PIF3 is a negative regulator of the *CBF* pathway and freezing tolerance in *Arabidopsis*

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Light and temperature are major environmental factors that coordinately control plant growth and survival. However, how plants integrate light and temperature signals to better adapt to environmental stresses is poorly understood. PHYTOCHROME-INTERACTING FACTOR 3 (PIF3), a key transcription factor repressing photomorphogenesis, has been shown to play a pivotal role in mediating plants' responses to various environmental signals. In this study, we found that PIF3 functions as a negative regulator of *Arabidopsis* freezing tolerance by directly binding to the promoters of *C-REPEAT BINDING FACTOR* (*CBF*) genes to down-regulate their expression. In addition, two F-box proteins, EIN3-BINDING F-BOX 1 (EBF1) and EBF2, directly target PIF3 for 26S proteasome-mediated degradation. Consistently, *ebf1* and *ebf2* mutants were more sensitive to freezing than were the wild type, and the *pif3* mutation suppressed the freezing-sensitive phenotype of *ebf1*. Furthermore, cold treatment promoted the degradation of EBF1 and EBF2, leading to increased stability of the PIF3 protein and reduced expression of the *CBF* genes. Together, our study uncovers an important role of PIF3 in *Arabidopsis* freezing tolerance by negatively regulating the expression of genes in the *CBF* pathway.

freezing tolerance | CBFs | PIF3 | EBF1 | EBF2

Plants are equipped with sophisticated mechanisms to cope with unpredictable environmental conditions. Low temperature is a major environmental factor that adversely affects plant growth and development. To survive under cold stress, a suite of biochemical and physiological changes is triggered in plants, which is termed cold acclimation (1). The three *C-REPEAT BINDING FACTOR/DROUGHT RESPONSE ELEMENT BINDING FACTOR 1B* (*CBF/DREB1*) genes, encoding AP2/ERF family transcription factors, play central roles in plant cold acclimation. The expression of *CBF* genes is promptly induced by cold, which is mediated by several types of transcription factors, including INDUCER OF CBF EXPRESSION 1 (ICE1), CALMODULIN BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3), CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1), and LATE ELONGATED HYPOCOTYL (LHY) (2–6). The cold-induced CBF proteins directly bind to the *CRT/DRE cis*-elements in the promoters of downstream *COLD-REGULATED* (*COR*) genes and activate their expression, thus enhancing plant tolerance to freezing stress (5, 7). The *cbfs* triple mutants generated by the CRISPR/Cas9 technique are defective in cold acclimation, and extremely sensitive to freezing stress after cold acclimation (8, 9).

Light not only provides the primary energy source for photosynthesis but also serves as a key environmental signal for regulating multiple facets of plant growth and development throughout the plants' life cycle. The ability of plants to integrate external signals (e.g., light and temperature) with internal regulatory pathways is vital for their survival. Phytochromes, photoreceptors for red (R) and far-red (FR) wavelengths in plants, have been shown to regulate cold acclimation in *Arabidopsis* (10). Furthermore, light quality and photoperiod also regulate plant freezing tolerance through phytochromes (11, 12). Phytochrome B (phyB) and two PHYTOCHROME-INTERACTING FACTORS (PIFs), that is, PIF4 and PIF7, repress the *CBF* pathway under long-day (LD) conditions (11), whereas a low ratio of red to far-red (R/FR) light increases *COR* gene expression (12). Intriguingly, two recent reports demonstrated that phyB acts as a thermosensor of ambient temperature (13, 14), but the precise role of phytochromes in the cold stress response awaits further investigation. CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and ELONGATED HYPOCOTYL 5 (HY5), two central regulators of photomorphogenesis, have been shown to integrate light and cold signaling to optimize plant survival under freezing temperatures (15). Therefore, plants have evolved a delicate system that perceives interweaved light and temperature signals, allowing plants to modulate development and stress tolerance appropriately for better adaptation to cold environments.

The PIF proteins have been shown to play pivotal roles in repressing photomorphogenesis and in mediating plants' responses to various environmental conditions (16, 17). PIF3, a basic helix–loop–helix (bHLH) family transcription factor, is the foundation member of the PIF proteins (18). It has been well documented that light-activated phyA and phyB both interact with PIF3 and induce its rapid phosphorylation and degradation upon light exposure (19, 20). Recently, PIF3 was shown to be phosphorylated directly by photoregulatory protein kinases (PPKs), and targeted by LRB Cullin 3 E3 ligases together with phyB for ubiquitination and degradation (21, 22). DELLAs promote the degradation of PIF3 and PIF4 in a light-independent manner by recruiting an unknown E3 ligase (23). BIN2, a protein kinase involved in BR signaling, has also been shown to mediate phosphorylation and degradation of PIF3 via the 26S proteasome pathway (24). Thus, it is evident that

Significance

PHYTOCHROME-INTERACTING FACTORS (PIFs) are central integrators of plants' responses to various environmental signals. In this study, we show that PIF3 acts as a negative regulator of plant cold acclimation by directly repressing the expression of *CBF* genes, whereas its protein stability is negatively regulated by two F-box proteins, EBF1 and EBF2, via the 26S proteasome pathway. Moreover, EBF1 and EBF2 are degraded under cold stress, which enhances the stability of PIF3 protein. Collectively, our study establishes an important regulatory paradigm for PIF3 in preventing runaway expression of the *CBF* genes at low temperature, which allows plants to adapt to and withstand harsh environments.

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multiple signaling pathways modulate plant growth and development by regulating the protein stability of PIF3.

In this study, we show that PIF3 acts as a negative regulator of plant freezing tolerance, whereas cold stress stabilizes PIF3 protein by facilitating the degradation of EBF1 and EBF2, two F-box proteins that directly target PIF3 for degradation via the 26S proteasome pathway. Moreover, our data indicate that PIF3 directly binds to the promoters of *CBF* genes and represses their expression. Thus, our study reveals that the EBF1/2-PIF3 module regulates the expression of *CBF* genes to fine-tune the CBF signaling pathway in the plant's response to cold stress.

Results

PIF3 Is a Negative Regulator of Plant Freezing Tolerance. To examine whether PIF3 functions in the plant's response to cold stress, we performed freezing assays using the *pif3* loss-of-function mutants (*pif3-1* and *pif3-3*) and *35S:PIF3-Myc* transgenic plants (25–27). The plants were first grown on 1/2 Murashige and Skoog (MS) medium for 12 d under LD conditions, and then subjected to freezing treatment with or without cold acclimation. The *pif3* mutants displayed a freezing-tolerant phenotype under both nonacclimated (NA) and cold-acclimated (CA) conditions, and their survival rates were much higher than those of the WT (Fig. 1 *A* and *B*). By contrast, the *35S:PIF3-Myc* plants displayed impaired freezing tolerance, and their survival rates were lower than that of the WT (Fig. 1 *D* and *E*). Relative electrolyte leakage can be used as an indicator of cell membrane integrity damage caused by freezing stress. Our data show that electrolyte leakage was reduced in the *pif3* mutants but significantly increased in *35S:PIF3-Myc* plants (Fig. 1 *C* and *F*). These combined results indicate that PIF3 negatively regulates freezing tolerance in *Arabidopsis*.

PIF3 Interacts with EBF1/2 both in Vitro and in Vivo. We previously showed that ethylene signaling regulates plant freezing tolerance (28) and that cold stress promotes the degradation of EBF1 (28), an F-box protein that targets EIN3/EIL1 (two key transcription factors in ethylene signaling) for proteasome-mediated degradation (28–31). We conducted yeast two-hybrid assays to screen for possible targets of EBF1 and its homolog EBF2 that might be involved in cold signaling, and found that PIF3 interacted with both EBF1 and EBF2 (Fig. 2*A*). Consistently, in vitro pull-down assays showed that His-tagged EBF1/2 interacted with GST-tagged PIF3, but not GST alone (Fig. 2 *B* and *C*). Coimmunoprecipitation (co-IP) assays were performed by cotransforming *pSuper:EBF1-Myc* or *pSuper:EBF2-Myc* with *35S:PIF3-HA-FLAG*, respectively, in *Arabidopsis* protoplasts, and strong interactions were detected between EBF1/2 and PIF3 (Fig. 2 *D* and *E*). These results demonstrate that EBF1 and EBF2 physically interact with PIF3 both in vitro and in vivo. *Arabidopsis* Skp1-related proteins (ASKs) are components of the SCF complex that associate with EBF1/2, and we also observed an interaction between PIF3 and ASK1/2 in pull-down assays (Fig. S1).

It has been reported that phosphorylation of several PIF3 Ser residues affects its binding affinity toward LRB E3 ligases (22). To dissect whether these light-induced Ser phosphorylation sites mediates the interaction between PIF3 and EBF1/2, we generated vectors expressing activation domain (AD)-tagged PIF3^{6D} (phosphorylation-mimic form by mutating the six Ser residues to aspartic acids) or PIF3^{6A} (nonphosphorylatable form by mutating

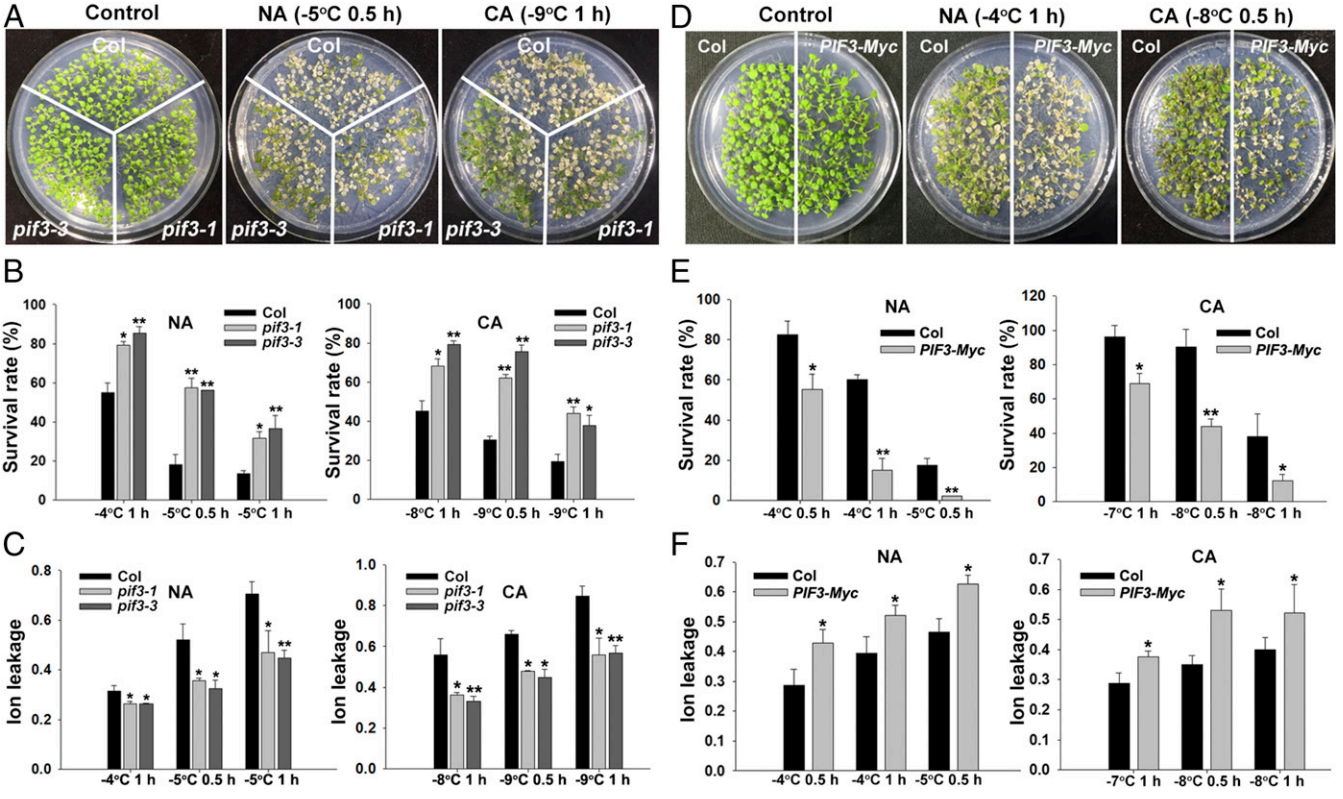

Fig. 1. PIF3 negatively regulates freezing tolerance in *Arabidopsis*. (*A*–*C*) The freezing phenotypes (*A*), survival rates (*B*), and ion leakage assays (*C*) of 12-d-old *pif3* mutants. (*D*–*F*) The freezing phenotypes (*D*), survival rates (*E*), and ion leakage assays (*F*) of 12-d-old *35S:PIF3-Myc* (*PIF3-Myc*) transgenic plants. The procedures for performing NA and CA treatments are described in *Materials and Methods*. After the freezing assays, representative photographs (*A* and *D*) were taken, the survival rates (*B* and *E*) were calculated, and the ion leakages (*C* and *F*) were measured for the indicated plants at different freezing temperatures. In *B*, *C*, *E*, and *F*, data are means of three replicates ± SD. Asterisks indicate significant differences compared with the WT Col under the same conditions (*$P < 0.05$, **$P < 0.01$, Student's *t* test).

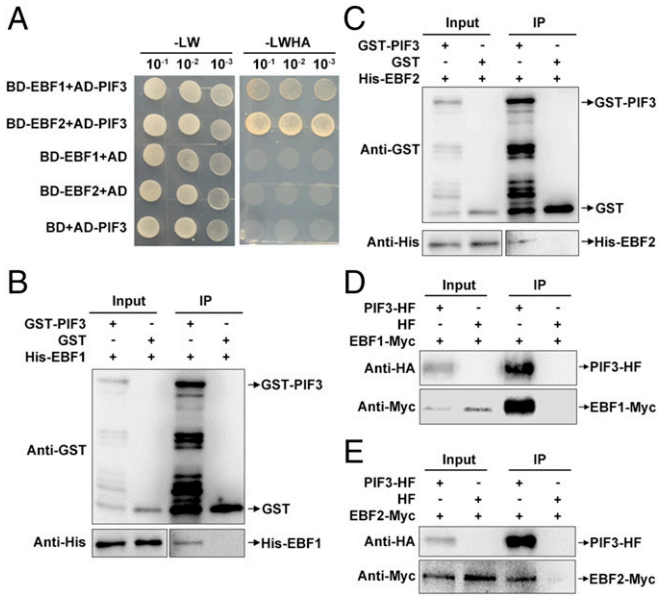

Fig. 2. PIF3 interacts with EBF1/2 in vitro and in vivo. (*A*) Yeast two-hybrid analyses showing the interactions between BD-EBF1/2 and AD-PIF3. The yeast strain AH109 was cotransformed with the pGBKT7 vectors expressing BD-EBF1 or BD-EBF2 and the pGADT7 vector expressing AD-PIF3. Yeast cells harboring both vectors were selected on SD/–Leu/–Trp (–LW) medium, and transferred to SD/–Leu/–Trp/–His/–Ade (–LWHA) medium for testing interactions between BD-EBF1/2 and AD-PIF3. (*B* and *C*) GST pull-down assays showing interactions between His-EBF1/2 and GST-PIF3 in vitro. Purified His-EBF1 (*B*) or His-EBF2 (*C*) fusion proteins were incubated with GST-PIF3 or GST alone, and then GST pull-down assays were performed using glutathione-agarose beads, followed by immunoblot analysis with anti-GST and anti-His antibodies. (*D* and *E*) Co-IP assays showing the interaction between EBF1/EBF2 and PIF3 proteins in vivo. *Arabidopsis* mesophyll protoplasts were cotransformed with *pSuper:EBF1-Myc* (*D*) or *pSuper:EBF2-Myc* (*E*) and *35S:PIF3-HA-FLAG* (*PIF3-HF*) or *35S:HA-FLAG* (*HF*), respectively. Protoplasts were incubated at 22 °C for 16 h in darkness. Total protein extracts were immunoprecipitated with HA Sepharose beads. Crude lysate proteins (input) and immunoprecipitated proteins (IP) were detected using anti-HA and anti-Myc antibodies.

the six Ser residues to alanines) proteins, and cotransformed them into yeast cells with the vector expressing binding domain (BD)-tagged EBF1, respectively. However, we found that the interaction was not affected by altering the PIF3 phosphorylation sites to either aspartic acid or alanine residues (Fig. S2).

EBF1/2 Are Positive Regulators of Plant Freezing Tolerance. We next examined the role of EBF1/2 in regulating plant freezing tolerance. The *ebf1-1* and *ebf2* loss-of-function mutants (*ebf2-1* and *ebf2-3*) exhibited impaired freezing tolerance with or without cold acclimation (Fig. 3 *A* and *B* and Fig. S3 *A* and *B*). Electrolyte leakage in *ebf1-1* and *ebf2* mutants was significantly higher than that of the WT, which is consistent with the survival rates of these plants (Fig. 3*C* and Fig. S3*C*). Conversely, transgenic plants overexpressing *EBF1* or *EBF2* showed enhanced freezing tolerance with or without cold acclimation, accompanied by higher survival rates and lower electrolyte leakage than the WT (Fig. 3 *D–F* and Fig. S3 *D–H*). These results indicate that EBF1 and EBF2 are positive regulators of plant tolerance to freezing stress.

PIF3 Acts Downstream of EBF1 to Negatively Regulate *CBF* Expression. Given the central role of the *CBF* genes in regulating plant freezing tolerance, we examined whether PIF3 and EBF1 mediate freezing tolerance through the *CBF* pathway. Our qRT-PCR data show that cold treatment dramatically induced the expression of *CBFs* and their regulons (Fig. 4 *A–F*), consistent with previous reports (32, 33). Moreover, cold induction of *CBF*s and their regulons (*COR15a*, *KIN1*, and *RD29A*) was up-regulated in the *pif3-1* mutant but down-regulated in the *ebf1-1* mutant compared with that in the WT after cold treatment (Fig. 4 *A–F*). Consistently, the expression of *CBFs* and their regulons was lower in the *35S:PIF3-Myc* plants but higher in the *35S:EBF1-TAP* plants than in the WT after cold treatment (Fig. S4). Therefore, *CBF* expression is negatively regulated by PIF3, but positively regulated by EBF1 under cold stress.

We also examined whether PIF3 and EBF1 regulate the expression of *CBF* genes and their regulon at a warm temperature (22 °C). The expression of *CBFs* and their target genes (such as *COR15b*, *COR47*, and *GOSL3*) was significantly higher in the *pif3* mutant than in the WT at Zeitgeber time 20 (ZT20) (nighttime) but not at ZT8 (daytime) (Fig. S5 *A–F*), which is consistent with the previous report that PIF3 accumulates to high levels in darkness but to low levels in the light (19, 34, 35) (Fig. S5*G*). Consistently, the expression of *CBFs* and their target genes in the *ebf1-1* mutant was much lower than that of the WT at ZT20 but not at ZT8 (Fig. S5 *A–F*). These results indicate that PIF3 and EBF1 also modulate *CBF* expression at 22 °C in the nighttime.

To further investigate the genetic interaction between PIF3 and EBF1, we generated *pif3-1 ebf1-1* double mutant plants by genetic crossing. The *pif3 ebf1* double mutant largely behaved like the *pif3-1* single mutant in terms of freezing tolerance and ion leakage (Fig. 4 *G–I*). Moreover, the expression of *CBFs* and their target genes in *ebf1-1* was partially rescued by the *pif3-1* mutation either after cold treatment or during the nighttime at warm temperatures (Fig. 4 *A–F* and Fig. S5 *A–F*). These results demonstrate that PIF3 acts downstream of EBF1 to negatively regulate the *CBF* pathway and freezing tolerance.

PIF3 Directly Represses the Expression of *CBF* Genes. Next, we investigated whether PIF3 could directly regulate the expression of *CBF* genes. A previous study showed that the PIF proteins recognize both the G-box and the E-box motifs (25), and promoter analysis revealed the presence of both G-box and the E-box motifs in all three *CBF* gene promoters (11) (Fig. 5*A*). Thus, we performed chromatin immunoprecipitation (ChIP) assays to test whether PIF3 is associated with the *CBF* promoters in vivo. The qPCR data showed that PIF3 was associated with the specific regions of the *CBF* promoters (fragments A, B, E, G, H, K, and L) but not with the coding regions of *CBF* genes (fragments D, I, and N) in vivo (Fig. 5 *A* and *B*).

We next performed electrophoretic mobility shift assays (EMSAs) to test whether PIF3 can directly bind to the G-box– or E-box–containing fragments of these gene promoters in vitro. The recombinant His-tagged PIF3 protein was expressed in *Escherichia coli* and used for EMSAs, and it was shown that His-PIF3 protein could bind directly to the G-box–containing fragment of the *CBF1* promoter (fragment A), and the E-box–containing fragments of the *CBF2* promoter (close to L) and of the *CBF3* promoter (fragment G), respectively, in vitro (Fig. 5*C*). Moreover, increasing amounts of unlabeled WT probe markedly decreased PIF3 binding to the biotin-labeled probes, whereas unlabeled mutant probes were unable to compete for PIF3 binding (Fig. 5*C*), indicating that PIF3 directly binds to the G-box or E-box motifs in the *CBF* gene promoters. Taken together, these data demonstrate that PIF3 represses *CBF* gene expression by directly binding to their promoters.

Light-Induced Degradation of PIF3 Is Inhibited by Cold Stress. Next, we used *35S:PIF3-Myc* transgenic plants to examine how cold stress regulates the PIF3 protein level. The seedlings were first grown on 1/2 MS medium for 12 d at 22 °C under LD conditions, and were then kept in darkness for 2 d, followed by pretreatment with the protein synthesis inhibitor cycloheximide (CHX) for 2 h. Then, these 14-d-old seedlings were exposed to light either at 4 °C or maintained at 22 °C. The immunoblot data showed that PIF3 was rapidly degraded upon exposure to light for the indicated time at 22 °C (Fig. 6*A*), which is consistent with previous reports (19, 36). By contrast, light-induced PIF3 degradation was strongly inhibited

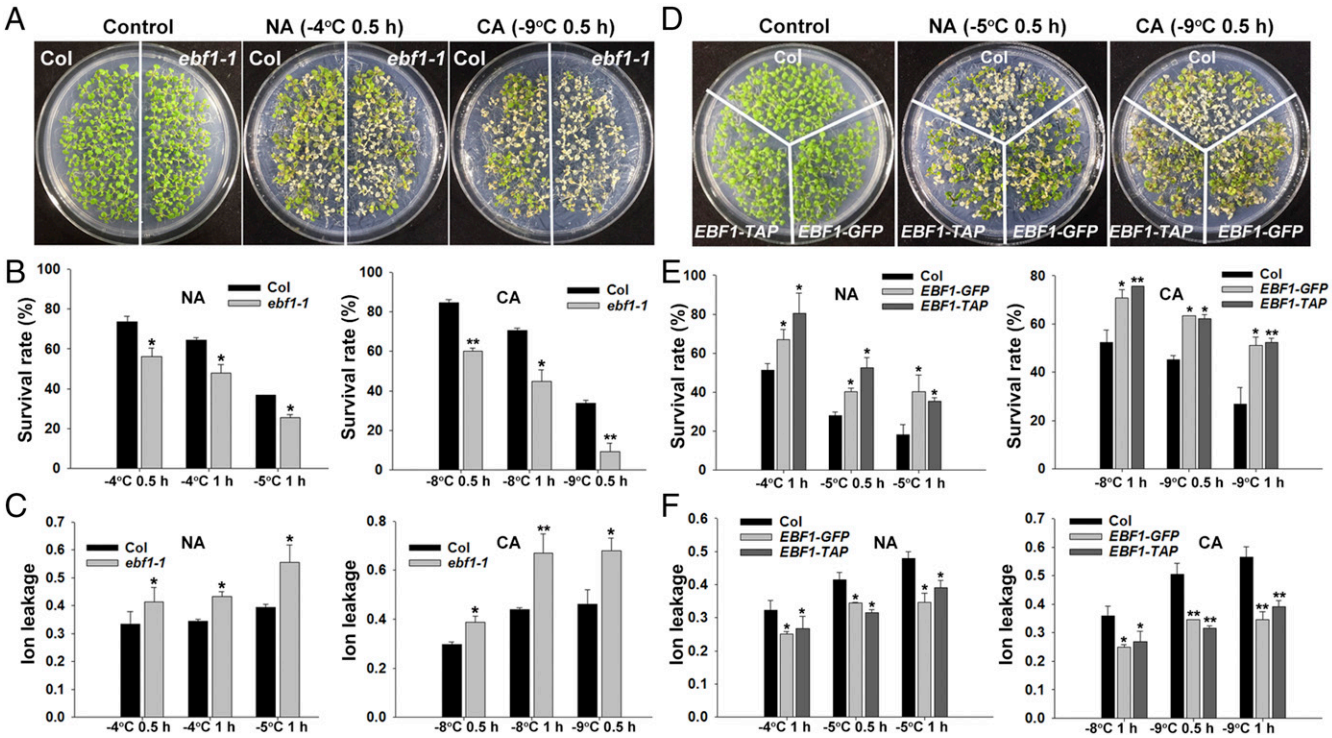

Fig. 3. EBF1 is a positive regulator of freezing tolerance. (*A–C*) The freezing phenotypes (*A*), survival rates (*B*), and ion leakage assays (*C*) of 12-d-old *ebf1-1* mutants. (*D–F*) The freezing phenotypes (*D*), survival rates (*E*), and ion leakage assays (*F*) of 12-d-old *35S:EBF1-TAP* (*EBF1-TAP*) and *35S:EBF1-GFP* (*EBF1-GFP*) transgenic plants. The procedures for performing NA and CA treatments are described in *Materials and Methods*. After the freezing assays, representative photographs (*A* and *D*) were taken, the survival rates (*B* and *E*) were calculated, and the ion leakages (*C* and *F*) were measured for the indicated plants at different freezing temperatures. In *B*, *C*, *E*, and *F*, data are means of three replicates ± SD. Asterisks indicate significant differences compared with the WT Col under the same conditions (*$P < 0.05$, **$P < 0.01$, Student's *t* test).

by cold treatment (Fig. 6*A*), whereas the expression of *PIF3* was only slightly reduced after cold treatment (Fig. S6*A*). These results suggest that cold stress inhibits light-induced degradation of PIF3.

EBF1/2 Target PIF3 for 26S Proteasome-Mediated Degradation. To explore the antagonistic relationship between EBF1/2 and PIF3, we first adopted an in vitro cell-free degradation assays to examine whether PIF3 protein stability is negatively regulated by EBF1. GST-tagged PIF3 proteins were first expressed in *E. coli*, and then incubated with total proteins extracted from 12-d-old seedlings of the WT, *ebf1-1* mutant, and *35S:EBF1-TAP* transgenic plants, respectively. Immunoblot analyses showed that GST-PIF3 protein was degraded rapidly in the WT within 30 min in the presence of ATP (Fig. 6*B*). By contrast, the degradation of GST-PIF3 was much faster in the *35S:EBF1-TAP* plants, and significantly slower in the *ebf1-1* mutants than in the WT within this observation period (Fig. 6*B*). However, the degradation of GST-PIF3 was inhibited in all extracts by the addition of MG132, a 26S proteasome-specific inhibitor (Fig. 6*B*), indicating that PIF3 is degraded through the 26S proteasome pathway.

To assess whether EBF1/2 regulate PIF3 protein stability in planta, we generated *35S:PIF3-Myc 35S:EBF1-TAP* plants by genetic crossing. Immunoblot analysis showed that PIF3-Myc protein level was much lower when EBF1-TAP was overexpressed under light conditions at 22 °C, whereas cold treatment significantly stabilized PIF3-Myc protein in the *35S:EBF1-TAP* background in the light (Fig. 6*C*). Next, we examined endogenous PIF3 protein levels in dark-grown *ebf1-1* or *ebf2-1* single mutants and the *35S:EBF1-TAP* or *35S:EBF2-TAP* plants using anti-PIF3 antibody. We found that endogenous PIF3 abundance was increased in *ebf1-1* or *ebf2-1* single mutants, but decreased in *35S:EBF1-TAP* or *35S:EBF2-TAP* transgenic plants compared with the WT seedlings (Fig. 6*D* and Fig. S6*B*). In addition, qRT-PCR analysis showed that *PIF3* transcript levels were essentially unaffected by changes in EBF1/2 proteins with or without cold treatment (Fig. S6*C*). Collectively, our data indicate that PIF3 is targeted by EBF1/2 for 26S proteasome-mediated degradation and that this degradation is inhibited by cold treatment.

To further investigate how light and cold coordinately regulate PIF3 protein stability, we performed a light/temperature shift assay in which the seedlings were first grown under a 12-h-light/12-h-dark photoperiod at 22 °C for 12 d, and were then transferred to 4 °C from either ZT4 (daytime) or ZT16 (nighttime), respectively. The seedlings kept at 22 °C were collected at the same time points and used as the control. The immunoblot data showed that EBF1 was stable during the daytime but degraded during the nighttime at 22 °C (Fig. 6*E* and Fig. S6*D*), which is consistent with our previous study (37). Notably, cold treatment induced the degradation of EBF1 during both the daytime and nighttime (Fig. 6*E*). Conversely, PIF3 was degraded by light but accumulated in darkness at 22 °C (Fig. 6*E* and Fig. S6*D*), in agreement with a previous report (38). However, PIF3 protein remained stable in plants during both the daytime and nighttime under cold stress (Fig. 6*E*). These results support the notion that PIF3 is negatively regulated by EBF1, whereas cold stress facilitates the degradation of EBF1, which leads to the promotion of PIF3 protein stability regardless of light conditions.

Discussion

The *CBF* signaling pathway plays a major role in plant cold acclimation and freezing tolerance (5, 7, 39), and plants have evolved sophisticated mechanisms to control this signaling pathway. Here, we provide several lines of evidence showing that the EBF1/2-PIF3 module is involved in regulating *CBF* expression and freezing

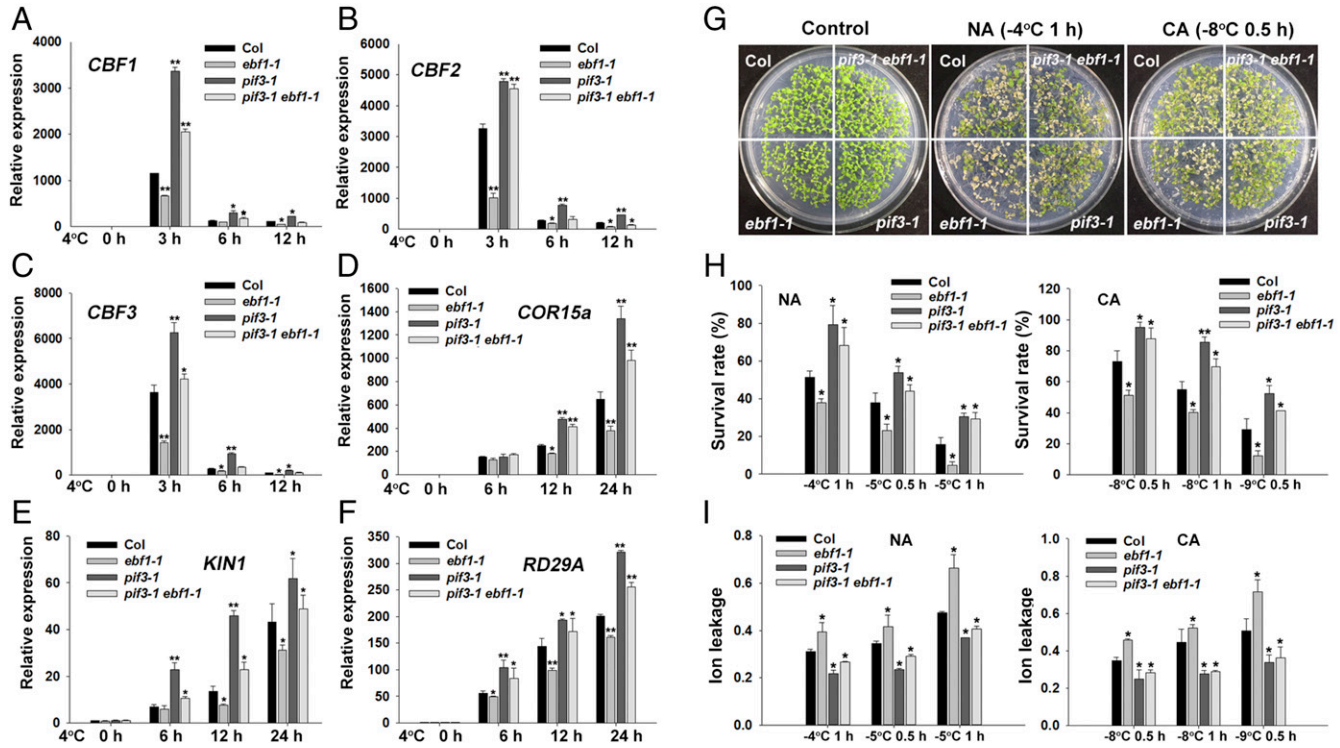

Fig. 4. *CBF* expression regulated by PIF3 and EBF1 under cold stress. (*A–F*) The expression of *CBF* genes and their regulons in *ebf1-1*, *pif3-1*, and *pif3-1 ebf1-1* mutants under cold stress. Twelve-day-old seedlings grown under LD conditions were treated with cold starting at ZT3 and harvested at the indicated time points, respectively. Relative expression in untreated WT (Col) plants was set to 1.00. (*G–I*) Freezing phenotypes (*G*), survival rates (*H*), and ion leakage assays (*I*) of *ebf1-1*, *pif3-1*, and *pif3-1 ebf1-1* mutants. The mutants were grown at 22 °C for 12 d and then subjected to the freezing assay. In *A–F*, *H*, and *I*, data are means of three replicates ± SD. Asterisks indicate significant differences compared with the WT Col under the same conditions (*$P < 0.05$, **$P < 0.01$, Student's *t* test).

tolerance in *Arabidopsis* (Fig. 7). First, the cold induction of *CBF* genes is increased in the *pif3* mutant and *EBF1/2*-overexpression lines, which both display enhanced freezing tolerance, but is decreased in *PIF3*-overexpression lines and *ebf1/ebf2* mutants, which both exhibit impaired freezing tolerance (Figs. 1, 3, and 4, and Figs. S1 and S4). Second, PIF3 directly binds to the G/E-boxes of the *CBF* promoters in vitro, and is associated with the promoters of *CBF* genes in vivo (Fig. 5). Third, cold stress promotes the degradation of EBF1/2 proteins and enhances the protein stability of PIF3 (Fig. 6). Finally, PIF3 is targeted by the $SCF^{EBF1/2}$ complex for degradation through the 26S proteasome pathway (Fig. 6). Together, our results identify PIF3 as a negative regulator of the cold response in plants that represses the expression of *CBF* genes.

The prompt induction of *CBF* genes in response to cold stress is mediated by the key transcriptional activators ICE1 and CAMTA3 (2, 3). In addition, circadian clock components CCA1/LHY were shown to act synergistically with ICE1 and CAMTA3 to activate *CBF* expression at low temperature in the morning (6), whereas evening-expressed PSEUDO-RESPONSE REGULATORS (PRRs), such as PRR5, PRR7, and PRR9, were found to negatively regulate cold-induced *CBF* expression during the night (40). In addition, MYB15 and EIN3 were shown to repress *CBF* gene expression in response to cold (28, 41). In this study, we found that PIF3 represses the cold induction of *CBF* genes. We speculate that PIF3-repressed *CBF* gene expression at low temperatures may help plants prevent runaway expression of the *CBF* pathway, which could have deleterious effects, such as excessive retardation of growth. Considering the pivotal role of PIF3 in mediating plants' responses to light signals, our study identifies PIF3 as an integrator between light and temperature signaling.

Our results also indicate that PIF3 acts as a transcriptional repressor of *CBF* genes in darkness at warm temperatures (Fig. S5). It was shown that PIF3 promotes hypocotyl growth during the night (38, 42), whereas the *CBF* pathway represses plant growth by promoting the accumulation of DELLAs (43, 44). We speculate that PIF3-mediated repression of *CBF* genes in darkness at warm temperatures not only relieves the repressive effect of the *CBF* pathway on plant growth, but also limits the amount of valuable resources that are channeled into freezing tolerance when it is not essential. However, upon light exposure at warm temperatures, photoactivated phytochromes induce rapid phosphorylation and degradation of PIF3 and other PIF proteins, which relieves their repression of photomorphogenesis (17, 18, 41), thereby resulting in a minor effect on *CBF* expression (Fig. S5).

Our biochemical data demonstrated that cold-induced degradation of EBF1/2 proteins is required for increased stability of PIF3 protein (Fig. 6). A recent report showed that COP1, an E3 ubiquitin ligase, directly targets EBF1/2 proteins for ubiquitination and degradation in darkness at warm temperatures (37). Interestingly, it was reported that cold treatment regulates COP1 abundance in the nucleus (15); however, it remains unclear whether COP1 is responsible for degrading EBF1/2 proteins in response to cold. In addition, phyB was shown to interact with EBF1/2 to promote the degradation of EIN3 (45). Given the recent finding that phyB is a thermosensor of ambient temperature (13, 14), it will be intriguing to explore whether phyB also serves as a cold sensor that mediates the EBF1/2–PIF3–CBF pathway.

Materials and Methods

Plant Material and Growth Conditions. Unless otherwise indicated, *Arabidopsis thaliana* plants were grown at 22 °C on 1/2 MS medium (Sigma-Aldrich) containing 0.8% agar and 1.5% sucrose under a 16-h-light/8-h-dark photoperiod. Plants at warm temperature (22 °C) were grown under 80–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white fluorescent illumination and transferred to low temperature (4 °C)

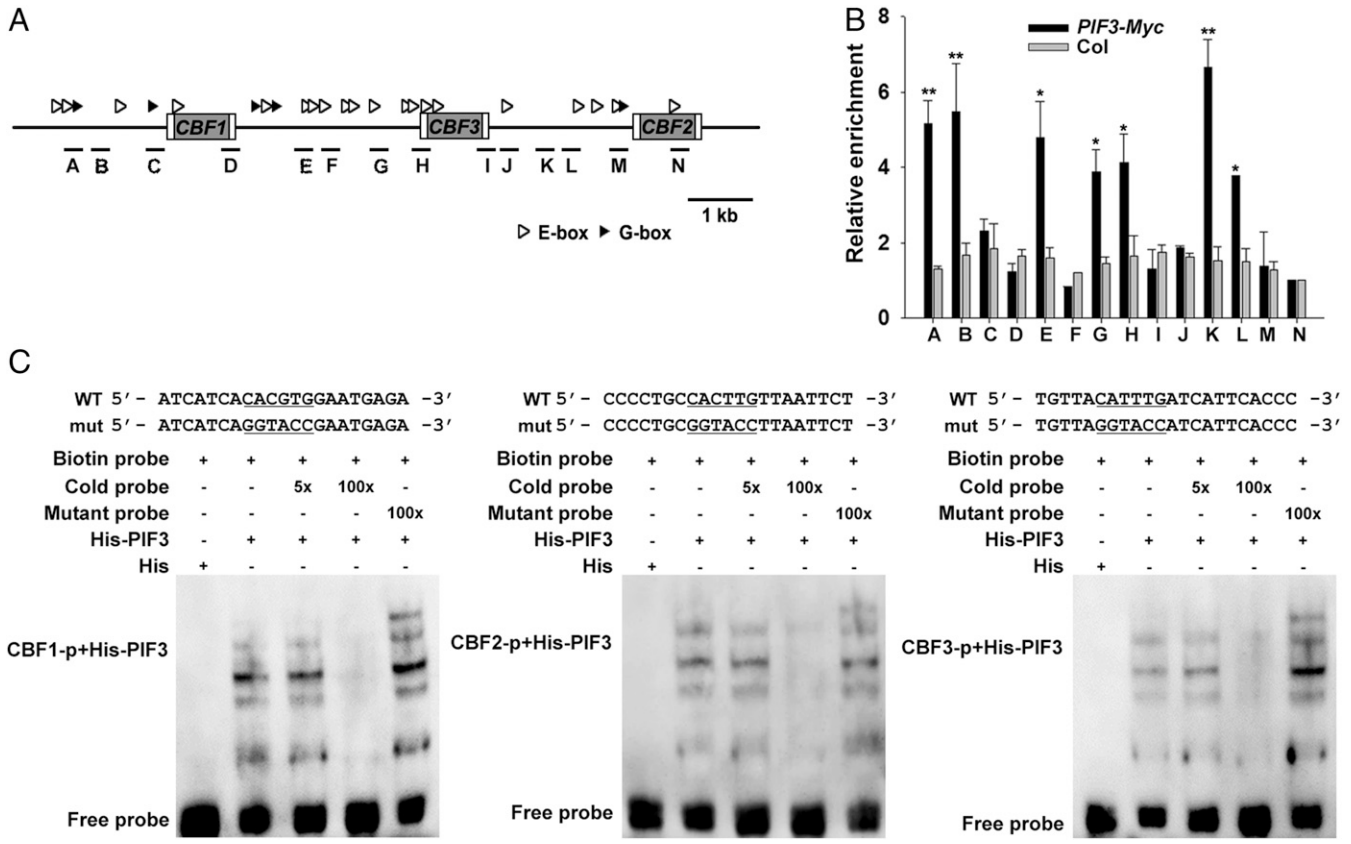

Fig. 5. PIF3 directly binds to the promoters of *CBFs*. (*A*) Schematic diagram showing the *CBF* genomic region and location of G-boxes (CACGTG, filled triangles) and E-boxes (CANNTG, open triangles) motifs. Filled squares indicate ORFs, and open squares indicate 5′- or 3′-untranslated regions (UTRs). (*B*) ChIP assays showing PIF3 binding to the *CBF* promoters in vivo. Twelve-day-old *35S:PIF3-Myc* or WT (Col) seedlings grown under LD conditions were harvested for ChIP analysis using anti-Myc antibody, and the precipitated DNA was analyzed by qPCR assays. Fragments of the *CBF* coding regions (D, I, and N) were used as controls. Data are means ± SD; $*P < 0.05$ and $**P < 0.01$ (Student's *t* test) for the indicated pair of seedlings. (*C*) EMSA showing that PIF3 binds to the promoters of *CBF1* (G-box of fragment A), *CBF2* (E-box next to fragment L), and *CBF3* (E-box of fragment G). Each biotin-labeled DNA fragment was incubated with His-PIF3 or His proteins. Competition assays for the labeled promoter sequences were performed by adding an excess of unlabeled WT or mutated probes.

under 20–25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white fluorescent illuminations. The *ebf1-1*, *ebf2-1* (30), *ebf2-3* (SALK_092571), *pif3-1* (20), *pif3-3* (26), and *pif3-1 ebf1-1* mutants, and *35S:EBF1-TAP*, *35S:EBF2-TAP* (30) and *35S:PIF3-Myc* (27) transgenic plants used in this study were reported previously. For the seedling treatments, 100 μM CHX (Sigma-Aldrich) and 80 μM MG132 (Sigma-Aldrich) were used.

Freezing Assays. Freezing tolerance assays were performed as described previously (46). *Arabidopsis* seedlings were grown at 22 °C for 12 d on 1/2 MS plates containing 0.8% agar, then subjected to the freezing assay conducted in a freezing chamber (RuMED4001). For NA treatment, the seedlings were directly subjected to the freezing assay. For CA treatment, the seedlings were first grown for 3 d at 4 °C under a 16-h-light/8-h-dark photoperiod, and then subjected to the freezing assay. The freezing assays for both NA and CA seedlings were conducted as follows: seedlings were maintained under white light (20–25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 0 °C for 1 h, and temperatures were then dropped by 1 °C per h 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 d. After recovery, the survival rates were determined by counting the number of seedlings that still generated new leaves.

Electrolyte Leakage Assays. Electrolyte leakage assays were performed as described previously (28). After the freezing treatment, the seedlings were collected in 15-mL tubes containing 8 mL of deionized water, and the electrical conductivity (EC) was measured as S_0. The samples were gently shaken at 22 °C for 15 min, and the resulting EC was measured as S_1. Then, the samples were boiled for at least 15 min and shaken at 22 °C for another 20 min, and the resulting EC was measured as S_2. Electrolyte leakage was calculated as follows: $(S_1 - S_0)/(S_2 - S_0)$.

Protein Extraction and Immunoblots. Total proteins were extracted into Mops buffer [containing 100 mM Mops, pH 7.6, 125 mM EDTA, pH 8.0, 5% SDS, 10% glycerol, 1× protease inhibitor mixture (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Amresco)] supplemented with 80 μM MG132 (Sigma-Aldrich). The total proteins from various materials were quantified, and then separated on 8% SDS/PAGE gels and subsequently transferred to PVDF membranes (Bio-Rad). Immunoblotting was performed using anti-PIF3, anti-HA, or anti-Myc antibody (Sigma-Aldrich).

RNA Extraction and RT-PCR. Twelve-day-old plants grown at 22 °C were transferred to 4 °C at ZT3 or ZT4 (ZT, 3 or 4 h after dawn). Total RNA was extracted from the materials 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 (qPCR) was performed using the SYBR Green PCR Master Mix Kit (TaKaRa). Relative expression levels were calculated as described previously (47). The specific primers used in the reactions are listed in Table S1.

Yeast Two-Hybrid Assays. The bait and prey plasmids were generated using fragments specifically amplified by PCR with different primers (Table S2). The full-length *EBF1/2* cloned into the pGBKT7 vector and the full-length *PIF3* in the pGADT7 vector were cotransformed into the yeast strain AH109. Interactions between the bait and prey proteins were examined on SD/−Trp/−Leu/−His/−Ade medium (Clontech).

Co-IP Assays. The plasmids used for co-IP assays were generated using different primers (listed in Table S2) and full-length *PIF3* was cloned into *35S:HA-FLAG* and full-length *EBF1/2* were cloned into *pSuper:Myc* (pCAMBIA1300-Myc vector harboring a Super promoter) (48, 49), respectively. The plasmids were purified

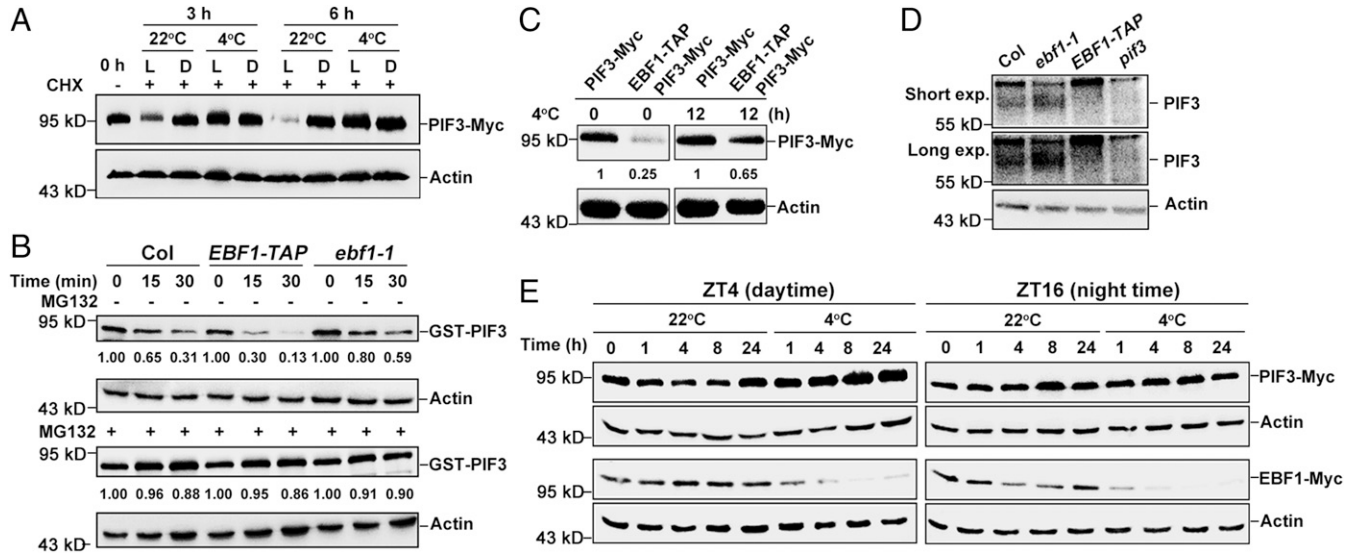

Fig. 6. EBF1 promotes PIF3 degradation through the 26S proteasome pathway. (*A*) Stability of PIF3 protein under low temperature. The *35S:PIF3-Myc* (*PIF3-Myc*) transgenic plants were grown for 12 d at 22 °C under LD conditions, and then transferred to a dark regime for 2 d, followed by treatment with cycloheximide (CHX) for 2 h. Then the seedlings were exposed to 4 °C or maintained at 22 °C for 3 or 6 h. Total proteins were extracted and examined by immunoblot analysis with anti-Myc antibody. D, dark; L, light. (*B*) PIF3 protein stability in cell-free assays. Total proteins were extracted from WT (Col), *35S:EBF1-TAP* (*EBF1-TAP*) transgenic plants, and *ebf1-1* mutant, and incubated with recombinant GST-PIF3 protein at room temperature with or without MG132 treatments. GST-PIF3 was detected by immunoblot analysis with anti-GST antibody. (*C*) The PIF3 protein levels in *PIF3-Myc* and *PIF3-Myc EBF1-TAP* transgenic plants. The transgenic seedlings were grown at 22 °C for 12 d and treated at 4 °C for 0 and 12 h, respectively. Total proteins were extracted and examined by immunoblot analysis with anti-Myc antibody. (*D*) Endogenous PIF3 protein levels in the *ebf1-1* mutant and *EBF1-TAP* plants. Total proteins were extracted from 4-d-old seedlings grown in darkness at 22 °C, and examined by immunoblot analysis with anti-PIF3 antibody. (*E*) The protein levels of PIF3 and EBF1 proteins in *PIF3-Myc* and *EBF1-TAP* transgenic plants under cold stress. The plants were grown at 22 °C for 12 d under a 12-h-light/12-h-dark photoperiod. The transgenic plants were transferred to 4 °C at either ZT4 (daytime) or ZT16 (nighttime), or continuously grown at 22 °C for 1, 4, 8, or 24 h. Total proteins were extracted and examined by immunoblot analysis with anti-Myc antibody. Actin served as a loading control. The protein levels were quantified using ImageJ software, and the relative intensity of the desired bands to that of the respective Actin without treatment was set to 1.00.

and transformed into *Arabidopsis* mesophyll protoplasts. The total proteins were extracted with extraction buffer containing 150 mM NaCl, 10 mM Tris·HCl, pH 7.5, 2 mM EDTA, 0.5% Nonidet P-40, and 1× protease inhibitor mixture (Roche). Then, the protein extracts were incubated with anti-Myc 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 antibodies (Sigma-Aldrich).

In Vitro Pull-Down Assays. The plasmids for expressing the fusion proteins were generated using different primers (listed in Table S2) and full-length *PIF3* was cloned into the pGEX4T-1 vector and full-length *EBF1/2* or *ASK1/2* were cloned into the pET32a vector, respectively. The constructs were transformed into *E. coli* BL21 or BL21 codon plus, and the GST-PIF3 and His-ASK1/2 fusion proteins were induced at 37 °C for 3 h using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The His-EBF1 and His-EBF2 proteins were expressed at 25 °C for 4 h using 0.5 mM IPTG. GST pull-down assays were performed using glutathione-agarose beads and then analyzed with anti-His antibody (Sigma-Aldrich).

Cell-Free Protein Degradation Assays. Cell-free protein degradation assays were performed as described (46). Total proteins extracted from the WT Col, *ebf1-1*, and *35S:EBF1-TAP* plants were incubated with GST-PIF3 recombinant protein purified from *E. coli* in the presence of ATP at 25 °C for the indicated time, and GST-PIF3 proteins were detected with anti-GST antibody.

EMSAs. EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher) according to the manufacturer's instructions with minor modifications. Briefly, 2 μg of purified His-PIF3 protein (expressed by the pET28a vector) was added to the binding reaction. The binding reactions were allowed to proceed at 25 °C for 25 min in a thermal cycler (Bio-Rad). The sequences of the complementary oligonucleotides used to generate the biotin-labeled and unlabeled probes are shown in Table S1.

ChIP Assays. ChIP assays were performed as described previously (50) with minor modifications. The *35S:PIF3-Myc* or WT (Col) seedlings were first grown for 2 wk at 22 °C on 1/2 MS medium under LD conditions. Then, the seedlings

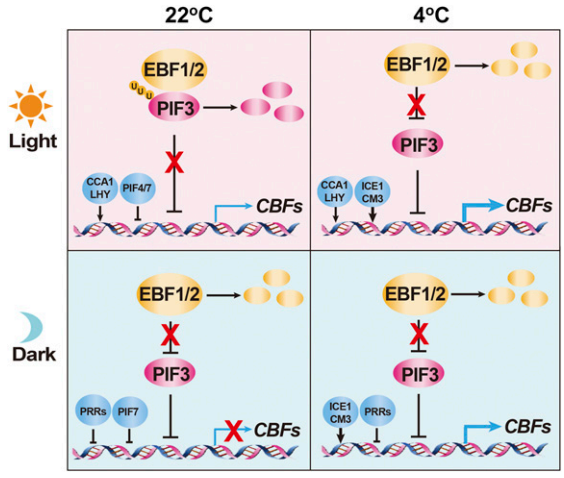

Fig. 7. A working model depicting the regulation of the EBF1/2-PIF3 module in the expression of *CBF* genes under different conditions. At a warm temperature (22 °C), PIF3 protein is degraded by EBF1/2 through the 26S proteasome pathway in the light, whereas EBF1/2 proteins are degraded and PIF3 protein is stabilized in darkness. Under cold stress (4 °C), EBF1/2 proteins are degraded regardless of the light conditions, which increases the protein stability of PIF3. PIF3 directly binds to the promoters of *CBF* genes and represses their expression. It was shown that CCA1, LHY, PIF4/7, and PRRs are involved in circadian- or photoperiod-mediated regulation of *CBF* genes. In response to cold stress, ICE1 and CAMTA3 (CM3) play key roles in activating *CBF* gene expression.

were grown in darkness at 22 °C for 2 d, fixed with 1% formaldehyde under a vacuum for 10 min, stopped by the addition of 2 M glycine to a final concentration of 0.125 M, and incubated for another 5 min under a vacuum at 22 °C. The chromatin was isolated and sonicated, and DNA fragments associated with PIF3-Myc protein were coimmunoprecipitated using anti-Myc antibody (Sigma-Aldrich). The enrichment of DNA fragments was quantified by qPCR using the primers listed in Table S1.

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