The Phytochrome-Interacting Factor PIF7 Negatively Regulates *DREB1* Expression under Circadian Control in Arabidopsis[1][W][OA]

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Transcription factors of the DRE-Binding1 (DREB1)/C-repeat binding factor family specifically interact with a cis-acting dehydration-responsive element/C-repeat involved in low-temperature stress-responsive gene expression in Arabidopsis (*Arabidopsis thaliana*). Expression of *DREB1*s is induced by low temperatures and is regulated by the circadian clock under unstressed conditions. Promoter sequences of *DREB1*s contain six conserved motifs, boxes I to VI. We analyzed the promoter region of *DREB1C* using transgenic plants and found that box V with the G-box sequence negatively regulates *DREB1C* expression under circadian control. The region around box VI contains positive regulatory elements for low-temperature-induced expression of *DREB1C*. Using yeast one-hybrid screens, we isolated cDNA encoding the transcriptional factor Phytochrome-Interacting Factor7 (PIF7), which specifically binds to the G-box of the *DREB1C* promoter. The *PIF7* gene was expressed in rosette leaves, and the PIF7 protein was localized in the nuclei of the cells. Transactivation experiments using Arabidopsis protoplasts indicated that PIF7 functions as a transcriptional repressor for *DREB1C* expression and that its activity is regulated by PIF7-interacting factors TIMING OF CAB EXPRESSION1 and Phytochrome B, which are components of the circadian oscillator and the red light photoreceptor, respectively. Moreover, in the *pif7* mutant, expression of *DREB1B* and *DREB1C* was not repressed under light conditions, indicating that PIF7 functions as a transcriptional repressor for the expression of *DREB1B* and *DREB1C* under circadian control. This negative regulation of *DREB1* expression may be important for avoiding plant growth retardation by the accumulation of DREB1 proteins under unstressed conditions.

Plants respond and adapt to abiotic stresses, such as low temperature, drought, and high salinity, at the molecular and cellular levels. The expression of many genes is induced by these stresses, and their gene products function directly in stress tolerance and in the regulation of gene expression and signal transduction in stress responses. The cis-acting elements that function in stress-responsive gene expression have been analyzed to elucidate the molecular mechanisms of gene expression in response to these stresses (Thomashow, 1999; Chinnusamy et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). The dehydration-responsive element (DRE) containing the core sequence A/GCCGAC was identified as a cis-acting element that regulates gene expression in response to both dehydration and low temperature in Arabidopsis (*Arabidopsis thaliana*; Yamaguchi-Shinozaki and Shinozaki, 1994). DREs are also found in the promoter regions of many drought- and low-temperature-inducible genes (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006). A similar motif was identified as the C-repeat and low-temperature-responsive element in promoter regions of low-temperature-inducible genes (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006).

Arabidopsis cDNAs encoding the ethylene-responsive element-binding factor/APETALA2-type DRE-binding (DREB) proteins, *CBF1*, *DREB1A*, and *DREB2A* were isolated by yeast one-hybrid screens (Stockinger et al., 1997; Liu et al., 1998). We isolated two

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cDNA clones homologous to *DREB1A/CBF3* (*DREB1B/CBF1* and *DREB1C/CBF2*) and one cDNA homologous to *DREB2A* (*DREB2B*; Liu et al., 1998; Shinwari et al., 1998). Both DREB1/CBF and DREB2 proteins bind to the DRE to activate the expression of stress-responsive genes. But DREB1/CBFs are thought to function in low-temperature-responsive gene expression, whereas DREB2s are involved in dehydration-responsive gene expression. Overexpression of DREB1/CBFs driven by the cauliflower mosaic virus (*CaMV*) *35S* promoter increases stress tolerance to freezing, dehydration, and high salinity in transgenic Arabidopsis (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). More than 100 downstream target genes of DREB1A have been identified by microarrays (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004, 2009; Vogel et al., 2005). Moreover, the levels of many metabolites (e.g. carbohydrates, amines, and organic acids) were reported to increase significantly in both low-temperature-exposed plants and transgenic lines overexpressing the *DREB1A* gene (Kaplan et al., 2007; Maruyama et al., 2009). However, overexpression of the DREB1A protein also caused severe growth retardation under normal growth conditions. Use of the stress-inducible *rd29A* promoter instead of the constitutive *CaMV 35S* promoter for the overexpression of DREB1A is reported to minimize the negative effects on plant growth (Kasuga et al., 1999).

Expression of the *DREB1A* gene is rapidly and significantly induced by low temperature, and its induction is gated by the circadian clock (Shinwari et al., 1998; Fowler et al., 2005). Three *DREB1* genes lie in tandem in the order *DREB1B*, *DREB1A*, and *DREB1C*, and their promoter sequences from translation initiation sites up to −500 bp are highly conserved. Shinwari et al. (1998) reported six conserved motifs, boxes I to VI, found in the *DREB1* promoters. On the other hand, Doherty et al. (2009) reported seven other conserved motifs, CM1 to CM7, between the *DREB1C/CBF2* and *ZAT12* promoters. These conserved regions are thought to include cis-acting elements of *DREB1* expression. Zarka et al. (2003) identified two sequences that contribute to low-temperature induction, ICEr1 and ICEr2, in the conserved regions of the *CBF/DREB1* genes, which are identical to boxes IV and VI, respectively. On the other hand, some proteins, including transcription factors, have been reported to regulate low-temperature induction of *DREB1*s directly or indirectly. ICE1/SCRM and MYB15 are basic helix-loop-helix (bHLH)- and Myb-type transcription factors, respectively, that regulate *DREB1A* expression under low-temperature conditions (Chinnusamy et al., 2003; Agarwal et al., 2006). CAMTA3/AtSR1, a calmodulin-binding transcription factor, binds to the CG-1 element in ICEr2 and activates the expression of *DREB1B* and *DREB1C* (Doherty et al., 2009). ICE1 was also reported to function in stomatal differentiation, and CAMTA3 negatively regulates the accumulation of salicylic acid (Kanaoka et al., 2008; Du et al., 2009).

DREB1A expression under unstressed conditions is regulated by the circadian clock (Harmer et al., 2000; Maruyama et al., 2004). In addition, the expression of many low-temperature-inducible genes, including *DREB1*s and their downstream genes, is also regulated by the circadian clock (Bieniawska et al., 2008). Mutants of pseudoresponse regulators (*PRR9*, *PRR7*, and *PRR5*) and phytochromes (*PhyB* and *PhyD*) upregulate the expression of *DREB1*s and the downstream genes under circadian control (Franklin and Whitelam, 2007; Nakamichi et al., 2009). PRRs and phytochromes are important components of light signaling pathways and the circadian oscillator (Yanovsky and Kay, 2001; Mizuno and Nakamichi, 2005). The light and circadian signaling pathways are also important for the regulation of *DREB1* expression. Phytochrome-Interacting Factor (PIF) family proteins are bHLH transcription factors that directly interact with the photoactivated phytochromes under specific light conditions (Castillon et al., 2007). They are negative regulators of the phytochrome-mediated signaling pathway and function in photomorphogenic development, such as hypocotyl elongation and chloroplast development. Most of them accumulate in the dark and rapidly degrade in response to light by the 26S proteasomal pathway. Particularly, the accumulation of both PIF4 and PIF5 mRNA and protein is regulated under light/dark (LD) cycles (Huq and Quail, 2002; Fujimori et al., 2004). Furthermore, they interact with TIMING OF CAB EXPRESSION1 (TOC1)/PRR1, a component of the light signaling pathway or the circadian oscillator. Some of them are suggested to function in the rhythmic growth pattern of hypocotyl elongation under circadian regulation (Nozue et al., 2007; Niwa et al., 2009).

In this study, we analyzed the promoter region of *DREB1C* and found that the conserved box V sequence functions as a negative regulatory element for circadian expression of *DREB1C*. The region around box VI contains positive regulatory elements for its low-temperature-induced expression. We isolated cDNA for one of the PIF family proteins, PIF7, which interacts with the G-box sequence in box V as detected using yeast one-hybrid screens. We characterized the *PIF7* gene in plant cells and analyzed the role of PIF7 in the expression of *DREB1* genes under circadian control.

RESULTS

Transcriptional Regulation in Boxes V and VI of the *DREB1C* Promoter

To identify the promoter regions including cis-acting elements involved in low-temperature-responsive gene expression of *DREB1* genes *DREB1A*, *DREB1B*, and *DREB1C*, we conducted promoter deletion analyses of the *DREB1C* gene, which, among the three *DREB1* genes, is induced at the highest level under low-temperature conditions (Shinwari et al., 1998). We

constructed a chimeric gene with the promoter region from −46 to the translation initiation site of *DREB1C*, including a 5′ untranslated region, fused to the *GUS* reporter gene as the minimal promoter. Then, four 5′ *DREB1C* promoter regions deleted to −394, −256, −190, and −113, which were tandemly repeated twice, were fused to the minimal promoter. The fused genes were transformed into Arabidopsis, and at least 10 independent transgenic lines for the expression of the *GUS* reporter gene of each construct were examined using RNA gel-blot analysis. Transgenic Arabidopsis plants grown for 2 weeks were treated at 4°C for 3 h and used for the analysis of *GUS* expression (Supplemental Fig. S1). Low-temperature treatment clearly increased accumulation of the *GUS* transcript in the transgenic plants carrying the −394, −256, −190, and −113 constructs but did not increase its accumulation in the transgenic plants carrying the minimal promoter. These results indicate that the 65-bp fragment containing boxes V and VI between positions −113 and −47 of the *DREB1C* promoter is important for its low-temperature-responsive gene expression (Supplemental Fig. S1).

To analyze the 65-bp promoter fragment of *DREB1C* in more detail, we constructed chimeric genes with the fragment (wild type) and its base-substituted fragments, which are tandemly repeated five times, fused to the minimal promoter and the *GUS* reporter gene (Fig. 1A). We analyzed four mutated 65-bp fragments (M1–M4) with one or two base substitutions. In this experiment, we analyzed more than 10 independent transgenic lines for each construct. The M1 mutation is in a G-box sequence of box V, and the M2 mutation is in the region between boxes V and VI. The M3 and M4 mutations are in the 5′ and 3′ regions of box IV, respectively. In the transgenic plants carrying the M1 fragment, *GUS* expression under low-temperature conditions was similar to that of the wild type. However, the *GUS* transcript had already mildly accumulated in the plants before the low-temperature treatment. The M2 fragment functioned in a manner similar to that of the wild-type fragment. In contrast, neither the M3 nor the M4 fragment allowed for *GUS* expression at all under low-temperature conditions (Fig. 1B).

Because *DREB1A* has been shown to be regulated by the circadian clock (Harmer et al., 2000; Maruyama et al., 2004), we tried to detect the *GUS* transcripts under circadian control. The transgenic Arabidopsis plants carrying the wild-type, M1, and M4 constructs were grown for 2 weeks under 12-h-light/12-h-dark conditions (LD cycle); we analyzed *GUS* expression every 3 h in the LD cycle and after transferring to continuous light (LL cycle). The *GUS* reporter gene driven by the wild-type fragment showed rhythmic expression in both the LD and LL cycles, similar to the endogenous *DREB1A* expression (Fig. 1C). The transcript levels peaked at 1 or 4 h after the subjective dawn (Zeitgeber time [ZT] 1 or 4 h). The *GUS* reporter gene driven by M4 showed the same rhythmic expression as that driven by the wild type, although the low-temperature-induced expression was completely lost (Fig. 1C). On the other hand, *GUS* expression was also rhythmic in the plants carrying M1, but the expression level was much higher than that in the plants carrying the wild type (Fig. 1C).

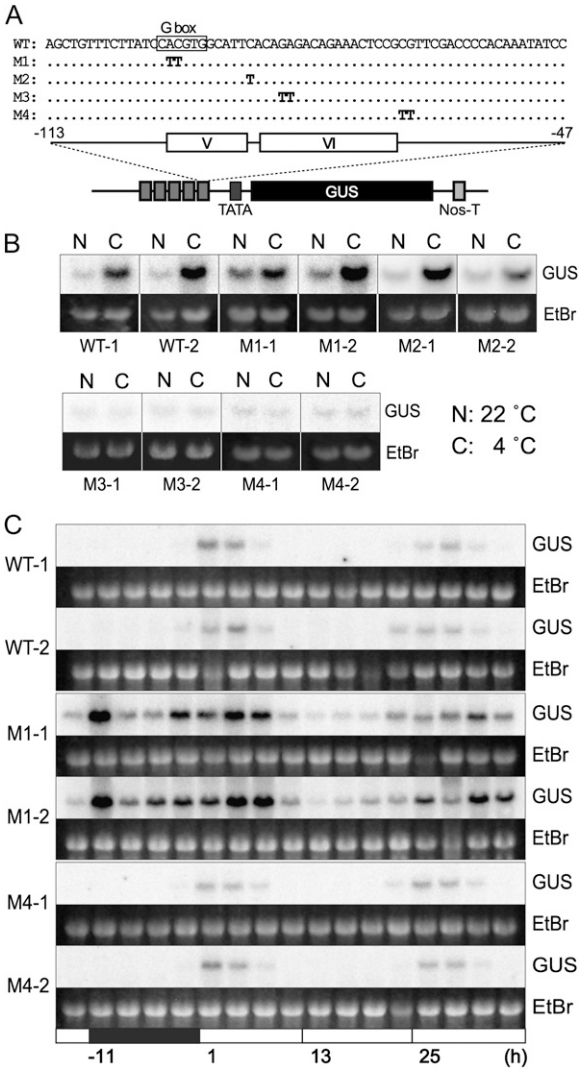

Figure 1. Mutation analysis of the 65-bp fragment in the *DREB1C* promoter. A, Schematic diagram of the reporter constructs and the sequences of the 65-bp fragment of the *DREB1C* promoter (wild type [WT]) and its mutated fragments (M1–M4). The *GUS* reporter construct contains five copies of the 65-bp fragment and the minimal TATA box of the *DREB1C* promoter fused to the *GUS* reporter gene. Nos-T indicates the nopaline synthase terminator. The wild-type G-box sequence is boxed. B, Expression of the *GUS* reporter gene in response to low temperature. Two representative lines are shown. Each lane was loaded with 7.5 μg of total RNA from 2-week-old seedlings. rRNAs are shown as equal loading controls. N and C indicate the treatments for 3 h at 22°C and 4°C, respectively. EtBr, Ethidium bromide. C, Expression of the *GUS* reporter gene under circadian regulation. Two-week-old seedlings grown in LD cycle conditions were then transferred to LL cycle conditions. Zero marks the start of the LL cycles. The lines are the same as those shown in B, and each lane was loaded with 15 μg of total RNA.

PIF7 Is Isolated as a Protein Bound to the 65-bp Region of the *DREB1C* Promoter

To isolate cDNAs encoding DNA-binding proteins that interact with the 65-bp fragment (−113 to −47) of the *DREB1C* promoter including boxes V and VI, we performed yeast one-hybrid screens. We first constructed a parental yeast strain carrying the dual reporter genes with integrated copies of *HIS3* and *lacZ* with four-times tandemly repeated 65-bp fragments. The yeast cells were then transformed with expression libraries containing cDNA fragments of mRNAs prepared from plants that were untreated or treated at low temperature (4°C) for 1 h. The cDNA fragments were fused to the transcriptional activation domain of yeast *GAL4*. We screened 1.5×10^6 yeast transformants of the mixed cDNA libraries. Thirty-six clones were resistant to 15 mM 3-amino-1,2,4-triazole. Eleven of them induced lacZ activity and formed blue colonies on filter papers containing X-gal (Supplemental Fig. S2). All 11 positive cDNA clones encoded the bHLH-type transcription factor PIF7, which is reported to be a negative regulator of PhyB signaling (Leivar et al., 2008).

PIF7 Recognizes the G-Box in Box V of the 65-bp Fragment of the *DREB1C* Promoter

The bHLH-type transcription factors recognize DNA with the consensus sequence CANNTG (Meshi and Iwabuchi, 1995), and PIF7 has been reported to recognize the G-box sequence, CACGTG (Leivar et al., 2008). In the 65-bp fragment of the *DREB1C* promoter, there is one potential element for PIF7 binding around box V. To confirm that PIF7 recognizes this sequence, the binding ability of the PIF7 recombinant protein was examined by an electrophoresis mobility shift assay. A fragment (residues 1–223) of PIF7, including the DNA-binding domain, was expressed and purified as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli*. The binding ability of the GST-PIF7 fusion protein to the wild-type, M1, and M4 fragments was examined. With the wild-type and M4 fragments, shifted bands were detected and the addition of competitors led to their disappearance, indicating that the fusion protein could bind the wild-type and M4 fragments (Fig. 2A). In contrast, the fusion protein could not bind the M1 fragment, which might be due to the mutation in the G-box sequence (Fig. 2A).

The G-box sequence is also found in the *DREB1B* promoter but not in the *DREB1A* promoter (TACGTG; Fig. 2B). The binding ability of PIF7 to the fragments containing boxes V and VI of the other *DREB1* promoters was also examined using competition experiments. The fragment (−111 to −47) of the *DREB1B* promoter competed with that of the *DREB1C* promoter for binding to the PIF7 fusion protein, but that (−114 to −48) of the *DREB1A* promoter did not (Fig. 2C), suggesting that PIF7 binds to the G-box in the *DREB1B* and *DREB1C* promoters but not as efficiently in the *DREB1A* promoter.

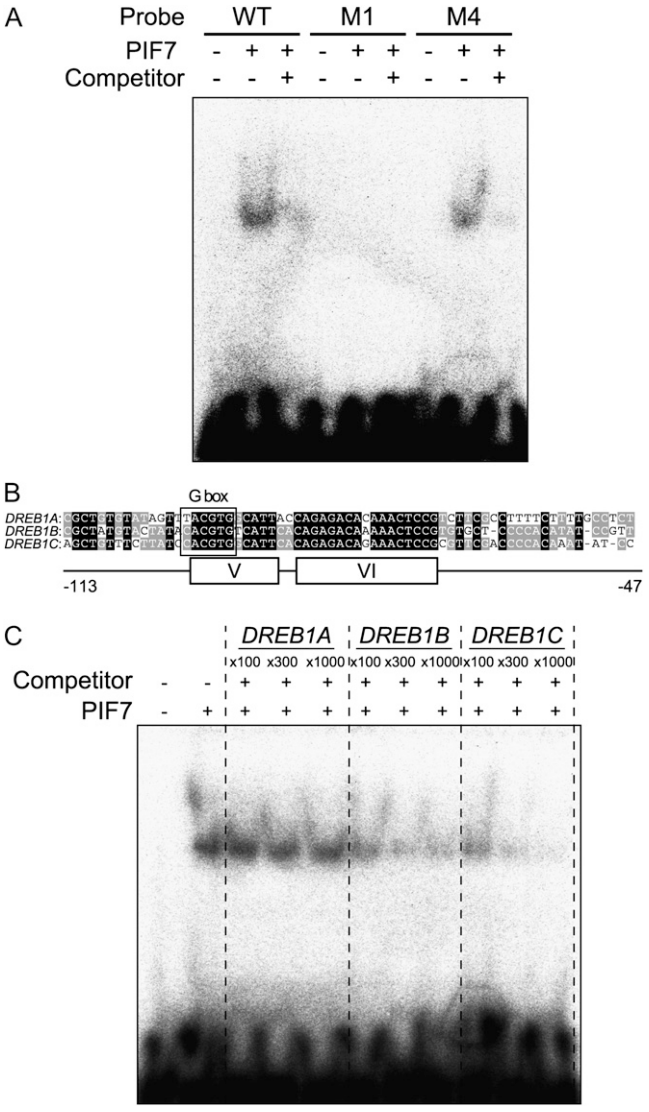

Figure 2. Binding assay of PIF7 to the *DREB1* promoters. A, The electrophoresis mobility shift assay of sequence-specific binding of the recombinant PIF7 protein. The radioactive probes (wild type [WT], M1, and M4) were incubated with or without 1,000 times competitors in the presence of the recombinant PIF7 protein. B, Alignment of the fragments containing boxes V and VI among the *DREB1* promoters. The G-box sequence is boxed. C, Electrophoresis mobility shift assay of binding to each *DREB1* promoter by the recombinant PIF7 protein. The radioactive 65-bp probe of *DREB1C* was incubated with or without each competitor of *DREB1A* (−114 to −48), *DREB1B* (−111 to −47), and *DREB1C* (−113 to −47) in the presence of the recombinant PIF7 protein.

Expression of the *PIF7* Gene and Subcellular Localization of the PIF7 Protein

To compare the expression patterns of *PIF7* and *DREB1*, we analyzed their transcript levels under circadian control and low-temperature conditions, respectively. We grew Arabidopsis plants at 22°C under LD cycle conditions for 10 d and then transferred them

to LL cycle conditions. We harvested the aerial parts of the plants every 3 h in the LD (−11 to −2 h; Supplemental Fig. S3) and subsequent LL (1–46 h) cycles. We measured the transcript levels using quantitative reverse transcription (RT)-PCR and found that the *DREB1A* and *DREB1B* genes showed circadian expression both in the LD and LL cycles (Supplemental Fig. S3). Their transcript levels peaked at 7 h after the subjective dawn (ZT 7 h). In contrast, the *DREB1C* transcripts displayed two peaks at ZT 1 and 7 h in the LD cycle but only one peak was exhibited in the LL cycle, similar to the *DREB1A* and *DREB1B* transcripts (Supplemental Fig. S3). On the other hand, *PIF7* also showed circadian expression, and its peak occurred at ZT 4 h, similar to *PIF4* and *PIF5/PIL6*, homologous genes to *PIF7* (Yamashino et al., 2003). Next, we subjected Arabidopsis plants to a low temperature (4°C) from ZT 2 h under dim light. However, the expression pattern of *PIF7* was almost the same as that under room temperature (22°C; data not shown).

To clarify the tissue-specific expression of *PIF7*, the *GUS* reporter gene was expressed under the control of the *PIF7* promoter in transgenic plants. GUS staining was detected in cotyledons and rosette leaves, but it was not clearly expressed in the roots or hypocotyls (Fig. 3A). To analyze the subcellular localization of PIF7, the *PIF7-sGFP* fusion gene was expressed under the control of the *PIF7* promoter in the *pif7-2* mutant background (Leivar et al., 2008), and two generated transgenic lines were used for further analysis (lines R6 and R7; Fig. 3B). In the transgenic Arabidopsis plants, which were grown for 2 weeks under 16-h-light/8-h-dark conditions, GFP fluorescence was clearly observed in the nuclei on the upper side of the leaves but not on the lower side (Fig. 3C). Whereas the fluorescence was diffused in the nuclei under the dark, speckles were formed in the nuclei after transferring to light conditions (Fig. 3D). When plants were kept under continuous light for 24 h, the speckles became unclear but the fluorescence could still be observed. Under the low-temperature condition, there was no significant change in the fluorescence (data not shown).

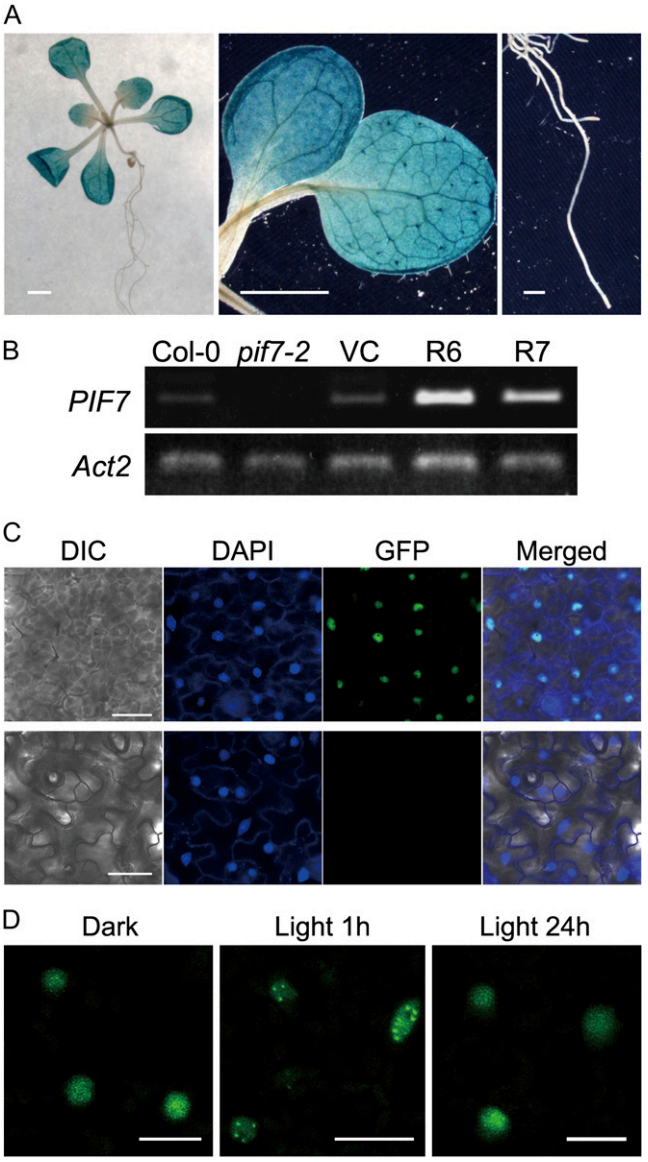

Figure 3. Tissue distribution and localization of PIF7. A, Patterns of GUS expression driven by the *PIF7* promoter in 2-week-old seedlings: whole plant (left), rosette leaves (middle), and roots (left) are shown. Bars = 1 mm. B, The RT-PCR analysis of *PIF7* expression in Col-0, *pif7-2*, vector control (VC), and two representative lines of complementation plants of *pif7-2* (R6 and R7). *Act2* was detected as a control in each experiment. C, Fluorescence of PIF7-GFP driven by the *PIF7* promoter on the top (top row) and bottom (bottom row) sides of leaves. Images shown are differential interference contrast (DIC), fluorescence by 4′,6-diamino-phenylindole (DAPI) staining, GFP fluorescence, and the merged image. Bars = 20 μm. D, Response of PIF7-GFP to light in 2-week-old seedlings: seedlings in the dark (left) and exposed to white light for 1 h (middle) and 24 h (right). Bars = 5 μm.

PIF7 Interacts with PIF4, PhyB, and PRR Proteins in Vivo

PIF family proteins have been reported to interact with TOC1/PRR1 and PhyA and/or PhyB (Yamashino et al., 2003; Castillon et al., 2007). To analyze whether PIF7 interacts in vivo with these factors and other regulators of *DREB1*, such as ICE1, MYB15, and CAMTA3 (Chinnusamy et al., 2003; Agarwal et al., 2006; Doherty et al., 2009), we performed a two-hybrid screen using Arabidopsis mesophyll protoplasts (Walter et al., 2004; Ehlert et al., 2006). For the two-hybrid system, we constructed effector plasmids containing the selected genes (PIF7, PhyB, ICE1, MYB15, CAMTA3, and PRRs) fused to the *GAL4* DNA-binding domain (DBD) under the control of the *CaMV 35S* promoter as baits (effector 1; Supplemental Fig. S4A). We also constructed an effector plasmid containing *PIF7* fused to the *VP16* transcriptional activation domain (AD) under the control of the *CaMV 35S* promoter as the prey (effector 2; Supplemental Fig. S4A). The GAL4-*GUS* plasmid, which contains nine copies of a GAL4-binding site and the minimal promoter of

CaMV 35S fused to the *GUS* reporter gene, and the 35S-*LUC* plasmid, which has the *luciferase* (*LUC*) reporter gene under the control of the *CaMV 35S* promoter, were used as a reporter and an internal control, respectively (Fujita et al., 2005). These constructs were transiently expressed in Arabidopsis mesophyll protoplasts (Supplemental Fig. S4A), and the transfected protoplasts were incubated overnight under white light. Coexpression of PIF7 or PhyB activated GUS activities by more than a factor of 20 (Supplemental Fig. S4B). Coexpression of TOC1 also activated GUS activity, but the level of activation was not as high as that of PIF7 or PhyB. We suspected that the interaction between PIF7 and TOC1 was indirect and mediated by other factors such as PhyB. To investigate the involvement of PhyB in the interaction between PIF7 and TOC1, we performed the experiment using PIF7 mutated in its APB motif, which is the PhyB-interacting domain, as a prey (Khanna et al., 2004; Castillon et al., 2007). This PIF7 mutant had a clearly reduced interaction with PhyB, but its association with TOC1 was not significantly affected (Supplemental Fig. S4C).

To confirm the interaction between PIF7 and some potential binding partners in the plant cells, we conducted a bimolecular fluorescence complementation (BiFC) assay. We constructed PIF7 and the candidates (PIF7, PIF4, PhyB, TOC1, and ICE1) fused to the N-terminal region of yellow fluorescent protein (YFP; YFP^N1–155; SPYNE) or to the C-terminal region of YFP (YFP^C156–239; SPYCE) under the control of the *CaMV 35S* promoter. These constructs were transfected into Arabidopsis mesophyll protoplasts and transiently expressed. We cotransfected cyan fluorescent protein (CFP) fused to H2B and driven by the *CaMV 35S* promoter as the positive control of transfection and nuclear localization (Dixit et al., 2006). For PIF7-PIF7 (SPYNE-SPYCE), PIF7-PIF4, PIF7-PhyB, and TOC1-PIF7, YFP fluorescence was merged with CFP in the nuclei; however, fluorescence was not detected with PIF7-ICE1 (Fig. 4).

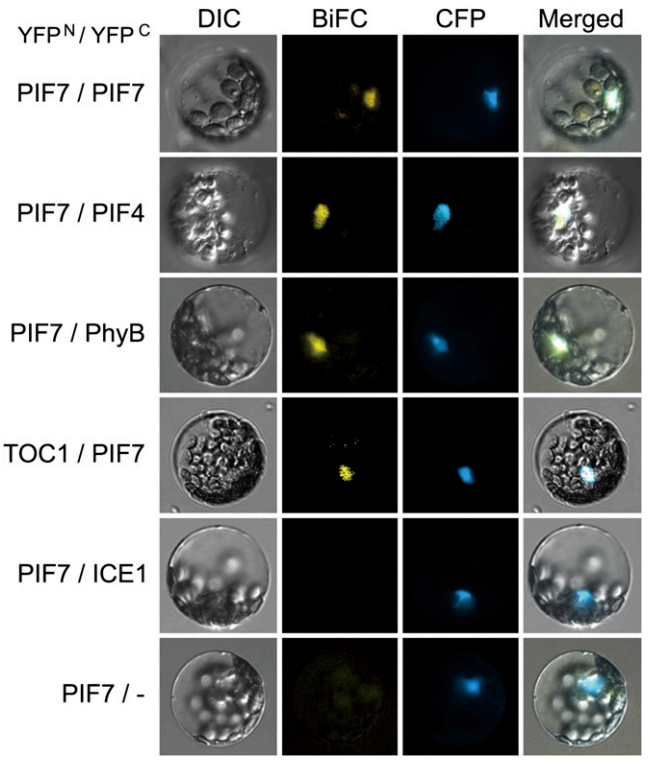

Figure 4. Interactions of PIF7 in Arabidopsis protoplasts revealed by the BiFC system. Each combination of constructs fused to YFP^N and YFP^C was transfected into Arabidopsis mesophyll protoplasts and transiently expressed. The dash indicates YFP^C expression only. CFP fused to H2B was cotransfected as the positive control of transfection and nuclear localization. Images shown are differential interference contrast (DIC), YFP fluorescence by BiFC, CFP fluorescence as the control of transfection and nuclear localization, and the merged image.

Transcriptional Activity of PIF7

In our analysis of the *DREB1C* promoter, mutation of the PIF7-binding site caused activation of *GUS* expression (Fig. 1, B and C). Therefore, we suspected that PIF7 functions as a transcription repressor for the *DREB1C* promoter. To examine the transcriptional activity of PIF7, we performed a transactivation assay using mesophyll protoplasts. We constructed full-length and deletion fragments of *PIF7* fused to GAL4-DBD under the control of the *CaMV 35S* promoter as effectors (Fig. 5A). These constructs were transiently expressed with the reporter and internal control in Arabidopsis mesophyll protoplasts and were incubated overnight under white light. Transient expression of PIF7 (FL; 1–366) reduced GUS activity as compared with that of the DBD control, suggesting that PIF7 can function as a transcriptional repressor. Coexpression of N-terminal deletion mutants of PIF7 (89–366, 89–327, and 328–366) also reduced the activities. In contrast, coexpression of C-terminal deletion mutants of PIF7 (1–327, 1–223, and 1–88) increased GUS activity comparable to DBD (Fig. 5B). These results suggest that PIF7 has a repression domain in the C-terminal region and an activation domain in the N-terminal region.

To address how PIF7 regulates the *DREB1C* promoter, we conducted a transactivation assay with PIF7 and its interacting factors PhyB and TOC1 as effectors under the control of the *CaMV 35S* promoter. We used the *GUS* reporter gene under the control of the 1-kb region upstream from the translation initiation site of *DREB1C* as a reporter (Fig. 5C). Coexpression of *PIF7* reduced GUS activity as compared with the control without the effector. In particular, coexpression of both PIF7 and TOC1 reduced GUS activity significantly more than PIF7 expression alone (Fig. 5D).

DREB1 Expression in the *pif7* Mutant

To characterize the role of PIF7 in *DREB1* expression, we compared the expression levels of *DREB1*s between wild-type plants (ecotype Columbia [Col-0])

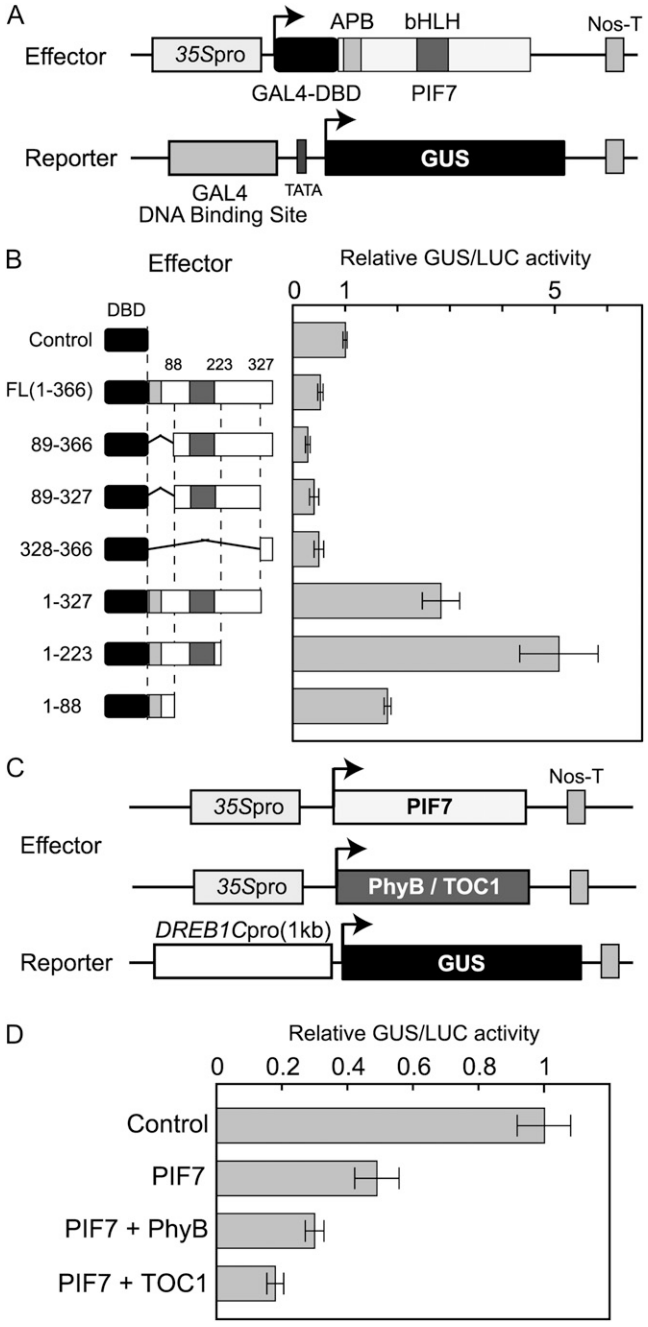

Figure 5. Transactivation activity of PIF7 in mesophyll protoplasts. A, Schematic diagram of the effector and reporter constructs used in the transactivation analysis with the GAL4-DBD. The effector constructs contain the *CaMV 35S* promoter fused to the GAL4-DBD and PIF7 fragments with or without deletion. The *GUS* reporter construct contains nine copies of the GAL4-binding site fused to the *CaMV 35S* minimal TATA box. To normalize for transfection efficiency, the *LUC* reporter gene driven by the *CaMV 35S* promoter was cotransfected as a control in each experiment. B, Transactivation or repression domain analysis of PIF7 using deletion constructs. Relative activity indicates the multiples of expression compared with the value obtained with the vector control. Error bars indicate the SD of three technical replicates. C, Schematic diagram of the effector and reporter constructs used in the transactivation analysis using the *DREB1C* promoter. The effector construct contains the *CaMV 35S* promoter fused to the PIF7, PhyB, and TOC1 fragments. The reporter construct contains the 1-kb region upstream of the translation initiation site of *DREB1C* fused to the *GUS* reporter gene. D, Transactivation analysis of PIF7 with interacting factors of the *DREB1C* promoter. Relative activity indicates the multiples of expression as compared with the value obtained with the vector control. Error bars indicate the SD of three technical replicates.

and the *pif7-2* mutant. We grew both plants under LD cycle conditions for 10 d and then transferred them to LL cycle conditions. We harvested the aerial parts of the plants every 4 h in the LD cycle (−16 to −4 h; Fig. 6) and subsequent LL cycles (0–36 h) and measured the transcript levels by semiquantitative PCR. In the LD cycle, we could not observe significant expression differences between the wild type and the *pif7-2* mutant for the three *DREB1* genes (Fig. 6). However, between 12 and 24 h in the LL cycle, expression levels of *DREB1*s, especially *DREB1B* and *DREB1C*, in the mutant were clearly higher than those in the wild-type plants. Expression patterns of *DREB1*s in the mutant after 24 h were similar to those in the wild-type plants. We also measured transcripts of other circadian-controlled genes. In the mutant *AtGolS3*, which is downstream of *DREB1* (Maruyama et al., 2004), a similar expression pattern of *DREB1* was displayed in both the wild type and the mutant. In contrast, expression levels of *HFR1*, which is downstream of PIF4 and PIF5 (Lorrain et al., 2008), were lower in the mutant than in the wild type, except at 16 and 20 h in the LL cycle. Expression patterns of *CCA1*, which is a component of the central oscillator (Green and Tobin, 2002), did not show significant differences between the wild type and the mutant.

To confirm that the elevated *DREB1B* and *DREB1C* gene expression in the mutant was caused by a disruption of the *PIF7* locus, we used plants expressing the *PIF7-sGFP* fusion gene under the control of the *PIF7* promoter in the *pif7-2* mutant as a means to complement the gene expression (Fig. 3B). In two independent complementation lines (R6 and R7), none of the *DREB1* genes exhibited different expression patterns as compared with the wild-type plants (Supplemental Fig. S5B). Recovery of *PIF7* gene function clearly complemented the expression of *DREB1* genes. We also performed expression analysis of *DREB1*s under low-temperature conditions, because induction of *DREB1* expression by low-temperature stress has been reported to be gated by the circadian clock (Fowler et al., 2005). However, under low-temperature conditions for 2 h, which began at subjective morning (ZT 2 h) or evening (ZT 14 h), no significant difference was observed in the expression of *DREB1* (Supplemental Fig. S5C).

DISCUSSION

Previously, we reported that there are six conserved sequences from boxes I to VI in the three *DREB1* promoters and hypothesized that these conserved

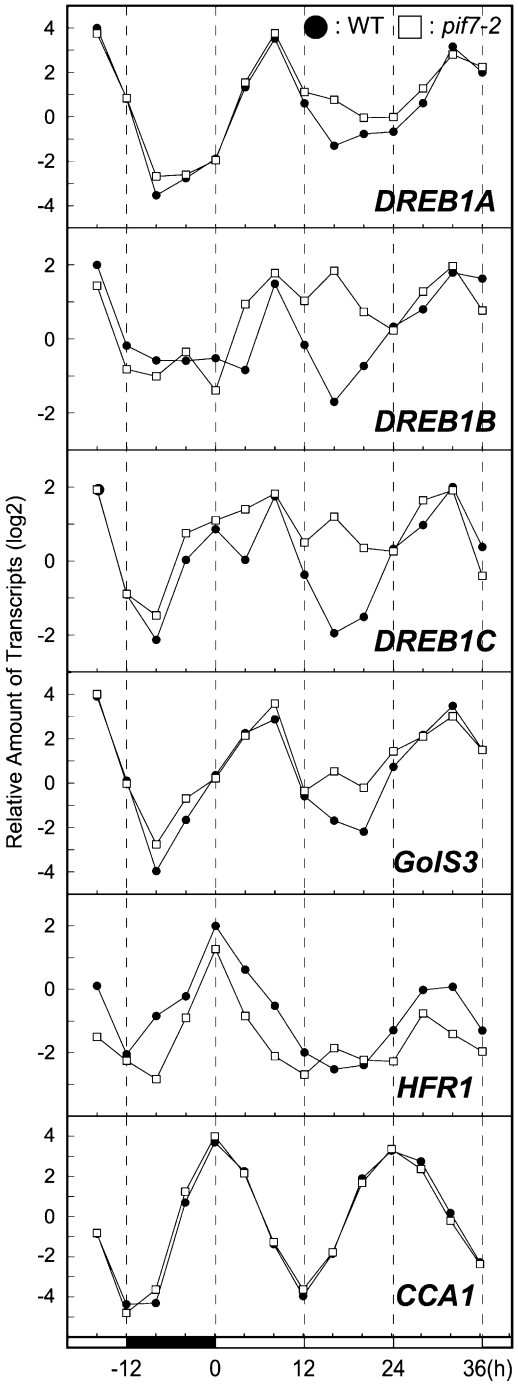

Figure 6. The expression of *DREB1* and circadian-controlled genes in the *pif7* mutant under circadian control. We analyzed the expression profile in *DREB1* and some circadian-controlled genes with semiquantitative RT-PCR under circadian control. Ten-day-old seedlings of wild-type (WT; Col-0) and *pif7* mutant plants were grown in LD cycle conditions and then transferred to LL cycle conditions. Zero marks the start of the LL cycles. Black circles and white squares indicate Col-0 and the *pif7* mutant, respectively. The relative amount indicates the multiples of expression as compared with the value obtained with the expression peaks.

sequences include cis-acting elements involved in *DREB1* expression (Shinwari et al., 1998). In this study, we analyzed the promoter region of *DREB1C* using transgenic plants and found that a 65-bp region containing boxes V and VI is sufficient for its induction by low temperature (Supplemental Fig. S1A). Moreover, we demonstrated that this 65-bp fragment responded not only to low temperature but also to the circadian clock (Fig. 1, B and D). We analyzed the promoter activity of the mutated fragments and found that the region around the M1 mutation functions as a negative regulatory element for circadian expression and that the regions around the M3 and M4 mutations function as positive regulatory elements for low-temperature-inducible gene expression of the *DREB1C* gene (Fig. 1, B and D). In the plants carrying M1, *GUS* expression levels were much higher than those in the plants carrying the wild type, indicating that the G-box sequence in the fragment negatively regulates *DREB1C* expression under circadian control. However, we still detected rhythmic expression and significant accumulation of *GUS* expression at the start of the dark period. This anomalous expression pattern of *GUS* in the plants carrying M1 suggests the presence of negative regulatory elements that influence circadian expression other than the G-box in the 65-bp fragment.

By yeast one-hybrid screening, *PIF7* was isolated as cDNA for a protein that binds to the 65-bp fragment of the *DREB1C* promoter (Supplemental Fig. S2). PIF7 was previously reported to function as a negative regulator of PhyB signaling in a deetiolated seedling under prolonged red light, similar to PIF3 and PIF4 (Leivar et al., 2008). PIF3 is known to function in anthocyanin accumulation and chloroplast development, and PIF4 plays a role in shade avoidance (Monte et al., 2004; Shin et al., 2007; Lorrain et al., 2008; Stephenson et al., 2009). However, the physiological function of PIF7 has not yet been revealed in detail. PIF7 contains the bHLH-type DNA-binding domain and can bind specifically to the G-box sequence around the M1 mutation in the 65-bp fragment of the *DREB1C* promoters (Fig. 2A). Therefore, we hypothesized that PIF7 might regulate the expression of *DREB1*s, particularly the negative regulation of *DREB1B* and *DREB1C*, under circadian control, as the G-box sequence is not completely conserved in the *DREB1A* promoter (Fig. 2B). Both GUS activity and GFP fluorescence driven by the *PIF7* promoter were mainly detected in cotyledons and rosette leaves (Fig. 3, A and C). GUS activity driven by the *DREB1* promoters was weakly detected in cotyledons but not at all in rosette leaves under unstressed conditions (Novillo et al., 2007). Expression of *PIF7* was regulated by the circadian clock but not by low temperature (Supplemental Fig. S3). These findings are consistent with the hypothesis that PIF7 negatively regulates *DREB1* expression under circadian control. This negative regulation of *DREB1* expression may be important for avoiding plant growth retardation by the accumulation of DREB1 proteins under unstressed

conditions, as overexpression of the DREB1A protein is reported to cause severe growth retardation under normal growth conditions (Kasuga et al., 1999).

Furthermore, we showed that the PIF7-sGFP protein driven by the *PIF7* promoter was localized in the nuclei of the leaves and formed speckles in the nucleus in response to short-term light, similar to other PIFs (Fig. 3D; Leivar et al., 2008). Fluorescence speckles of PIF-GFP were reported to be the sites for the degradation of PIF proteins (Chen, 2008). PIF7-sGFP in the speckles might also be degraded under long-term light. However, diffuse fluorescence of PIF7-sGFP in the nucleus was still detected after 24 h of incubation under white light (Fig. 3D). These results coincide with the report that the PIF7 protein is stable under long-term light, whereas most other PIF proteins are rapidly degraded under the long-term light condition (Castillon et al., 2007; Leivar et al., 2008), suggesting that the PIF7 function is dominant under light conditions. Under the subjective night condition in the LL cycle (12–24 h in LL), expression of *DREB1B* and *DREB1C* in the *pif7-2* mutant was significantly higher than in the wild type (Fig. 6; Supplemental Fig. S5A). However, expression levels of these genes did not exceed the expression peak (at 8 h in LL). These results indicate that expression of *DREB1B* and *DREB1C* is not repressed in the mutant under the light condition. A PIF7 mutation affected their expression only in LL cycles but not in LD cycles. In the LD cycles, especially the dark conditions, the other PIFs, such as PIF4, seem to function redundantly to repress the expression of these *DREB1* genes. Indeed, we showed that PIF7 formed not only homodimers but also heterodimers with PIF4, using two-hybrid and BiFC systems in Arabidopsis protoplasts (Fig. 4; Supplemental Fig. S4B).

Nakamichi et al. (2009) reported that expression of the *DREB1* genes in LD cycles is up-regulated in the triple mutant of *PRR9*, *PRR7*, and *PRR5* (*d975*). The expression patterns of *DREB1*s in the *d975* mutant were similar to that of the *GUS* reporter gene driven by the M1 fragment of the *DREB1C* promoter (Fig. 1C; Nakamichi et al., 2009). Therefore, we supposed that these *PRR*s are upstream factors of PIF7 that regulate the circadian expression of *DREB1C*. We analyzed the interactions between PIF7 and each PRR protein with the two-hybrid assay using Arabidopsis protoplasts, but direct association was not detected in the plant cells (Supplemental Fig. S4B). On the other hand, *TOC1* expression has been reported to be completely repressed in the *d975* mutant (Nakamichi et al., 2009). Our two-hybrid and BiFC assays revealed that PIF7 interacts with TOC1 directly in plant cells (Fig. 4; Supplemental Fig. S4B). Moreover, coexpression of TOC1 with PIF7 showed stronger repression of the reporter gene driven by the *DREB1C* promoter (Fig. 5D), suggesting that up-regulation of *DREB1*s in *d975* is partly caused by PIF7 interacting with TOC1.

CAMTA3 has been reported to be a transcriptional activator to regulate the expression of *DREB1B* and *DREB1C* under low-temperature conditions, and this protein has been shown to bind the CG-1 element in their promoters (Doherty et al., 2009). The CG-1 element and G-box are proximal, especially in the *DREB1C* promoter (Fig. 1A). We could not detect a direct interaction between PIF7 and CAMTA3 in the plant cells (Supplemental Fig. S4B) or between PIF7 and other regulators in the low-temperature-responsive expression of *DREB1*, such as ICE1 and MYB15. These results suggest that PIF7 functions independently from the transcription factors that regulate *DREB1* expression in response to low temperature. These ideas are consistent with the result that *PIF7* disruption did not affect low-temperature-induced expression of *DREB1* (Supplemental Fig. S5C). However, both circadian- and low-temperature-regulated gene expression were reported to be involved in calcium signaling (Catala et al., 2003; Dodd et al., 2006; Doherty et al., 2009). A common upstream factor might coordinately regulate *DREB1* expression under circadian control and the low-temperature response.

Leivar et al. (2008) reported that PIF7 functions as an activator under the dark conditions in etiolated seedlings. However, in our transient experiments using mesophyll protoplasts, expression of PIF7 fused to GAL4-DBD reduced the relative reporter activity under light conditions, and deletion of the N-terminal region of PIF7 further decreased the relative reporter activity (Fig. 5B). In contrast, deletion of its C-terminal region raised the activity higher than the control. These results suggest that PIF7 contains separate transcriptional activation and repression domains and can

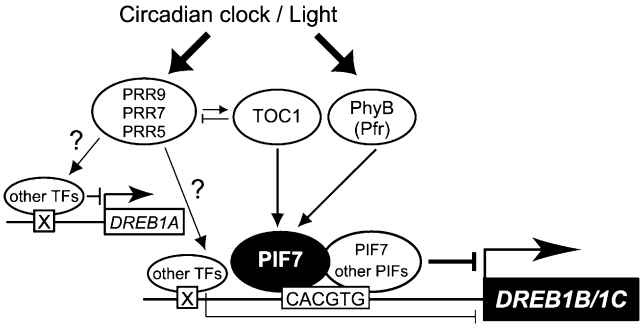

Figure 7. A model of the regulation of *DREB1* expression under circadian control. PIF7 binds to the G-box sequence in the promoter regions of *DREB1B* and *DREB1C* and represses their expression under circadian control. PIF7 forms both homodimers and heterodimers with other PIFs, such as PIF4, in Arabidopsis cells. These other PIFs may function redundantly to repress the expression of these *DREB1* genes. PIF7 activity is regulated by one of its binding partners, TOC1, downstream of PRR9, PRR7, and PRR5 (Nakamichi et al., 2009). PIF7 also interacts with and is regulated by PhyB. Another transcriptional regulatory pathway is expected to exist downstream of PRR9, PRR7, and PRR5 to repress the expression of *DREB1A*. Thus, the expression of *DREB1*s is repressed by the central oscillator under the unstressed conditions. TFs, Transcription factors.

function as a transcriptional activator or repressor. When the reporter gene was fused to the *DREB1C* promoter, PIF7 reduced the relative reporter activity under light conditions (Fig. 5D), indicating that PIF7 functions as a transcriptional repressor to *DREB1* expression. In the *pif7-2* mutant, expression of *DREB1B*, *DREB1C*, and their downstream genes was up-regulated but that of *HFR1* was down-regulated (Fig. 6B; Supplemental Fig. S5A). On the other hand, Franklin and Whitelam (2007) reported that the circadian expression of *DREB1* genes was higher under a low-red to far-red light compared with a high-red to far-red light. We showed that coexpression of both PIF7 and PhyB reduced the reporter activity of the *DREB1C* promoter under light conditions to a greater extent than did PIF7 expression alone (Fig. 5D), suggesting that the interaction between PIF7 and the Pfr form of PhyB enhanced the activity of PIF7 to repress *DREB1* expression (Leivar et al., 2008). Transcriptional effects of PIF7 might depend on the downstream genes and/or light conditions.

In conclusion, we showed that the G-box sequence in the *DREB1C* promoter negatively regulates *DREB1C* expression under circadian control. We isolated the cDNA for PIF7, which binds to the G-box sequence and functions under circadian control as a transcriptional repressor of *DREB1B* and *DREB1C*. PIF7 forms not only homodimers but also heterodimers with other PIFs, such as PIF4, in Arabidopsis cells; these other PIFs might function redundantly to repress the expression of these *DREB1* genes. Moreover, PIF7 activity is regulated by the interacting factor TOC1 downstream of PRR9, PRR7, and PRR5 (Nakamichi et al., 2009). PIF7 also interacts with the Pfr form of PhyB, which regulates the activity of PIF7 under the light conditions (Leivar et al., 2008). It is expected that other transcriptional regulatory pathways exist downstream of PRR9, PRR7, and PRR5 to repress *DREB1A* expression. The expression of *DREB1*s is strongly repressed by the central oscillator, which prevents the accumulation of DREB1 to avoid growth retardation under unstressed conditions (Fig. 7).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana* Col-0) plants were grown for 1 to 2 weeks at 22°C on GM agar plates containing 3% Suc and 0.83% agar under 16-h-light (40 μmol m^{-2} s^{-1})/8-h-dark conditions and then transferred to 4°C under continuous dim light conditions (2.5 μmol m^{-2} s^{-1}) for the low-temperature treatment. Plants were grown on GM agar plates containing 1% Suc and 1.2% agar under 12-h-light (80 μmol m^{-2} s^{-1})/12-h-dark conditions and then transferred to the continuous light condition for circadian control. For the protoplasts, plants were grown on soil at 22°C for 6 weeks under 12-h-light (40 μmol m^{-2} s^{-1})/12-h-dark conditions.

A T-DNA insertion line of *PIF7*, *pif7-2* (SAIL_622_G02), was obtained from Syngenta (Sessions et al., 2002). Isolation of the homozygous *pif7-2* line was confirmed by PCR with Ampdirect Plus (Shimadzu; http://www.shimadzu.co.jp/) and ExTaq polymerase (TaKaRa; http://www.takara-bio.co.jp/) using the T-DNA left-border primer (5′-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3′) and the *PIF7*-specific primers (5′-TTGAAACAGCTACAAGCACAAGTACAG-3′ and 5′-GATTCGAAGAACTTGAAGGCATG-3′).

Yeast One-Hybrid Screening of Arabidopsis cDNA Libraries

Yeast one-hybrid screens were performed as described previously (Liu et al., 1998). The titer of the cDNA library was 4.5×10^6 plaque-forming units. We used the yeast strain YM4271 and the Fast Yeast Transformation Kit (TaKaRa) for yeast transformation. For isolation of the full-length cDNA, 5′-RACE was performed using the 5′-RACE system (Invitrogen; http://www.invitrogen.co.jp/) according to the manufacturer's instructions.

Fusion Protein Preparation and Purification and Gel Mobility Shift Assay

The coding region of *PIF7* was inserted into the pGEX-4T-1 vector (GE Healthcare Life Sciences; http://www.gelifesciences.co.jp/) at the *Bam*HI site. The plasmid was expressed in *Escherichia coli* Rosetta (DE3) cells and purified using Glutathione Sepharose (GE Healthcare Life Sciences) according to the manufacturer's instructions with minor modifications. The fusion protein was eluted from the beads by incubation with an elution buffer (50 mM Tris-HCl [pH 8.0] and 20 mM glutathione) overnight at 4°C.

Probe labeling and gel mobility shift assays were performed as described previously (Urao et al., 1993) with minor modifications. The oligonucleotides of the wild-type fragments of *DREB1A*, *-1B*, and *-1C* and the mutation fragments, fused to the *Hin*dIII site, were amplified by PCR and digested by *Hin*dIII. A mixture of 20,000 dpm of ^{32}P-labeled probe and 2 μg of the fusion proteins was incubated in 1× binding buffer [25 mM HEPES-KOH (pH 7.9), 40 mM KCl, 1 mM dithiothreitol, 1 mM ETDA, 10% glycerol, 1% bovine serum albumin, and 1 μg of poly(dI-dC)] for 30 min at room temperature. The reaction mixtures were resolved by electrophoresis through a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 50 V for 2.5 h and visualized by autoradiography. For competition experiments, the competitors were incubated with the fusion protein for 5 min at room temperature prior to the addition of labeled probes.

Construction of Transgenic Plants

For the deletion analysis, the minimal promoter region of *DREB1C* (198 bp upstream of the translation initiation site) was amplified from Arabidopsis genomic DNA as a template by PCR with PrimeStar HS polymerase (TaKaRa). The fragment was then digested with *Hin*dIII and *Xho*I and inserted into the pGK-GUS vector (Qin et al., 2008). The partial fragments of the *DREB1C* promoter were also amplified by PCR and inserted into the *Hin*dIII site.

The *PIF7* promoter region (1,019 bp upstream of the translation initiation site) was amplified from genomic DNA by PCR. The fragment was then inserted into the *Xba*I and *Sma*I sites of the pGK-GUS vector. For the GFP fusion constructs expressed under the control of the promoter region, the *PIF7* promoter and coding regions were amplified by PCR from genomic DNA and cDNA, respectively, with primer sets containing the overlapping sequence between the promoter and coding regions. The fragments were annealed and polymerized as described previously (Fujita et al., 2005). The fused fragment was inserted into the *Hin*dIII and *Sma*I sites of the pGK-35SsGFP vector, which had its *CaMV 35S* promoter removed.

All constructs were then introduced into *Agrobacterium tumefaciens* strain GV3101 with the pSoup helper plasmid (Hellens et al., 2000). Arabidopsis plants were transformed with these constructs as described previously (Liu et al., 1998).

RNA Gel-Blot and Quantitative RT-PCR Analyses

Total RNA from Arabidopsis plants was isolated with the RNAiso reagent (TaKaRa) according to the manufacturer's instructions. The RNA gel-blot analysis was conducted as described (Satoh et al., 2004). Probes of *DREB1C* and *GUS* for the RNA gel-blot analysis were prepared as described previously (Shinwari et al., 1998). For cDNA synthesis, 500 ng of total RNA was used with SuperScriptIII reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR analysis was performed as described previously (Sakuma et al., 2006). The *PIF7* and

DREB1 primers are described in Supplemental Table S1, and the *HFR1* and *CCA1* primers were as described by Lorrain et al. (2008) and Yamashino et al. (2008), respectively. At4g32680 was quantified as an internal control (Maruyama et al., 2009). In the quantitative real-time RT-PCR, all reactions were performed in triplicate.

Construction of Transient Expression Assays

For the protoplast two-hybrid system, the transcriptional activation domain was amplified by PCR from pVP16 and inserted into a *Bam*HI site of the pBI35SΩ vector (pBI35SΩ-*VP16*AD; Hollenberg et al., 1995). Effector constructs for the transactivation assays and the protoplast two-hybrid system were constructed with PCR-amplified coding sequences of PIF7, which were inserted into the *Spe*I site of p35S-564, which contains the GAL4-DBD, or pBI35SΩ-*VP16*AD. The pGUS-558 and pBI35SΩ-LUC vectors were used as the reporter and internal control constructs, respectively (Fujita et al., 2005). For BiFC analysis, full-length fragments of *PIF7*, *PIF4*, *PhyB*, *TOC1*, and *ICE1* were amplified by PCR and cloned into the *Xba*I site of the pUC-SPYNE and pUC-SPYCE vectors (Walter et al., 2004). As an experimental control, a full-length fragment of *H2B* was amplified by PCR and cloned into the *Xba*I and *Sma*I sites of the pGK-CFP vector (Fujita et al., 2009).

Transient Expression Assay Using Arabidopsis Protoplasts

The transient expression assays using protoplasts derived from Arabidopsis mesophyll were performed as described previously (Yoo et al., 2007) with minor modifications. Leaves from plants grown for 6 weeks were cut into 0.5- to 1-mm pieces using a fresh razor blade. Twenty-five to 30 leaves were digested in 25 mL of enzyme solution, which was vacuumed for 15 min, and incubated in the dark for 3.5 h at 22°C to 25°C. The protoplasts were washed and resuspended to approximately 1.2×10^5 cells mL^{-1}. For transfection, a plasmid mixture (3–5 μg each of effector and reporter and 3 μg of internal control) was added to a protoplast and polyethylene glycol solution [40% (w/v) polyethylene glycol 4000 (Fluka; http://www.sigma-aldrich.co.jp/fluka/first.htm), 0.2 M mannitol, and 100 mM $Ca(NO_3)_2$]. The transfected protoplasts were incubated under light conditions (25 μmol m^{-2} s^{-1}) for more than 16 h. LUC activity was assayed using the Bright-Glo Luciferase Assay System (Promega; http://www.promega.co.jp/). Both the luminescence and fluorescence intensities were measured with a multilabel counter (Wallac AVROsx 1420; Perkin-Elmer; http://www.perkinelmer.com/).

Staining and Fluorescence Observation

Histochemical staining of GUS activity was determined as described previously (Qin et al., 2008). GUS staining was observed with an MZ APO stereomicroscope (Leica; http://www.leica-microsystems.co.jp/). The images were captured with Axiovision4.4 digital imaging processing software (Zeiss; http://www.zeiss.com/).

Fluorescence was observed with a LSM5 PASCAL confocal laser-scanning microscope (Zeiss). For nuclear staining, samples were incubated with a 4′,6-diamino-phenylindole solution (4 μg mL^{-1} 4′,6-diamino-phenylindole [Sigma; http://www.sigmaaldrich.com/japan.html], 0.25× phosphate-buffered saline, and 0.2% methanol) for 15 min.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Deletion analysis of the *DREB1C* promoter fragment.

Supplemental Figure S2. Isolation of PIF7, the protein bound to the 65-bp fragment of the *DREB1C* promoter.

Supplemental Figure S3. Gene expression patterns of *PIF7* and *DREB1*s.

Supplemental Figure S4. Examination of possible interactions with PIF7.

Supplemental Figure S5. Expression of *DREB1*s under circadian control and low-temperature conditions in the *pif7* mutant.

Supplemental Table S1. Oligonucleotides used for the experiments.

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