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Supplementary Information for

Posttranslational regulation of multiple clock-related transcription factors triggers cold-inducible gene expression in *Arabidopsis*

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This PDF file includes:

Figures S1 to S13
Tables S1 to S2
Legends for Datasets S1
SI References

Other supplementary materials for this manuscript include the following:

Datasets S1

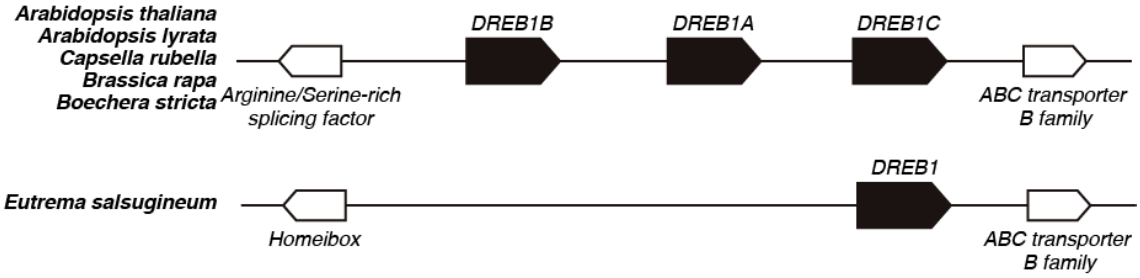

Fig. S1. Genomic structure of the *DREB1* genes in five Brassicaceae species.

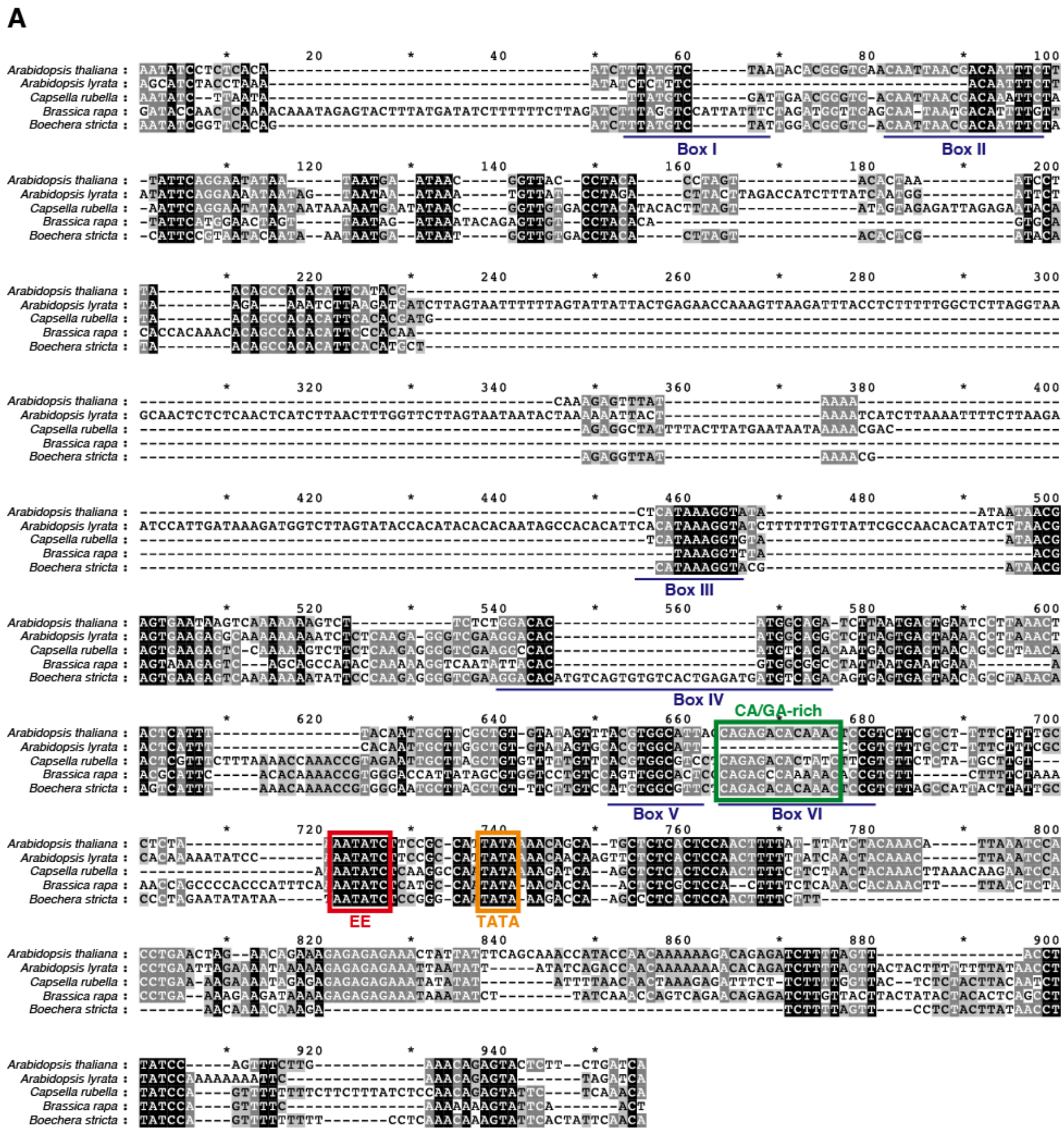

Fig. S2. Alignment of promoter sequences of the *DREB1* genes in Brassicaceae species. Promoter sequences of *DREB1A* (A), *DREB1B* (B) and *DREB1C* (C) in five Brassicaceae species are aligned. Blue lines indicate the conserved regions of the three *DREB1* promoters in *Arabidopsis thaliana*, named Box I to VI (1).

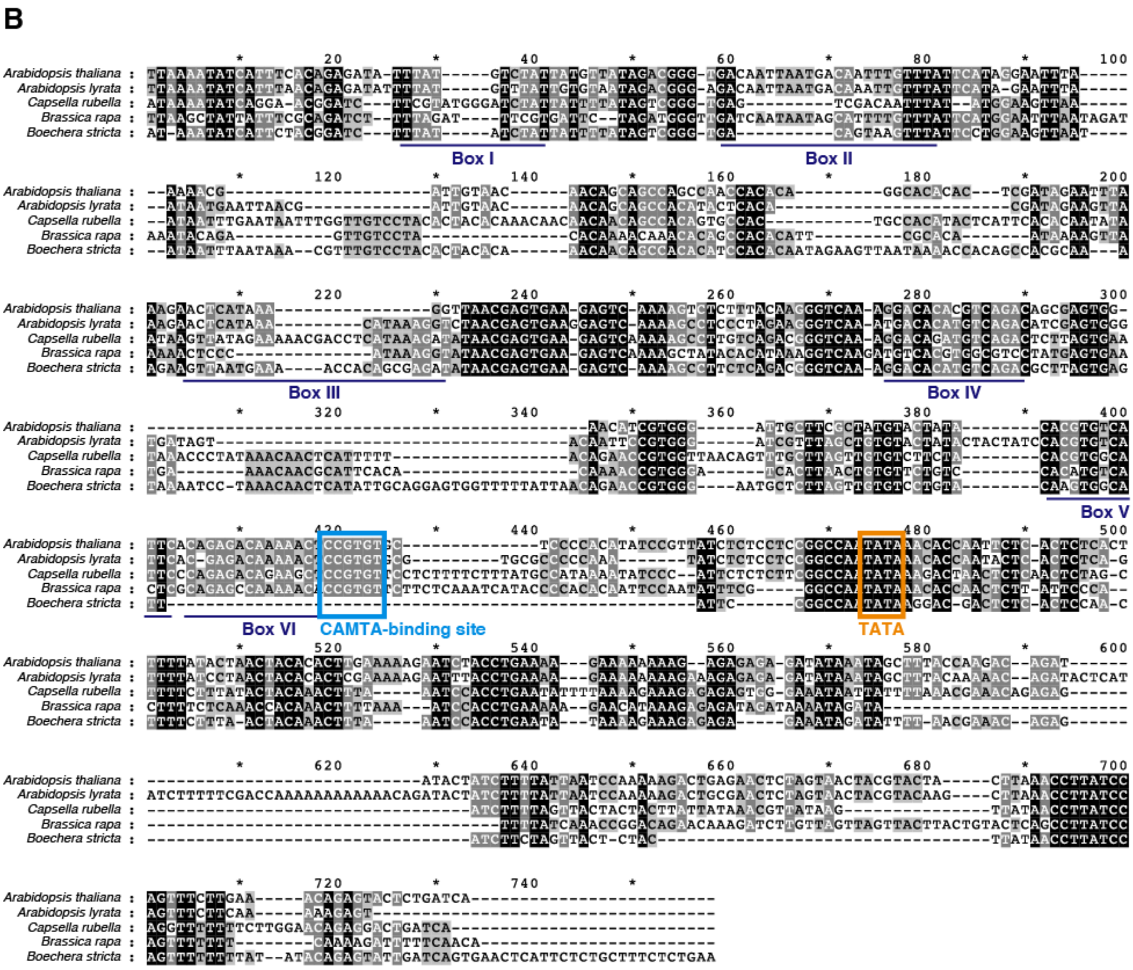

Fig. S2. (continued)

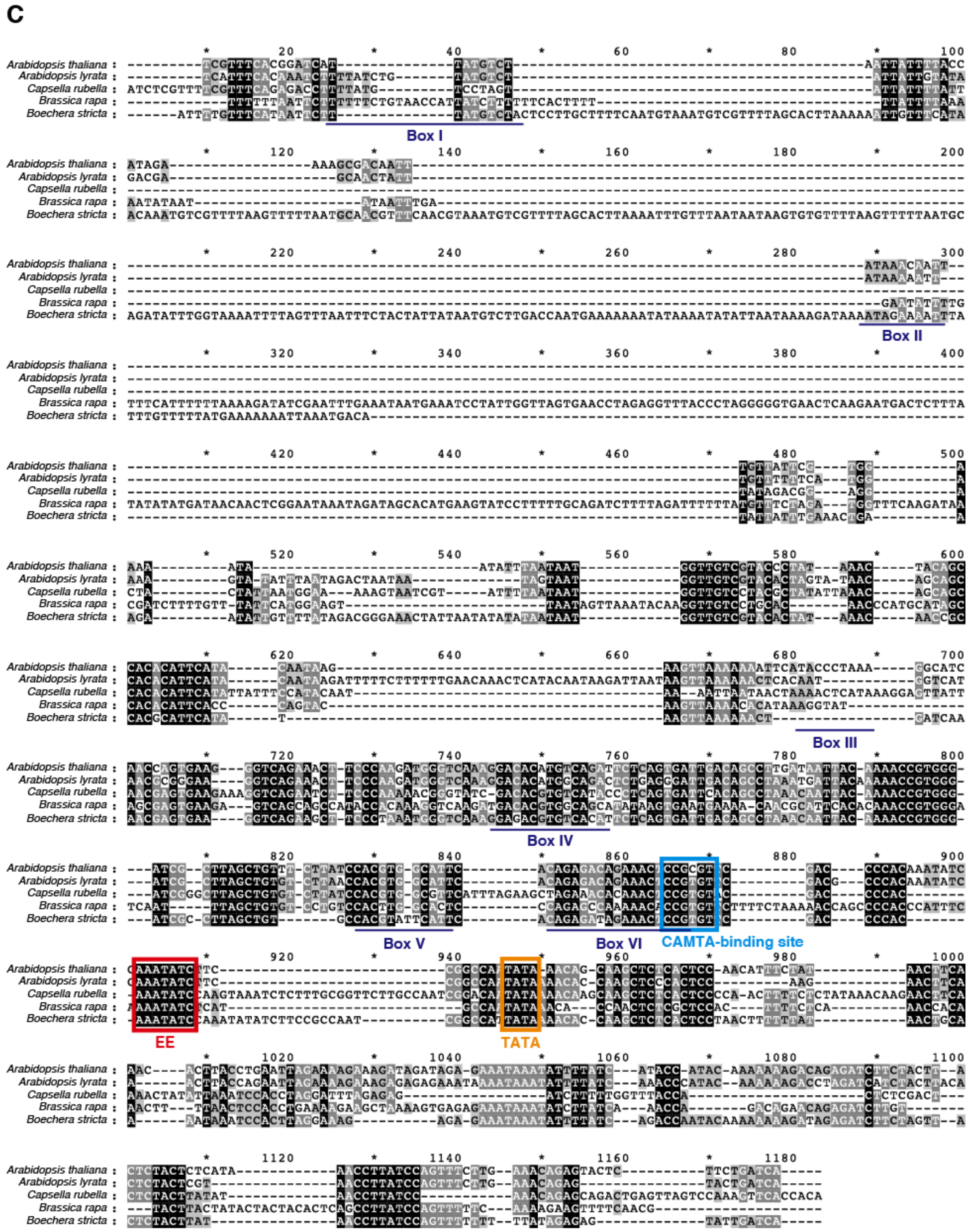

Fig. S2. (continued)

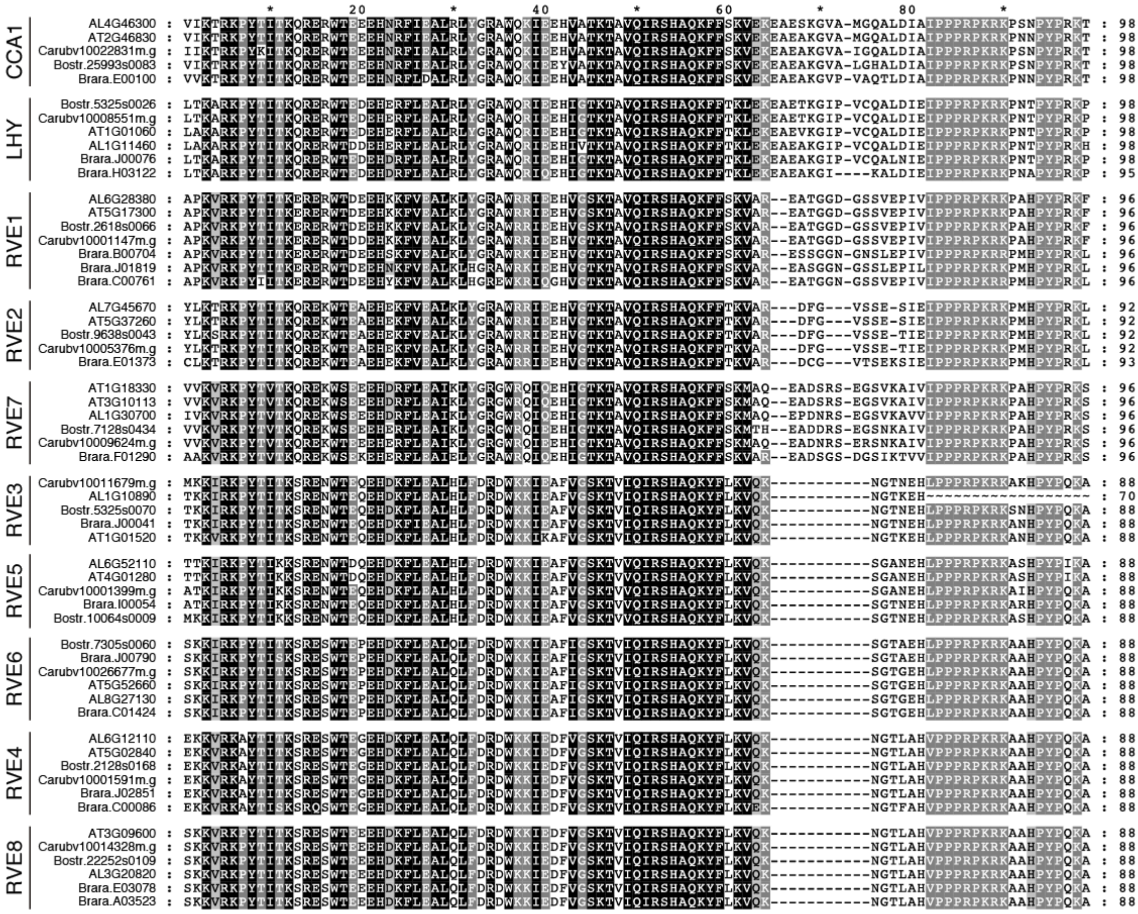

Fig. S3. Alignment of the MYB binding domains of the CCA1, LHY, and RVE transcription factors in Brassicaceae species.

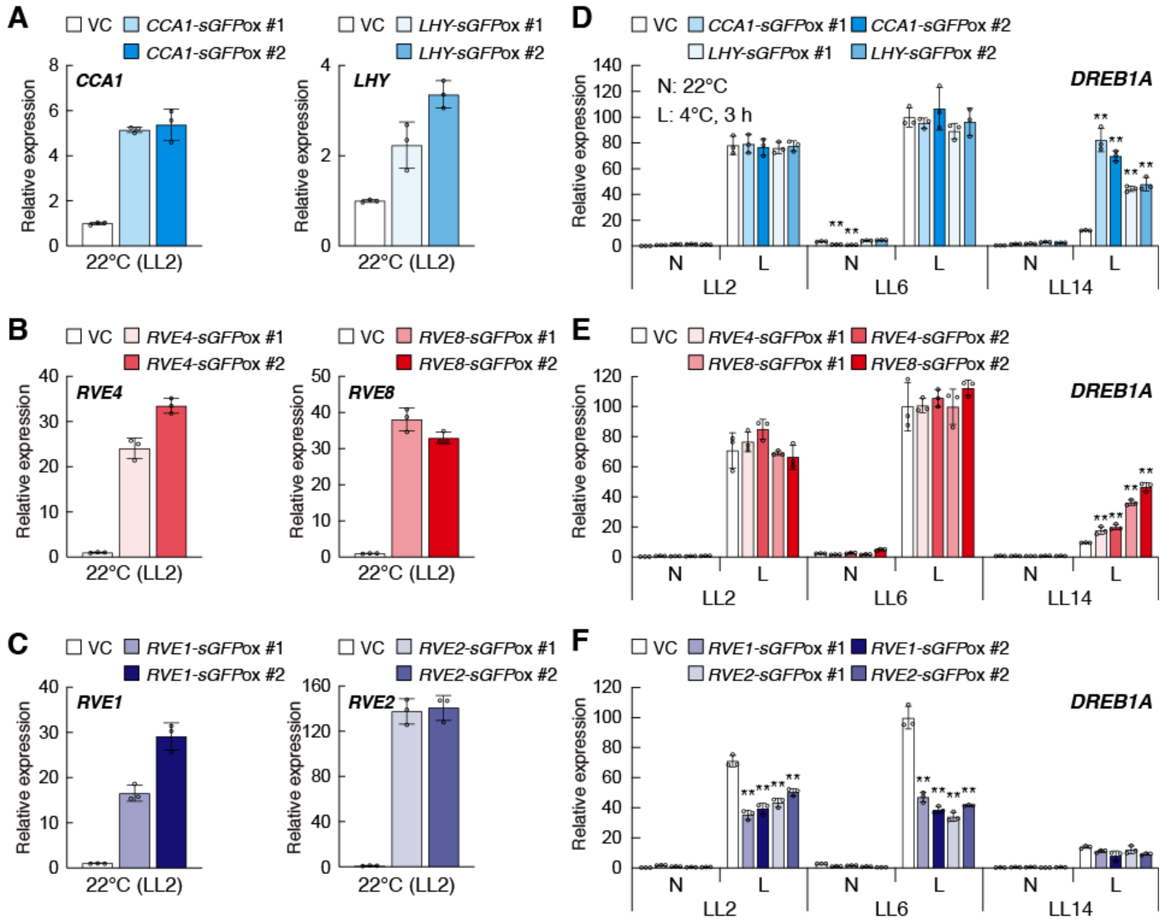

Fig. S4. Cold-inducible *DREB1A* expression in the *CCA1, LHY* and *RVEs* overexpression plants. (A-C) Expression levels of each transgene in transgenic *Arabidopsis* overexpressing the *GFP* fusion genes of *CCA1, LHY* and *RVEs*. (D-F) Cold-inducible *DREB1A* expression in the overexpression plants. The *GFP* fusion genes of *CCA1/LHY* (A, D), *RVE4/RVE8* (B, E) and *RVE1/RVE2* (C, F) were overexpressed using the CaMV *35S* promoter in transgenic *Arabidopsis*. Two-week-old seedlings grown on agar plates were gradually chilled at 4°C for 3 h beginning 2 h (LL2), 6 h (LL6) and 14 h (LL14) after dawn. Gene expression before (N) and after cold stress (L) was measured. The asterisks indicate significant differences (**P<0.01 as analyzed by one-way ANOVA followed by a Tukey's post hoc test) compared with the expression levels in the VC plants at each time point.

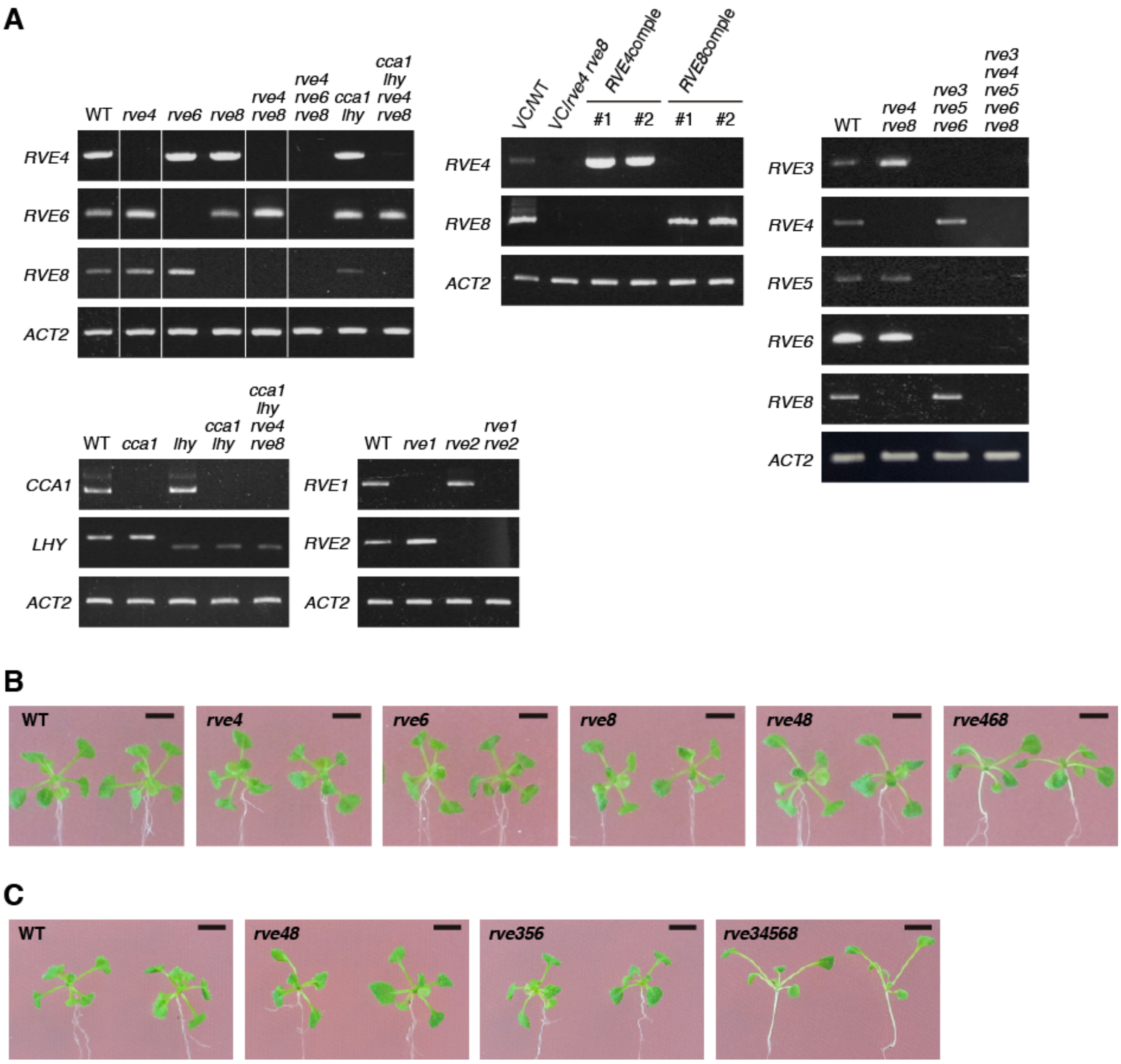

Fig. S5. Generation of multiple mutant plants of *CCA1, LHY* and *RVEs.* (A) Presence or absence of each transcript in each mutant plant was determined by derived cleaved amplified polymorphic sequences (dCAPS) assay for the *LHY* genes and RT-PCR for the *CCA1* and *RVE* genes. (B, C) Plant growth of the mutant plants of *RVE* genes used in Fig. 2A (B) and 2C (C). Sixteen-day-old seedlings grown on agar medium are shown. Black bars indicate 5 mm.

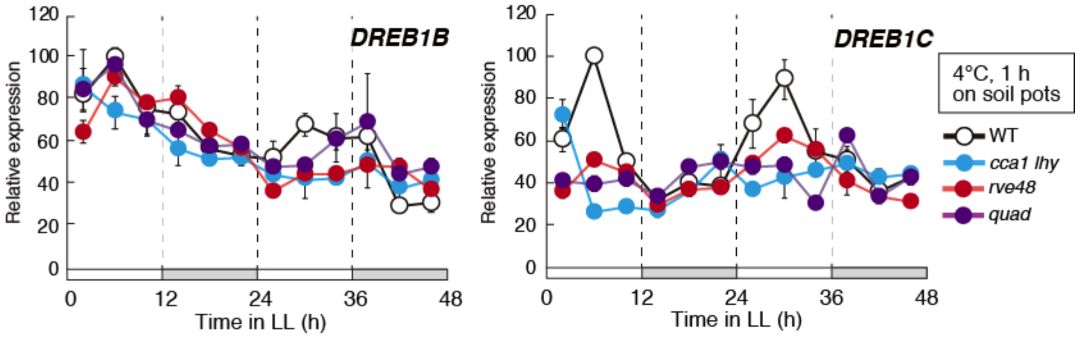

Fig. S6. Effects of the circadian clock on the expression of the *DREB1B* and *DREB1C* genes in response to the rapid temperature decrease. Two-week-old seedlings grown in soil pots under a 12-h light/12-h dark cycle were transferred to free-running conditions under continuous light from dawn. Cold treatments were initiated every 4 h from 2 h to 46 h after the beginning of the free-running conditions. At each time point, the seedlings were rapidly cooled at 4°C for 1 h. The transcript levels of each gene in the plants treated at 4°C were measured using the same samples as in Fig. 2G.

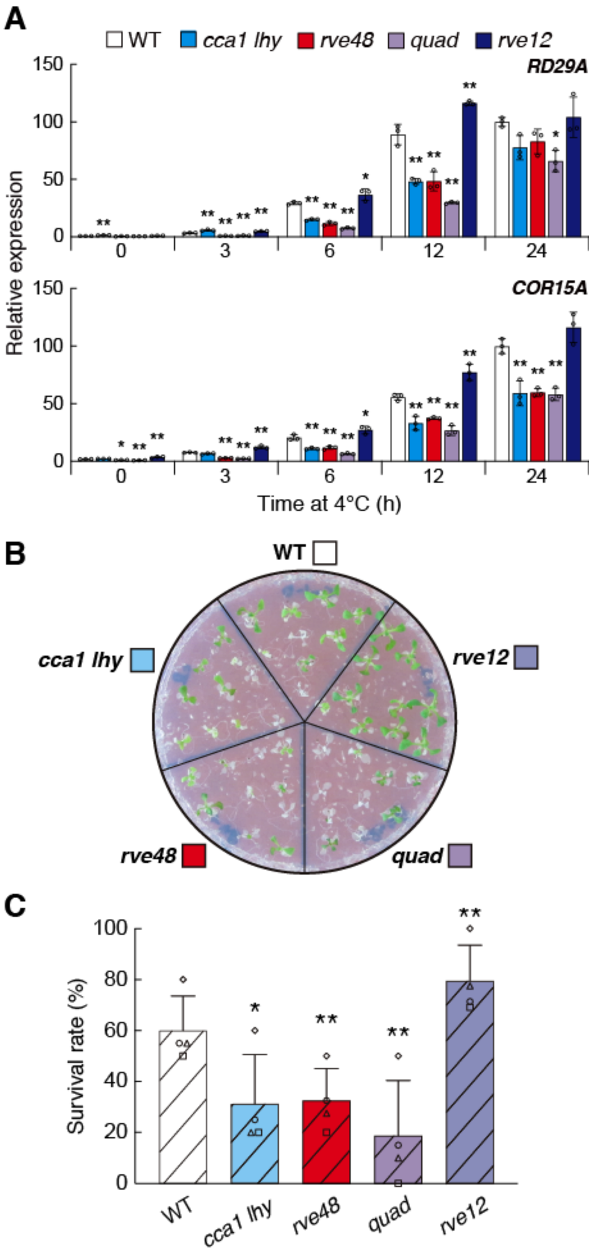

Fig. S7. Freezing tolerance of the multiple mutant plants of *CCA1, LHY* and *RVEs*. (A) Cold-inducible expression of genes downstream of DREB1 in the mutant plants of *CCA1, LHY* and *RVEs*. The asterisks indicate significant differences (**P*<0.05, ***P*<0.01 as analyzed by one-way ANOVA followed by a Tukey's post hoc test) compared with the WT plants at each time point. (B, C) Freezing tolerance test. Non-acclimated seedlings were treated at −9°C for 0.5 h. Representative images (B) and survival rates (C) of the plants after recovery are shown. The symbols in the graph indicate the survival rate in four independent experiments. The asterisks indicate significant differences (**P*<0.05, ***P*<0.01 as analyzed by one-way repeated measures ANOVA followed by a Tukey's post hoc test) compared with the WT plants at each time point.

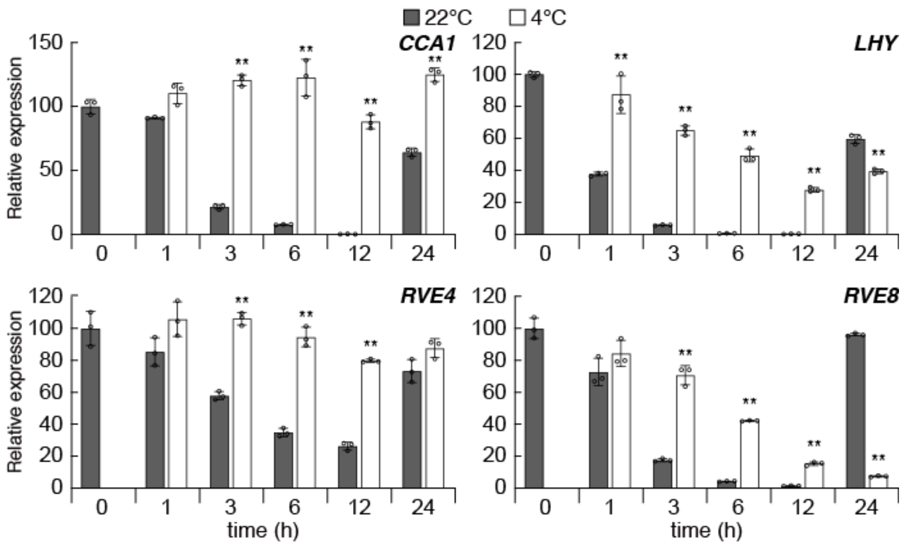

Fig. S8. Expression of *CCA1, LHY* and *RVEs* under cold stress conditions. Two-week-old seedlings grown in agar plates under a 12-h light/12-h dark cycle were incubated at 22°C or 4°C under continuous light from LL2. The bars refer to the means ± standard deviation of three biological replicates. The asterisks indicate significant differences (***P* < 0.01 according to Student's *t*-test) compared with the expression in the WT plants at 22°C.

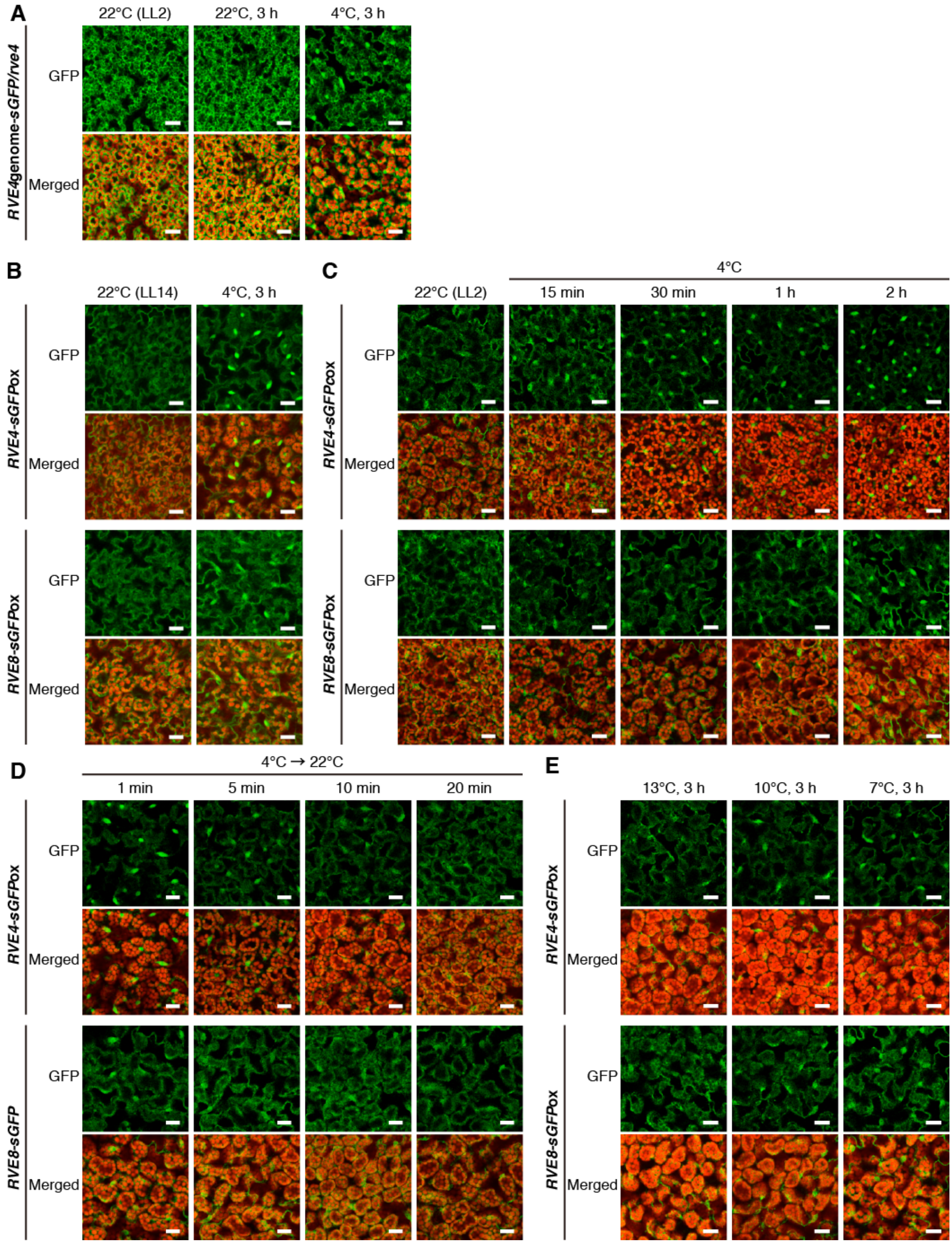

Fig. S9. Subcellular localization of RVE4 and RVE8 in transgenic *Arabidopsis* plants. (A) Subcellular localization of RVE4-sGFP driven by its own promoter in transgenic *Arabidopsis* plants. (B, C) Subcellular localization of RVE4-sGFP and RVE8-sGFP under cold stress conditions from 14 h (B, LL14) and 2 h (C, LL2) after dawn. (D) Subcellular localization of RVE4-sGFP and RVE8-sGFP under the recovery treatment from 4°C to 22°C. (E) Subcellular localization of RVE4-sGFP and RVE8-sGFP in response to various temperature decreases. Two-week-old seedlings overexpressing *RVE4-sGFP* and *RVE8-sGFP* driven by the CaMV *35S* promoter were used for GFP observation. Bars indicate 20 μm.

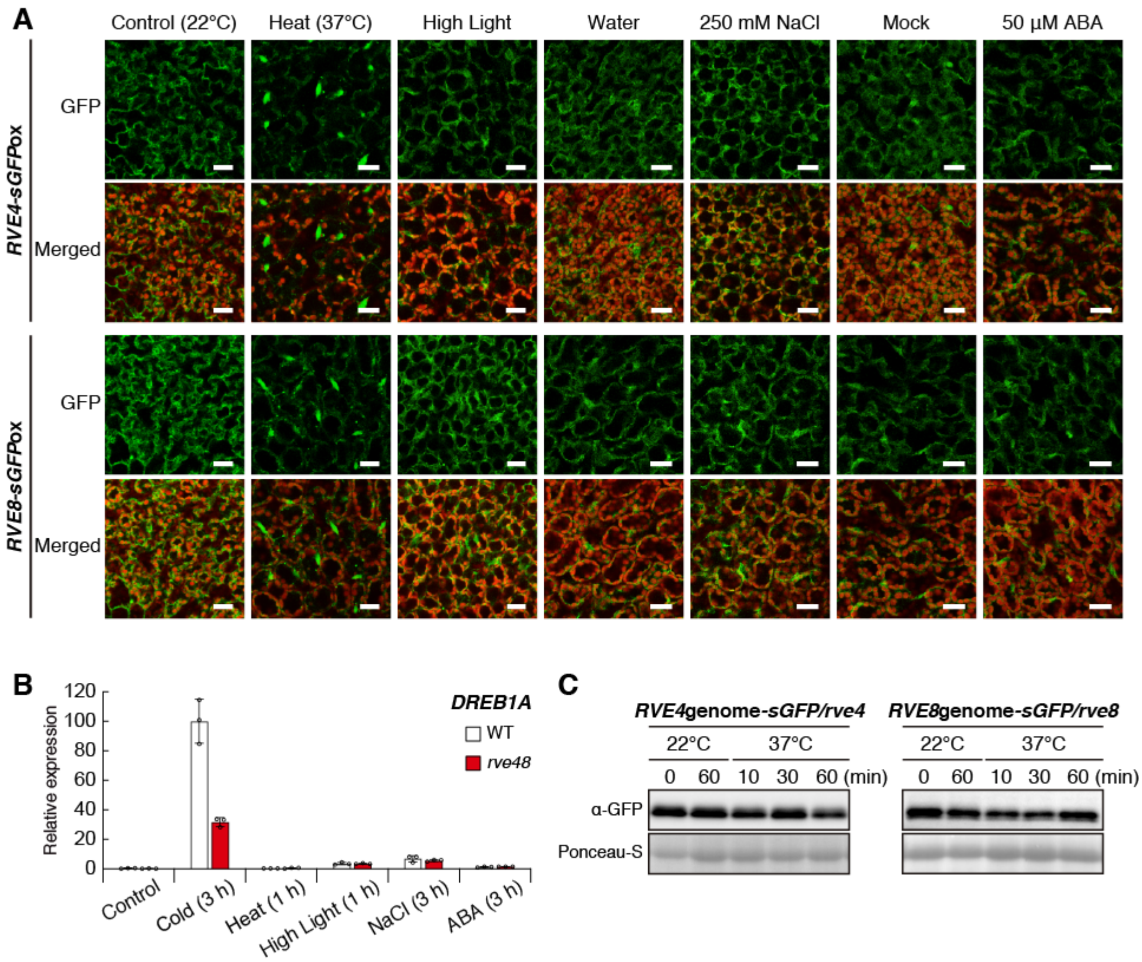

Fig. S10. Subcellular localization of RVE4-sGFP and RVE8-sGFP and expression of *DREB1A* under various stress treatments. (A) Subcellular localization of RVE4-sGFP and RVE8-sGFP under various stress treatments, such as heat stress (37°C), high light stress (1000 µE), high salinity stress (250 mM NaCl) and ABA (50 µM) treatment. Bars indicate 20 µm. (B) Expression levels of *DREB1A* under various stress treatments. The bars refer to the means ± standard deviation of triplicates. Two-week-old seedlings overexpressing *RVE4-sGFP* and *RVE8-sGFP* driven by the CaMV *35S* promoter were used for GFP observation. (C) Immunoblot analyses using transgenic *Arabidopsis* plants expressing *RVE4-sGFP* and *RVE8-sGFP* driven by their own promoters. Two-week-old seedlings grown on agar plates were warmed at 37°C up to 1 h from LL2.

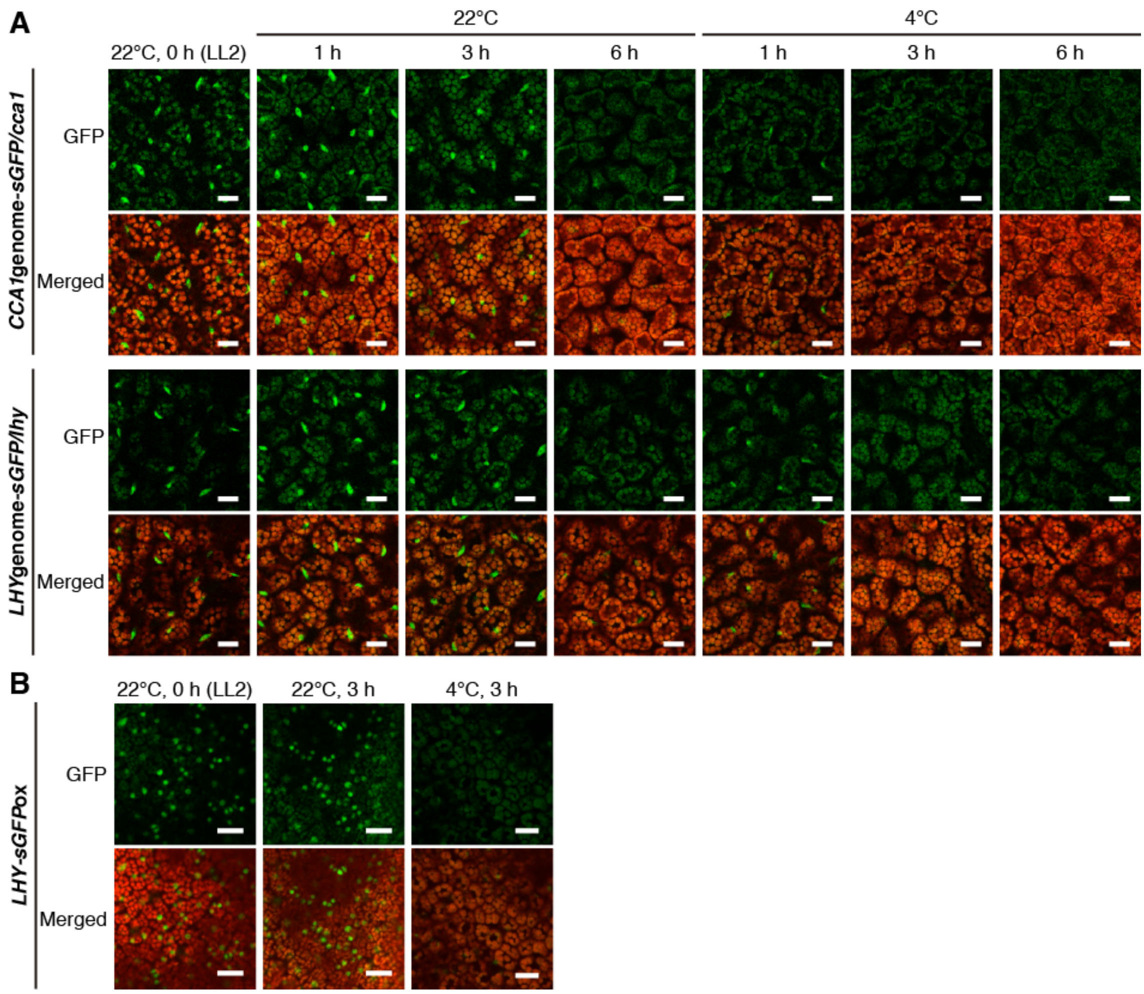

Fig. S11. GFP fluorescence of CCA1-sGFP and LHY-sGFP in response to cold stress. (A) GFP fluorescence of CCA1-sGFP and LHY-sGFP driven by their own promoters in transgenic *Arabidopsis*. Two-week-old seedlings overexpressing the *GFP* fusion genes of the genomic fragments, including the promoter regions of *CCA1* and *LHY,* were used for GFP observation. (B) GFP fluorescence of LHY-sGFP in transgenic *Arabidopsis* plants. Two-week-old seedlings overexpressing *LHY-sGFP* driven by the CaMV *35S* promoter in Fig. S4 were used for GFP observation. Bars indicate 20 µm.

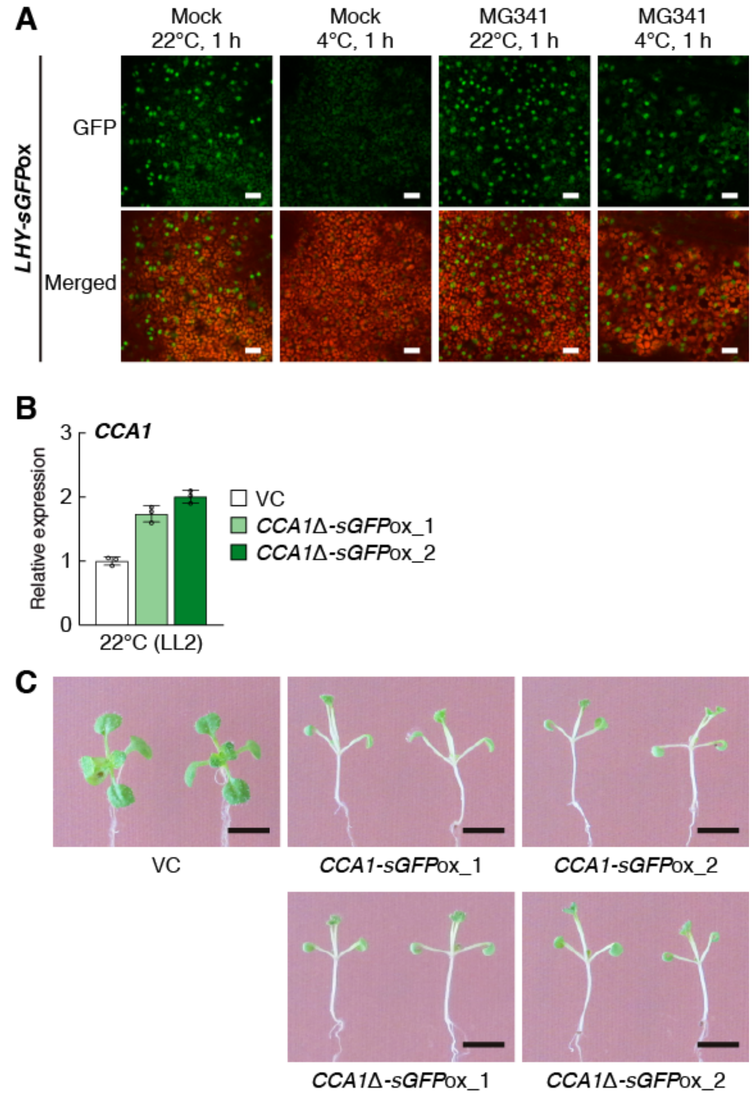

Fig. S12. Regulation of CCA1 and LHY stability. (A) Effect of a 26S proteasome inhibitor on the cold response of LHY-sGFP. Two-week-old seedlings overexpressing *LHY-sGFP* driven by the CaMV *35S* promoter were used for GFP observation. White bars indicate 20 µm. (B) Expression levels of the transgene in transgenic *Arabidopsis* overexpressing the s*GFP* fusion gene of *CCA1Δ*. (C) Plant growth of the transgenic plants overexpressing the s*GFP* fusion gene of *CCA1* and *CCA1Δ*. Ten-day-old seedlings grown on agar medium are shown. The *CCA1-sGFP*-overexpressing plants are the same lines as shown in Fig. S4. Black bars indicate 5 mm.

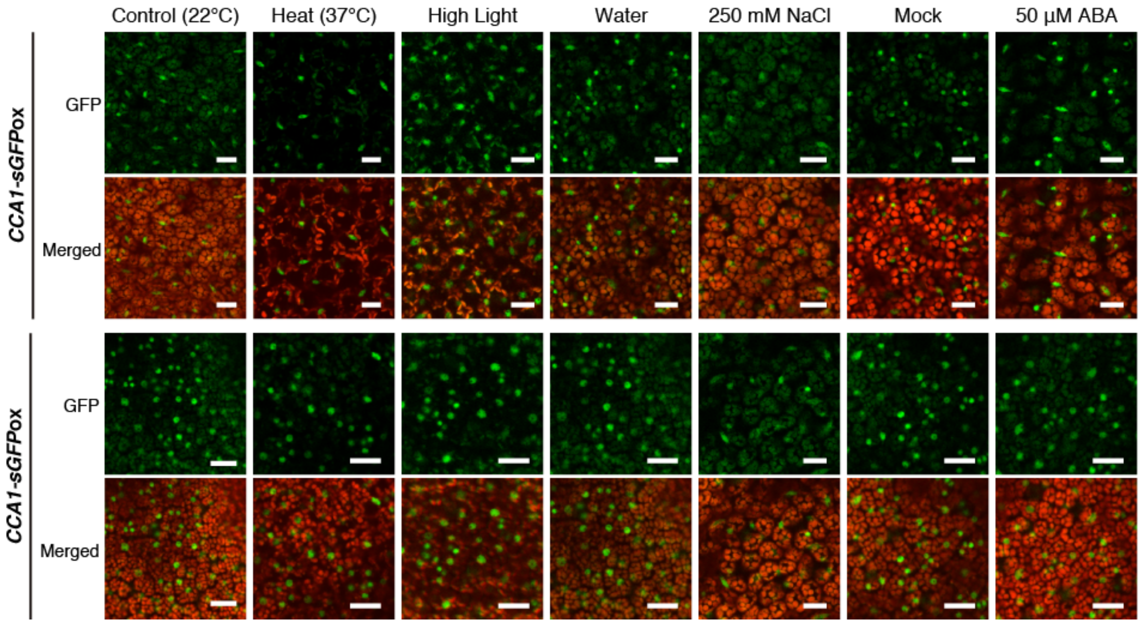

Fig. S13. Subcellular localization of CCA1-sGFP and LHY-sGFP under various stress treatments, such as heat stress (37°C), high light (1000 μE), high salinity stress (250 mM NaCl) and ABA (50 μM) treatment. Two-week-old seedlings overexpressing *CCA1-sGFP* and *LHY-sGFP* driven by the CaMV *35S* promoter were used for GFP observation. Bars indicate 20 μm.

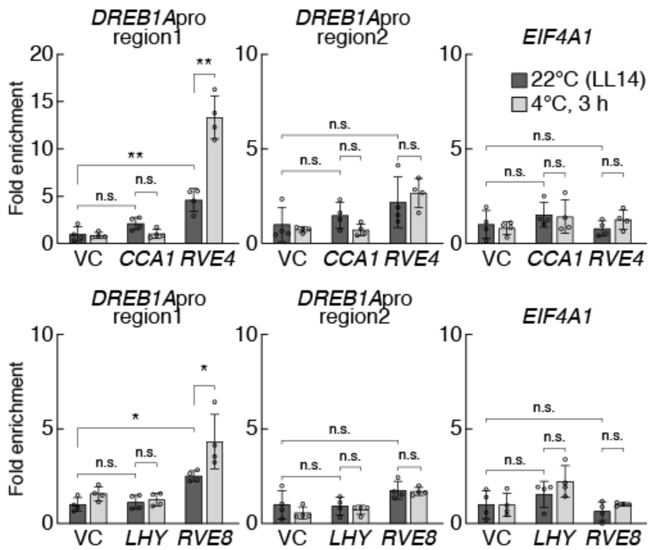

Fig. S14 ChIP assays of the CCA1, LHY and RVE proteins under the cold stress at subjective night. Enrichment of two regions around EEs in the *DREB1A* promoter (1 and 2; Fig. 6A) was measured using ChIP-qPCR analyses. The transgenic *Arabidopsis* plants used in Fig. 4A and Fig. 5A were analyzed. The cold stress treatment was started 14 h after dawn (LL14). The bars refer to the means ± standard deviation in four replicates. The asterisks and "n.s." indicate significant (**P*<0.05, ***P*<0.01) and no differences (*P*>0.05), respectively, as analyzed by one-way ANOVA followed by a Tukey's post hoc test) between two samples.

Table S1. Number of the differential expression genes in the cold stress response and in the mutants.

# DEG	Cold (WT)	
	3 h	12 h
UP	557	1503
DOWN	463	3133

# DEG	*cca1 lhy*			*rve48*			*quad*		
	0 h	3 h	12 h	0 h	3 h	12 h	0 h	3 h	12 h
UP	791	877	1005	92	282	480	429	760	765
DOWN	1273	1206	1246	758	693	653	529	955	884

Table S2. Oligomers used in this study.

Name	Sequence (5'-3')
RT-qPCR	
DREB1A_qRT_F	CGCTGACTCGGCTTGGA
DREB1A_qRT_R	GCATCACACATCTCATCCTGAAAC
DREB1B_qRT_F	AGTCAACATGCGCCAAGGAT
DREB1B_qRT_R	ATGTCCAGGCCATGATTCG
DREB1C_qRT_F	TGACGTGTCCTTATGGAGCTA
DREB1C_qRT_R	CTGCACTCAAAAACATTTGCA
RD29A_qRT_F	CTTGATGGTCAACGGAAGGT
RD29A_qRT_R	CAATCTCCGGTACTCCTCCA
COR15A_qRT_F	GTCAGAGTCGGCCAGAAAACTC
COR15A_qRT_R	AACAACGTAGTCTTTCGCTTTCTCA
CCA1_qRT_F	GGTGGACTGAGGAAGAAC
CCA1_qRT_R	GGAGAAAAATTTCTGAGCGTGAC
LHY_qRT_F	GAAGTCTCCGAAGAGGGTCG
LHY_qRT_R	TATTCACATTCTCTGCCACTTGAG
RVE4_qRT_F	GGCGGAAACTTCTACAGATGC
RVE4_qRT_R	AGCTTTCCTCACCTTCTTCTCC
RVE8_qRT_F	TCGTGGAGCAGAAGCTGATA
RVE8_qRT_R	TGGAGGCTGTTTAGCCTTTCTTAC
ELUC_qRT_F	AGGATCATCATACTGGACTCTGAA
ELUC_qRT_R	GAAGTTGTGCAGGCTCTCG
IPP2_qRT_F	GTATGAGTTGCTTCTCCAGCAAAG
IPP2_qRT_R	GAGGATGGCTGCAACAAGTGT
Construction	
CCA1pro_F_ApaI	ATTGGGCCCATGCATGGTTAGCTTAG
CCA1_F+1_EcoRV	GGGATATCCATGGAGACAAATTCG
CCA1_R-3_EcoRV	GGGATATCTGTGGAAGCTTGAGTTTC
CCA1_1014R_EcoRV	GGGATATCACCACCTGAACTAAG
CCA1_1327_inverse_F	AGGCAAGAGGATGGCACCAATG
CCA1_1134_inverse_R	CTTTGATGCCTCGGAGTGTTC
LHYpro_F_ApaI	ATTGGGCCCGGTTATTTCAATTAGATTC
LHY_F+1_SmaI	TCCCCCGGGCATGGATACTAATACATC
LHY_R-3_SmaI	TCCCCCGGGTGTAGAAGCTTCTCCTTC
RVE1_F+1_SmaI	TCCCCCGGGCATGGCGTCGTCTCCG
RVE1_R-3_SmaI	TCCCCCGGGTAAGTGGAGATGAATCTC
RVE2_F+1_SmaI	TCCCCCGGGGATGGCTATGCAGGAAC
RVE2_R-3_SmaI	TCCCCCGGGCCACAAAGGATATGA
RVE4pro_F_ApaI	ATTGGGCCCTTTCAACACAACTCTTTAC
RVE4_F+1_SmaI	TCCCCCGGGGATGACCTCAACCAA
RVE4_R-3_SmaI	TCCCCCGGGAGAGCTTAAGTGTTC
RVE8pro_F_ApaI	ATTGGGCCCTTGTTGTATTTGTTTCG

RVE8_F+1_SmaI	TCCCCCGGGGATGAGCTCGTCGCCG
RVE8_R-3_SmaI	TCCCCCGGGTATGATAAGAGGAC
SV40NLS_XbaI_S	CTAGAATGACTAGTCCTAAGAAGAAGAGGAAGGTTGGAACTAGTT
SV40NLS_XbaI_AS	CTAGAACTAGTTCCAACCTTCCTCTTCTTCTTAGGACTAGTCATT
1AR_F_XbaI	GCTCTAGAGCTTCGCTGTGTATAG
1AR_R_XbaI	GCTCTAGAAATGGCGGAAGATATTTTAG
1AR_mCA/GA_inverse_F	TTTTTTAAACTCCGTCTTCGCCTTTTC
1AR_mCA/GA_inverse_R	AAAGTAATGCCACGTAAACTATACAC
1AR_mEE_R_XbaI	GCTCTAGAAATGGCGGAAAAAAAAAAAAGAGG

Genotyping

LBb1.3_SALK	ATTTTGCCGATTTCGGAAC
LB1_SAIL	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
rve1_LP	AAGTGGAGATGAATCTCATGCTC
rve1_RP	CAAAGACCGCAGTTCAGATTC
rve2_LP	CAGATGCCTTTTACCTCAAGG
rve2_RP	ACGAACACTTCACCACAAAGG
rve3_LP	TGGCTTTCCACAACTTTCTTG
rve3_RP	TGTTAGTCCCACGCTCTGAAC
rve4_LP	AAATCAAACAGGCACAGGATG
rve4_RP	TTTTTACAACTTCCACACCGG
rve5_LP	TTTTGGCAGATTTGTCGATTC
rve5_RP	TCCACGCGGACATCTTTATAG
rve6_LP	GTGAAGAACGAAACAGGCAAG
rve6_RP	GGAGGGGAGTGAAGCTAATTG
rve8_LP	TTCAGCAAAATCAGGAACACC
rve8_RP	AGAGCTGGACAGAGGAAGAGC

RT-PCR

CCA1_RT_F	ATGGAGACAAATTCGTCTGGAG
CCA1_RT_R	CTTTGATGCCTCGGAGTGTTC
RVE1_RT_F	ATGGCGTCGTCTCCGTTGACTGC
RVE1_RT_R	TTATAAGTGGAGATGAATCTC
RVE2_RT_F	ATGGCTATGCAGGAACG
RVE2_RT_R	TCACCACAAAGGATATGATAA
RVE3_RT_F	ATGGTGACTGTAAACCCTAG
RVE3_RT_R	CTAACTGGCGTTGTAAGATG
RVE4_RT_F	ATGACCTCAACCAATCCG
RVE4_RT_R	CTAAGAGCTTAAGTGTTCATG
RVE5_RT_F	ATGGTGTCCGTAAACCCTAG
RVE5_RT_R	CTATTTCAAAGCTTTAGCGC
RVE6_RT_F	ATGGTCTCTAGAAATTCTG
RVE6_RT_R	TTAAGTAGAGATTTCAGGTGG
RVE8_RT_F	ATGAGCTCGTCGCCGTCAAG
RVE8_RT_R	TCATATGATAAGAGGACTTTC

dCAPs	
lhy_dCAPs_F	CTCCAATCTTATTATGTCAACTCTC
lhy_dCAPs_R_BglII	AGAATTCCCGACACTCGCATAGATC
EMSA	
1AR-C_WT_S	CTAGACTTCGCCTTTTCTTTTGCCTCTAAAATATCTT
1AR-C_WT_AS	CTAGAAGATATTTTAGAGGCAAAAGAAAAGGCGAAGT
1AR-C_mEE_S	CTAGACTTCGCCTTTTCTTTTGCCTCTTTTTTTTTTT
1AR-C_mEE_AS	CTAGAAAAAAAAAAAGAGGCAAAAGAAAAGGCGAAGT
ChIP-qPCR	
DREB1A_ChIP1_F	TCTCGTTGTCGTCTTGCTTCTC
DREB1A_ChIP1_R	GTCTGCCTCGTTACCTACCTCTCA
DREB1A_ChIP2_F	CAAACTCCGTCTTCGCCTTTTC
DREB1A_ChIP2_R	GTTGGAGTGAGAGCATGCTGTTTTA
EIF4A1_ChIP_F	TGTTTTGCTTCGTTTCAAGGA
EIF4A1_ChIP_R	GCATTTTCCCGATTACAAC

Under bars indicate restriction enzyme sites.

Dataset S1 (separate file). List of the changes in gene expression in the cold stress response and mutants.

SI References

1. S. Kidokoro, K. Yoneda, H. Takahashi, F. Takahashi, K. Shinozaki, K. Yamaguchi-Shinozaki, Different cold-signalling pathways function in the responses to rapid and gradual decreases in temperature. *Plant Cell* **29**, 760–774 (2017).