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

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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. 

Introduction
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., 1994). 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
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 fcl 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 epigenetic regulation. Screens for mutants that are insensitive to vernalization and to vernalization exposure (Lang, 1965); this mitotic stability is a hallmark of epigenetic regulation. Screens for mutants that are insensitive to vernalization and to vernalization exposure (Lang, 1965); this mitotic stability is a hallmark of epigenetic regulation. Screens for mutants that are insensitive to vernalization and to vernalization exposure (Lang, 1965); this mitotic stability is a hallmark of epigenetic regulation.

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. fril1 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, fdl-3, fcl-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.biotec.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’-CACCATGGCGAAATTCCGAGATTTACGTTTTA-3’) and FES-PGR (5’-TTAC-
CATACTTTTTCGACATCCCCCTGCA-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’-CACCATTGCTCTGATTCGAGAATGCATTTGA-3’) and FES-OXR (5’-AGTCTTGTTTGTGATAACTCAGGGTTTACCA-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’-GTCTGATTCCGACATGGACATTGA-3’). PCR amplification was performed with Platinum Taq DNA Polymerase (Invitrogen Life Technologies), according to the manufacturer’s recommendations. FES1 (5’-CCAAGTTCCTCAACCGATGGTAAGA-3’ and 5’-CAGACCCGATACCA-CCGTTCTCCAGC-3’), FLC (5’-TCTCTCAACGTTGCAAGCT-3’ and 5’-GATTITGGTCAAGGTGACATC-3’), FRI (5’-GCAAAACGGAAAGCCCAGTC-3’ and 5’-CGATGAGGAA-AAGATGTTGACGGG-3’), and UBIQUITIN (UBQ; 5’-GATCTTGGCCGAAAAACATTGAGGATGTTG-3’ and 5’-CGACTTTGGCATTAGAAGAAGAGATACAGG-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) 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, whereas 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.

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-autonomous-pathway mutants act similarly to Col FRI, expression (data not shown). Therefore, FES1 is specifically FES1 is necessary for the FRI-mediated increase of FLC mRNA levels. In addition, FES1 is not regulated by FRI (Fig. 1C).

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 cosegregated 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. fes1-3 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.](Image)
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.

**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. 5B).

![Fig. 4](image1.png) 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.

![Fig. 5](image2.png) 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.
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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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