FRIGIDA-ESSENTIAL 1 interacts genetically with *FRIGIDA* and *FRIGIDA-LIKE 1* to promote the winter-annual habit of *Arabidopsis thaliana*

Robert J. Schmitz[1], Lewis Hong[2], Scott Michaels[2,*] and Richard M. Amasino[1,2,†]

[1]Laboratory of Genetics, University of Wisconsin, Madison, WI 53706, USA
[2]Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA
*Present address: Department of Biology, Indiana University, Bloomington, IN 47405, USA
†Author for correspondence (e-mail: amasino@biochem.wisc.edu)

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Summary

Studies of natural variation have revealed that the winter-annual habit of many accessions of *Arabidopsis* is conferred by two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), whose activities impose a vernalization requirement. To better understand the mechanism underlying the winter-annual habit, a genetic screen was performed to identify mutants that suppress the late-flowering behavior of a non-vernalized winter-annual strain. We have identified a locus, *FRIGIDA-ESSENTIAL 1* (*FES1*), which, like *FRI*, is specifically required for the upregulation of *FLC* expression. *FES1* is predicted to encode a protein with a CCCH zinc finger, but the predicted sequence does not otherwise share significant similarity with other known proteins. *fes1* is a complete suppressor of *FRI*-mediated delayed flowering, but has little effect on the late-flowering phenotype of autonomous-pathway mutants. Thus, *FES1* activity is required for the *FRI*-mediated winter-annual habit, but not for the similar phenotype resulting from autonomous-pathway mutations. Epistasis analysis between *FES1*, *FRI* and another specific suppressor of *FRI*-containing lines, *FRIGIDA-LIKE 1* (*FRL1*), indicates that these genes do not function in a linear pathway, but instead act cooperatively to promote the expression of *FLC*.

Key words: Winter annuals, *FLOWERING LOCUS C* (*FLC*), *FRIGIDA* (*FRI*), Flowering time, CCCH zinc finger

Introduction

The transition from vegetative to reproductive development is a highly regulated event in the plant life cycle, involving the perception and integration of a variety of endogenous signals and environmental cues. The ability to coordinate these cues permits plants to flower during times that maximize reproductive success. Summer-annual accessions of *Arabidopsis thaliana* complete the life cycle in a single growing season; when grown in the laboratory under long day-length conditions that are inductive for flowering, such accessions are often rapid cycling. By contrast, winter-annual accessions of *Arabidopsis* typically begin vegetative growth in the fall and transition to flowering the following spring. A crucial feature of the winter-annual habit is a vernalization requirement for rapid spring flowering. Vernalization is the acquisition of competence to flower that is achieved by long periods of cold exposure. A vernalization requirement prevents untimely flowering in the fall before the onset of winter, and also permits rapid flowering in the spring after the cold of winter has satisfied the vernalization requirement. Thus, when winter-annual accessions of *Arabidopsis* are grown in the laboratory without vernalization, they often exhibit an extreme late-flowering habit even when grown under inductive long days.

One of the first examples of the use of natural variation to explore the genetic basis of the differences in *Arabidopsis* life-history traits was the study of the flowering habit in winter-versus summer-annual accessions. Napp-Zinn first identified *FRIGIDA* (*FRI*), as a locus that plays a major role in conferring a vernalization requirement upon certain winter-annual accessions (Napp-Zinn, 1979). Later studies indicated that a second locus, *FLOWERING LOCUS C* (*FLC*), is required for *FRI* to confer the vernalization requirement (Koornneef et al., 1994; Lee et al., 1994b). *FRI* encodes a plant-specific protein (Johanson et al., 2000) that elevates *FLC* expression to a level that effectively represses flowering (Michaels and Amasino, 1999). *FLC* encodes a MADS-box transcriptional regulator that is a potent floral repressor, and vernalization promotes flowering by repressing *FLC* expression (Michaels and Amasino, 1999; Sheldon et al., 1999). Many naturally occurring rapid-cycling accessions have weak or non-functional alleles of *FRI* (Gazzani et al., 2003; Hagenblad and Nordborg, 2002; Johanson et al., 2000; Le Corre et al., 2002; Werner et al., 2005) and/or *FLC* (Michaels et al., 2003; Werner et al., 2005); without *FRI* or *FLC* activity these accessions no longer require vernalization for rapid flowering.

FLC inhibits flowering, at least in part, by repressing the expression of a set of floral promotion genes, including

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FLOWERING LOCUS T (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*; AGL20 – The Arabidopsis Information Resource) (Borner et al., 2000; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Samach et al., 2000). *FT* and *SOC1* are promoters of flowering, and are often referred to as floral integrators because their expression is also positively regulated by other flowering pathways, such as the photoperiod pathway (Borner et al., 2000; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Samach et al., 2000). Thus in a typical winter-annual life cycle, elevated *FLC* expression represses the expression of the floral integrators in the fall season. Exposure to prolonged cold during the winter represses *FLC* and alleviates the repression of the floral integrators, permitting rapid flowering in response to the lengthening days of the spring.

Genetic screens in rapid-cycling accessions have identified a class of mutants, known as the autonomous-pathway mutants, that have a late-flowering phenotype similar to that of *FRI*-containing winter annuals. Autonomous-pathway mutants display elevated levels of *FLC*; therefore, the autonomous-pathway components are negative regulators of *FLC* (Michaels and Amasino, 1999; Michaels and Amasino, 2001; Sheldon et al., 1999). Lesions in *flc* completely suppress the effects of the autonomous-pathway mutants, suggesting that the products of autonomous-pathway genes affect flowering solely by the downregulation of *FLC* (Michaels and Amasino, 2001). *FRI* activity is epistatic to the autonomous pathway and acts by promoting *FLC* expression to levels that are sufficient to block the floral transition. The delayed flowering and high levels of *FLC* observed in *FRI*-containing lines or in autonomous-pathway mutants are both eliminated by vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999).

In many species, the vernalized state is stably maintained throughout cell divisions in the absence of continued cold exposure (Lang, 1965); this mitotic stability is a hallmark of epigenetic regulation. Screens for mutants that are insensitive to vernalization have revealed aspects of how vernalization occurs at a molecular level. Such screens have identified genes, such as *VERNALIZATION INSENSITIVE 3* (*VIN3*) and *VERNALIZATION 2* (*VRN2*), that encode proteins that are likely to be present in chromatin-remodeling complexes (Sung and Amasino, 2004). Vernalization transiently induces the expression of *VIN3*, which facilitates histone modifications at the *FLC* locus, resulting in a silent chromatin state (Bastow et al., 2004; Sung and Amasino, 2004). This repression is maintained throughout the remainder of the life cycle after vernalization by the involvement of VERNALIZATION 1 (VRN1), a DNA-binding protein, and VRN2, a homolog of the *Drosophila* Suppressor of Zeste 12, which is part of the Enhancer of Zeste transcriptional repressor complex (Gendall et al., 2001; Levy et al., 2002).

Two classes of gene have recently been identified that are required for the elevated expression of *FLC* and that are therefore necessary to establish the vernalization-requiring, winter-annual habit in *Arabidopsis*. One class is required in all situations in which *FLC* expression is elevated (i.e. in both *FRI*-containing lines and in autonomous-pathway mutants); the other class, which is a subset of the first class, is required for *FRI* to elevate *FLC* expression, but not for elevated *FLC* expression in autonomous-pathway mutants. Many loci of the first class are components of the *Arabidopsis PAF1* transcriptional activator complex (He et al., 2004; Oh et al., 2004; Zhang and van Nocker, 2002). This complex is required for the methylation of lysine 4 on histone 3 in *FLC* chromatin (He et al., 2004), a modification associated with an active chromatin state. Mutations in members of this complex also affect the expression of other members of the *FLC* clade, such as *FLOWERING LOCUS M* and *MADS AFFECTING FLOWERING 2*, and, as a result, such mutations cause early flowering in short days (He et al., 2004; Oh et al., 2004). A member of the second class, *FRIGIDA-LIKE 1* (*FRL1*), is necessary for the promotion of *FLC* expression in a *FRI*-dependent manner; i.e. *frl1* mutations are unable to suppress mutants in the autonomous or the photoperiod pathways, indicating that *FRL1* might act specifically with *FRI* to promote *FLC* expression (Michaels et al., 2004).

Here, we report the identification of a gene, *FRIGIDA-ESSENTIAL 1* (*FES1*), that, like *FRL1*, is required for the upregulation of *FLC* in the presence of *FRI*, and hence is necessary for conferring the winter-annual habit in *Arabidopsis*. *FES1* encodes a protein with a CCCH zinc finger, and promotes the expression of *FLC* in a *FRI*-dependent manner. Epistasis analysis between *FES1*, *FRL1* and *FRI* indicate that these genes do not function in a linear pathway, but instead act cooperatively to promote the expression of *FLC*.

Materials and methods

Plant material

FRI-SF2 in the Columbia (Col) background, *ld-1*, *fca-9*, *fld-3*, *flc-3* and *gi-2* have been described previously (He et al., 2003; Lee et al., 1994a; Lee et al., 1994b; Macknight et al., 1997; Michaels and Amasino, 1999; Redei, 1962). *fes1-3 and fes1-4* insertion lines in the Columbia background were isolated from the SALK Collection (http://signal.salk.edu/; *fes1-3* and *fes1-4* are SALK_100573 and SALK_137525, respectively) (Alonso et al., 2003).

Growth conditions

Plants were grown under long days (16 hours light/8 hours dark) or short days (8 hours light/16 hours dark) at 22°C under cool-white fluorescent lights. For experiments involving vernalization, seeds were plated on agar-solidified medium containing 0.65 g/liter Peters Excel 15-5-15 fertilizer (Grace Sierra, Milpitas, CA), and were kept at room temperature overnight to allow seeds to become metabolically active before being transferred to 2°C for 40 days. During cold treatment, samples were kept under short-day conditions (8 hours light/16 hours dark).

T-DNA flanking-sequence analysis

The sequence flanking the T-DNA of *fes1-1* and *fes1-2* was obtained by thermal asymmetric interlaced PCR (Liu et al., 1995), the details of which are described elsewhere (Schomburg et al., 2003). T-DNA borders were defined by sequencing PCR products obtained using a T-DNA border primer and a gene-specific primer. The T-DNA border primers used for each T-DNA insertion population are described on the Arabidopsis Knockout Facility web site (see http://www.biotech.wisc.edu/Arabidopsis/Index2.asp).

Histochemical β-glucuronidase assays and overexpression analyses

The *FES1* β-*glucuronidase* fusion construct was generated by PCR amplification of the 2.8 kb genomic region plus 600 bp of the promoter region of *FES1*, using FES-PGF (5′-CACCATGGCGAA-ATTGCGGAGGATTCTTAGGGTTTA-3′) and FES-PGR (5′-TTAC-

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CATACTTTTCGACATACCCCTGCA-3′) as primers. The *FES1* 35S Cauliflower Mosaic Virus construct was generated by PCR amplification of a 2.8 kb section of the *FES1* genomic region beginning at the start codon, using FES-OXF (5′-CACCATGTCTGATTCCGACATGGACATTGA-3′) and FES-OXR (5′-AGTGACATTTGGTTTGATAACTCAGGGTTTACCA-3′) as primers. The resulting PCR product was subcloned into D-TOPO (Invitrogen Life Technologies). Gateway Technologies were used to generate *FES1*::GUS in pMDC163 and 35S::*FES1* in pMDC32 (Curtis and Grossniklaus, 2003). *Arabidopsis* (ecotype Col) plants were transformed with the *Agrobacterium tumefaciens* strain LBA4404 by infiltration (Clough and Bent, 1998). Transgenic lines were selected on agar-solidified medium containing 0.65 g/l Peter's Excel 15-5-15 fertilizer and 25 μg/ml Hygromycin. Staining for β-glucuronidase activity was performed as described previously (Schomburg et al., 2001).

RT-PCR

RT-PCR analysis, first-strand cDNA synthesis was performed on 2 μg of RNA by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) and a primer containing the M13 primer sequence with an oligo dT extension (5′-GTAAAACGACGGCCAGTCCCT$_{15}$-3′). PCR amplification was performed with Platinum *Taq*DNA Polymerase (Invitrogen Life Technologies), according to the manufacturer's recommendations. *FES1* (5′-CAATTCCCACAACGATGTGAAGA-3′ and 5′-CAGACCGGATACCACCGTTTCCAGC-3′), *FLC* (5′-TTCTCCAAACGTCGCAACGGTCTC-3′ and 5′-GATTTGTCCAGCAGGTGACATCTC-3′), *FRI* (5′-GCAAAACGGAAAGCCCAGTC-3′ and 5′-CGATGAGGAAAAGATGTTGACGG-3′), and *UBIQUITIN* (UBQ; 5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′) were amplified using the indicated primers. Cycling was performed as follows: 95°C for 4 minutes, followed by 22 (for *UBQ*) or 30 (for *FRI, FES1* and *FLC*) cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds. Amplified fragments were separated on a 1.2% agarose gel.

Results

Identification of a mutant that suppresses the *FRI*-mediated winter-annual habit of *Arabidopsis*

To identify positive regulators of the winter-annual habit in *Arabidopsis*, T-DNA mutagenesis was performed on a late-flowering line (Col *FRI*), created by introgressing a functional *FRI* locus from the San-Feliu (Sf-2) accession into the Columbia accession (Lee and Amasino, 1995). Without vernalization, Col *FRI* flowering is significantly delayed because of elevated levels of *FLC* (Michaels and Amasino, 1999). The Col *FRI* line was mutagenized by T-DNA insertion and screened for mutants that flowered early under inductive photoperiods. Two independent mutant lines were chosen for further study that completely suppressed *FRI*-mediated late flowering (Fig. 1A,B). Both mutants had T-DNA insertions in a locus we named *FRIGIDA-ESSENTIAL 1* (*FES1*). The mutations in these lines behaved recessively in the F1 and F2 generations when crossed to the parental line, and F2 populations segregate for suppression of late flowering in a Mendelian manner (data not shown).

The ability of lesions in *FES1* to suppress *FRI*-mediated late flowering suggested that *FES1* is required for *FLC* expression. A number of genes, in addition to *FRI*, have been identified as being required for *FLC* expression (Bezerra et al., 2004; Doyle et al., 2005; He et al., 2004; Michaels et al., 2004; Noh et al., 2004a; Noh and Amasino, 2003; Noh et al., 2004b; Oh et al., 2004; Zhang and van Nocker, 2002). Some of these loci are specifically involved in *FLC* expression, whereas others affect the expression of additional members of the *FLC* clade, such as *FLOWERING LOCUS M* (*FLM*; *MAF1* – The Arabidopsis Information Resource) and *MADS AFFECTING FLOWERING 2* (*MAF2*) (Doyle et al., 2005; He et al., 2004; Oh et al., 2004). Mutants in which the expression of the entire *FLC* clade is affected flower early in non-inductive photoperiods, mainly as a result of the decreased expression of *FLM* (He et al., 2004; Oh et al., 2004; Ratcliffe et al., 2001; Scortecci et al., 2001) and perhaps *MAF2*, whereas loss of only *FLC* expression does not have a large effect on flowering in the short-day conditions that we used (Michaels and Amasino, 1999). Therefore, *fes1-1* and *fes1-2* mutants were grown in short days to assess whether or not the *fes1* lesion might affect expression of the *FLC* clade. *fes1* alleles grown in short days flowered similarly to, but not earlier than, Columbia and a loss-of-function *flc* allele, *flc-3* (Fig. 1B). These data indicate that *FES1* does not affect the entire *FLC*

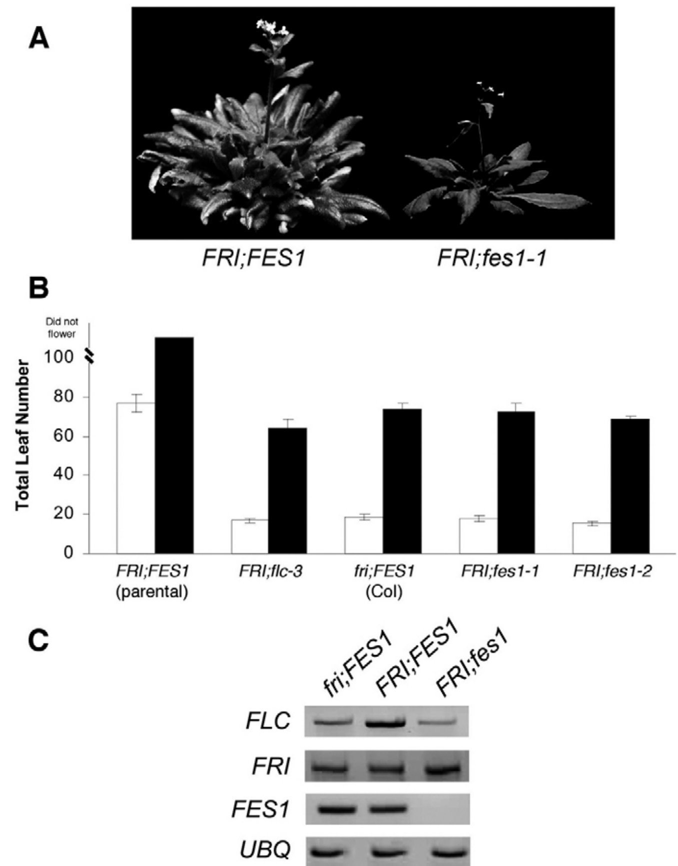

Fig. 1. Suppression of *FRI*-mediated late flowering by mutations in *fes1*. (A) Col *FRI* (left) and *fes1-1* (right) grown in long days. The only observable phenotype associated with *fes1* lesions is an inability to delay flowering. (B) Total leaf numbers when grown in both inductive (white bars) and non-inductive (black bars) photoperiods. *fes1* mutants have a similar flowering-time phenotype to *flc-3* in both conditions tested. *fes1* mutants do not flower early in non-inductive photoperiods, unlike *flm* and *maf2* mutants, suggesting that the suppression of late-flowering may be *FLC* specific. Error bars represent s.d. (C) *fes1-2* lesions reduce steady-state levels of *FLC* mRNA.

clade, but more likely specifically affects the expression of *FLC*.

To evaluate the level at which *fes1* caused an early-flowering phenotype in Col *FRI*, expression studies of *FRI* and *FLC* were performed. In *fes1* mutants, there is no detectable difference in the expression of *FRI*, but there is a significant reduction in the expression of *FLC* when compared with the Col *FRI* parental line (Fig. 1C). As expected from the *fes1* short day phenotype, there was no change in *FLM* expression (data not shown). Therefore *FES1* is necessary for the *FRI*-mediated increase of *FLC* mRNA levels. In addition, *FES1* is not regulated by *FRI* (Fig. 1C).

fes1 mutations are unable to suppress the late flowering of autonomous- or photoperiod-pathway mutants

Mutations in autonomous-pathway genes delay flowering in non-vernalized plants owing to the increased expression of *FLC*, and, as is the case with *FRI*-containing lines, exposure to vernalization promotes rapid flowering by suppressing *FLC* expression (Michaels and Amasino, 2001). Therefore, the autonomous-pathway mutants act similarly to Col *FRI* in that they require vernalization treatment to flower rapidly. Double-mutant analyses with autonomous-pathway mutants and *fes1* were performed to determine whether *FES1* is specifically required for *FRI*-mediated late flowering, or whether *FES1* plays a more general role in *FLC* upregulation. *fes1-4* was unable to suppress the late flowering of autonomous-pathway mutants *fld-3* and *fca-9*, and only slightly suppressed another autonomous-pathway mutant *ld-1*. Nor was *fes1-4* able to suppress the late flowering of *gigantea*, a photoperiod mutant (Fig. 2A). Furthermore, the inability of *fes1* to suppress the late flowering of autonomous-pathway mutants is due to a failure to reduce the level of *FLC* mRNA (Fig. 2B). These data indicate that *FES1* is part of a pathway, involving *FRI*, that elevates *FLC* expression (Fig. 2C).

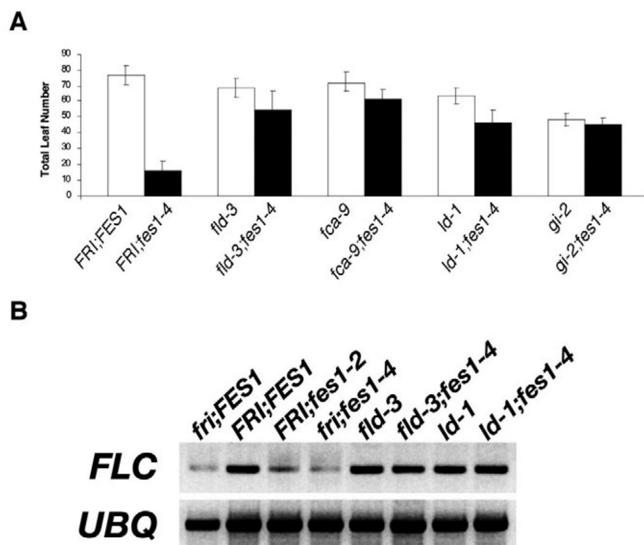

Fig. 2. *fes1* lesions are not suppressors of late-flowering autonomous-pathway or photoperiod mutants. (A) *fes1-4* completely suppresses *FRI*-mediated late flowering. *fes1-4* is unable to suppress the late-flowering behavior of *fld-3*, *fca-9*, *ld-1* and *gi-2*. Error bars represent s.d. (B) The suppression observed in Col *FRI* is due to a reduction in steady-state levels of *FLC* mRNA. No decrease in *FLC* mRNA is observed in the autonomous-pathway mutants *fld-3* and *ld-1*. These data suggest two distinct mechanisms associated with promotion of *FLC* expression.

FES1 encodes a protein with a CCCH zinc finger

Flanking sequences from *fes1-1* and *fes1-2* align with At2g33835 on chromosome 2 (Fig. 3A). Analysis of the early-flowering plants in a segregating F2 population of each line indicated that the early-flowering phenotype completely co-segregated with a T-DNA insertion at At2g33835 (data not shown). To confirm that *FES1* was At2g33835, two additional alleles, *fes1-3* and *fes1-4*, were isolated by screening the SALK T-DNA collection (Alonso et al., 2003). Complementation tests indicate that *fes1-1*, *fes1-2*, *fes1-3* and *fes1-4* were allelic (data not shown). Furthermore, when introduced into the Col *FRI* background, *fes1-3* and *fes1-4* are able to suppress *FRI*-mediated late flowering (data not shown). Therefore, *FES1* is At2g33835.

A BLAST search with the *FES1* protein-coding region failed to identify any proteins with extensive sequence identity in *Arabidopsis*, although a sequence was identified from poplar that encodes a protein containing a CCCH zinc finger and that shares ~30% identity with the carboxy-terminal of *FES1*. *FES1* does share ~60% sequence identity in an ~20 amino-acid CCCH zinc finger with other CCCH zinc fingers in *Arabidopsis* (Fig. 3), but the similarity only extends approximately 50 amino acids N- and C-terminal of the CCCH zinc finger.

FES1 genetically interacts with *FRI* and *FRL1*

Three genes, *FRI*, *FRL1* and *FES1*, are required specifically for the upregulation of *FLC* that is characteristic of the winter-annual habit in *Arabidopsis* (Johanson et al., 2000; Michaels et al., 2004). Double mutants were isolated to determine whether *FRI* and *FES1* are involved in the same flowering pathway, as was shown with *FRI* and *FRL1* (Michaels et al.,

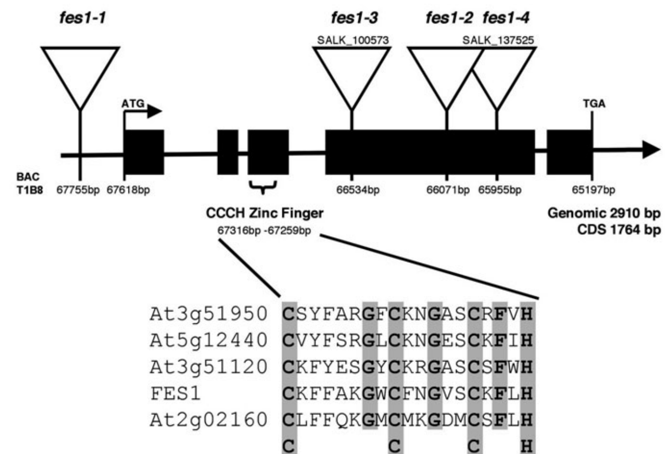

Fig. 3. Schematic of the genomic structure and isolated lesions in *FES1*. *fes1-1* and *fes1-2* were isolated in a genetic screen for early flowering mutants in a Col *FRI* background. *fes-1-3* and *fes1-4* were isolated from the SALK T-DNA collection. *FES1* contains a C-×8-C-×5-C-3×-H zinc finger. The closest four matches are represented in the alignment.

2004). Double mutants displayed phenotypes identical to the single mutants (Fig. 4A). The failure to observe an additive phenotype between these mutants suggests that they have non-redundant roles in delaying the floral transition and are likely to act in the same genetic pathway.

To further examine the relationship between *FRI*, *FRL1* and *FES1*, constitutively expressed alleles were created by driving the expression of these genes with the 35S CaMV promoter (Odell et al., 1985), and transgenic plants containing combinations of these alleles and mutants were analyzed for their flowering time. Introduction of a 35S::*FES1* transgene into a *fes1-2* mutant caused late flowering, indicating that the transgene can rescue the mutant phenotype (Fig. 4A). However, introduction of 35S::*FES1* into a *fri* or *frl1* mutant did not affect flowering. Thus, *FES1* alone is not sufficient to delay the floral transition, indicating that *FES1* is not a sole downstream component of a *FRI* or *FRL1* pathway (Fig. 4A). The reciprocal experiments were also performed in which transgenic lines with functional 35S::*FRI* or 35S::*FRL1* transgenes were crossed to *fes1-2*, and lines were identified in which the 35S::*FRI* or 35S::*FRL1* transgenes were present in a *fes1-2* mutant. Neither 35S::*FRI* nor 35S::*FRL1* could restore late flowering to a homozygous *fes1*-2 mutant. Therefore, neither *FRI* nor *FRL1* appears to be downstream of *FES1*. The combination of these genetic experiments provides evidence against a simple linear pathway in which *FRI* or *FRL1* promotes expression of *FES1*, or *FES1* promotes expression of *FRI* or *FRL1*.

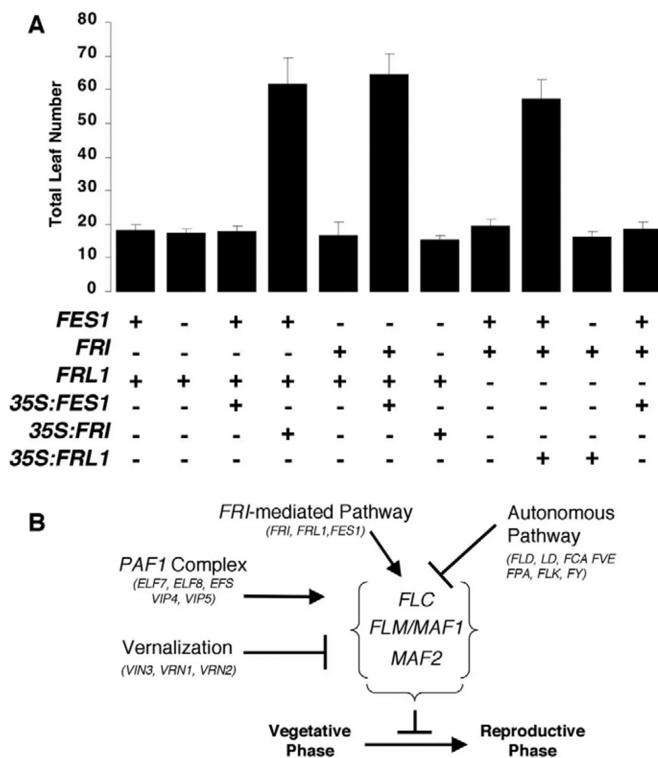

Fig. 4. Genetic analyses between *FRI*, *FRL1* and *FES1*. (A) *fri;fes1* double mutants have the same flowering time as the parent plants do. Overexpression of *FES1* is unable to delay the floral transition in a *fri* (Col) or *frl1* (Col) genetic background. Overexpression of either *FRI* or *FRL1* is suppressed by *fes1* lesions. Error bars represent s.d. (B) Pathways that affect the expression of *FLC* and the *FLC* clade. These data provide evidence against a linear pathway, and favor a model in which *FRI*, *FRL1* and *FES1* form a complex to promote the expression of *FLC*.

Vernalization occurs downstream of *FES1*

Vernalization promotes rapid flowering of winter-annual *Arabidopsis* by silencing the floral repressor *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). Lines that express *FLC* from a constitutive promoter are not responsive to vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999). Lines in which 35S::*FES1* rescued the *fes1* lesion are fully responsive to vernalization (Fig. 5A). Furthermore, *FES1* mRNA levels are not affected by vernalization. Thus, vernalization acts downstream of *FES1* (Fig. 5B). This is not surprising considering that autonomous-pathway mutants are vernalization responsive, and that mutations in *fri*, *frl1* and *fes1* are unable to suppress mutants in the autonomous pathway.

FES1 is expressed in the shoot/root apex and the vascular system

FLC is expressed in the apex of the shoot/root and in the vascular tissue (Michaels and Amasino, 2000). Translational fusions to β-*glucuronidase* (*GUS*) were used to evaluate the relationship of the *FES1* and *FLC* expression pattern. The *FES1*::GUS translational fusion comprised the entire genomic sequence (minus the stop codon and the 3′ untranslated region) plus 600 bp upstream of the start codon. The FLC::GUS construct has been described previously (Michaels et al., 2005). The *FES1*::GUS translational fusion was functional; transformants were identified for GUS analysis in which the transgene rescued the early-flowering behavior of the *fes1-2* lesion. *FES1*::GUS activity was detected most strongly in the shoot and root apex, as well as in the vascular system (Fig.

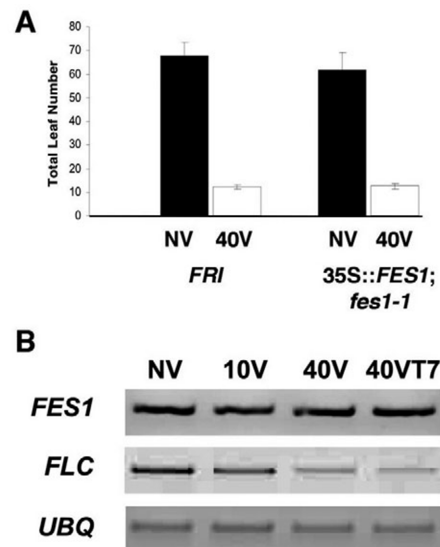

Fig. 5. Vernalization acts downstream of *FES1*. (A) 35S::*FES1* plants are completely responsive to vernalization. Error bars represent s.d. (B) Steady-state levels of *FES1* mRNA are not affected by vernalization. NV, no vernalization; 10V, 10 days of vernalization; 40V, 40 days of vernalization; 40VT7, 40 days of vernalization plus 7 days return to warm growth conditions.

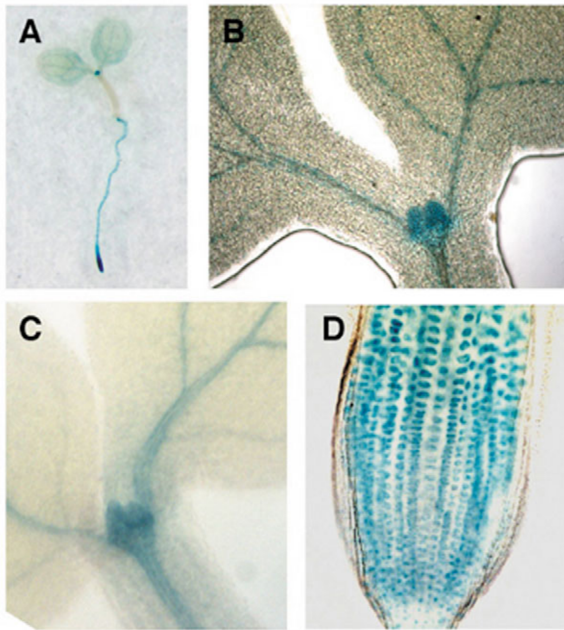

Fig. 6. *FES1* spatial expression pattern. (A) GUS fusions to *FES1*. GUS expression is mainly detected in the root and shoot apex and throughout the vascular system. (B) A close-up view of *FES1*::GUS expression in the shoot apex and vascular system. (C) A close-up view of *FLC*::GUS expression in the shoot apex and vascular system. (D) Nuclear expression pattern in the root of *FES1*::GUS.

6A,B). This expression pattern is similar to the expression pattern of *FLC*::GUS (Fig. 6C). In addition, *FES1*::GUS was localized to the nucleus (Fig. 6D).

Discussion

FES1 was identified in a screen for rapid-flowering mutants in the late-flowering Col *FRI* background. Interestingly, the *fes1* lesion has little effect on the late-flowering phenotype caused by autonomous-pathway mutations, demonstrating that the loss of autonomous-pathway genes must promote *FLC* expression by a largely *FES1*-independent mechanism. Thus, *FES1* is required to increase the expression of *FLC* to levels sufficient to block flowering, prior to vernalization, in a *FRI*-mediated manner.

It is interesting that there are mutations that repress *FLC* expression in both *FRI* and autonomous-pathway mutants, and other mutations, like *fes1*, that suppress *FRI*, but not autonomous-pathway mutants. *FRL1* is another example of a gene that is specifically required for *FLC* activity in a *FRI* background but is not required for *FLC* expression in autonomous-pathway mutants (Michaels et al., 2004). Thus, *FRL1* and *FES1* comprise a class of gene that is highly *FRI* specific for the upregulation of *FLC* expression. In addition, lesions in *ABH1*, the large subunit of the mRNA cap-binding protein, strongly suppress the *FRI*-mediated promotion of *FLC*, like *fes1* and *frl1*, but cause a weak suppression of certain autonomous-pathway mutants (Bezerra et al., 2004). In other words, the *abh1* mutation exhibits a degree of *FRI* specificity, but does not affect the *FRI* pathway as specifically as *fes1* and *frl1* do.

Autonomous-pathway mutants are late flowering as a result of the elevated expression of *FLC*, but the natural route to elevated *FLC* expression and the associated delay of flowering characteristic of winter-annual *Arabidopsis* is most often due to the presence of *FRI*. How *FRI*, *FRL1* and *FES1* increase *FLC* expression at a biochemical level is not known. Genetic analyses using both recessive and dominant alleles of *FRI*, *FRL1* and *FES1* revealed that they do not appear to act in a linear pathway to promote *FLC* expression; rather, *FRI*, *FRL1* and *FES1* appear to act in parallel, perhaps in a common protein complex. However, we found no evidence for an interaction between *FRI* and *FES1*, or *FRL1* and *FES1*, by yeast two-hybrid analysis.

FES1 encodes a protein with a CCCH zinc finger. This class of zinc fingers is typically found in proteins that bind to RNA, and such proteins can participate in mRNA production or degradation. For example, in the mouse, tristetraprolin (TTP; ZFP36 – Mouse Genome Informatics), a protein containing two CCCH zinc fingers, binds directly to AU-rich elements within the 3′ untranslated region of target transcripts to facilitate mRNA degradation (Carballo et al., 1998). The *Arabidopsis HUA1* protein contains six CCCH zinc fingers and is thought to participate in the pre-mRNA processing of target RNAs (Cheng et al., 2003). If FES1 binds to *FLC* RNA, the phenotype of *fes1* mutants is consistent with *FES1* stabilizing or facilitating the processing of *FLC* mRNA.

Studies of natural variation in flowering time have revealed that lesions that reduce or eliminate *FRI* activity have arisen independently several times, and that allelic variation in the *FRI* gene accounts for most of the natural variation in flowering time (Gazzani et al., 2003; Hagenblad and Nordborg, 2002; Johanson et al., 2000; Le Corre et al., 2002; Shindo et al., 2005; Werner et al., 2005). Mutations in *FES1* or *FRL1* appear to specifically affect flowering in cooperation with *FRI*, and the phenotype caused by these mutations appears to be identical to that caused by *fri* loss-of-function mutations. Thus, *FES1* and *FRL1* could, in principle, be targets for natural variation. Recently Werner et al. identified several early-flowering accessions that appear to contain functional *FRI* and *FLC* alleles (Werner et al., 2005). However, sequence analysis of *FES1* in these accessions did not reveal any obvious changes that may account for the early-flowering phenotype. Perhaps, *fes1* and *frl1* lesions have deleterious affects that have not yet been recognized. Alternatively, there may be undiscovered examples of allelic variation in *FES1* and *FRL1* that account for the natural variation in flowering time.

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References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003).

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Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**, 653-657.

Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A. and Dean, C. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature* **427**, 164-167.

Bezerra, I. C., Michaels, S. D., Schomburg, F. M. and Amasino, R. M. (2004). Lesions in the mRNA cap-binding gene ABA HYPERSENSITIVE 1 suppress FRIGIDA-mediated delayed flowering in Arabidopsis. *Plant J.* **40**, 112-119.

Borner, R., Kampmann, G., Chandler, J., Gleissner, R., Wisman, E., Apel, K. and Melzer, S. (2000). A MADS domain gene involved in the transition to flowering in Arabidopsis. *Plant J.* **24**, 591-599.

Carballo, E., Lai, W. S. and Blackshear, P. J. (1998). Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science* **281**, 1001-1005.

Cheng, Y., Kato, N., Wang, W., Li, J. and Chen, X. (2003). Two RNA binding proteins, HEN4 and HUA1, act in the processing of AGAMOUS pre-mRNA in Arabidopsis thaliana. *Dev. Cell* **4**, 53-66.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.

Curtis, M. D. and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133**, 462-469.

Doyle, M. R., Bizzell, C. M., Keller, M. R., Michaels, S. D., Song, J., Noh, Y. S. and Amasino, R. M. (2005). HUA2 is required for the expression of floral repressors in Arabidopsis thaliana. *Plant J.* **41**, 376-385.

Gazzani, S., Gendall, A. R., Lister, C. and Dean, C. (2003). Analysis of the molecular basis of flowering time variation in Arabidopsis accessions. *Plant Physiol.* **132**, 1107-1114.

Gendall, A. R., Levy, Y. Y., Wilson, A. and Dean, C. (2001). The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. *Cell* **107**, 525-535.

Hagenblad, J. and Nordborg, M. (2002). Sequence variation and haplotype structure surrounding the flowering time locus FRI in Arabidopsis thaliana. *Genetics* **161**, 289-298.

He, Y., Michaels, S. D. and Amasino, R. M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. *Science* **302**, 1751-1754.

He, Y., Doyle, M. R. and Amasino, R. M. (2004). PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. *Genes Dev.* **18**, 2774-2784.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R. and Dean, C. (2000). Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. *Science* **290**, 344-347.

Kardailsky, I., Shukla, V. K., Ahn, J. H., Dagenais, N., Christensen, S. K., Nguyen, J. T., Chory, J., Harrison, M. J. and Weigel, D. (1999). Activation tagging of the floral inducer FT. *Science* **286**, 1962-1965.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**, 1960-1962.

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C. J., Soppe, W. and Peeters, T. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. *Plant J.* **6**, 911-919.

Lang, A. (1965). Physiology of flower initiation. In *Encyclopedia of Plant Physiology* (ed. W Ruhland), pp. 1371-1536. Berlin: Springer-Verlag.

Le Corre, V., Roux, F. and Reboud, X. (2002). DNA polymorphism at the FRIGIDA gene in Arabidopsis thaliana: extensive nonsynonymous variation is consistent with local selection for flowering time. *Mol. Biol. Evol.* **19**, 1261-1271.

Lee, H., Suh, S. S., Park, E., Cho, E., Ahn, J. H., Kim, S. G., Lee, J. S., Kwon, Y. M. and Lee, I. (2000). The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. *Genes Dev.* **14**, 2366-2376.

Lee, I. and Amasino, R. M. (1995). Effect of vernalization, photoperiod, and light quality on the flowering phenotype of Arabidopsis plants containing the FRIGIDA gene. *Plant Physiol.* **108**, 157-162.

Lee, I., Aukerman, M. J., Gore, S. L., Lohman, K. N., Michaels, S. D., Weaver, L. M., John, M. C., Feldmann, K. A. and Amasino, R. M. (1994a). Isolation of LUMINIDEPENDENS: a gene involved in the control of flowering time in Arabidopsis. *Plant Cell* **6**, 75-83.

Lee, I., Michaels, S. D., Masshardt, A. S. and Amasino, R. (1994b). The late-flowering phenotype of *FRIGIDA* and mutations in *LUMINIDEPENDS* is suppressed in the Landsberg *erecta* strain of Arabidopsis. *Plant J.* **6**, 903-909.

Levy, Y. Y., Mesnage, S., Mylne, J. S., Gendall, A. R. and Dean, C. (2002). Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. *Science* **297**, 243-246.

Liu, Y. G., Mitsukawa, N., Oosumi, T. and Whittier, R. F. (1995). Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* **8**, 457-463.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C. et al. (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell* **89**, 737-745.

Michaels, S. D. and Amasino, R. M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.

Michaels, S. D. and Amasino, R. M. (2000). Memories of winter: vernalization and the competence to flower. *Plant Cell Environ.* **23**, 1145-1153.

Michaels, S. D. and Amasino, R. M. (2001). Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-941.

Michaels, S. D., He, Y., Scortecci, K. C. and Amasino, R. M. (2003). Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **100**, 10102-10107.

Michaels, S. D., Bezerra, I. C. and Amasino, R. M. (2004). FRIGIDA-related genes are required for the winter-annual habit in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **101**, 3281-3285.

Michaels, S. D., Himelblau, E., Kim, S. Y., Schomburg, F. M. and Amasino, R. M. (2005). Integration of flowering signals in winter-annual Arabidopsis. *Plant Physiol.* **137**, 149-156.

Napp-Zinn, K. (1979). On the genetical basis of vernalization requirement in *Arabidopsis thaliana* (L.) Heynh. In *La Physiologie de la Floraison* (ed. P. Champagnat and R. Jaques), pp. 217-220. Paris: Collogues Internationaux Centre National de la Recherche Scientifique.

Noh, B., Lee, S. H., Kim, H. J., Yi, G., Shin, E. A., Lee, M., Jung, K. J., Doyle, M. R., Amasino, R. M. and Noh, Y. S. (2004a). Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. *Plant Cell* **16**, 2601-2613.

Noh, Y. S. and Amasino, R. M. (2003). PIE1, an ISWI family gene, is required for FLC activation and floral repression in Arabidopsis. *Plant Cell* **15**, 1671-1682.

Noh, Y. S., Bizzell, C. M., Noh, B., Schomburg, F. M. and Amasino, R. M. (2004b). EARLY FLOWERING 5 acts as a floral repressor in Arabidopsis. *Plant J.* **38**, 664-672.

Odell, J. T., Nagy, F. and Chua, N. H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**, 810-812.

Oh, S., Zhang, H., Ludwig, P. and van Nocker, S. (2004). A mechanism related to the yeast transcriptional regulator Paf1c is required for expression of the Arabidopsis FLC/MAF MADS box gene family. *Plant Cell* **16**, 2940-2953.

Ratcliffe, O. J., Nadzan, G. C., Reuber, T. L. and Riechmann, J. L. (2001). Regulation of flowering in Arabidopsis by an FLC homologue. *Plant Physiol.* **126,** 122-132.

Redei, G. P. (1962). Supervital mutants of Arabidopsis. *Genetics* **47**, 443-460.

Samach, A., Onouchi, H., Gold, S. E., Ditta, G. S., Schwarz-Sommer, Z., Yanofsky, M. F. and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science* **288**, 1613-1616.

Schomburg, F. M., Patton, D. A., Meinke, D. W. and Amasino, R. M. (2001). FPA, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. *Plant Cell* **13**, 1427-1436.

Schomburg, F. M., Bizzell, C. M., Lee, D. J., Zeevaart, J. A. and Amasino, R. M. (2003). Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. *Plant Cell* **15**, 151-163.

Scortecci, K. C., Michaels, S. D. and Amasino, R. M. (2001). Identification of a MADS-box gene, FLOWERING LOCUS M, that represses flowering. *Plant J.* **26**, 229-236.

Sheldon, C. C., Burn, J. E., Perez, P. P., Metzger, J., Edwards, J. A., Peacock, W. J. and Dennis, E. S. (1999). The FLF MADS box gene: a

repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell* **11**, 445-458.

Shindo, C., Aranzana, M. J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M. and Dean, C. (2005). Role of FRIGIDA and FLOWERING LOCUS C in determining variation in flowering time of Arabidopsis. *Plant Physiol.* **138**, 1163-1173.

Sung, S. and Amasino, R. M. (2004). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. *Nature* **427**, 159-164.

Werner, J. D., Borevitz, J. O., Uhlenhaut, H., Ecker, J. R., Chory, J. and Weigel, D. (2005). FRIGIDA-independent variation in flowering time of natural A. thaliana accessions. *Genetics* **170**, 1197-1207.

Zhang, H. and van Nocker, S. (2002). The VERNALIZATION INDEPENDENCE 4 gene encodes a novel regulator of FLOWERING LOCUS C. *Plant J.* **31**, 663-673.

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