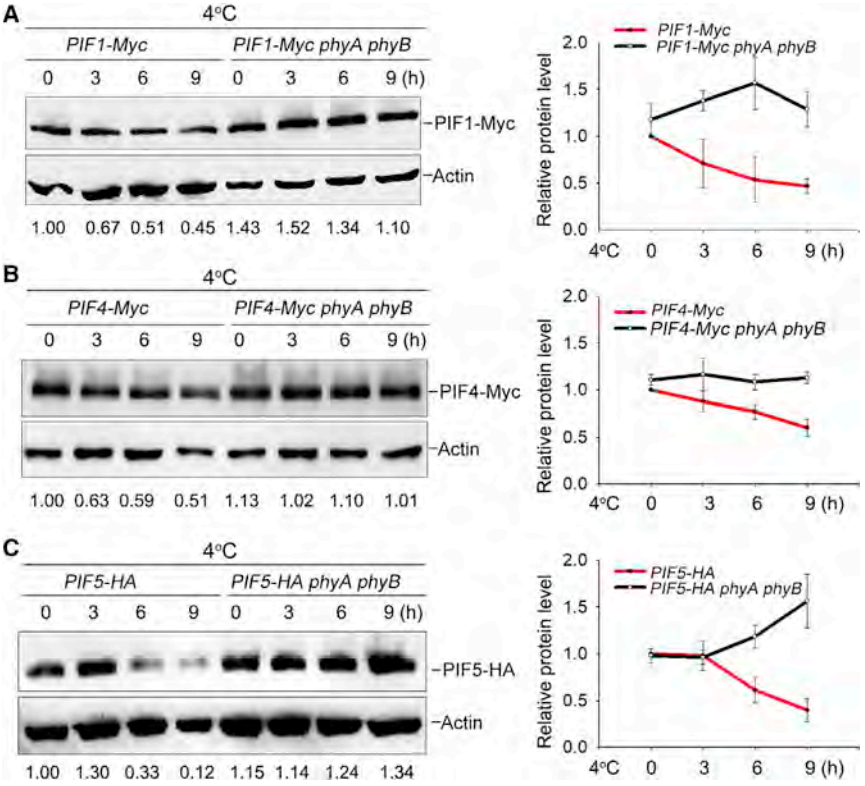

Figure 5. Cold Stress Promotes the Phytochrome-dependent Degradation of PIF1, PIF4, and PIF5.

(A–C) Stability of PIF1 **(A)**, PIF4 **(B)**, and PIF5 **(C)** proteins in *PIF*-overexpressing plants in wild-type and *phyA phyB* mutant backgrounds under cold treatment as described in Figure 3. Quantitative data are shown as means of three biological replicates ±SEM.

degraded gradually after cold treatment (Figure 5A–5C and Supplemental Figure 7A–7C). By contrast, the degradation of the PIF1, PIF4, and PIF5 proteins was largely inhibited when the plants were transferred to darkness at 4°C (Supplemental Figure 7D–7F). Consistent with these results, cold-induced degradation of these three PIFs under light was fully suppressed in the *phyA phyB* mutant (Figure 5A–5C). These results indicate that cold stress negatively regulates the stability of the PIF1, PIF4, and PIF5 proteins in a phytochrome-dependent manner. In addition, the expression of *PIF1*, *PIF4*, and *PIF5* in the wild-type plants was also downregulated by cold stress, but independently of phyA or phyB (Supplemental Figure 7G).

We then examined the freezing tolerance of the *pif1*, *pif4*, and *pif5* single mutants, as well as the *pif1 pif3 pif4 pif5* (*pifq*) quadruple mutant. The *pif4*, *pif5*, and *pifq* mutants exhibited increased survival rates and reduced ion leakage compared with the wild type with or without cold acclimation, with *pif4* and *pifq* mutants showing a phenotype similar to that of *pif5* but with higher freezing tolerance (Figure 6A–6C). The *pif1* mutant showed no obvious freezing phenotype when compared with the wild type (Supplemental Figure 7H). By contrast, transgenic plants overexpressing *PIF1*, *PIF4*, or *PIF5* showed impaired freezing tolerance and increased ion leakage after freezing treatment (Figure 6D–6F). These results further indicate that PIF1, PIF4, and PIF5 are negative regulators of plant freezing tolerance, with PIF4 playing a more prominent role than PIF1 and PIF5.

To explore the genetic interaction between *phyB* and *PIFs*, we compared the freezing tolerance phenotypes of the *phyB*, *pifq*, and *phyB pifq* mutants. The freezing-sensitive phenotype of the *phyB* mutant was partially compromised in the *pifq* mutant background (Figure 7A–7C). This observation is consistent with previous findings that hypocotyls of *phyB pifq* mutant seedlings are of intermediate length compared with those of Col and *phyB* mutants under both continuous white and red light (Leivar et al., 2008; Lorrain et al., 2008), which might be explained by a mutually negative regulatory loop between phyB and PIFs (Leivar et al., 2008). Interestingly, downregulation of *BZS1* and *ZAT10* expression in the *phyB* mutant was almost fully rescued in the *pifq* mutant background before and after cold treatment (Figure 7D). These data together suggest that phyB promotes the degradation of the PIF1, PIF4, and PIF5 proteins under cold stress, thereby enhancing *Arabidopsis* freezing tolerance.

DISCUSSION

Previous studies have demonstrated the roles of PIF3, PIF4, and PIF7 in repressing the expression of *CBF* genes, which encode the key transcription factors positively regulating cold acclimation (Lee and Thomashow, 2012; Jiang et al., 2017). In this study, we demonstrate an intricate molecular link between PIF3 and CBFs, the master transcription regulators in the light- and cold-signaling pathways, respectively. On one hand, PIFs directly repress *CBF* genes at low temperatures, which presumably helps plants avoid an excessive cold response and growth retardation. On the other hand, cold-induced CBF proteins relieve this repression by interacting with PIF3 and repressing the co-degradation of the PIF3–phyB module (Figure 7E). This feedback regulatory mechanism likely fine-tunes cold signaling, allowing plants to withstand cold environments.

Accumulating evidence indicates that CBFs are transcriptional activators of *COR* genes (Stockinger et al., 1997; Liu et al., 1998; Jia et al., 2016; Zhao et al., 2016). In this study, we uncover a new biological function of CBFs as molecular partners of PIF3. Several interacting partners have been reported for PIF3 in previous studies. It was shown that DELLA proteins interact with PIF3 and PIF4 and coordinate hypocotyl elongation (de Lucas et al., 2008; Feng et al., 2008). HY5 and PIF1/3 directly interact with each other and antagonistically regulate the expression of reactive oxygen species-responsive genes upon light irradiation (Chen et al., 2013). TIMING OF CAB

EXPRESSION1 (TOC1), an essential component of the circadian clock system, interacts with the PIFs and represses their transcriptional activity, thereby regulating diverse downstream morphogenic responses (Soy et al., 2016). Intriguingly, we show that CBF1 modulates PIF3 protein abundance, rather than its transcript level or transcriptional activity (Figure 2 and Supplemental Figure 2). This finding provides a framework for future studies aiming to understand how CBFs function as a node that integrates cold response and other physiological processes important for optimal plant fitness.

Recent studies showed that phyB senses ambient temperature changes through conversion from the inactive Pr form to the active Pfr form (Jung et al., 2016; Legris et al., 2016). In this study, we demonstrate that the *phyB* mutant shows decreased basal and acquired freezing tolerance under both white- and red-light conditions (Figure 3 and Supplemental Figure 4). Furthermore, cold stabilized phyB promotes the degradation of PIF1, PIF4 and PIF5, all of which negatively regulate plant freezing tolerance . Thus, this study expands our understanding of the predominant role of phyB in adaptation to environmental temperature variation. Notably, it was previously shown that *COR15a* expression was repressed by phyB in high R/FR light at 16°C under a 12-h photoperiod (Franklin and Whitelam, 2007), and that phyB suppresses the expression of *CBF* genes and their target genes, *COR15a* and *GolS3*, at 22°C under LD conditions, thereby negatively regulating basal freezing tolerance (Lee and Thomashow, 2012). The discrepancy in the basal freezing tolerance of the *phyB* mutant between studies is possibly due to differences in the experimental conditions (seedling age, growth in soil versus plates, and freezing measurement methods). In our current study, we indeed observed that phyB negatively regulates the expression of *CBFs* and *COR15a* at 22°C under LD conditions; however, the repression of *CBFs* and *COR15a* by phyB was fully abolished after a 4°C cold treatment (Supplemental Figure 8B). These observations prompted us to hypothesize that the impaired freezing tolerance of the *phyB* mutant might not be due to decreased expression of *CBFs* and *COR15a*. Consistent with this notion, our RNA-seq analysis indicated that phyB regulates freezing tolerance possibly through other stress- and growth-related genes such as *ZAT10*, *RGL3*, and *BZS1* (Achard et al., 2008; Fan et al., 2012; Park et al., 2015) (Supplemental Figure 5 and Supplemental Tables 1, 2, 3, 4, and 5). Moreover, the repression of these genes in the *phyB* mutant was almost completely released by mutations of four *PIFs* (Figure 7). These data strongly suggest that phyB–PIF signaling regulates both basal and acquired freezing tolerance through mediating the expression of a set of stress- and growth-related genes.

A previous study reported that under LD conditions the transcript levels and protein stabilities of PIF4 and PIF7 are higher compared with those under short-day conditions , and that they directly repress the expression of *CBF* genes and freezing tolerance (Lee and Thomashow, 2012). In this study, we established that both gene expression and protein stability of PIF1, PIF4, and PIF5 were downregulated by cold stress (Figure 5 and Supplemental Figure 7). In agreement with these results, PIF4 and PIF5 were found to negatively regulate freezing tolerance in *Arabidopsis* (Figure 6). By contrast, although PIF3 was also shown to negatively regulate freezing tolerance, its protein stability is enhanced by cold stress (Jiang et al., 2017). These findings suggest that PIF3 plays a distinct role in plants' response to cold stress. Indeed, in this study we showed that PIF3, but not PIF1, PIF4, or PIF5 interacts with CBFs under cold stress (Figure 1 and Supplemental Figure 1); this interaction promotes the stability of phyB, thereby resulting in the degradation of the PIF1, PIF4, and PIF5 proteins and increased freezing tolerance (Figures 5 and 6). These findings, combined with the central role of PIF4 in thermomorphogenesis (Koini et al., 2009; Kumar et al., 2012), suggest that plants adapt to environmental temperature changes by utilizing the diverse functions of different PIFs.

While we demonstrated that the interaction between CBFs and PIF3 stabilizes the PIF3–phyB module under cold conditions, the underlying mechanism still remains unclear. Photoregulatory protein kinases (PPKs) were shown to be responsible for photoactivated-phy-induced phosphorylation of PIF3 (Ni et al., 2017). Given that light-induced phosphorylation of PIF3 was reduced during cold stress and that cold-induced CBFs had a limited effect on the interaction of PIF3 and phyB (Supplemental Figure 3), future studies should examine whether the interaction of CBFs and PIF3 under cold stress affects the phosphorylation of PIF3 by PPKs or the interaction of PIF3 with LRBs. In addition, the F-box proteins EBF1 and EBF2 were shown to directly mediate PIF3 degradation through the 26S proteasome pathway, and cold induces the ubiquitin-mediated degradation of EBF1 and EBF2 (Dong et al., 2017; Jiang et al., 2017). Considering the earlier evidence showing that phyB physically associates with EBF1 and EBF2 and enhances the substrate–E3 ligase interactions in a light-dependent manner to directly control the stability of EIN3 at warm temperatures (Shi et al., 2016), it will be interesting to investigate whether EBF1 and EBF2 are involved in the CBF-mediated stabilization of PIF3 and phyB.

To summarize, our study defines CBFs as PIF3-interacting partners that stabilize the PIF3–phyB module and provides genetic and molecular evidence that the CBFs–PIF3 module acts as a molecular hub to integrate the cold- and phytochrome-mediated light-signaling networks, allowing plants to better adapt to cold environmental conditions.

METHODS

Plant Materials and Growth Conditions

A. thaliana seeds were sterilized in 10% sodium hypochlorite solution and washed at least five times, then grown on half-strength Murashige and Skoog medium (Sigma-Aldrich) containing 0.8% agar and 1.5% sucrose under a 16-h-light/8-h-dark LD photoperiod. *phyB* (*phyB-9*) (Reed et al., 1993), *phyA phyB* (Strasser et al., 2010), *pif1* (*pif1-1*) (Leivar et al., 2008), *pif3-1* (Ni et al., 2013), *pif4* (*pif4-2*) (Leivar et al., 2008), *pif5* (*pif5-3*) (Leivar et al., 2008), *pifq* (Leivar et al., 2008), *cbfs* (Jia et al., 2016), *CBF1*:*CBF1-Myc* (Jia et al., 2016), *35S*:*PIF1-Myc* (Dong et al., 2014), *35S*:*PIF3-Myc* (*PIF3-His-Myc*) (Park et al., 2004), *35S*:*PIF4-Myc* (Sakuraba et al., 2014), *35S*:*PIF5-HA* (Shen et al., 2007), and *35S*:*phyB-GFP* (Medzihradszky et al., 2013; Yue et al., 2016) were used in this study.

For plants grown in cool white light generated by a fluorescent light bulb (Philips), the intensity of white light was 60–80 μmol m^{-2} s^{-1}, and the composition of white light was UV (0.40%), blue light (20.5%), green light

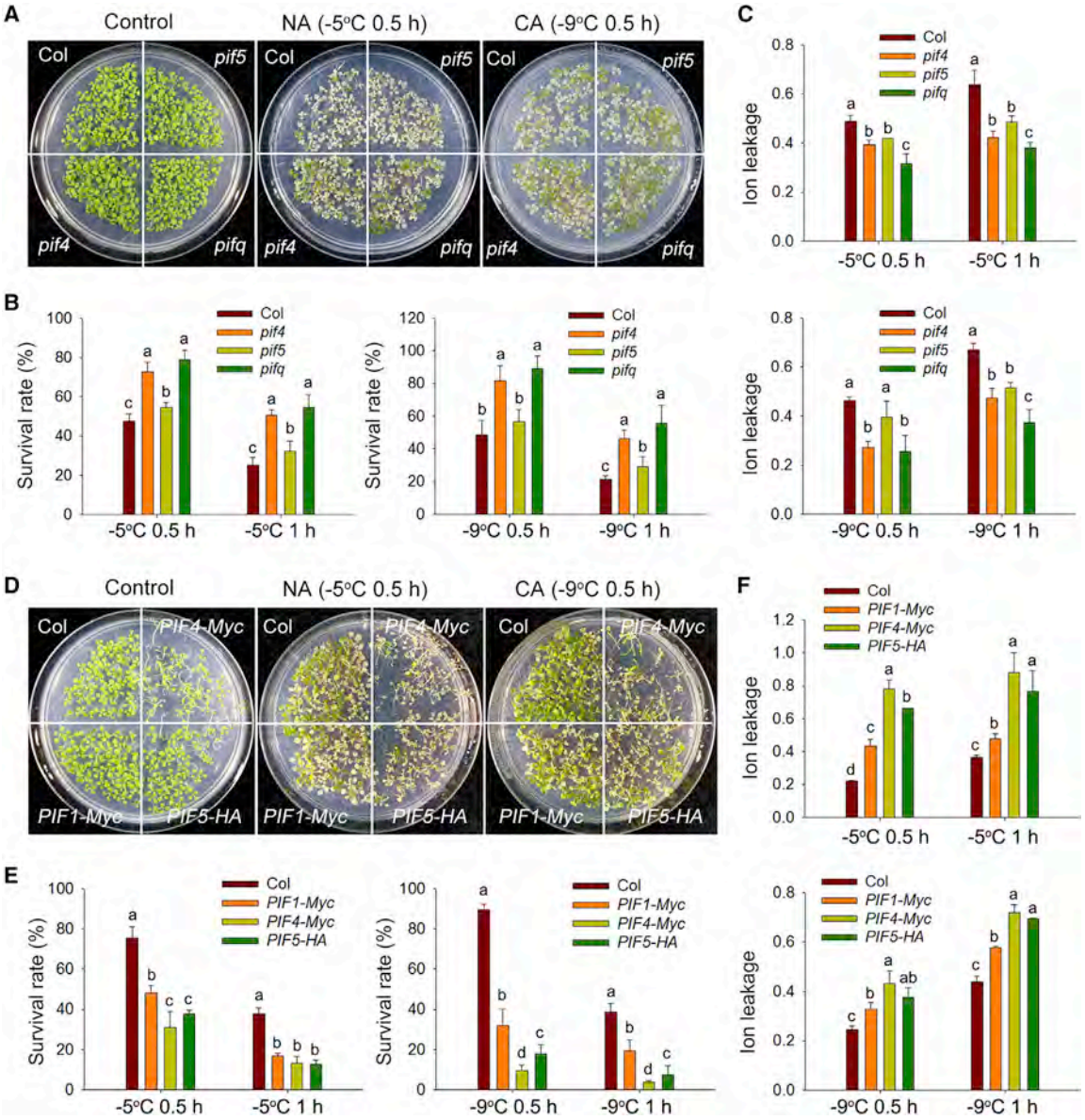

Figure 6. PIF1, PIF4, and PIF5 Negatively Regulate Plant Freezing Tolerance.
(A–F) Freezing phenotypes **(A and D)**, survival rates **(B and E)**, and ion leakage **(C and F)** of 12-day-old plants. Data are the means of three biological replicates ±SEM. Lowercase letters indicate statistically significant differences based on one-way ANOVA with Tukey's HSD post hoc analysis.

(41.3%), red light (34.1%), and near infrared (3.7%). For plants grown in red light, the intensity was 20–25 μmol m^{-2} s^{-1}.

Freezing Tolerance and Electrolyte Leakage Assays

Freezing tolerance assays were performed as described previously (Shi et al., 2012). For cold-acclimation treatment, seedlings were first grown at 22°C under 60–80 μmol m^{-2} s^{-1} cool white fluorescent illumination for 12 days, transferred to 4°C under 10–15 μmol m^{-2} s^{-1} cool white fluorescent illumination for 3 days, and then transferred to a freezing chamber (RuMED4001) for freezing assays, which were conducted as follows. Seedlings were maintained at 0°C for 1 h and temperatures were then dropped by 1°C per hour until the temperatures described in the figure legends were reached. After the freezing treatment, the seedlings were shifted to 4°C and kept in darkness for 12 h before being transferred to normal conditions (16-h-light/8-h-dark photoperiod) at 22°C for another 3 days of recovery. The survival rates were determined by counting the number of seedlings that still generated new leaves. After recovery from the freezing treatment, the seedlings were collected and electrolyte leakage was measured as described by Shi et al. (2012).

In Vitro Pull-Down Assays

The plasmids for expressing the fusion proteins were generated using different primers (listed in Supplemental Table 6), and full-length *CBF1/2/3* were cloned into the pMAL-c2 or pMAL-c5x vector and full-length *PIF3*, *PIF4* and *PIF5* were cloned into the pET28a or pGEX4-1 vectors. The constructs were transformed into *Escherichia coli* BL21 or BL21 codon plus, and expression of the PIF3 and CBF1/2/3 fusion proteins was induced at 37°C for 3 h using 0.5 mM IPTG (isopropyl β-D-1-thiogalacto-pyranoside). Pull-down assays were performed using maltose binding protein agarose or glutathione-agarose beads (Sigma-Aldrich), and the pulled-down proteins were analyzed with anti-His, anti-GST or anti-MBP antibodies (Sigma-Aldrich).

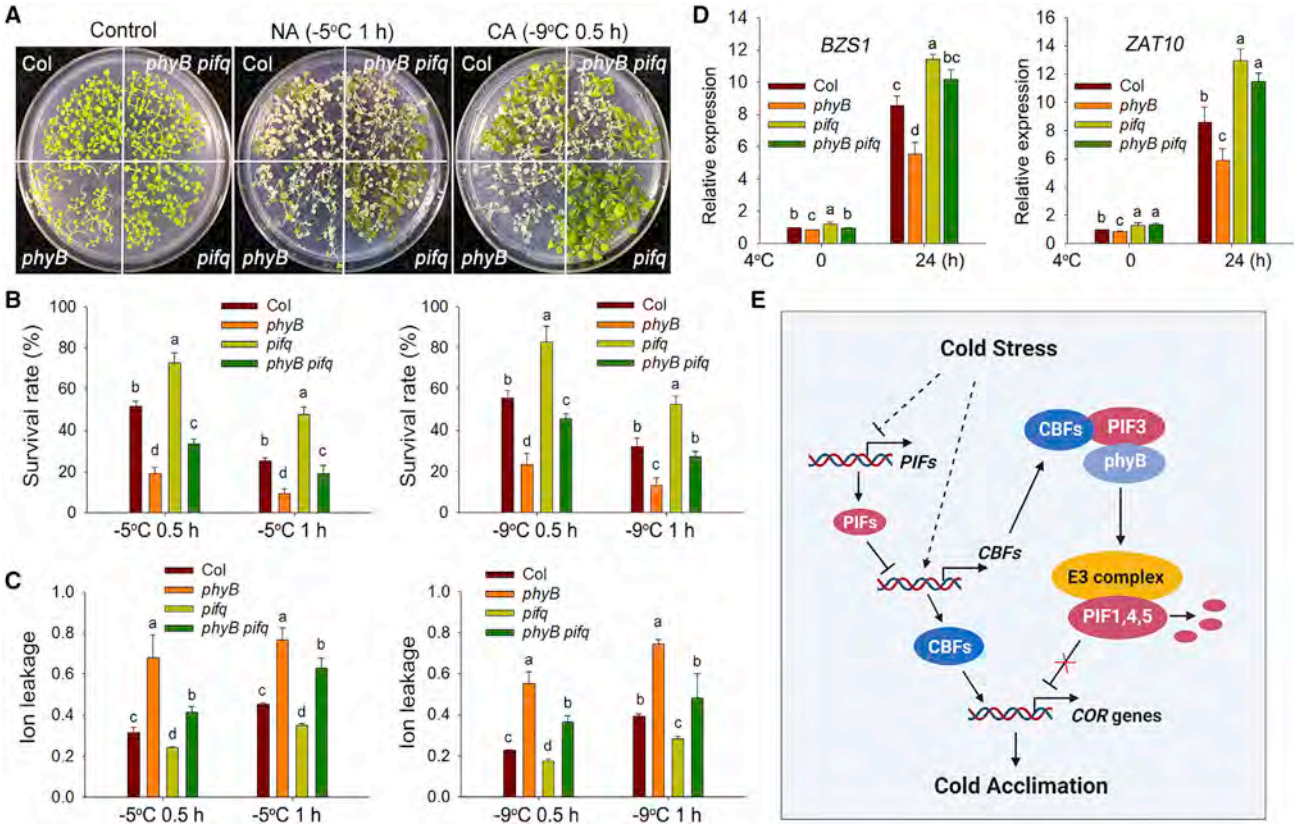

Figure 7. phyB Positively Regulates Plant Freezing Tolerance Partially through PIFs.

(A–C) Freezing phenotypes **(A)**, survival rates **(B)**, and ion leakage **(C)** of 12-day-old *phyB*, *pifq*, and *phyB pifq* mutants. Data are means of three biological replicates ±SEM. Lowercase letters indicate statistically significant differences based on one-way ANOVA with Tukey's HSD post hoc analysis.

(D) Expression of *BZS1* and *ZAT10* in *phyB*, *pifq*, and *phyB pifq* plants as described in Figure 4D. Data are means of three replicates ±SD. Lowercase letters indicate statistical significance based on one-way ANOVA with Tukey's HSD post hoc analysis.

(E) A proposed model depicting the role of the CBF–PIF3–phyB module in regulating freezing tolerance in *Arabidopsis*. Cold stress induces the expression of *CBF* genes, and the accumulated CBF proteins interact with PIF3. This interaction prevents PIF3 and phyB proteins from undergoing the co-degradation that occurs under light at warm temperatures. Cold-stabilized phyB promotes the degradation of PIF1, PIF4, and PIF5, and consequently mediates the expression of a set of stress-related and growth-related *COR* genes, thus increasing freezing tolerance in *Arabidopsis*.

Co-immunoprecipitation Assays

The plasmids used for Co-IP assays were generated using different primers (listed in Supplemental Table 6), and full-length *CBF1/2/3* were cloned into *35S:HA-FLAG* and full-length *PIF3* was cloned into *Super:Myc* (pCAMBIA1300-Myc vector harboring a Super promoter) (Yang et al., 2010). The plasmids were purified and transformed into *Arabidopsis* mesophyll protoplasts. Total proteins were extracted with extraction buffer containing 150 mM NaCl, 10 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5% NP-40, and 1× protease inhibitor cocktail (Roche). The protein extracts were then incubated with anti-HA beads (Sigma-Aldrich) at 4°C for 2 h. Samples were washed five times with the extraction buffer and then used for immunoblotting with anti-HA or anti-Myc antibody (Sigma-Aldrich).

For *PIF3-His-Myc CBF1:CBF1-Myc* transgenic plants, total proteins were extracted and protein extracts were incubated with anti-His beads (Sigma-Aldrich) at 4°C for 2 h. Samples were washed five times with the extraction buffer and then used for immunoblotting with anti-His or anti-Myc antibody (Sigma-Aldrich).

Luciferase Complementation Imaging Assay

The plasmids for expressing the fusion proteins were generated using different primers (listed in Supplemental Table 6), and full-length *CBF1/2/3, phyB* and *β-glucuronidase (GUS)* were cloned into the pCAMBIA1300-cLUC vector and full-length *PIF1*, *PIF3, PIF4, PIF5* and *GUS* were cloned into the pCAMBIA1300-nLUC vector. Pairs of constructs were transiently expressed in *N. benthamiana* leaves as described by Chen et al. (2008), and the luciferase (LUC) signal was detected by a cold charge-coupled device camera (Nikon-L936; Andor Tech) after 2 days of growth.

Protein Extraction and Immunoblots

For the protein stability experiments, protein extraction and immunoblotting were performed as described by Li et al. (2016) with minor modifications. Total proteins were extracted with denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl [pH 8.0], 8 M urea, 1× protease inhibitor cocktail [Roche], 1 mM phenylmethylsulfonyl fluoride [PMSF; Amresco]) supplemented with 80 μM MG132 (Sigma-Aldrich). For detecting the phosphorylation of PIF3, total proteins were extracted in extraction buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Tween 20, 1 mM PMSF, and 1× complete protease inhibitor cocktail. The total proteins from various materials were quantified and then subjected to immunoblotting analysis with anti-Myc, anti-HA (Sigma-Aldrich), or anti-phyB antibody (Zhang et al., 2018).

RNA Extraction and RT–PCR

Unless otherwise indicated, 12-day-old plants grown at 22°C were transferred to 4°C at ZT3. Total RNAs were extracted at the indicated time

points using the RNAprep Pure Plant Kit (Tiangen), followed by reverse transcription using M-MLV reverse transcriptase (Promega). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix Kit (Takara). Relative expression levels were calculated as described previously (Shi et al., 2012). The specific primers used in the reactions are listed in Supplemental Table 6.

Cell-Fractionation Assay

The cell-fractionation assay was performed as described previously (Liu et al., 2017). The nuclear proteins were extracted and subjected to immunoblot analysis with anti-phyB or anti-H3 antibody.

High-Throughput mRNA-Sequencing Analysis

The wild type and *phyB* mutants were grown at 22°C for 12 days and then treated at 4°C for 0 h, 3 h or 24 h. Total RNAs were isolated and used for RNA deep sequencing on an Illumina Hiseq platform. Results from two independent experiment were collected for the analysis. After removing low-quality reads and adapter sequences, about 4.0 GB of clean reads were generated from each sample. TopHat (version 2.1.0) was used for mapping the clean reads to the reference genome of *Arabidopsis* (TAIR10) (Trapnell et al., 2012). Cuffdiff (version 2.1.0) was used to calculate the differentially expressed genes between two samples (Trapnell et al., 2013). The comparisons with a fold change ≥ 2 and P value <0.05 were considered as differentially expressed genes. Heatmaps were drawn based on the multiplied relative expression levels using the software MultiExperiment Viewer (MeV version 4.9.0) (Saeed et al., 2003). GO-term enrichment was analyzed with agriGO v2.0, which contains up-to-date GO annotation data for *Arabidopsis* (Tian et al., 2017).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This work was supported by grants from the Ministry of Agriculture of China, China (2016ZX08009003-002), the National Natural Science Foundation of China, China (31872658, 31921001), and Discipline Program of Beijing Outstanding University, China.

AUTHOR CONTRIBUTIONS

S.Y. directed the project. B.J. performed the experiments with the help of Y.S., Y.P., and J.D. All authors discussed and interpreted the results. B.J., Y.S., and S.Y. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Peter H. Quail (University of California, Berkeley), Suiwen Hou (Lanzhou University), and Hongtao Liu (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) for providing the *35S*: *PIF5-HA*, *35S*: *phyB-GFP*, *phyB pifq*, and *35S*:*PIF4-Myc* seeds. No conflict of interest declared.

Received: November 28, 2019
Revised: March 31, 2020
Accepted: April 15, 2020
Published: April 18, 2020

REFERENCES

Achard, P., Gong, F., Cheminant, S., Alioua, M., Hedden, P., and Genschik, P. (2008). The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell **20**:2117–2129.

Al-Sady, B., Ni, W., Kircher, S., Schafer, E., and Quail, P.H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Mol. Cell **23**:439–446.

Al-Sady, B., Kikis, E.A., Monte, E., and Quail, P.H. (2008). Mechanistic duality of transcription factor function in phytochrome signaling. Proc. Natl. Acad. Sci. U S A **105**:2232–2237.

Bae, G., and Choi, G. (2008). Decoding of light signals by plant phytochromes and their interacting proteins. Annu. Rev. Plant Biol. **59**:281–311.

Casal, J.J., and Questa, J.I. (2018). Light and temperature cues: multitasking receptors and transcriptional integrators. New Phytol. **217**:1029–1034.

Chen, H.M., Zou, Y., Shang, Y.L., Lin, H.Q., Wang, Y.J., Cai, R., Tang, X.Y., and Zhou, J.M. (2008). Firefly luciferase complementation imaging assay for protein-protein interactions in plants. Plant Physiol. **146**:368–376.

Chen, D., Xu, G., Tang, W., Jing, Y., Ji, Q., Fei, Z., and Lin, R. (2013). Antagonistic basic helix-loop-helix/bZIP transcription factors form transcriptional modules that integrate light and reactive oxygen species signaling in *Arabidopsis*. Plant Cell **25**:1657–1673.

Crosatti, C., de Laureto, P.P., Bassi, R., and Cattivelli, L. (1999). The interaction between cold and light controls the expression of the cold-regulated barley gene *cor14b* and the accumulation of the corresponding protein. Plant Physiol. **119**:671–680.

de Lucas, M., Daviere, J.M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blazquez, M.A., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature **451**:480–484.

Ding, Y., Jia, Y., Shi, Y., Zhang, X., Song, C., Gong, Z., and Yang, S. (2018). OST1-mediated BTF3L phosphorylation positively regulates CBFs during plant cold responses. EMBO J. **37**:e98228.

Ding, Y., Shi, Y., and Yang, S. (2020). Molecular regulation of plant responses to environmental temperatures. Mol. Plant. **13**:544–564.

Dong, J., Tang, D., Gao, Z., Yu, R., Li, K., He, H., Terzaghi, W., Deng, X.W., and Chen, H. (2014). *Arabidopsis* DE-ETIOLATED1 represses photomorphogenesis by positively regulating phytochrome-interacting factors in the dark. Plant Cell **26**:3630–3645.

Dong, J., Ni, W., Yu, R., Deng, X.W., Chen, H., and Wei, N. (2017). Light-dependent degradation of PIF3 by $SCF^{(EBF1/2)}$ promotes a photomorphogenic response in *Arabidopsis*. Curr. Biol. **27**:2420–2430.

Fan, X.Y., Sun, Y., Cao, D.M., Bai, M.Y., Luo, X.M., Yang, H.J., Wei, C.Q., Zhu, S.W., Sun, Y., Chong, K., et al. (2012). BZS1, a B-box protein, promotes photomorphogenesis downstream of both brassinosteroid and light signaling pathways. Mol. Plant **5**:591–600.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J.M., Kircher, S., et al. (2008). Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. Nature **451**:475–479.

Franklin, K.A., and Whitelam, G.C. (2007). Light-quality regulation of freezing tolerance in *Arabidopsis thaliana*. Nat. Genet. **39**:1410–1413.

Guy, C.L., Hummel, R.L., and Haskell, D. (1987). Induction of freezing tolerance in spinach during cold acclimation. Plant Physiol. **84**:868–871.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998). *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science **280**:104–106.

Jia, Y., Ding, Y., Shi, Y., Zhang, X., Gong, Z., and Yang, S. (2016). The *cbfs* triple mutants reveal the essential functions of *CBFs* in cold acclimation and allow the definition of CBF regulons in *Arabidopsis*. New Phytol. **212**:345–353.

Jiang, B., Shi, Y., Zhang, X., Xin, X., Qi, L., Guo, H., Li, J., and Yang, S. (2017). PIF3 is a negative regulator of the *CBF* pathway and freezing

tolerance in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A **114**:E6695–E6702.

Jiao, Y., Lau, O.S., and Deng, X.W. (2007). Light-regulated transcriptional networks in higher plants. Nat. Rev. Genet. **8**:217–230.

Jung, J.H., Domijan, M., Klose, C., Biswas, S., Ezer, D., Gao, M., Khattak, A.K., Box, M.S., Charoensawan, V., Cortijo, S., et al. (2016). Phytochromes function as thermosensors in *Arabidopsis*. Science **354**:886–889.

Kim, H.J., Kim, Y.K., Park, J.Y., and Kim, J. (2002). Light signalling mediated by phytochrome plays an important role in cold-induced gene expression through the C-repeat/dehydration responsive element (C/*DRE*) in *Arabidopsis thaliana*. Plant J. **29**:693–704.

Koini, M.A., Alvey, L., Allen, T., Tilley, C.A., Harberd, N.P., Whitelam, G.C., and Franklin, K.A. (2009). High temperature-mediated adaptations in plant architecture require the bHLH transcription factor PIF4. Curr. Biol. **19**:408–413.

Kumar, S.V., Lucyshyn, D., Jaeger, K.E., Alos, E., Alvey, E., Harberd, N.P., and Wigge, P.A. (2012). Transcription factor PIF4 controls the thermosensory activation of flowering. Nature **484**:242–245.

Lau, O.S., Song, Z., Zhou, Z., Davies, K.A., Chang, J., Yang, X., Wang, S., Lucyshyn, D., Tay, I.H.Z., Wigge, P.A., et al. (2018). Direct Control of *SPEECHLESS* by PIF4 in the high-temperature response of stomatal development. Curr. Biol. **28**:1273–1280.

Lee, C.M., and Thomashow, M.F. (2012). Photoperiodic regulation of the C-repeat binding factor (*CBF*) cold acclimation pathway and freezing tolerance in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U S A **109**:15054–15059.

Legris, M., Klose, C., Burgie, E.S., Rojas, C.C., Neme, M., Hiltbrunner, A., Wigge, P.A., Schafer, E., Vierstra, R.D., and Casal, J.J. (2016). Phytochrome B integrates light and temperature signals in *Arabidopsis*. Science **354**:897–900.

Leivar, P., and Monte, E. (2014). PIFs: systems integrators in plant development. Plant Cell **26**:56–78.

Leivar, P., and Quail, P.H. (2011). PIFs: pivotal components in a cellular signaling hub. Trends Plant Sci. **16**:19–28.

Leivar, P., Monte, E., Oka, Y., Liu, T., Carle, C., Castillon, A., Huq, E., and Quail, P.H. (2008). Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. Curr. Biol. **18**:1815–1823.

Li, S.N., Wang, W.Y., Gao, J.L., Yin, K.Q., Wang, R., Wang, C.C., Petersen, M., Mundy, J., and Qiu, J.L. (2016). MYB75 phosphorylation by MPK4 is required for light-induced anthocyanin accumulation in *Arabidopsis*. Plant Cell **28**:2866–2883.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell **10**:1391–1406.

1.. **Liu, Z., Jia, Y., Ding, Y., et al.** (2017). Plasma membrane CRPK1-mediated phosphorylation of 14-3-3 proteins induces their nuclear import to fine-tune *CBF* signaling during cold response. Mol. Cell **66**:117–128.

Liu, J., Shi, Y., and Yang, S. (2018). Insights into the regulation of CBF cold signaling in plants. J. Integr. Plant Biol. **9**:780–795.

Lorrain, S., Allen, T., Duek, P.D., Whitelam, G.C., and Fankhauser, C. (2008). Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant J. **53**:312–323.

Lu, X., Zhou, Y., Fan, F., Peng, J., and Zhang, J. (2019). Coordination of light, circadian clock with temperature: the potential mechanisms regulating chilling tolerance in rice. J. Integr. Plant Biol. https://doi.org/10.1111/jipb.12852.

Medzihradszky, M., Bindics, J., Adam, E., Viczian, A., Klement, E., Lorrain, S., Gyula, P., Merai, Z., Fankhauser, C., Medzihradszky, K.F., et al. (2013). Phosphorylation of phytochrome B inhibits light-induced signaling via accelerated dark reversion in *Arabidopsis*. Plant Cell **25**:535–544.

Ni, W., Xu, S.L., Chalkley, R.J., Pham, T.N., Guan, S., Maltby, D.A., Burlingame, A.L., Wang, Z.Y., and Quail, P.H. (2013). Multisite light-induced phosphorylation of the transcription factor PIF3 is necessary for both its rapid degradation and concomitant negative feedback modulation of photoreceptor phyB levels in *Arabidopsis*. Plant Cell **25**:2679–2698.

Ni, W., Xu, S.L., Tepperman, J.M., Stanley, D.J., Maltby, D.A., Gross, J.D., Burlingame, A.L., Wang, Z.Y., and Quail, P.H. (2014). A mutually assured destruction mechanism attenuates light signaling in *Arabidopsis*. Science **344**:1160–1164.

Ni, W., Xu, S.L., Gonzalez-Grandio, E., Chalkley, R.J., Huhmer, A.F.R., Burlingame, A.L., Wang, Z.Y., and Quail, P.H. (2017). PPKs mediate direct signal transfer from phytochrome photoreceptors to transcription factor PIF3. Nat. Commun. **8**:15236.

Park, E., Kim, J., Lee, Y., Shin, J., Oh, E., Chung, W.I., Liu, J.R., and Choi, G. (2004). Degradation of phytochrome interacting factor 3 in phytochrome-mediated light signaling. Plant Cell Physiol **45**:968–975.

Park, S., Lee, C.M., Doherty, C.J., Gilmour, S.J., Kim, Y., and Thomashow, M.F. (2015). Regulation of the *Arabidopsis CBF* regulon by a complex low-temperature regulatory network. Plant J. **82**:193–207.

Qiu, Y., Li, M., Kim, R.J., Moore, C.M., and Chen, M. (2019). Daytime temperature is sensed by phytochrome B in *Arabidopsis* through a transcriptional activator HEMERA. Nat. Commun. **10**:140.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell **5**:147–157.

Rockwell, N.C., Su, Y.S., and Lagarias, J.C. (2006). Phytochrome structure and signaling mechanisms. Annu. Rev. Plant Biol. **57**:837–858.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., et al. (2003). TM4: a free, open-source system for microarray data management and analysis. Biotechniques **34**:374–378.

Sakuraba, Y., Jeong, J., Kang, M.Y., Kim, J., Paek, N.C., and Choi, G. (2014). Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in *Arabidopsis*. Nat. Commun. **5**:4636.

Shen, Y., Khanna, R., Carle, C.M., and Quail, P.H. (2007). Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation. Plant Physiol. **145**:1043–1051.

Shen, H., Zhu, L., Castillon, A., Majee, M., Downie, B., and Huq, E. (2008). Light-induced phosphorylation and degradation of the negative regulator PHYTOCHROME-INTERACTING FACTOR1 from *Arabidopsis* depend upon its direct physical interactions with photoactivated phytochromes. Plant Cell **20**:1586–1602.

Shi, Y., Tian, S., Hou, L., Huang, X., Zhang, X., Guo, H., and Yang, S. (2012). Ethylene signaling negatively regulates freezing tolerance by repressing expression of *CBF* and type-A *ARR* genes in *Arabidopsis*. Plant Cell **24**:2578–2595.

Shi, H., Shen, X., Liu, R.L., Xue, C., Wei, N., Deng, X.W., and Zhong, S.W. (2016). The red light receptor phytochrome B directly enhances substrate-E3 ligase interactions to attenuate ethylene responses. Dev. Cell **39**:597–610.

Shi, Y., Ding, Y., and Yang, S. (2018). Molecular regulation of CBF signaling in cold acclimation. Trends Plant Sci. **23**:623–637.

Soitamo, A.J., Piippo, M., Allahverdiyeva, Y., Battchikova, N., and Aro, E.M. (2008). Light has a specific role in modulating *Arabidopsis* gene expression at low temperature. BMC Plant Biol. **8**:13.

Soy, J., Leivar, P., Gonzalez-Schain, N., Martin, G., Diaz, C., Sentandreu, M., Al-Sady, B., Quail, P.H., and Monte, E. (2016). Molecular convergence of clock and photosensory pathways through PIF3-TOC1 interaction and co-occupancy of target promoters. Proc. Natl. Acad. Sci. U S A **113**:4870–4875.

Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997). *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. U S A **94**:1035–1040.

Strasser, B., Sanchez-Lamas, M., Yanovsky, M.J., Casal, J.J., and Cerdan, P.D. (2010). *Arabidopsis thaliana* life without phytochromes. Proc. Natl. Acad. Sci. U S A **107**:4776–4781.

Thomashow, M.F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**:571–599.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W., and Su, Z. (2017). agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. **45**:W122–W129.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. **7**:562–578.

Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat. Biotechnol. **31**:46–53.

Yang, H., Shi, Y., Liu, J., Guo, L., Zhang, X., and Yang, S. (2010). A mutant CHS3 protein with TIR-NB-LRR-LIM domains modulates growth, cell death and freezing tolerance in a temperature-dependent manner in *Arabidopsis*. Plant J. **63**:283–296.

Yue, J., Qin, Q., Meng, S., Jing, H., Gou, X., Li, J., and Hou, S. (2016). TOPP4 regulates the stability of PHYTOCHROME INTERACTING FACTOR5 during photomorphogenesis in *Arabidopsis*. Plant Physiol. **170**:1381–1397.

Zhang, S.M., Li, C., Zhou, Y.Y., Wang, X.J., Li, H., Feng, Z.Y., Chen, H.D., Qin, G.J., Jin, D., Terzaghi, W., et al. (2018). TANDEM ZINC-FINGER/PLUS3 is a key component of phytochrome A signaling. Plant Cell **30**:835–852.

Zhao, C., Zhang, Z., Xie, S., Si, T., Li, Y., and Zhu, J.K. (2016). Mutational evidence for the critical role of *CBF* transcription factors in cold acclimation in *Arabidopsis*. Plant Physiol. **171**:2744–2759.