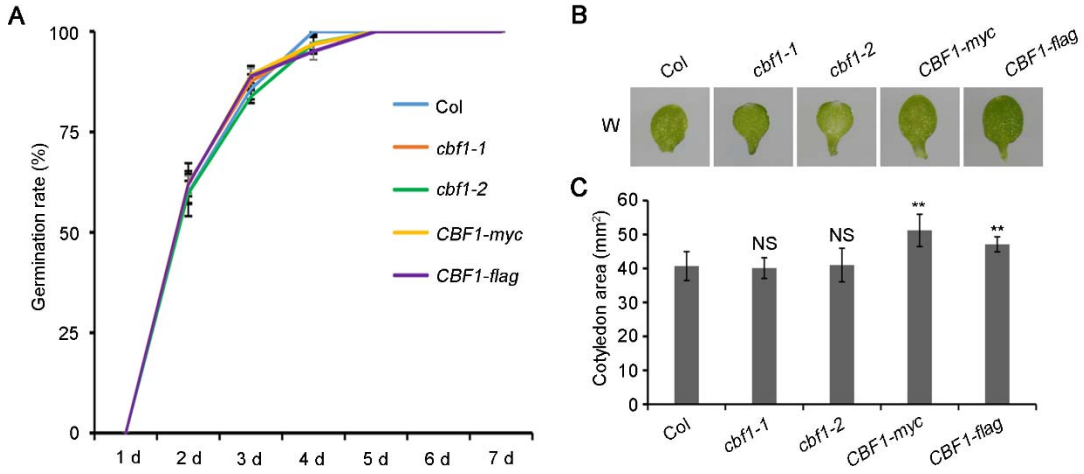
Appendix

CBF1, a key cold response regulator, promotes *Arabidopsis* hypocotyl growth at ambient temperatures

This PDF file includes:

Appendix Figures S1 to S11

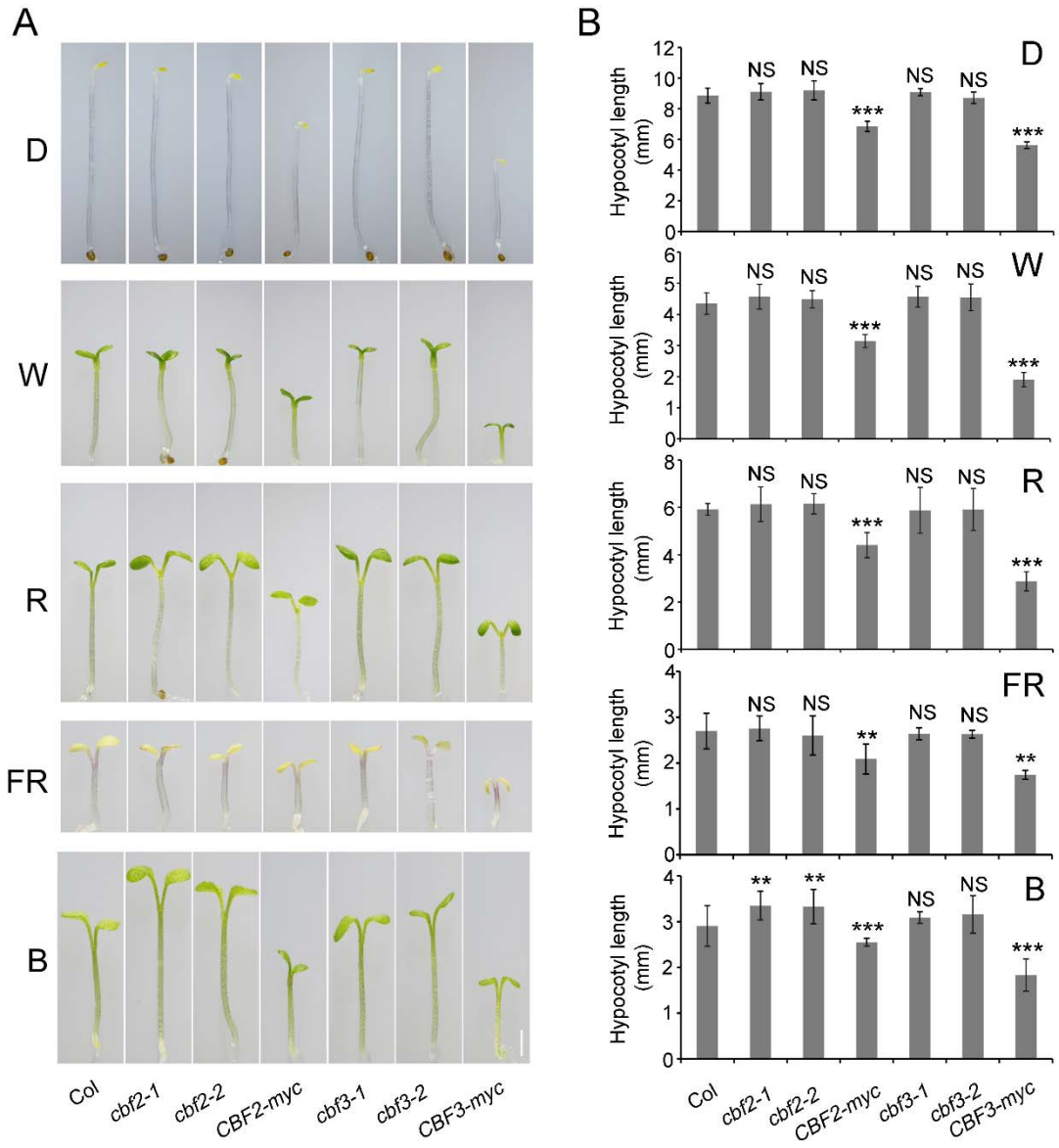
Appendix Table S1



Appendix Figure S1. Germination rates and cotyledon areas of Col, *cbf1* mutants and CBF1-OE lines grown in continuous white light.

A Germination rates of Col, two *cbf1* mutants (*cbf1-1* and *cbf1-2*) and two CBF1-OE lines (*CBF1-myc* and *CBF1-flag*) grown at 22°C in continuous W light for the indicated days.

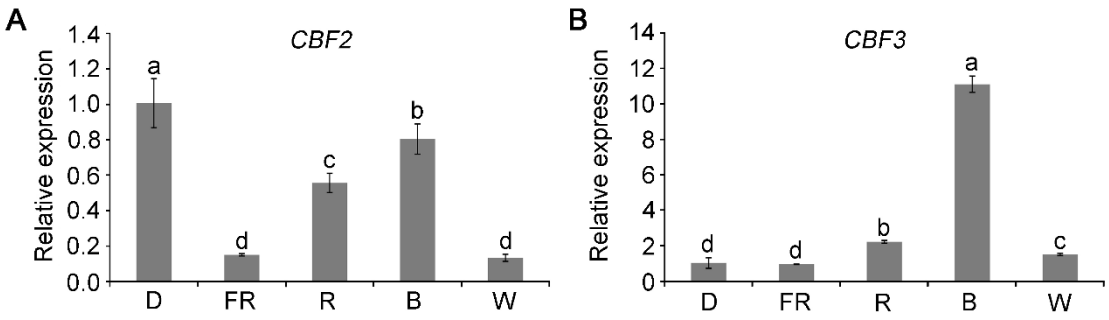
B, C Cotyledon phenotypes (B) and areas (C) of Col, two *cbf1* mutants (*cbf1-1* and *cbf1-2*) and two CBF1-OE lines (*CBF1-myc* and *CBF1-flag*) grown at 22°C in continuous W light for 5 d. Error bars represent SD from 10 seedlings. $^{**}P < 0.01$ (two-tailed *t*-test) for the indicated genotype compared with Col. NS, not significant.



Appendix Figure S2. Phenotypes of loss-of-function mutants and overexpression lines of *CBF2* and *CBF3* grown under different light conditions.

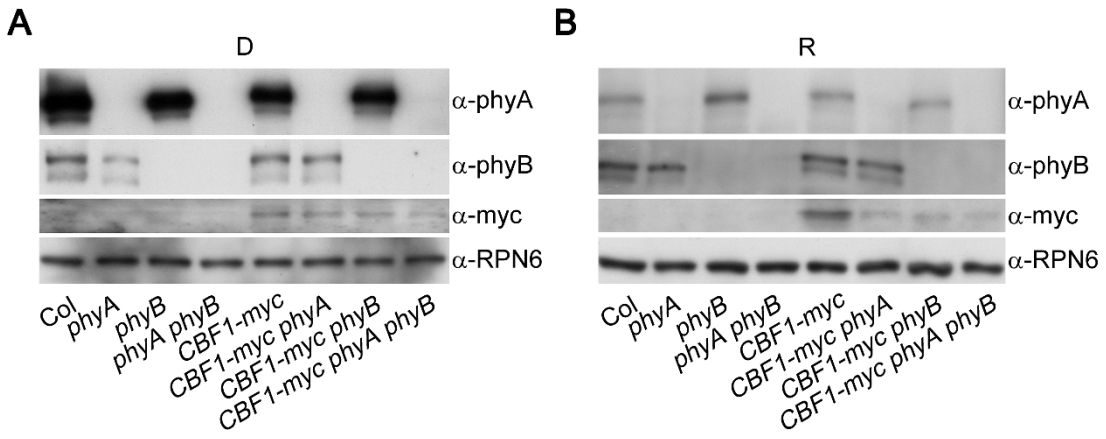
A Phenotypes of 4-d-old Col, *cbf2-1*, *cbf2-2*, *CBF2-myc*, *cbf3-1*, *cbf3-2*, and *CBF3-myc* seedlings grown at 22°C in darkness (D) or continuous FR, R, B, and W light. Bar = 1 mm.

B Hypocotyl lengths of 4-d-old Col, *cbf2-1*, *cbf2-2*, *CBF2-myc*, *cbf3-1*, *cbf3-2*, and *CBF3-myc* seedlings grown at 22°C in darkness (D) or continuous FR, R, B, and W light. Error bars represent SD from 20 seedlings. $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-tailed *t*-test) for the indicated genotype compared with Col.



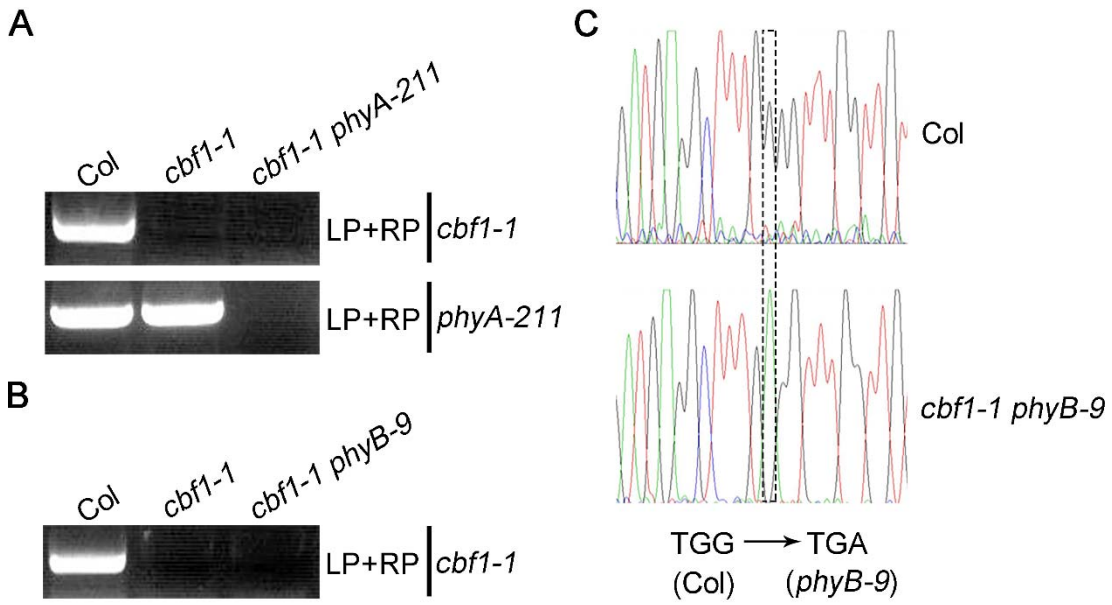
Appendix Figure S3. The expression levels of *CBF2* and *CBF3* in different light conditions.

qRT-PCR data showing the relative expression of *CBF2* (A) and *CBF3* (B) in 4-d-old Col grown at 22°C in darkness (D) or continuous FR, R, B, and W light. Error bars represent SD of three technical replicates. Different letters represent significant differences by one-way ANOVA with Duncan's post hoc test ($P < 0.05$).



Appendix Figure S4. Genotyping of *CBF1-myc phyA*, *CBF1-myc phyB* and *CBF1-myc phyA phyB* seedlings by immunoblotting.

Immunoblots showing the levels of phyA, phyB, and CBF1-myc proteins in 4-d-old Col, *phyA*, *phyB*, *phyA phyB*, *CBF1-myc*, *CBF1-myc phyA*, *CBF1-myc phyB*, and *CBF1-myc phyA phyB* seedlings grown at 22°C in darkness (A) or continuous R light (B). Anti-RPN6 was used as a sample loading control.

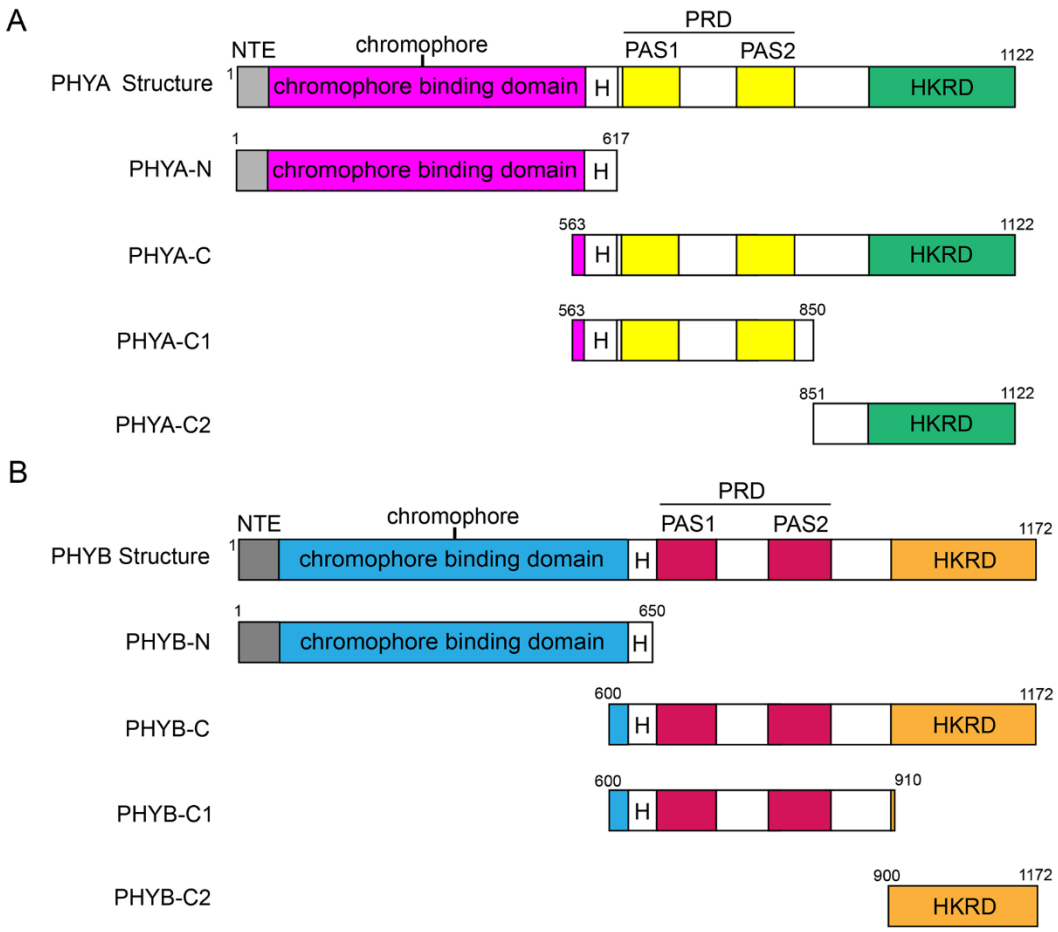


Appendix Figure S5. Genotyping of *cbf1-1 phyA-211* and *cbf1-1 phyB-9* mutants.

A Genotyping of the *cbf1-1* and *phyA-211* loci in *cbf1-1 phyA-211* mutants by PCR using the LP and RP primers shown in Appendix Table S1.

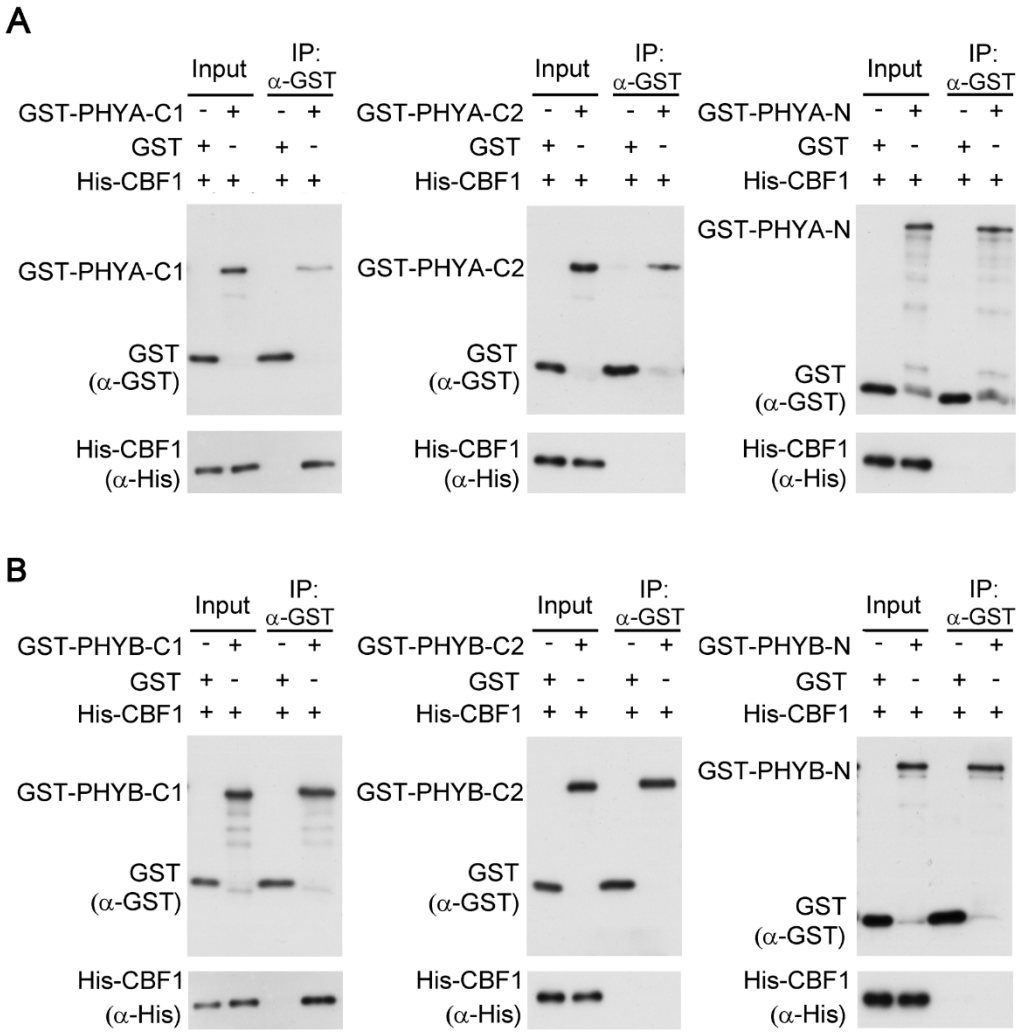
B Genotyping of the *cbf1-1* locus in *cbf1-1 phyB-9* mutants by PCR.

C Genotyping of the *phyB-9* locus in *cbf1-1 phyB-9* mutants by sequencing the PCR products containing the Trp397 (TGG) to TGA mutation.



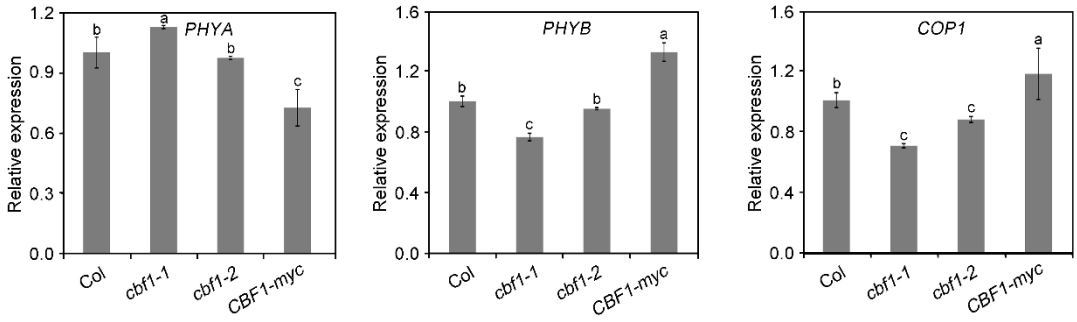
Appendix Figure S6. Different domains of PHYA and PHYB used for *in vitro* pull-down assays.

Domain structures of PHYA (A) and PHYB (B), and different domains of PHYA and PHYB fused with GST or His for *in vitro* pull-down assays. The domain structures were based on previous studies (Mathews *et al*, 1995; Quail *et al*, 1997; Seo *et al*, 2004). NTE, N-terminal extension; PRD, PAS-related domain; HKRD, histidine kinase-related domain; H, hinge.



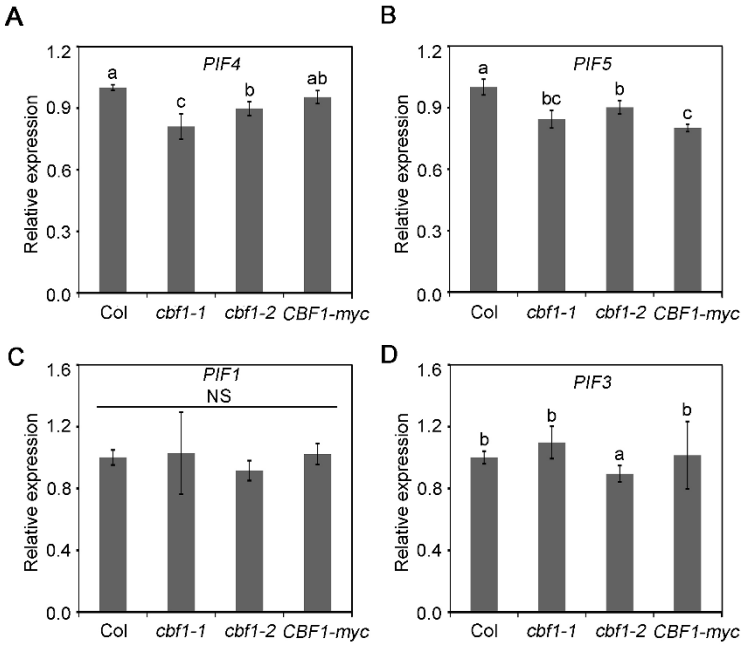
Appendix Figure S7. Pull-down of CBF1 with the PRD domains of PHYA and PHYB *in vitro*.

The His-tagged CBF1 proteins pulled down with GST-tagged PHYA/B-N, PHYA/B-C1 (PAS-related domain), PHYA/B-C2 (histidine kinase-related domain), or GST were detected by anti-His antibody. Input, 6% of the His-tagged purified target proteins used in pull-down assays.



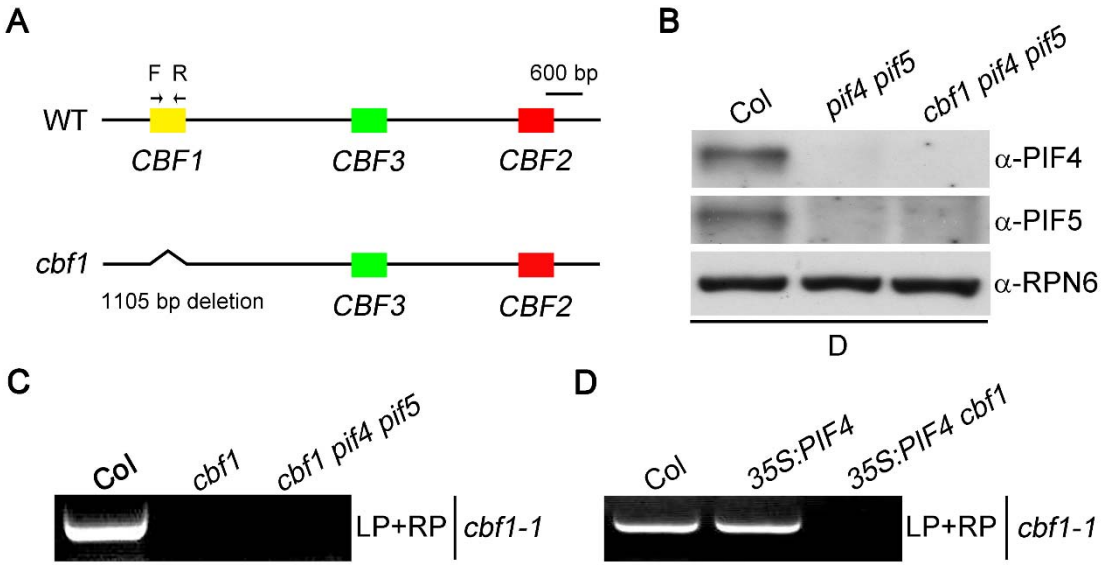
Appendix Figure S8. Expression of *PHYA*, *PHYB*, and *COP1* in *cbf1* mutants and CBF1-OE seedlings grown in the light.

qRT-PCR assays showing the relative expression of *PHYA*, *PHYB*, and *COP1* in 4-d-old Col, *cbf1-1*, *cbf1-2* and *CBF1-myc* seedlings grown at 22°C in continuous W light for 4 d. Error bars represent SD of three technical replicates. Different letters represent significant differences by one-way ANOVA with Duncan's post hoc test ($P < 0.05$).



Appendix Figure S9. Expression of *PIF4*, *PIF5*, *PIF1* and *PIF3* in *cbf1* mutants and CBF1-OE seedlings grown in darkness.

qRT-PCR assays showing the expression levels of *PIF4*, *PIF5*, *PIF1* and *PIF3* in 4-d-old Col, *cbf1-1*, *cbf1-2* and *CBF1-myc* seedlings grown at 22°C in darkness. Error bars represent SD of three technical replicates. Different letters represent significant differences by one-way ANOVA with Duncan's post hoc test ($P < 0.05$). NS, not significant.



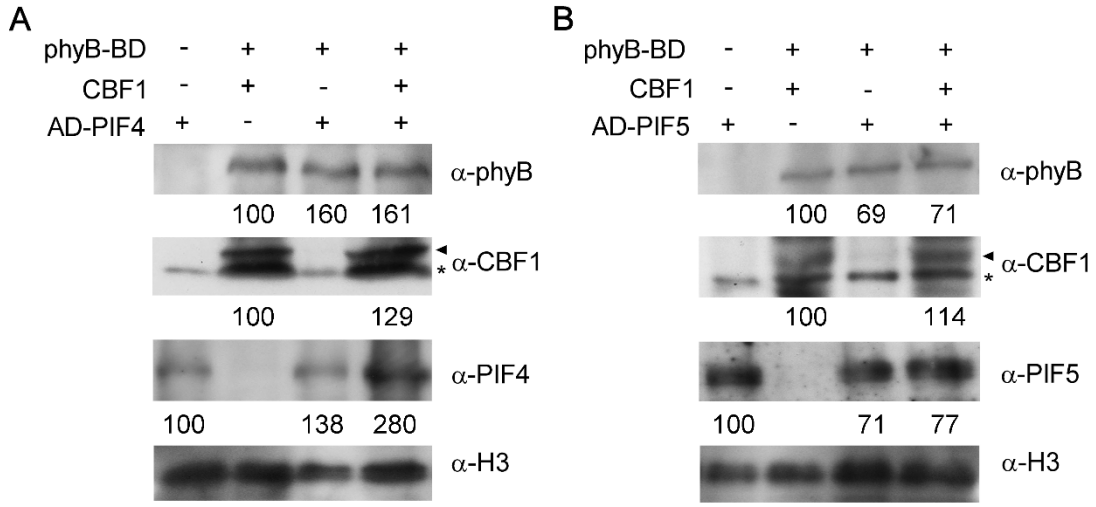
Appendix Figure S10. Genotyping of *cbf1 pif4 pif5* and *35S:PIF4 cbf1* seedlings.

A Schematic representation of the CBF1-CBF3 cluster, and of the mutation in the *cbf1-1* mutant (Zhao *et al*, 2016).

B Immunoblots showing the levels of PIF4 and PIF5 proteins in 4-d-old Col, *pif4 pif5* and *cbf1 pif4 pif5* seedlings grown at 22°C in darkness. Anti-RPN6 was used as a sample loading control.

C Genotyping of the *cbf1-1* locus in *cbf1 pif4 pif5* mutants by PCR.

D Genotyping of the *cbf1-1* locus in *35S:PIF4 cbf1* seedlings by PCR.



Appendix Figure S11. The levels of phyB-BD, CBF1, AD-PIF4/PIF5 proteins in yeast cells used for yeast three-hybrid assays.

Immunoblots showing the levels of phyB-BD, CBF1, and AD-PIF4 (A) or AD-PIF5 (B) proteins in yeast cultures co-expressing the indicated combinations of proteins. Anti-H3 was used as a sample loading control. The asterisk indicates a cross-reacting band recognized by our anti-CBF1 antibodies. Numbers below the immunoblots indicate the relative band intensities of phyB-BD, CBF1, and AD-PIF4/PIF5 normalized to those of loading control, respectively. The ratio of the first band was set to 100 for each blot.

Appendix References

Mathews S, Lavin M, Sharrock RA (1995) Evolution of phytochrome gene family and its utility for phylogenetic analyses of angiosperms. *Anal Missouri Bot Garden* 82: 296–321

Quail PH (1997) The phytochromes: a biochemical mechanism of signaling in sight? *Bioessays* 19: 571–579

Seo HS, Watanabe E, Tokutomi S, Nagatani A, Chua NH (2004) Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. *Genes Dev* 18: 617–622

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

Appendix Table S1. Summary of primers used in this study.		
Purpose		Primer Sequence (5'-3')
Plasmid Constructs (Note: The underlined nucleotides indicate the restriction sites for cloning.)		
AD fusions (pGADT7 vector, used in GAL4 Y3H system)	PIF4	TGGATCGAATTCATGGAACACCAAGGTTGG
		TGGATCCTCGAGCTAGTGGTCCAAACGAGA
	PIF5	TGGATCGAATTCATGGAACAAGTGTTTGCT
		TGGATCCTCGAGTCAGCCTATTTTACCCAT
BD fusions (D153 vector, used in GAL4 Y3H system)	phyB	TGGATCGCGGCCGCATGGTTTCCGGAGTCGGGGGT
		TGGATCGCGGCCGCCATATGGCATCATCAGCAT
pRS423-JL vector	Modification of Multiple Cloning Sites	ACCGACGAGCTCGCACTAGTCGCTGCAGTGCCGCGGAACCCGGGTTGAATTTTCAAAAATTCTTAC
		CGATTGCTCGAGTGGCGGCCGCTCGTCGACCCATCGATCGGGATCCGTTTTTTCTCCTTGACGTTAAAG
	CBF1	TGGATCGGATCCATGAACTCATTTTCAGCT
		TGGATCCTCGAGTTAGTAACTCCAAAGCGA
cLUC fusions (35S:cLuc vector)	CBF1	TGGATCGGTACCATGAACTCATTTTCAGCT
		TGGATCGGATCCTTAGTAACTCCAAAGCGA
	phyB	TGGATCGGTACCATGTCAGGCTCTAGGCCGAC
		TGGATCGGTACCCTAATATGGCATCATCAGCAT
nLUC fusions (35S:nLuc vector)	phyA	GGAAGGATCCAATGTCAGGCTCTAGGCCGAC
		GGAAGTCGACCTTGTTTGCTGCAGCGAGTTC
	PIF4	TGGATCGGTACCATGGAACACCAAGGTTGG
		TGGATCGTCGACGTGGTCCAAACGAGAACC
	PIF5	TGGATCGGTACCATGGAACAAGTGTTTGCT
		TGGATCGTCGACGCCTATTTTACCCATATG
pSuper1300-GFP vector	CBF1	TGGATCAAGCTTATGAACTCATTTTCAGCT
		TGGATCGTCGACGGTAACTCCAAAGCGACAC

pCAMBIA1381 vector	CBF1p:GUS	GCGAATTCGTTTTCCTCTTCATTGGATTG
		GCGGATCCTGATCAGAGTACTCTGTTTC
pK7WFWG2 vector	CBF1-FLAG	TGGATCTCTAGAATGAACTCATTTTCAGCT
		TGGATCGGTACCGGTAACTCCAAAGCGACAC
GST fusions (pGEX-4T-1 vector) / His fusion (pET28a vector)	phyA-C1	GAATTCGGATCCACAAGGAGTTTACCTTGGAAGG
		CTCGAGGTCGACCTACTCCACATACTTGCCACCTC
	phyA-C2	GAATTCGGATCCTGTCTGTTGTGTGTGAGTAAG
		CTCGAGGTCGACCTACTTGTTTGCTGCAGCGAGTTC
	phyA-N	GAATTCGGATCCATGTCAGGCTCTAGGCCGACT
		CTCGAGGTCGACCTAAGCTTCTAGTTCTTGTATACC
	phyB-C1	TTGATCGGATCCGAAACTGCGGAAATGGATG
		TTGATCGCGGCCGCCTACTCAGGGCTCGGGATTTG
	phyB-C2	TGGATCGGATCCGCTTTCTGTTTCTTGCAAATC
		TGCAGCCTCGAGCTAATATGGCATCATCAGCAT
	phyB-N	TTGATCGAATTCATGGTTTCCGGAGTCGGG
		TTGATCGCGGCCGCCTAACCTAACTCATCAATCCC
GST fusions (pGEX-4T-1 vector)	CBF1	TTGATCGGATCCATGAACTCATTTTCAGCT
		TTGATCGAATTCTTAGTAACTCCAAAGCGA
6His fusions (pET28a vector)	CBF1	TGGATCGAATTCATGAACTCATTTTCAGCT
		TGGATCCTCGAGTTAGTAACTCCAAAGCGACAC
Genotyping		
CBF1		TGGATCGGTACCATGAACTCATTTTCAGCT
		TGGATCGTCGACGTAACTCCAAAGCGACAC
phyA		GACACGATGATTCCTGCATC
		CAGCTGTGCGGTGCTCTAA
phyB		CTACTCTTGAGAAACCTGAG
		GAGTCTCTCAGAATAAGCTG

Antibody		
6His fusions (pET28a vector)	phyB	TGGATCGGATCCGCTTTCTGTTTCTTGCAAATC
		TGCAGCCTCGAGCTAATATGGCATCATCAGCAT
Real-time qRT-PCR		
CBF1		GCATGTCTCAACTTCGCTGA
		ATCGTCTCCTCCATGTCCAG
CBF2		TGACGTGTCCTTATGGAGCTA
		CTGCACTCAAAAACATTTGCA
CBF3		GATGACGACGTATCGTTATGGA
		TACACTCGTTTCTCAGTTTTACAAAC
PIF1		CTCTTCAGCTTCAAATACAGATG
		GAATAGGCTGGTTCATACC
PIF3		CTCCAACTTCAAGTGCAGATC
		GCAAGCCCATTGCATAAG
PIF4		CAGCTTCAAGTGATGTGGATG
		CATAACCGGAAATCGAGGTAA
PIF5		CAACTCCAAGTGATGTGGATG
		CAATTGCATCTGACTTTGCAT
phyA		GAAGGATGCTTGGATTTGGA
		CCTCAGGGAAGCTGAAACAG
phyB		CGTGACATTCCCGAAGAGAT
		CTCTGGAGGCAGACCTTCAC
COP1		TCACGAGACCCGTGACATC
		GGACTATCACTCTTCCAGC
HY5		CCATCAAGCAGCGAGAGGTCATCAA
		CGCCGATCCAGATTCTCTACCGGAA
Tubulin3		ATCCGTGAAGAGTACCCAGAT
		AAGAACCATGCACTCATCAGC

ChIP-qPCR	
PIF4p-a	GCATTTTGATTTACATTT
	GACCCAATATATTTTCTT
PIF4-exon	ACCAAGGTTGGAGTTTTGAGGA
	CCACTTGTCCATCTCGCCAT
PIF5p-a	CGCCGCTTCTAGCCTTTC
	CCGAAAAATACAAATTTA
PIF5-exon	AATCGAGCCAACGGTCAGG
	GCTGCAGTGAGGTATGAGTTCT
EMSA Probes	
PIF4-GCC/DRE-WT	biotin-TTCCTCTCCGCCGCCAATCTGCCGACAAGTTTCTTTTCGT
	ACGAAAAGAAACTTGTCGGCAGATTGGCGGCGGAGAGGAA
PIF4-GCC/DRE-mut	biotin-TTCCTCTCCTCCTCCAATCTACTTATAAGTTTCTTTTCGT
	ACGAAAAGAAACTTATAAGTAGATTGGAGGAGGAGAGGAA
PIF5-GCC-WT	biotin-TACTTCCATCGCCGCCACCGCCGCCTGAATGTTTCAGAAC
	GTTCTGAAACATTCAGGCGGCGGTGGCGGCGATGGAAGTA
PIF5-GCC-mut	biotin-TACTTCCATCTCCTCCACCTCCTCCTGAATGTTTCAGAAC
	GTTCTGAAACATTCAGGAGGAGGTGGAGGAGATGGAAGTA