Supporting Information

Lee and Thomashow 10.1073/pnas.1211295109

SI Materials and Methods

Electromobility Shift Assay (EMSA). To prepare recombinant PIF4 and PIF7 protein for EMSA, the ORF of PIF4 or PIF7 was first cloned into pCR8/GW/TOPO vector (Invitrogen), and then moved to pET-60-DEST vector (Stratagene) through recombination reactions (Invitrogen). The recombinant proteins were induced in *E. coli* BL21-Gold (DE3) strain by addition of 0.1 mM of IPTG at 30 °C for 6 h. The crude protein was extracted with B-PER Protein Extraction Reagent (Pierce), and quantified with BCA Protein Assays (Pierce).

The probes for EMSA were prepared by amplification of *CBF1* (−224 to −1), *CBF2* (−189 to −1), and *CBF3* (−316 to −94) promoter regions by PCR from the genomic DNA, and then end-labeled with gamma ^{32}P. The WT and G-box mutated competitors were made by annealing of G-box (CACGTG), E-box (CATGTG) or mutated G-box (ggtacc) primers. The primers are listed in Table S2. The EMSAs were performed as described (1), except 20 ng of crude protein extract, 4 fmol of probes, and 20-fold and 100-fold competitors were used in the binding reactions. The samples were resolved on 5% (wt/vol) polyacrylamide as described (2).

***CBF2* Promoter Reporter Lines.** The *CBF2* promoter fragment from −207 bp to + 134 (just upstream of the ATG) was first cloned into pCR8/GW/TOPO vector (Invitrogen). The G-box at −112 to −107 bp, CACGTG, was converted to GGTACC by site-directed mutagenesis using the Quick Change kit (Stratagene). The WT and mutagenized promoter fragments were fused to the *GUS* reporter in the pMDC164 vector (3) using a recombination reaction (Invitrogen), and then transformed to WT *Arabidopsis* using the floral dip method (4). Transgenic lines in the T3 generation were used for experiments. The primers used for cloning are listed in Table S2.

Transgenic Lines Overexpressing *PIF7* or *PIF4*. For 35S::PIF7-CFP-HA, the open-reading frame (ORF) sequence of PIF7 was cloned into the pCR8/GW/TOPO vector (Invitrogen), transferred to the plant binary vector pEarleyGate102 (5) by recombination, and then transformed into WT *Arabidopsis* plants. The cloning primers are listed in Table S2. For 35S::PIF4-TAP lines, a plant binary vector containing the ORF of PIF4 fused to the TAP-tag (DKLAT2G43010) was obtained from the *Arabidopsis* Biological Resource Center (https://abrc.osu.edu) and transformed into WT *Arabidopsis* plants.

Protein Extraction and Immunoblots. Protein from *Arabidopsis* seedlings was obtained by heating samples at 70 °C for 10 min in extraction buffer [60 mM Tris·HCl, pH 8.5/2% (wt/vol) SDS/2.5% (vol/vol) Glycerol/0.13 mM EDTA, pH 8.0/protease inhibitor mixture; Roche]. The soluble protein was quantified with *DC* Protein Assay (Bio-Rad). One hundred micrograms of total soluble protein with 5% (vol/vol) β-mercaptoethanol was separated on 4–12% (wt/vol) NuPAGE SDS/PAGE (Invitrogen) followed by Western blotting analysis. Immunodetection of PIF4-TAP and PIF7-CFP-HA was done using rabbit anti-myc monoclonal antibodies (71D10, Cell Signaling) and mouse anti-GFP antibodies (11814460001, Roche), respectively. Histone H3 was detected with rabbit anti-Histone H3 antibodies (07-690, Millipore). Corresponding secondary antibodies conjugated with horseradish peroxidase (Thermo Scientific) and SuperSignal West Pico or Femto Chemiluminescent Substrate kits (Thermo Scientific) were used for detection.

1. Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell* 21:972–984.
2. Stead JA, McDowall KJ (2007) Two-dimensional gel electrophoresis for identifying proteins that bind DNA or RNA. *Nat Protoc* 2:1839–1848.
3. Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* 133:462–469.
4. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16:735–743.
5. Earley KW, et al. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* 45:616–629.

Fig. S1. The CBF pathway is regulated by photoperiod. WT plants were grown under SD or LD conditions and the transcript levels for *CBF1*, *CBF3*, and the CBF regulon genes *COR15a* and *GOLS3* were determined at the indicated times. The expression values were normalized with internal control gene *IPP2*. The results are mean values from three independent experiments (error bars indicate SEM).

Fig. S2. A G-box motif within the *CBF2* promoter confers photoperiodic regulated gene expression. (*A*) Diagram of *CBF2*::*GUS* reporter fusions used to test role of the G-box (−112 to −107 bp) in photoperiod-regulated gene expression. (*B*) Transgenic plants carrying the WT-pro and Gmut-pro constructs were grown under SD or LD conditions, and GUS transcript levels were determined at the indicated times. The results are mean values from three independent experiments (error bars indicate SEM). (*C*) Ratio of GUS transcript levels at ZT8 in transgenic plants carrying the WT-pro (WT) or Gmut-pro (Gmut) constructs grown under SD or LD conditions. Values are mean ratios from the eight independent transgenic lines presented in *B* (Student's *t* test, $P < 0.001$). (*D*) Relative expression levels of the WT-pro and Gmut-pro constructs in the experiments described in *B* (Student's *t* test, $P < 0.001$).

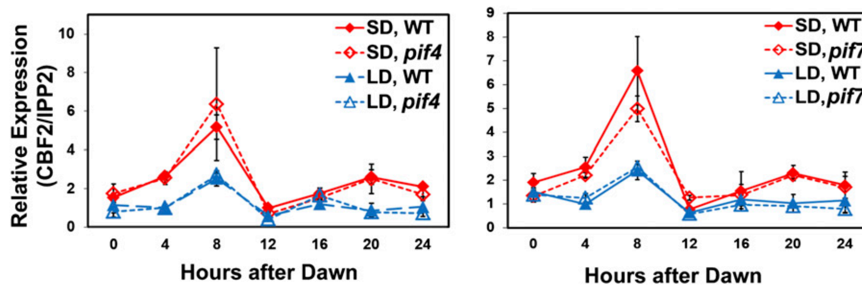

Fig. S3. Repression of the CBF pathway under LD conditions is not affected by single *pif4* and *pif7* null mutations. Plants were grown under SD or LD conditions, and the transcript levels for *CBF2* were determined at the indicated times in WT plants and in *pif4* and *pif7* single mutant plants as indicated. The results are mean values from three independent experiments (error bars indicate SEM).

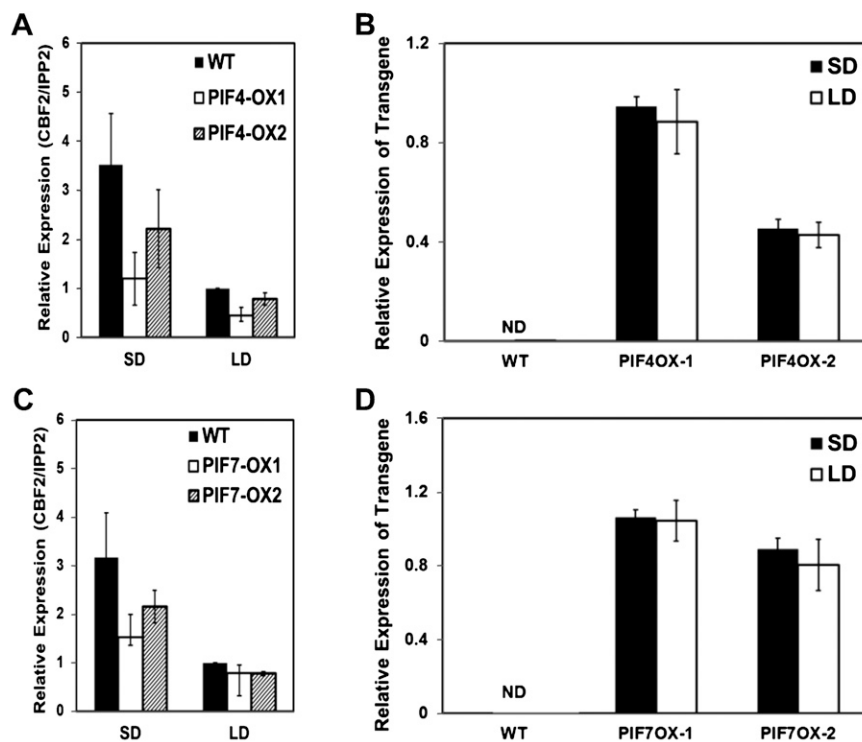

Fig. S4. Overexpression of *PIF4* and *PIF7* down-regulate expression of *CBF2* in plants grown under SD conditions. (*A* and *C*) Relative levels of *CBF2* transcripts at ZT8 in transgenic plants overexpressing PIF4-TAP or PIF7-CFP grown under LD and SD conditions. (*B* and *D*) Relative transcript levels for the PIF4-TAP (PIF4OX-1 and -2) and PIF7- CFP (PIF7OX-1 and -2) gene fusions at ZT8 in transgenic plants grown under SD or LD conditions. Transcripts for the transgenes were nondetectable (ND) in WT plants. All expression levels were normalized to *IPP2* and are mean values from three independent experiments (error bars indicate SEM).

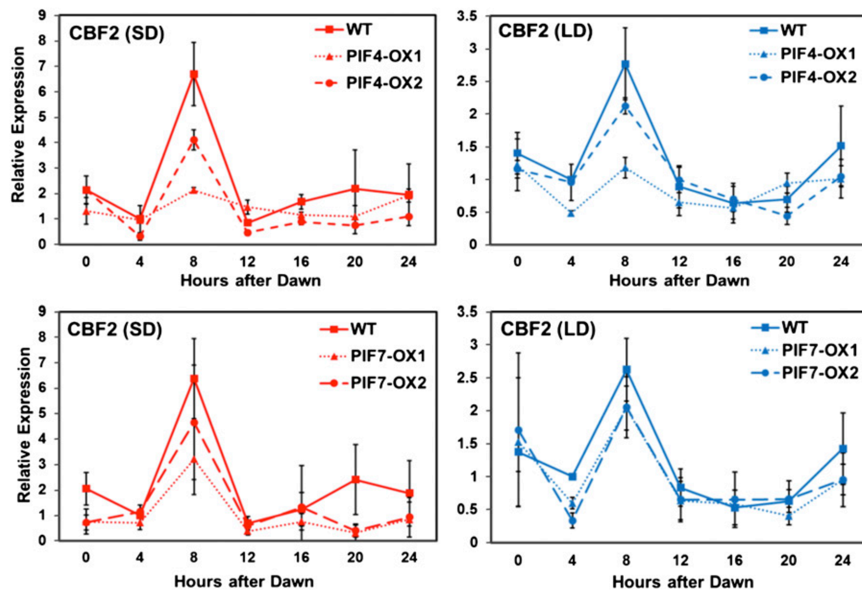

Fig. S5. *CBF2* transcript levels in WT plants and transgenic plants overexpressing PIF4-TAP (PIF4OX-1 and -2) and PIF7-CFP (PIF7OX-1 and -2) grown under SD and LD conditions. *CBF2* transcript levels were normalized against *IPP2*. The results are means from three independent experiments (error bars indicate SEM).

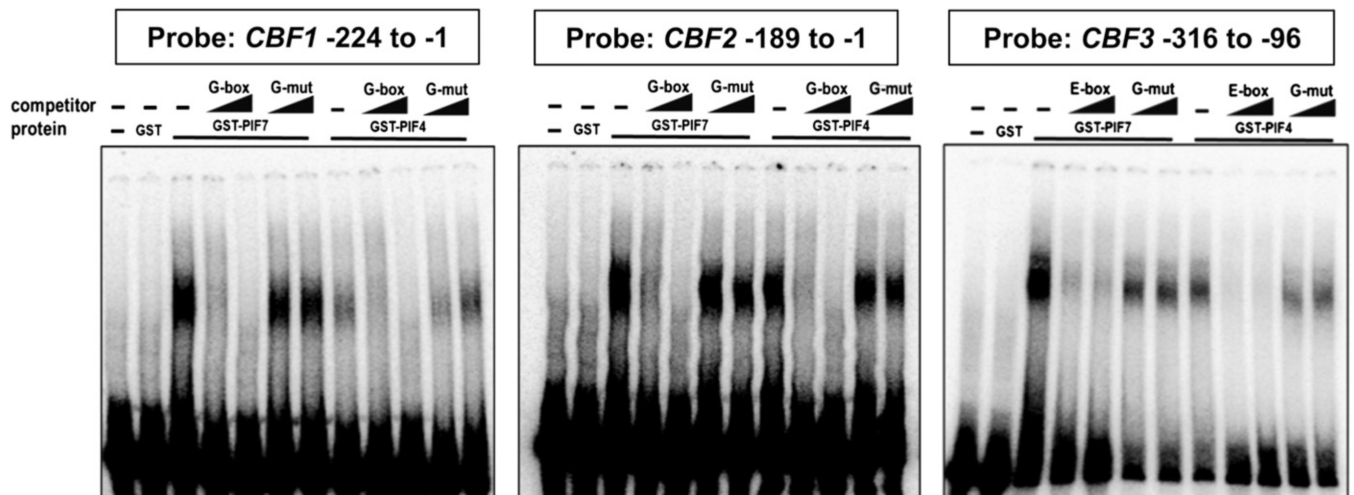

Fig. S6. PIF4 and PIF7 bind to *CBF1*, *CBF2*, and *CBF3* promoters through G-box and E-box motifs. The production of GST-PIF4 and GST-PIF7 recombinant proteins and information of how the EMSAs were performed are described in the *SI Materials and Methods*. The probes used are indicated at the top of the gels. The observed binding was specific as it decreased in response to addition of 20-fold and 100-fold G-box (CACGTG) or E-box (CATGTG) sequence as competitor, but not mutated G-box (ggtacc). The sequences of competitors are listed in Table S2.

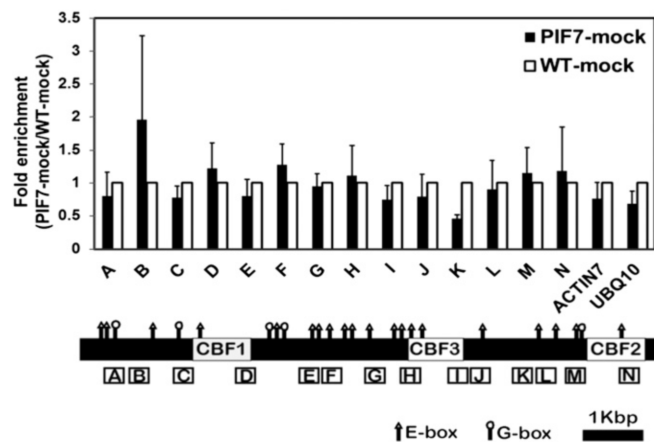

Fig. S7. Mock control experiment for ChIP assays determining binding of PIF7 at the *CBF* locus. WT plants and transgenic plants overexpressing PIF7-CFP were grown under LD conditions, tissue was harvested at ZT8, and ChIP assays were performed using nonspecific rabbit IgG. Precipitated DNA sequences were quantified using primer sets across *CBF* locus (boxes A through N). DNA sequences from ACTIN7 and UBQ10 were used as negative controls. The fold enrichment of precipitated DNA for each primer set in PIF7-OX (PIF7-mock, black bars) samples are relative to the level in the WT samples (WT-mock, open bars). The locations and sequences of primer sets are listed in Table S1. Data are presented as mean ± SEM; $n = 4$. No statistically significant enrichment was observed ($P < 0.05$, paired t test). The location of G-box (CACGTG, circle) and E-box (CANNTG, triangle) motifs are indicated.

Table S1. List of primers for qRT-PCR and ChIP assays

Name	Locus	Forward primer (Fw)	Fw position	Reverse primer (Rv)	Rv position	Source
qRT-PCR						
CBF1	AT4g25490	CCGCCGTCTGTTCAATGGAATCAT	+734	TCCAAAGCGACACGTCACCATCTC	+774	This study
CBF2	AT4g25470	CGACGGATGCTCATGGTCTT	+562	TCTTCATCCATATAAAACGCATCTTG	+630	This study
CBF3	AT4g25480	TTCCGTCCGTACAGTGGAAT	+694	AACTCCATAACGATACGTCGTC	+741	(1)
COR15a	AT2g42540	GAAAAAAACAGTGAAACCGCAGAT	+704	CCACATACGCCGCAGCTT	+750	(1)
GOLS3	AT1g09350	CTGACGAGCGAGGTTCTTGTC	+1090	AACAAATTCTAAGTAAACATCACCAGTT	+1137	(2)
IPP2	AT3g02780	ATTTGCCCATCGTCCTCTGT	+115	GAGAAAGCACGAAAATTCGGTAA	+155	This study
PIF4	AT2g43010	TCTCCGACCGGTTTGCTAGA	+1360	CGCGGCCTGCATGTGT	+1397	This study
PIF7	AT5g61270	CAAGTGCGAGTGGTACCAATATG	+484	TTCAAGCTCCGACCGGATT	+523	This study
GUS		TGGCCTGGCAGGAGAAACT		CGTATCCACGCCGTATTCG		This study
TAP (myc)		TGCAGCCTAGGGATTACGATATC		GGCCCCTGGAACAGAACTTC		This study
CFP		GTCCGCCCTGAGCAAAGA		TCCAGCAGGACCATGTGATC		This study
ChIP						
A	AT4g25490	TGCTTTCAAGGCCGAATGAT	−1312	CGTTCTCATTCCACGTGTGATG	−1247	(1)
B	AT4g25490	TTACCACTCTTTTTTTCCCTCTTTG	−845	CTCGCTCTCACGTTATTGACATTT	−801	(1)
C	AT4g25490	TCTTTACAAGGGTCAAAGGACACA	−186	GCGAAGCAATCCCACGAT	−142	(1)
D	AT4g25490	CCGCCGTCTGTTCAATGGAATCAT	+734	TCCAAAGCGACACGTCACCATCTC	+774	This study
E	AT4g25480	AGTTCTATCGGACTAATTCTTGGCTTA	−1859	GATGATCAAGCGTAATTGCTTTGT	−1752	(1)
F	AT4g25480	TGACTAAGGACGTGGTGGTTGA	−1235	AGCGCACTTCCTTCTCACTCA	−1178	(1)
G	AT4g25480	TGTTACATTTGATCATTCACCCAAA	−604	CGTATATAAGCACGTAAGTCACCAAGT	−550	(1)
H	AT4g25480	CGTGGCATTACCAGAGACACA	−124	GCGGAAGATATTTTAGAGGCAAAA	−83	(1)
I	AT4g25480	TTCCGTCCGTACAGTGGAAT	+694	AACTCCATAACGATACGTCGTC	+741	(1)
J	AT4g25470	CAAGAGAGCACTGTCCGTAGCTT	−1851	TGGTTACAAGAGGAGCCACGTA	−1811	(1)
K	AT4g25470	TTTGCCGGAAAACTCAACTCA	−1147	CCTTCTTTTTGGTCTGAAA	−1108	(1)
L	AT4g25470	GAGAGATGCTGGAAATTGTGATCA	−943	AAATATGGTAAGTGGTTAGGCGAAA	−897	(1)
M	AT4g25470	GGGTCAAAGGACACATGTCAG	−201	GAACGCGGAGTTTCTGTCTC	−102	Tiffany Liu
N	AT4g25470	CGACGGATGCTCATGGTCTT	+562	TCTTCATCCATATAAAACGCATCTTG	+630	This study
Actin7	AT5g09810	CGTTTCGCTTTCCTTAGTGTTA	+54	AGCGAACGGATCTAGAGACTC	+167	(3)
UBQ10	AT4g05320	TCCAGGACAAGGAGGTATTCCTCCG	+1616	CCACCAAAGTTTTACATGAAACGAA	+1796	(3)

1. Dong MA, Farré EM, Thomashow MF (2011) Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis. *Proc Natl Acad Sci USA* 108:7241–7246.
2. Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF (2009) Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell* 21: 972–984.
3. Pruneda-Paz JL, Breton G, Para A, Kay SA (2009) A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. *Science* 323:1481–1485.

Table S2. List of primers for cloning and EMSA assays

Name	Locus	Forward primer	Reverse primer
Cloning			
CBF2-pro	AT4g25470	CAAGATGGGTCAAAGGACACATGTCAGATT	TGATCAGAAGAGTACTCTGTTTCAAGAAACTGGA
CBF2-pro-Gmut	AT4g25470	TTAGCTGTTTCTTATCGGTACCGCATTCACAGAGACAGA	TCTGTCTCTGTGAATGCGGTACCGATAAGAAACAGCTAA
PIF4	AT2g43010	ATGGAACACCAAGGTTGGAGTTTTGAGGAGAA	CGCGGCCTGCATGTGT
PIF7	AT5g61270	CAAGTGCGAGTGGTACCAATATG	TTCAAGCTCCGACCGGATT
EMSA			
CBF1-pro	AT4g25490	AAGAACTCATAAAGGTTAACGAGTGAAGAGTCAAAAG	TGTGTAGTTAGTATAAAAAGTGAGAGTGAGAATTGGT
CBF2-pro	AT4g25470	CAAGATGGGTCAAAGGACACATGTCAGATT	GCCGGAAGATATTTGGATATTTG
CBF3-pro	AT4g25480	ACGGTTACCCTACACCTAGTACACTAAATCCT	ACGGAGTTTGTGTCTCTGGTAATGCCACGT
6X G-box		CACGTGCACGTGCACGTGCACGTGCACGTGCACGTG	
6X E-box		CATGTGCATGTGCATGTGCATGTGCATGTGCATGTG	
6X G-box mutated		GGTACCGGTACCGGTACCGGTACCGGTACCGGTACC	