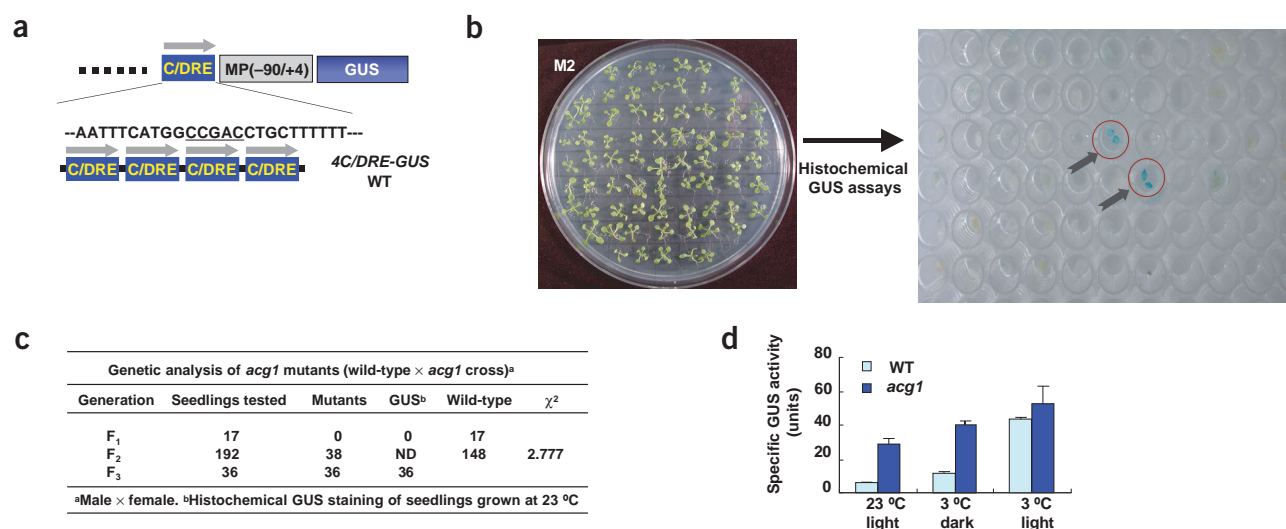


# A genetic link between cold responses and flowering time through *FVE* in *Arabidopsis thaliana*

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Cold induces expression of a number of genes that encode proteins that enhance tolerance to freezing temperatures in plants<sup>1,2</sup>. A *cis*-acting element responsive to cold and drought, the C-repeat/dehydration-responsive element (C/DRE), was identified in the *Arabidopsis thaliana* stress-inducible genes *RD29A*<sup>3</sup> and *COR15a*<sup>4</sup> and found in other cold-inducible genes in various plants<sup>1,5</sup>. C/DRE-binding factor/DRE-binding protein (CBF/DREB) is an essential component of the cold-acclimation response<sup>1,2</sup>, but the signaling pathways and networks are mostly unknown. Here we used targeted genetic approach to isolate *A. thaliana* mutants with altered cold-responsive gene expression (*acg*) and identify *ACG1* as a negative regulator of

the CBF/DREB pathway. *acg1* flowered late and had elevated expression of FLOWERING LOCUS C (*FLC*)<sup>6</sup>, a repressor of flowering encoding a MADS-box protein. We showed that *acg1* is a null allele of the autonomous pathway gene *FVE*. *FVE* encodes a homolog of the mammalian retinoblastoma-associated protein, a component of a histone deacetylase (HDAC) complex involved in transcriptional repression<sup>7,8</sup>. We also showed that plants sense intermittent cold stress through *FVE* and delay flowering with increasing expression of *FLC*. Dual roles of *FVE* in regulating the flowering time and the cold response may have an evolutionary advantage for plants by increasing their survival rates.



**Figure 1** Isolation and genetic analysis of *acg1* mutants with constitutive GUS expression at 23 °C and the late-flowering phenotype. **(a)** Diagram of the chimeric construct used for generating 4C/DRE-GUS transgenic *A. thaliana*. WT, wild-type. **(b)** A typical example of screening that shows putative 4C/DRE-GUS mutants with constitutive GUS expression. **(c)** Genetic analysis of *acg1* mutants by backcrossing with wild-type plants. ND, not determined. **(d)** Fluorometric quantification of GUS activities of *acg1* mutants and wild-type plants (WT). Specific activities (units) are shown as pmol reaction product (4-methylumbelliferone) generated per min per mg of total protein. Data are mean ± standard error of three independent treatments.

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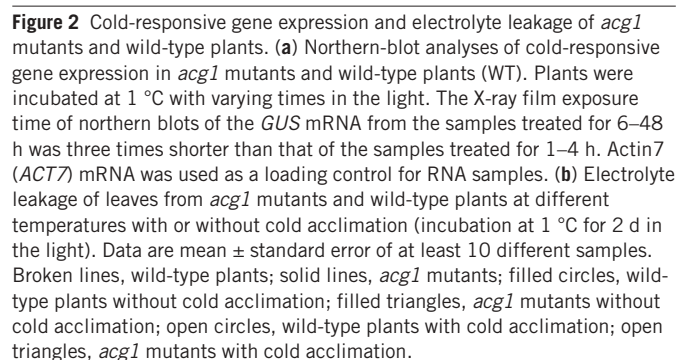


To investigate alteration of cold-responsive gene expression in *acg1* mutants, we examined the time course of *GUS* induction in response to cold in *acg1* and wild-type plants using northern-blot analysis (**Fig. 2a**). The level of *GUS* mRNA was higher in *acg1* mutants than in wild-type plants before cold treatment and began to increase in *acg1* mutants, but not wild-type plants, 4 h after cold treatment. By 6–8 h after cold treatment, the level of *GUS* mRNA was much higher in mutants than in wild-type plants. Longer cold treatment substantially increased *GUS* mRNA levels in mutants and wild-type plants to similar levels.

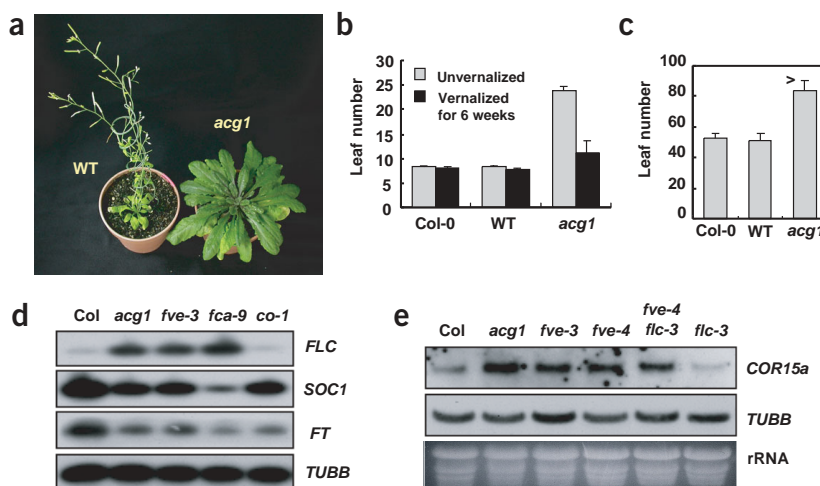
To test whether the mutation affected expression of *COR* genes containing the *C/DRE*, we analyzed induction kinetics of *COR15a* and *COR47* and observed an expression pattern similar to that of *GUS*, although expression of *COR47* was not as robust as that of *COR15a*. We could not detect the transcripts of three *CBF* genes at 23 °C in *acg1*, suggesting that the constitutive expression of *GUS* and *COR* in *acg1* at normal temperatures was not caused by enhanced expression of *CBF*. We observed similarly increased levels of *CBF* transcripts in response to cold in wild-type plants and mutants, but the transient increase in *CBF* expression occurred earlier in *acg1* mutants than in wild-type plants. Thus, enhanced expression of *COR15a* in response to cold may be due, in part, to earlier expression of *CBF*. These results indicate that the *acg1* mutation impairs the repression of the *CBF/DREB* pathway.

To test whether the *acg1* mutation had an effect on freezing tolerance, we conducted an electrolyte leakage test<sup>10</sup> on 2-week-old *acg1* mutant and wild-type plants before and after preincubation at 4 °C for 2 d (**Fig. 2b**). Nonacclimated *acg1* and wild-type plants showed similar tolerance to freezing, but when cold-acclimated, *acg1* mutants had greater freezing tolerance than wild-type plants, in the range of  $-7$  to  $-9$  °C. This difference is statistically significant and may be due to the enhanced upregulation of *COR* genes in response to cold.

*acg1* mutants flowered later than wild-type plants under both long days and short days, and the flowering time of *acg1* mutants was reduced to wild-type level by vernalization treatment (Fig. 3a–c), suggestive of a mutation in the autonomous pathway. In *A. thaliana*,



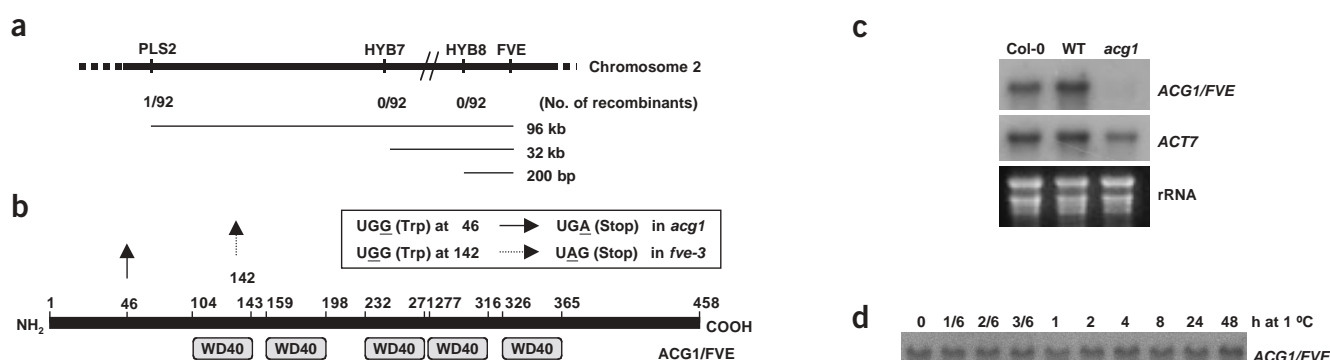
**Figure 3** Analysis of the flowering time and expression of floral pathway genes and *COR15a* in *acg1* and other late-flowering mutants. (a) 6-week-old wild-type (WT; left) and *acg1* (right) plants grown under long-day conditions (16 h light–8 h dark). (b) The rosette leaf numbers of *acg1* mutants and wild-type plants (WT) vernalized for 6 weeks (solid column) or not vernalized (open column) and grown under long-day conditions. Time to flowering was measured as the number of rosette leaves formed on the main shoot. (c) The rosette leaf numbers of *acg1* mutants and wild-type plants (WT) grown under short-day conditions (8 h light–16 h dark). Counting of the rosette leaf number of *acg1* mutants was stopped after growing the plants for 7 months. (d) RT-PCR analysis of expression of *FLC*, *SOC1* and *FT* in Col-0, *acg1* and other late-flowering mutants.  $\beta$ -tubulin (*TUBB*) mRNA was used as a loading control for RNA samples. (e) Northern-blot analysis of *COR15a* expression in *fve-3*, *fve-4*, *fve-4 flc-3* and *flc-3* mutants compared with *acg1* and Col-0 plants.



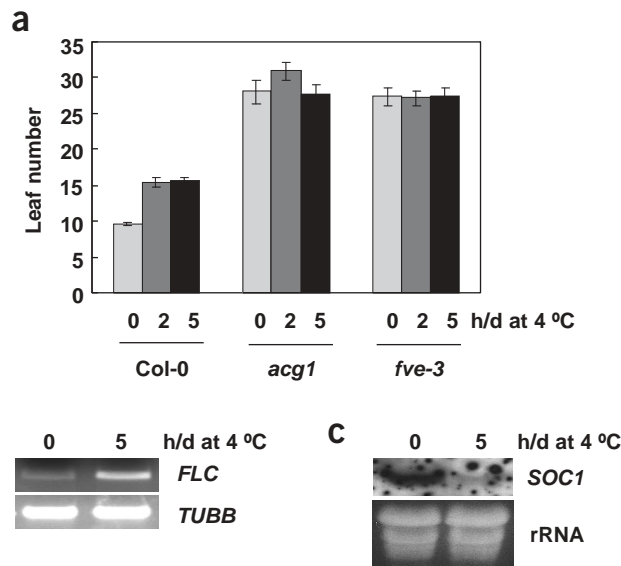
mutations in autonomous pathway genes cause elevated *FLC* expression and late flowering, but vernalization offsets this effect<sup>11</sup>. *FLC* expression was higher in *acg1* mutants than in Columbia-0 (Col-0) plants, similar to the level of expression seen in *fve-3* plants (Fig. 3d). *fca-9* (ref. 12) plants, which flowered later than *acg1* and *fve-3* (ref. 13) plants, had a higher *FLC* level, but *co-1* (ref. 14) plants, which are late-flowering only in long-day photoperiods, had a similar *FLC* level as Col-0. *FLC* negatively regulates the expression of SUPPRESSOR OF OVEREXPRESSION OF CO 1 (*SOC1*), encoding a MADS-box transcription factor, and FLOWERING LOCUS T (*FT*), encoding a protein with similarity to a Raf kinase inhibitor, both of which activate flower meristem identity genes<sup>15</sup>. We observed decreased levels of *SOC1* and *FT* in *acg1* mutants as well as in *fve-3* and *fca-9* plants. These results and complementation tests (Supplementary Fig. 1 online) suggest

that late flowering in *acg1* mutants is due to elevated *FLC* expression and that *acg1* is an allele of *fve*, an autonomous pathway gene<sup>13,16</sup>. Expression of *COR15a* at 23 °C was enhanced in *fve-3*, *fve-4*, *fve-4 flc-3* plants, as in *acg1* mutants, but expression in *flc-3* plants (Fig. 3e) was similar to that seen in Col-0 plants, suggesting that the effect of *FVE* on expression of cold-responsive genes is independent of *FLC* function.

*FVE* was isolated by positional cloning<sup>17</sup>. Linkage analysis confirmed that *acg1* might be *fve*, an allele of *FVE* (Fig. 4a). *acg1* or *fve-3* has a point mutation that converts Trp46 or Trp142, respectively, into a premature stop codon (Fig. 4b). The level of *FVE* mRNA was much lower in *acg1* mutants than in Col-0 and wild-type plants, suggestive of mRNA instability due to a premature termination (Fig. 4c). The protein encoded by *FVE* shows high sequence homology to the mammalian retinoblastoma-associated protein (RbAp; Supplementary Fig. 2 online) and is



**Figure 4** *acg1* is a null allele of *FVE* that encodes a plant homolog of the mammalian RbAp. (a) Linkage analysis of the late-flowering phenotype of *acg1* with *FVE*. Homozygous *acg1* mutants in the segregated  $F_2$  population obtained from crossing *acg1* with the ecotype Landsberg *erecta* were used for mapping. (b) Scheme of domain structures of the protein encoded by *FVE* and positions of nonsense mutations in *acg1* and *fve-3*. Numbers above the thick column denote the amino acid numbers. WD40 indicates WD-40-repeat domain predicted by a Motif Scan program<sup>25</sup>. (c) Northern blot showing the *FVE* mRNA level in Col-0, wild-type (WT) and *acg1* plants. (d) Northern-blot analysis of *FVE* expression in response to cold in Col-0 plants. Plants were incubated at 1 °C for varying periods of time (h). *COR15a* was analyzed for monitoring cold-inducible gene expression.



**Figure 5** Intermittent cold treatment delays the flowering of wild-type plants but not that of *acg1* and *fve* mutants. **(a)** Plants were incubated at 4 °C for 2 h or 5 h every day, and the rosette leaf numbers were measured at bolting. RT-PCR analysis of *FLC* expression **(b)** and northern-blot analysis of *SOC1* expression **(c)** in Col-0 plants subjected to intermittent cold-stress treatment.

predicted to contain five WD-40 repeats, domains that are found in multiprotein complexes involved in numerous cellular processes<sup>18</sup>. In mammals, the retinoblastoma (Rb) protein represses transcription by recruiting HDAC involved in repressing gene expression by deacetylation of histone residues<sup>7</sup>. RbAp interacts with HDAC associated with Rb and has a role in transcriptional repression<sup>8</sup>. These mechanisms seem to be conserved in plants, although the biological roles of the homologous proteins are not known<sup>19,20</sup>. A loss-of-function mutation in *FVE* could release the transcriptional repression mediated by HDAC, resulting in aberrant expression of *GUS* and *COR* at normal temperatures. This alteration of *COR* expression could affect a negative feedback loop of *CBF* expression, shifting it to an earlier time. Release of the transcriptional repression and earlier *CBF* expression could enhance the response of *COR* expression to cold in *acg1* mutants (Fig. 2a). *FVE* mRNA was not affected by cold (Fig. 4d).

Because the same mutation caused both enhanced response of cold-induced gene expression and late flowering, we tested whether cold stress affected flowering time by treating plants at 4 °C for 2 h or 5 h every day during the entire growth period. Intermittent cold treatment substantially delayed flowering in wild-type plants but had no effect on *acg1* and *fve* mutants (Fig. 5a). Moreover, intermittent cold treatment increased the level of *FLC* (Fig. 5b) and thereby decreased *SOC1* expression (Fig. 5c). Genetic and expression analyses of various flowering-time mutants have indicated that a low ambient growth temperature (such as 16 °C) is sensed through a genetic pathway requiring both *FCA* and *FVE*<sup>21</sup>. Our results suggest that plants sense intermittent cold stress through *FVE* and delay flowering time by increasing the level of *FLC*. Consistent with a proposed role of *FVE* as a component of HDAC, the *fve* mutant had an increase in acetylation of *FLC*, as shown by chromatin immunoprecipitation analysis of the acetylation state of histone H4 at *FLC*<sup>22</sup>. Dual roles of *FVE* in regulating the flowering time and the stress response to cold may provide evolutionary fitness to plants, particularly in enduring the cold spell of early spring.

## METHODS

**Plant materials and growth conditions.** We used the ecotype Columbia-0 for all experiments. We obtained the late-flowering mutants *fve-3*, *fca-9*, *ld-1* and *co-1* from Arabidopsis Biological Resource Center and *fld-3*, *fve-4*, *fve-4 flc-3* and *flc-3* seeds from R. Amasino (University of Wisconsin, Madison). We grew plants for 10 d on agar medium under long-day conditions (16 h light–8 h dark) at 23 °C and used them for cold treatment at 1–4 °C or for expression analysis of floral pathway genes. For flowering time analysis, we grew plants on soil under long-day (16 h light–8 h dark) or short-day conditions (8 h light–16 h dark). For analysis of the effect of intermittent cold stress on the flowering time, we incubated plants on soil every day at 4 °C for 0, 2 or 5 h, starting 4 h after dawn.

**Mutagenesis, mutant isolation and genetic analysis.** We mutagenized *4C/DRE-GUS* seeds (approximately 20,000 seeds) with ethylmethane sulfonate and harvested M2 seeds. We planted M2 seeds individually in 150-mm × 20-mm plates (69 seedlings per plate) containing agar medium and grew M2 seedlings for 10 d under long-day conditions. We excised two leaves from each seedling and then carried out histochemical GUS assays in the 96-well plates. We then transferred putative mutants with GUS staining in the leaves to soil to set seeds (M3). We carried out GUS assays for 5–10 seedlings of M3 to isolate mutants that inherited GUS expression. We backcrossed *acg1* mutants with wild-type plants and tested the resulting F<sub>1</sub> seeds for both the late-flowering phenotype and GUS expression. We scored the F<sub>2</sub> populations obtained from self-pollinated F<sub>1</sub> plants for segregation with the late-flowering phenotype. We tested the late-flowering F<sub>3</sub> populations for GUS expression by the histochemical method. For genetic mapping of the *acg1* mutation, we crossed *acg1* with the ecotype Landsberg *erecta*. We selected homozygous *acg1* mutants in the segregated F<sub>2</sub> population based on the late-flowering phenotype. We mapped the mutation using SSLP markers HYB7, HYB8 and PLS2. Primer sequences are available on request.

**Analysis of GUS expression.** We carried out histochemical assays of GUS activity by incubating the treated seedlings in 5-bromo-4-chloro-3-indolyl glucuronide (DUCHEFA) at 37 °C for 24 h and removing the chlorophyll from green tissues by incubation in 100% ethanol, as described<sup>23</sup>. We carried out quantitative GUS assays in tissue extracts by fluorometric measurement of 4-methylumbelliferone produced from the β-D-glucuronide precursor<sup>23</sup>.

**Analysis of gene expression.** We extracted total RNA using TRI Reagent (Molecular Research Center) and carried out northern-blot analysis as previously described<sup>9</sup>. We analyzed expression of the genes *FLC*, *SOC1*, *FT* and *TUBB* (encoding β-tubulin) by RT-PCR as described<sup>24</sup>. DNA probes used for northern-blot analysis and primer sequences and conditions for amplification are available on request.

**Electrolyte leakage test.** We determined freezing-induced electrolyte leakage from fully developed rosette leaves from 2-week-old plants grown in soil as described<sup>10</sup>. We placed one excised leaflet in a 5-ml test tube containing 100 μl of deionized water in a refrigerated circulator bath (PolyScience) at 0 °C and programmed the temperature of the bath to decrease to –9 °C in 1-°C increments over 30 min. We used at least 10 samples for each temperature.

**Sequencing of DNA from mutants.** We extracted genomic DNA of mutants using DNeasy plant mini-kit (Qiagen) and amplified the *FVE* genomic region by PCR using two sets of specific primers, HYB15F and MJH6-2, and HYB6 and HYB15R. Primer sequences are available on request. We sequenced six independent PCR products for each amplified DNA fragment.

*Note: Supplementary information is available on the Nature Genetics website.*

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## COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.



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