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The *FLX* Gene of *Arabidopsis* is Required for *FRI*-Dependent Activation of *FLC* Expression

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The *Arabidopsis FLOWERING LOCUS C* (*FLC*) gene encodes a MADS box protein that acts as a dose-dependent repressor of flowering. Mutants and ecotypes with elevated expression of *FLC* are late flowering and vernalization responsive. In this study we describe an early flowering mutant in the C24 ecotype, *flc expressor* (*flx*), that has reduced expression of *FLC*. *FLX* encodes a protein of unknown function with putative leucine zipper domains. *FLX* is required for *FRIGIDA* (*FRI*)-mediated activation of *FLC* but not for activation of *FLC* in autonomous pathway mutants. *FLX* is also required for expression of the *FLC* paralogs *MADS AFFECTING FLOWERING 1* (*MAF1*) and *MAF2*.

Keywords: *Arabidopsis* — FLC activator — Flowering time — *FLX Gene* — FRI pathway — *MADS AFFECTING FLOWERING*.

Abbreviations: ChIP, chromatin immunoprecipitation; *DFC*, *DOWNSTREAM OF FLC*; *FLC*, *FLOWERING LOCUS C*; *flx*, *flc expressor*; *FRI*, *FRIGIDA*; GFP, green fluorescent protein; LB, left border; *MAF*, *MADS AFFECTING FLOWERING*; ORF, open reading frame; RB, right border; RNAi, RNA interference; *SAM, S-adenosylmethionine*; TAIL-PCR; thermal asymmetric interlaced-PCR; *UFC, UPSTREAM OF FLC*.

Introduction

The transition from vegetative to reproductive growth is a major developmental event in the life cycle of a plant. The timing of this transition is coordinated to environmental conditions and the developmental state of the plant by a number of signaling pathways (Simpson and Dean 2002). The Arabidopsis flowering repressor gene *FLOWERING LOCUS C* (*FLC*) is regulated by several of these pathways. The late flowering phenotype of many ecotypes is due to elevated expression of *FLC* (Sheldon et al. 2000, Michaels et al. 2003). This *FLC*-induced late flowering can be overcome by vernalization (an extended period of cold temperatures), which leads to repression of *FLC* expression. *FLC* encodes a MADS box transcription factor (Michaels and Amasino 1999, Sheldon et al. 1999) that is thought to directly repress expression of the floral promoting genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Hepworth et al. 2002, Helliwell et al. 2006, Searle et al. 2006). The *FLC* clade of the MADS box gene family contains five other genes, known as *MADS AFFECTING FLOWERING 1* (*MAF1*)/*FLOWERING LOCUS M* (*FLM*) and *MAF2–MAF5* (Ratcliffe et al. 2001, Scortecci et al. 2001). The *MAF* genes can all act as repressors of flowering (Ratcliffe et al. 2003). Mutant analysis has defined specific roles for *MAF1* in repressing flowering in short days (Ratcliffe et al. 2001, Scortecci et al. 2001) and for *MAF2* in preventing flowering in response to short periods of cold (Ratcliffe et al. 2003). However, *FLC* is the major determinant of vernalization-responsive late flowering.

Much of the natural variation in *FLC* expression can be attributed to the presence of active alleles of *FRIGIDA* (*FRI*) which confer high levels of *FLC* expression (Johanson et al. 2000, Le Corre et al. 2002, Gazzani et al. 2003, Shindo et al. 2005). *FRI* appears to act specifically on *FLC* and does not regulate the *MAF* genes. Mutant screens for suppressors of the *FRI* phenotype have identified the *FRIGIDA ESSENTIAL 1* (*FES1*), *FRIGIDA LIKE 1* (*FRL1*), *FRL2* and *SUPPRESSOR OF FRI 4* (*SUF4*) genes (Michaels et al. 2004, Schmitz et al. 2005, Schlappi 2006, Kim and Michaels 2006, Kim et al. 2006). Mutants in these genes are early flowering in long day conditions and have reduced *FLC* expression, but they do not affect expression of the *MAF* genes. In short days these mutants are relatively late flowering due to the repressive effect of *MAF1* in these conditions (Sung et al. 2006). Protein interaction data, along with genetic evidence, suggest that the FRI, FES1, FRL1, FRL2 and SUF4 proteins act in a complex (Kim et al. 2006), binding to a region of the *FLC* promoter that has been shown to be important for high expression of the *FLC* gene (Sheldon et al. 2002).

A distinct group of genes required for *FLC* activation encodes proteins similar to components of the yeast SWR1 and Paf1C transcriptional activation complexes (Zhang and van Nocker 2002, Noh and Amasino 2003, He et al. 2004, Oh et al. 2004, Martin-Trillo et al. 2006, Choi et al. 2007,

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March-Díaz et al. 2007). Interaction data suggest that these proteins form complexes similar to SWR1 and Paf1c (Oh et al. 2004, Choi et al. 2007, March-Díaz et al. 2007). These genes form a group distinct from those encoding the components of the putative FRI complex; in addition to being required for *FLC* activation by *FRI* they are also required for high *FLC* expression in autonomous pathway mutants such as *fca*, and affect expression of *MAF* genes. Mutants in the SWR1- and Paf1c-like complexes have morphological defects besides flowering time, consistent with a more general role than *FRI* in promoting active gene expression.

We screened for early flowering mutants in the moderately late flowering ecotype C24 using an activation-tagged population. We identified an early flowering mutant, *flx*, which is a suppressor of *FRI* but not of autonomous pathway mutants. These data place it in the *FRI*-mediated pathway for *FLC* activation.

Results and Discussion

The flx mutant is early flowering but not via the vernalization pathway

We screened a T-DNA-tagged population of the late flowering C24 ecotype (Koiwa et al. 2002) for plants that flowered early. One seed pool produced three early flowering plants which were siblings. Progeny of these plants, derived from self-pollination, were early flowering and morphologically wild-type.When one of the early flowering plants was back-crossed to C24, the F_1 progeny flowered at the same time as C24. In the F_2 population, we found a 1 : 3 ratio of early to late flowering plants (8 early: 31 late), indicating that the early flowering *flx* mutant carries a recessive allele at a single locus we have designated *FLX* (*FLC EXPRESSOR*).

To test whether *FLX* is involved in vernalization or photoperiod responses, the flowering time of vernalized and non-vernalized plants was determined in long and short day conditions. The *flx* mutant flowered earlier than C24 under both vernalized and non-vernalized conditions in both long (16 h light: 8 h dark) and short day (8 h light: 16 h dark) photoperiods (Fig. 1A). *flx* flowered earlier in long day than short day conditions, suggesting that *FLX* is not required for the photoperiod response. *flx* retained a slight vernalization response under the short day regime, flowering with four fewer leaves than non-vernalized *flx* after vernalization (8 vs. 12). This suggests that *FLX* is not required for the vernalization response.

The early flowering phenotype of flx segregates with a single T-DNA tag

The *flx* mutant carries a single copy of the pSKI015 T-DNA tag. We used plasmid rescue to clone the genomic region flanking the right-border (RB) of this T-DNA and TAIL-PCR (thermal asymmetric interlaced-PCR) to determine the location of the T-DNA left border (LB) (Weigel et al. 2000). Both identified the same region of chromosome 2, but we found that the genomic DNA sequences flanking the T-DNA LB and RB are approximately 40 kb apart in wild-type Columbia (SeqViewer at www.arabidopsis.org). The orientation of these fragments indicated that the T-DNA insertion was associated with an inversion of a 40 kb genomic fragment flanking the T-DNA insert. We used PCR primers to confirm the inversion, and that regions of DNA 40 kb apart in C24

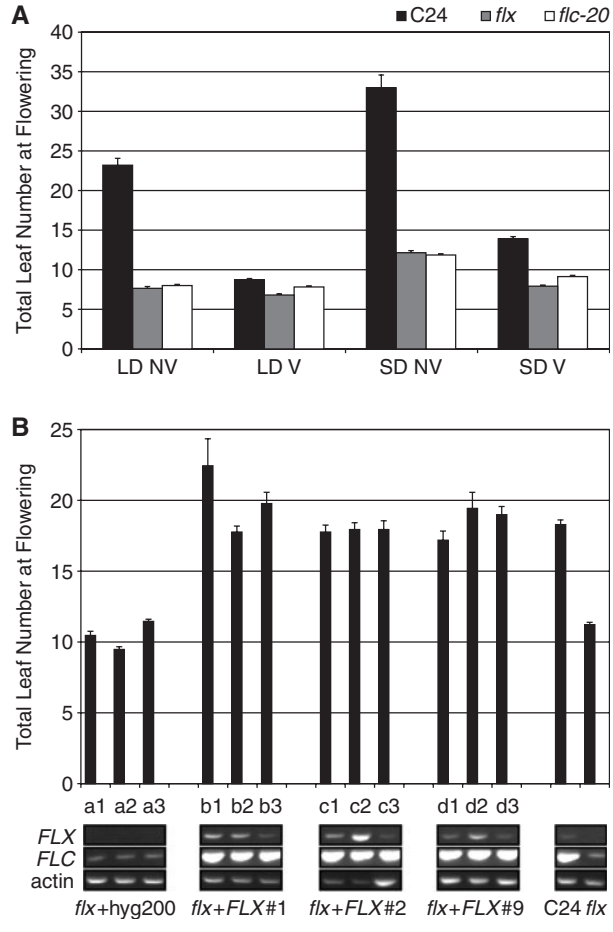
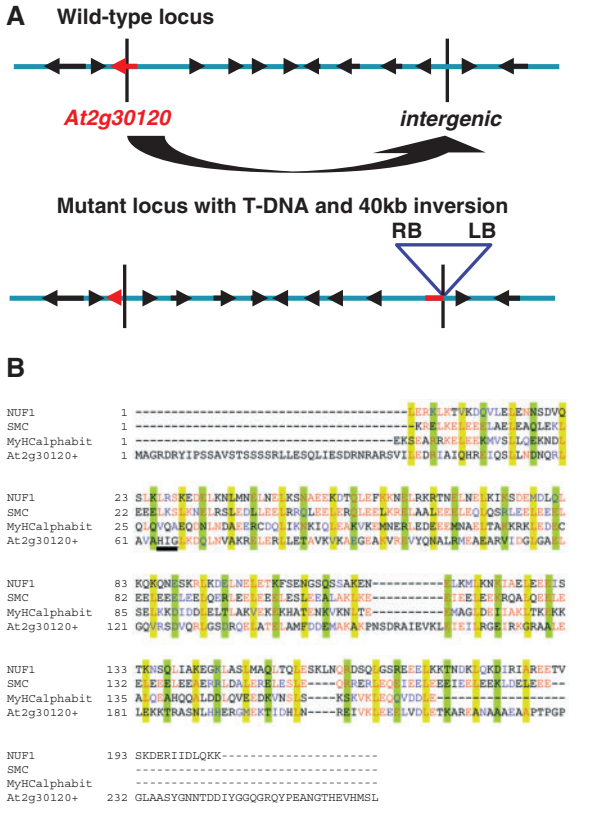


Fig. 1 The early flowering phenotype of the *flx* mutant. (A) Flowering time as measured by total leaf number at flowering of *flx* mutant and C24, unvernalized (NV) and vernalized (V), under long day (LD) and short day (SD) photoperiods, $n > 25$ for all categories. (B) Flowering time as measured by total leaf number at flowering of complemented T_2 families transformed with either an empty binary vector (hyg200) or a binary vector with a genomic fragment encompassing At2g30120. The mean flowering time of three independent lines for each construct was calculated from at least 10 HygR plants and is shown ±SEM. Semi-quantitative RT–PCR for *FLX, FLC* and actin was performed using RNA from complemented T_2 plants grown in a 16 : 8 LD and harvested on day 10. PCR products were visualized with ethidium bromide.



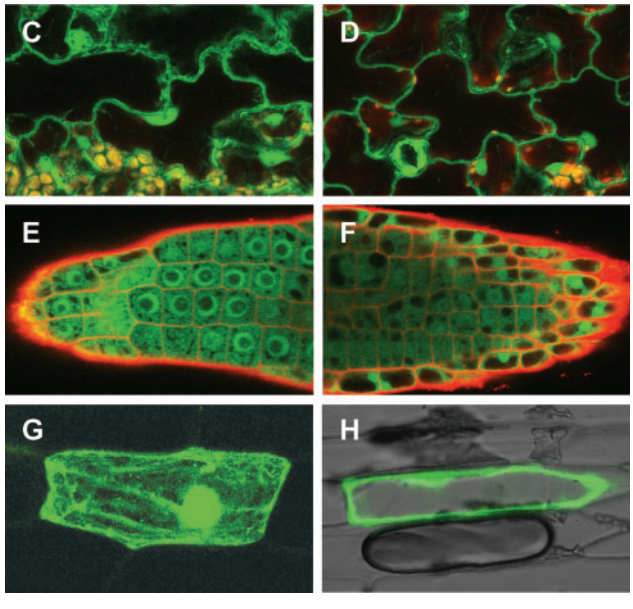


Fig. 2 The *FLX* gene, encoding a leucine zipper protein, is disrupted in the *flx* mutant. (A) Map of the *FLX* region in the early flowering *flx* mutant. Insertion of the single T-DNA is associated with an inversion of a 40 kb genomic region that disrupts the gene At2g30120. (B) Sequence alignment of FLX and the leucine zipper regions of some related proteins. The full-length predicted protein sequence of FLX is given, but only the leucine zipper regions of the yeast NUF1 (Mirzayan et al. 1992), the SMC (structural maintenance of chromosomes) domain (Hirano et al. 1995, Marchler-Bauer and Bryant 2004) and human myosin heavy chain cardiac muscle α form (Matsuoka et al. 1991) are shown. Amino acid residues highlighted with yellow tend to be leucine or other small, non-polar residues, such as valine, alanine or isoleucine. Residues highlighted with green tend to be acidic (aspartic or glutamic acid) or basic (lysine, arginine or histidine). The position of the T-DNA insertion and associated small deletion in the early-flowering mutant is underlined. Expression of g*FLX*::FLX–GFP in leaf (C) and primary root (E) cells is detected throughout the cytoplasm and nucleus (absent from the nucleolus). Expression of 35S::FLX–GFP is detected in the nucleus and cytoplasm of onion epidermal cells (G). Expression of 35S::GFP in leaf (D), primary root (F) and onion epidermal cells (H). GFP was visualized using a confocal microscope; the red in leaf images is chlorophyll autofluorescence, and in root images is propidium iodide-stained cell walls.

are in close proximity in *flx* (Fig. 2A). DNA sequencing of this region showed deletions of 8 bp within the first exon of At2g30120, and 13 bp from the region between At2g30220 and At2g30230. The T-DNA insertion segregated with the *flx* phenotype.

To determine whether the disruption of At2g30120 is the cause of the early flowering phenotype, we amplified the genomic region of C24 encompassing the predicted At2g30120 coding region extending ~2 kb upstream and ~500 bp downstream. We introduced this genomic clone, in parallel with an empty vector negative control, into *flx*. Under non-vernalized conditions almost all primary transgenic plants had a flowering time restored to that of wild type, whereas the empty vector controls retained the early flowering time of *flx* (Fig. 1B). T_2 lines segregating with a 3 : 1 ratio for the transgene had the same flowering time as wild-type C24 (Fig. 1C). We conclude that At2g30120 is *FLX*.

FLX encodes a protein of unknown function

The TAIR annotation of At2g30120, based on a single full-length cDNA (RAFL09-50-H06, gb NM_128567), predicts a relatively short open reading frame (ORF) and a long 3′ untranslated region (UTR). The MIPS annotation (http://mips.gsf.de) predicts two introns and an extended ORF in At2g30120. We used reverse transcription–PCR (RT–PCR) to determine which forms of At2g30120 mRNA are produced in C24, and found three products. The DNA sequence of the largest product matched the genomic sequence, apart from a single nucleotide polymorphism (SNP) between Col and C24, predicted to cause a threonine to asparagine change in exon 2. The intermediate product was consistent with one of the introns not being spliced out. The DNA sequence of the smallest PCR product was consistent with the two introns being spliced out, supporting the MIPS gene prediction.

The protein predicted to be encoded by the fully spliced mRNA has 270 amino acids. The protein is predicted to form an α-helix, and to contain regions that could form leucine zipper structures, with small, non-polar residues interspersed with charged or basic residues. The predicted At2g30120 protein aligns with the leucine zipper regions of some other proteins, such as myosin heavy chain α-subunit (Matsuoka et al. 1991), yeast SMC (chromosome-segregating ATPase involved in cell division and chromosome partitioning; Hirano et al. 1995, Marchler-Bauer and Bryant 2004) and yeast NUF1 (involved in spindle body function, Mirzayan et al. 1992) (Fig. 2B).

Translational fusions of the *FLX* gene to a green fluorescent protein (GFP) reporter complementing the early flowering phenotype of the *flx* mutant showed FLX–GFP protein in both the nucleus and cytoplasm of root and leaf cells with no obvious subcellular localization or tissue specificity (Fig. 2C, E). A similar localization was seen when a 35S::FLX–GFP construct was expressed in onion epidermal cells by particle bombardment (Fig. 2G).

The early flowering phenotype of flx is correlated with a loss of FLC expression

In order to understand further the role of *FLX* in previously defined flowering time pathways, we examined the mRNA expression levels of other flowering time genes in the *flx* mutant. In this mutant the expression of *FLC* showed a dramatic reduction (Fig. 3A), which largely accounts for the early flowering of *flx*. Expression of *FLC* is restored to wild-type levels in *flx* plants complemented with an *FLX* transgene (Fig. 1C).

Previous studies have shown that FLC acts by repressing the expression of two key activators of the floral transition: *SOC1*, encoding another MADS box protein; and *FT*, encoding a RAF kinase inhibitor-like protein (Lee et al. 2000, Michaels et al. 2005). Loss of *FLC* expression in *flx* or the *flc-20* null mutant was associated with an increase in the expression of *SOC1* and *FT* (Fig. 3A).

flx has lower expression of two *FLC* paralogs, *MAF1* and *MAF2* (Fig. 3A), genes shown to be repressors of flowering (Ratcliffe et al. 2001, Scortecci et al. 2001, Ratcliffe et al. 2003). However, the three other *FLC* paralogs, *MAF3*, *4* and *5*, did not have altered expression in the *flx* mutant (Fig. 3A). The lowered expression of *MAF1* and *MAF2*, in addition to the reduced expression of *FLC*, may account for the very early flowering phenotype of the *flx* mutant.

To establish whether the change in *MAF* expression is a consequence of the reduced expression of *FLC*, rather than reduced expression of *FLX* itself, we determined the expression levels of the *MAF* gene family in *flc-20*, a mutant allele with no expression of *FLC*. We found that expression

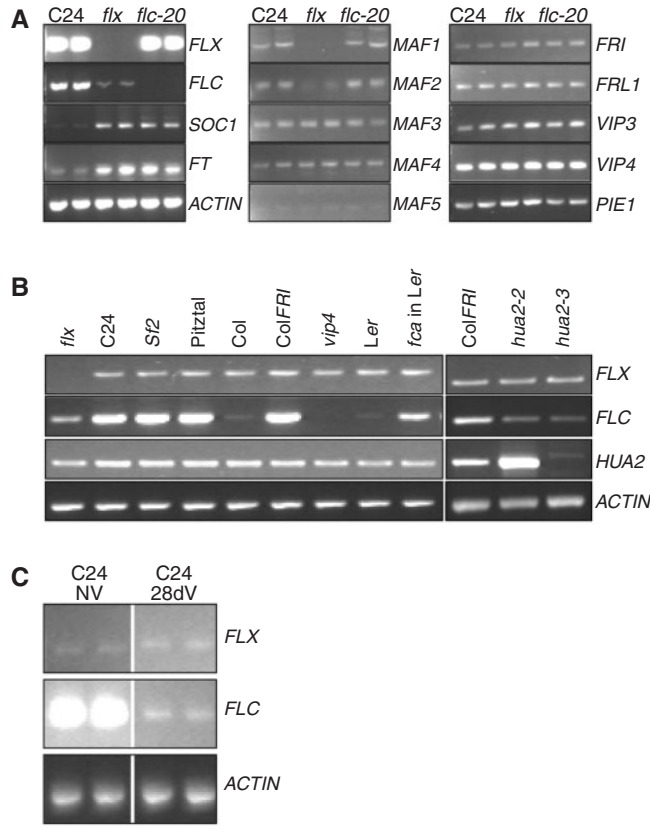


Fig. 3 Molecular phenotype of the *flx* mutant and control of *FLX* expression (A) Expression levels of key flowering time genes and known activators of *FLC* expression in the *flx* mutant. Semi-quantitative RT–PCR for *FLX*, *FLC*, *SOC1*, *FT*, *MAF1*, *MAF2*, *MAF3*, *MAF4*, *MAF5*, *FRI*, *FRL1*, *VIP3*, *VIP4*, *PIE1* and *ACTIN* was performed on RNA isolated from C24, *flx* and *flc-20* grown for 10 d under long day (LD; 16 : 8) conditions. Results from two biological replicates are shown. (B) Expression of *FLX*, *FLC* and *HUA2* in different flowering time mutants and ecotypes. RT–PCR analysis for *FLX*, *FLC*, *HUA2* and *ACTIN* was performed on RNA from 10-day-old non-vernalized plants grown under LD conditions. (C) Expression of *FLX* in vernalized plants. RT–PCR for *FLX*, *FLC* and *ACTIN* was carried out on mRNA isolated from C24 plants grown for 10 LD (NV) or 28 d vernalization plus 8 LD (V).

levels of the *FLC* paralogs were not altered (Fig. 3A), results similar to those reported by Oh et al. (2004).

FLX does not alter expression of known activators of FLC, and FLX expression is not affected in flowering time mutants

Our analysis clearly places *FLX* upstream of *FLC* in a genetic pathway to flowering. *FLC* expression has been shown to be activated by a variety of genes including members of a putative FRI complex (FRI, FRL1, FRL2, FES1 and SUF4) a Paf1c-like complex (VIP4, VIP5, ELF7 and ELF8), a SWR1 complex (PIE, ARP6 and SEF) as well as a number of other proteins that are likely to be involved in chromatin modification.

An active *FRI* allele and the *FRI* paralog *FRL1* are both required for expression of *FLC* (Johanson et al. 2000, Michaels et al. 2004). We could not find any difference in *FRI* or *FRL1* expression levels between C24 plants carrying FLX or *flx* mutant alleles (Fig. 3A)

Although *vip* mutants cause extremely early flowering by lowering expression of *FLC* and all the *MAF* family of genes (Zhang and Van Nocker 2002, Zhang et al. 2003, He et al. 2004, Oh et al. 2004), the expression levels of *VIP3* or *VIP4* were not altered in *flx* (Fig. 3A). Mutations in *PIE1* and *HUA2* also reduce *FLC* expression levels, leading to early flowering (Noh and Amasino 2003, Doyle et al. 2005), but the expression levels of these genes were not changed in the *flx* mutant (Fig. 3A, B).

Since the *flx* mutation does not alter expression of known activators of *FLC* expression, we investigated whether *FLX* transcript levels were altered in flowering time mutants that affect *FLC* expression levels.

In *fca*, which has elevated *FLC* expression (Sanda and Amasino 1996, Sheldon et al. 1999), there is no change in *FLX* expression relative to its L*er* parent (Fig. 3B); nor did we find any difference in *FLX* expression levels between Col and a late flowering Col*FRI* line carrying a dominant *FRI* allele introgressed from the Sf2 ecotype (Lee et al. 1994).

As the *flx* mutant affects expression of only a subset of the *FLC*/*MAF* family, whereas the *vip* mutations alter expression of all members of the *FLC*/*MAF* family (Oh et al. 2004), *FLX* may act downstream of VIP. We could not detect any difference in *FLX* message abundance between the *vip4* mutant and its Col*FRI* parent (Fig. 3B). Similarly, *FLX* may be downstream of *HUA2*, but no changes in *FLX* expression could be detected in *hua2* mutants (Fig. 3B). *FLX* expression levels in ecotypes, ranging from early (Col, L*er*) to intermediate (C24) and very late (Sf2, Pitztal) flowering, were equivalent (Fig. 3B).

Seedlings exposed to varying durations of cold temperatures did not demonstrate any change in *FLX* expression levels (Fig. 3C). The gene investigator web site (www.genevestigator.ethz.ch; Zimmermann et al. 2004) and the AtGenExpress expression atlas (www.weigelworld.org/resources/microarray/AtGenExpress/; Schmid et al. 2005) provide gene expression data for *Arabidopsis*. Both data sets show that *FLX* is expressed throughout development in all organs examined, with no apparent differential gene expression.

The flx mutation affects expression of a gene adjacent to FLC

Vernalization causes a coordinate down-regulation of *FLC* (At5g10140) and its two flanking genes, *UFC* (*UPSTREAM OF FLC*, At5g10150) and the downstream gene, *DFC* (*DOWNSTREAM OF FLC*, At5g10130). Coordinate regulation is limited to this three-gene cluster (Finnegan et al. 2004). RNA gel blot analysis showed that

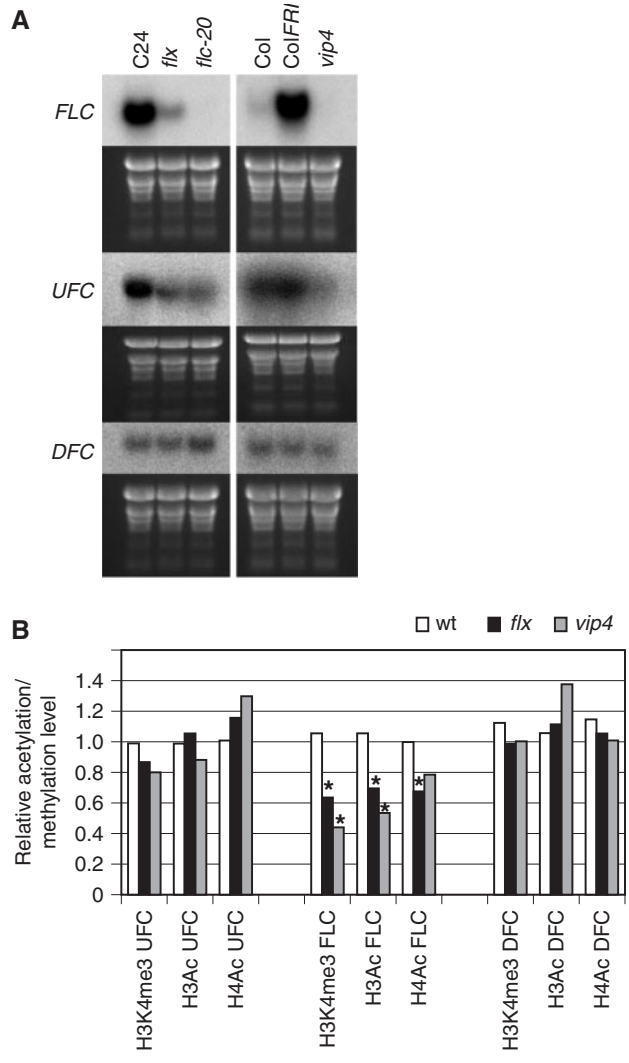


Fig. 4 The *flx* mutation affects expression of *FLC* and the flanking gene, *UFC*. (A) The *flx* mutant has reduced expression of *UFC* (At5g10150), the gene upstream of *FLC*, but not *DFC* (At5g10130), the gene downstream of *FLC*. Northern analysis of *FLC*, *UFC* and *DFC* expression in C24, *flx*, *flc-20*, Col, Col + *FRI* and *vip4* plants using gene-specific riboprobes on 10 μg of total RNA. (B) The *flx* mutation results in altered histone modifications at the *FLC* locus. Fifteen-day-old non-vernalized wild-type, *flx* and *vip4* plants were examined for histone modifications across the transcription–translation start regions of the *FLC*, *UFC* and *DFC* gene cluster using chromatin immunoprecipitation followed by quantitative real-time PCR analysis. Antibodies recognizing histone H3 trimethyl-K4 (H3K4me3), acetylated histone H3 (H3Ac) and tetra-acetylated H4 (H4Ac) were used. Each measurement is the average of at least three biological replicates. Pairs of bars annotated with an asterisk are significantly different ($P < 0.05$), as determined by a non-parametric Mann–Whitney U-test.

expression of *UFC* is lowered in the *flx* mutant (Fig. 4A), but we could not detect any differences in At5g10130 expression by either RNA gel blot (Fig. 4A) or quantitative real-time RT–PCR (data not shown).

Acetylation of both histone H3 and H4 in the region spanning the transcription–translation start site of *FLC* was decreased in the *flx* mutant (Fig. 4B). No change was detected in the transcription–translation start of the *UFC* or *DFC* genes (Fig. 4B and data not shown), suggesting that loss of FLX activity resulted in a localized effect on histone acetylation.

Activation of *FLC* transcription by the *Arabidopsis* Paf1C-like complex is associated with increased levels of histone H3 trimethylated at Lys4 (H3K4me3) (He et al. 2004) and acetylation of H3 and H4 (Fig. 4B). Trimethylation of Lys4 has been reported to mark actively transcribed genes in yeast (Ng et al. 2003). We found that the level of H3K4me3 was decreased across the transcription–translation start of *FLC* in the *flx* mutant, but not of *UFC* or *DFC* (Fig. 4B), despite the expression of *UFC* being reduced in the *flx* mutant. It is possible that any changes in histone state at the *UFC* gene are below the limits of sensitivity of the chromatin imunoprecipitation (ChIP) assay, consistent with the relative reduction in gene expression being lower for *UFC* than for *FLC*. In the *vip4* mutant, which also has reduced *FLC* expression, chromatin modifications similar to those observed in *flx* were seen in *FLC* chromatin.

flx is a strong suppressor of FRI but not of autonomous pathway mutants

The *flx* mutant was isolated in a C24 background which has a late allele of *FRI*, hence *flx* is an *FRI* suppressor. In the absence of *flx* alleles in other ecotypes, an RNA interference (RNAi) approach was used to test whether *FLX* could also suppress autonomous pathway mutants and whether the *FRI* suppression observed in the C24 *flx* mutant applied to other ecotypes. The siFLX RNAi construct caused early flowering in C24 (Fig. 5A), showing that the construct was effective. When the same construct was introduced into Col*FRI* it also caused early flowering, confirming that *flx* is a suppressor of *FRI*. In contrast, the siFLX construct did not suppress the late flowering phenotype of *fca-9*, *ld-1* or *fld-1* (these alleles are all in a Col background) in the T_1 generation (data not shown). T_2 lines for C24, Col*FRI*, *fca-9* and *ld-1* with reduced *FLX* mRNA content were identified by qRT–PCR. The flowering time of these lines confirmed that siFLX strongly suppresses the late flowering of Col*FRI*. *FLC* mRNA abundance was strongly reduced in siFLX lines in Col*FRI*. *FLC* expression was unchanged in *ld-1* transformed with siFLX and slightly reduced in *fca-9* + siFLX. This weak suppression of an autonomous pathway mutant is similar to that observed for the *FRI* suppressor *suf4* (Kim et al. 2006, Kim and Michaels 2006). These data support *FLX* being a part of the *FRI* pathway for activation of *FLC* expression but not being required for *FLC* activation in autonomous

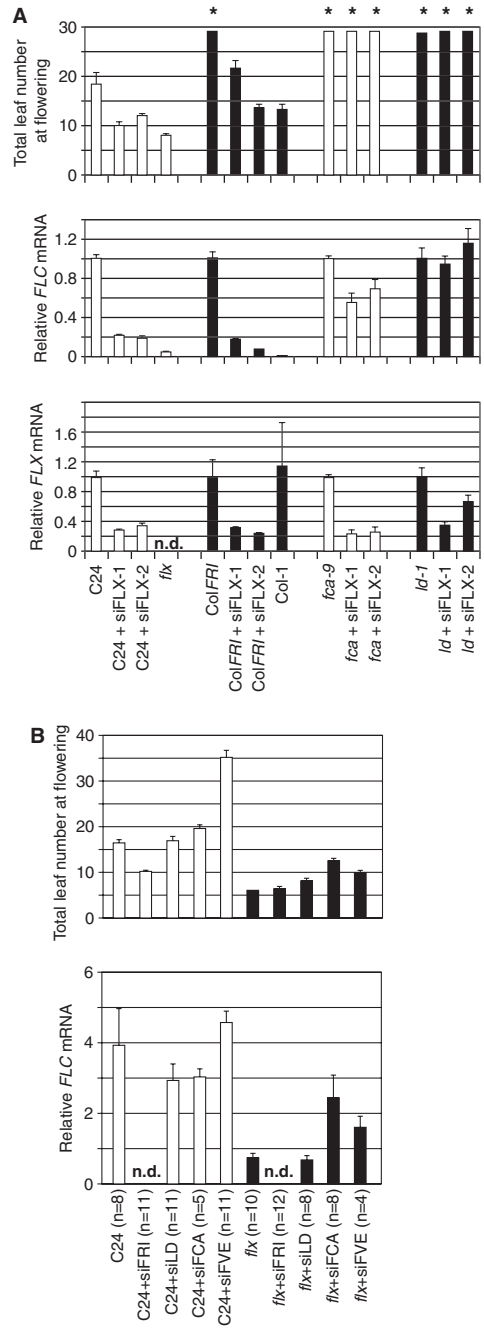


Fig. 5 FRI is suppressed by *flx*. (A) An RNAi construct targeting *FLX* was introduced into C24, Col*FRI*, *fca-9* and *ld-1*. Total leaf number at flowering in long day conditions of T_2 lines carrying the siFLX transgene and with reduced *FLX* mRNA was scored. *FLC* and *FLX* mRNA abundance relative to non-transformed controls was determined by qRT–PCR. (B) *FRI* and the autonomous pathway genes *LD*, *FCA* and *FVE* were silenced by RNAi. Total leaf number at flowering of T_1 plants from transformations of C24 and *flx* with the RNAi constructs was scored and average *FLC* mRNA abundance determined by qRT–PCR. Error bars are SEM, n.d., not determined, * plants had at least 28 leaves and had not flowered when the experiment was terminated due to drying of growth medium.

pathway mutants. Consistent with this, silencing of autonomous pathway genes in an *flx* mutant background delays flowering (Fig. 5B).

Conclusions

The *FLX* gene encodes a protein required for FLC expression. The FLX protein sequence contains putative leucine zipper structures and could therefore interact with other components of a protein complex. The strong suppression of the late flowering phenotype of Col*FRI* and weak suppression of autonomous pathway mutants by *FLX* places it in the *FRI* pathway for *FLC* activation. The *FRI* pathway components identified to date appear to act specifically on *FLC*, while mutants of other *FLC* activators such as the components of the Paf1c-like and SWR1-like complexes have phenotypes other than altered flowering time, suggesting they act on many genes. We have not observed phenotypes other than early flowering in *flx*. This is consistent with a role for *FLX* in the *FRI* activation pathway rather than it being associated with the Paf1c-like or SWR1-like complexes. We did not find evidence for regulation of the expression of components of the FRI-containing complex by *FLX*, hence FLX must either be a component of the FRI complex or act downstream of it. *FLX* regulates *MAF1* and *MAF2* as well as *FLC*. However, where tested, no other *FRI* activation pathway mutants have been shown to be required for activation of any of the *MAF* genes. The finding that *FLX* regulates *MAF1* and *MAF2* as well as *FLC* suggests that it could act in a complex with FRI-related proteins to regulate *MAF1* and *MAF2*.

Materials and Methods

Plant materials and growth conditions

A population of *Arabidopsis* ecotype C24 carrying the pSKI015 activation tag (Weigel et al. 2000, Koiwa et al. 2002) was obtained from the ABRC (stock number CS31400). Since these lines are likely to be T_3 or T_4, the T-DNA tag has the potential to reveal both dominant and recessive mutations. Seeds were surface sterilized, plated on MS (Murashige and Skoog) medium containing 3% (w/v) sucrose and stratified at 4°C for 2 d before transfer to a growth room maintained at 21°C with a 16 h: 8 h long day of cool white fluorescent lights (100 μE). Plants were scored for flowering time by counting the number of rosette leaves when floral buds were first visible. Putative mutants were recovered to soil and allowed to self-fertilize and set seed. Lines were re-scored in the next generation.

For subsequent flowering time experiments, seeds were vapor-phase surface sterilized and plated on MS medium containing 3% (w/v) sucrose. For non-vernalized conditions, seeds were stratified at 4°C under dim white fluorescent light for 2 d. For vernalization experiments, seeds were kept at 4°C in the dark for 28 d. Plants were then grown at 21°C under either long (16 : 8, 100 μE) or short day (8 : 16, 200 μE) conditions using cool white fluorescent light. Flowering time was scored as both total leaf number and number of days when the bolt had started to elongate.

The *hua2-2* and *hua2-3* mutants and their Col + *FRI* parent line were obtained from Rick Amasino (Lee et al. 1994, Doyle et al. 2005). The *vip4* mutant was obtained from Steve van Nocker (Zhang and Van Nocker 2002). The *fca* mutant in the L*er* background was obtained from Caroline Dean.

Identification of FLX

Genomic DNA isolated from the *flx* mutant was analyzed by Southern blot using a *Hin*dIII–*Eco*RI fragment from the LB of pSKI015 as a probe. LB TAIL-PCR using primers SKIL1, 2 and 3 (5′ ACGACGGATCGTAATTTGTCG, 5′ TTCATTTTATAA TAACGCTCGGG and 5′ CTTTCTTTTCCTCCATATTGACC, respectively) as described in Liu et al. (1995), and RB plasmid rescue with restriction enzymes *Eco*RI and *Hin*dIII was performed as described in Weigel et al. (2000). BLAST analysis of the flanking genomic sequence against the TAIR database (www.arabidopsis.org) identified the region of T-DNA insertion.

Segregating F_2 individuals from an *flx* × C24 backcross population were genotyped using primers ca144 (AGATGGT TCACAAATACAGC) and ca145 (TTTAACGTAGGAATCA GTCG) to identify the wild-type allele, and SKC12 (Weigel et al. 2000) plus ca145 for the mutant. The implied ~40 kb inversion of genomic DNA associated with the T-DNA was confirmed by PCR amplification from mutant DNA using primers ca144 (anneals between At2g30220 and At2g30230) and ca150 (ATCTCTC CTCGAAGAATCTC, within At2g30120), and ca145 (between At2g30220 and At2g30230) and SKC12 (LB of pSKI015, Weigel et al. 2000).

A genomic DNA fragment encompassing the *FLX* candidate gene At2g30120 was amplified with *Pfu*Turbo DNA Polymerase (Stratagene) using primers ca151 (CCTGGTACCCATTATTAC AACTTTTGG) and ca152 (GACGGATCCACTCTTTCCCAA GTGAAAC) in three independent amplification reactions, to obviate the need to verify sequence fidelity. These fragments were cloned as a *Kpn*I–*Bam*HI fragment into a binary vector, hyg200 [pPZP200 (Hajdukiewicz et al. 1994), modified to contain a 35S-driven hygromycin resistance gene]. The *flx* mutant was transformed with both the genomic At2g30120-containing vector and an empty binary vector as negative control using *Agrobacterium* (GV3101) and the floral dip method (Clough and Bent 1998). T_2 lines segregating for hygromycin resistance (30 μg ml^{-1}) with an approximately 3 : 1 ratio were scored for flowering time. All three independently amplified genomic fragments complemented the early flowering phenotype.

FLX cDNA was amplified from oligo(dT)-reverse transcribed RNA extracted from non-vernalized C24 using primers ca208 (CGCAAACCATGGCCGGACGAGATCGTTATATTC) and ca191 (AGACCCATGGGTACCTCATGAGTACCATTAGCC) with *Pfu*Turbo DNA polymerase (Stratagene).

Expression analysis

Total RNA was isolated from 10-day-old non-vernalized plants and 8-day-old vernalized plants (to match developmental stage) using the Qiagen RNeasy Plant Mini Kit. First-strand cDNA synthesis was performed using 3 μg of total RNA with an oligo(dT) primer and SuperScriptII RT according to the manufacturer's directions (Gibco). RT–PCR was performed using gene-specific primers as described: *FLC*, *MAF1*, *MAF2*, *MAF3*, *MAF4*, *MAF5*, *SOC1* and actin (Ratcliffe et al. 2001) [except for Fig. 4B, where FLCa#7 was replaced by ca218 (CTGGTCAAGATC CTTGATCG)]; *FT* (Blazquez and Weigel 1999); *FRI*, *FRL1*, *VIP3*, *VIP4*, *PIE1*, *VIN3* (Finnegan et al. 2005); *FLX*—ca208 and

ca150 (amplifying within the first exon and therefore including all three splicing variants); *HUA2* (GAGGTCTCGCCTATGG CTCC + TCTAATTTGGGGAAGCAAGG).

Northern analysis was performed using 10 μg of total RNA and riboprobes essentially as described in Sheldon et al. (1999) and Finnegan et al. (2004). Riboprobe templates specific for *FLC* and *AT5G10130* were generated by PCR amplification from C24 first-strand cDNA using the following primer pairs: FLCT7 (ttttttgagcgcgcgtaatacgactcactatagCTCACACGAATAAGGTAC AAAGTTC) and FLCcomp (GATCAAATGTCAAAAATGTGA GTATCG); and AT5G10130T7 (ttttttgagcgcgcgtaatacgactcactatag TCAAATAGCCCGGTTATCGCCGTCTG) and AT5G10130 comp (CATTGAAGGAAGTGTTTACTGCGACAC). The *UFC* riboprobe plasmid template was as described in Sheldon et al. (1999).

Real-time PCR analysis was carried out using FLC primers (CGGTCTCATCGAGAAGCTC and CCACAAGCTTGCTAT CCACA) and FLX primers (TCGAGCTATTGAGGTTAA TTGGA and GAGCAGCCCTTCCTTTACGA) normalized to formaldehyde dehydrogenase, At5g43940 (TGGGAAACCCAT TTATCACTTCA and CAGCAAGTCCAACAGTGCCAG) or At4g26410 (GAGCTGAAGTGGCTTCCATGAC and GGTCC GACATACCCATGATCC).

gFLX::FLX–GFP construct and expression

The *FLX* gene, along with approximately 2 kb of upstream region, was amplified from C24 genomic DNA using primers ca209 (GACCTGCAGACTCTTTCCCAAGTGAAAC) and ca191 (AGACCCATGGGTACCTCATGAGTACCATTAGCC) and PfuTurbo DNA Polymerase (Stratagene). This fragment was cloned as a *Pst*I–*Nco*I fragment into pA7-GFP [pA7-GFP is a pUC19 derivative with the GFP-containing cassette from the binary vector pJH-GFP (J. Harper, personal communication) from Sohlenkamp et al. (2002)] to make a g*FLX*::FLX–GFP fusion. Both the resulting g*FLX*::FLX–GFP fusion and a control pA7-GFP 35S:GFP construct were subcloned as Klenow-blunted *Eco*RI/*Hin*dIII fragments into Klenow-blunted *Acc*65I/*Pst*I hyg200 binary vector.

Both constructs were introduced into the *flx* mutant via *Agrobacterium* (GV3101) and the floral dip method (Clough and Bent 1998). T_2 lines segregating for hygromycin resistance (30 μg ml^{-1}) with an approximately 3 : 1 ratio were selected. Images of FLX–GFP fusion protein and free GFP in Fig. 5 were captured with a Leica SP2 confocal laser scanning microscope (Leica Microsystems, Sydney, Australia). Excitation was at 488 nm, and emission was collected between 500 and 550 nm. Roots were stained with 10 μg ml^{-1} propidium iodide to visualize cell walls. Chlorophyll autofluorescence and propidium iodide stain was collected between 650 and 720 nm. T_2 lines were also scored for flowering time under both non-vernalized and vernalized conditions. A 35S::FLX–GFP construct was also generated in pA7-GFP, coated onto gold particles and used to bombard onion epidermal cells. GFP was visualized by confocal microscopy as described above.

RNAi constructs

RNAi constructs were made in pHELLSGATE8 (Helliwell et al. 2002) using the following primers to amplify gene-specific fragments from genomic DNA: *FLX*, TCCTTTACGAATCTCT CCTCGAAG and TAATCGAATCCGATCGAAATCGAG, *FCA*, CAAAATCGGGCAGCTGGCCAG and CTGTTGTTTA CCAGCTCGGCC; *LD*, ATAACAAAGAACGCAGGAAAG TAC and CTGGTGGTACATGCCAATCAATC; *FRI*, ATGT CCAATTATCCACCGACG and ATCAACTCACATATACG TTCC; and *FVE*, GAAAATGGAGAGCGAGCAAGCAGC and CTGCAAGAGAGTGAAGGCCAG. Recombination reactions were carried out as described (Helliwell et al. 2002). Constructs were used to transform wild-type C24 and *flx* mutant plants. Flowering time of transformed plants (total leaf number at flowering) was measured for all T_1 plants selected.

Chromatin immunoprecipitation and real-time PCR

ChIP assays were performed according to Johnson et al. (2002). Antibodies recognizing acetylated histone H3 (K9 and K14), tetra-acetylated H4 (K5, K8, K12 and K16) and H3 trimethyl-K4 were purchased from Upstate Biotechnology, NY, USA. The amount of DNA precipitated in ChIP assays was quantified as described in Finnegan et al. (2005) using the primer pairs FLC2, ChUFCpc, At5g10130 and *SAM* (*S-ADENOSYL METHIONINE SYNTHASE*; At4g01850) that amplify across the transcription–translation start regions (Finnegan et al. 2005). The housekeeping gene, *SAM*, was used to normalize the amount of DNA precipitated in each sample (gene of interest/*SAM*), and then the ratio (gene of interest/*SAM*)^{mutant}/(gene of interest/*SAM*)$^{wild\ type}$ was determined. A ratio of 1 indicates no change from the control whereas ratios of <1 indicate a reduction in histone modification relative to the control. As the data are presented as the average of a ratio of ratios, they may not be distributed normally and so it is not appropriate to present either the standard deviation or standard error about the mean (Pfaffl 2002). The data presented are the average of at least three independent experiments. Significance was tested using the non-parametric Mann–Whitney U-test, which does not rely on the data being normally distributed (Pfaffl 2002).

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References

Blazquez, M.A. and Weigel, D. (1999) Independent regulation of flowering by phytochrome B and gibberellins in Arabidopsis. *Plant Physiol.* 120: 1025–1032.

Choi, K., Park, C., Lee, J., Oh, M., Noh, B. and Lee, I. (2007) *Arabidopsis* homologs of components of the SWR1 complex regulate flowering and plant development. *Development* 134: 1931–1941.

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.

Doyle, M.R., Bizzell, C.M., Keller, M.R., Michaels, S.D., Song, J.D., 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.

Finnegan, E.J., Kovac, K.A., Jaligot, E., Sheldon, C.C., Peacock, W.J. and Dennis, E.S. (2005) The down regulation of *FLOWERING LOCUS C* (*FLC*) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms. *Plant J*. 44: 420–432.

Finnegan, E.J., Sheldon, C.C., Jardinaud, F., Peacock, W.J. and Dennis, E.S. (2004) A cluster of *Arabidopsis* genes with a coordinate response to an environmental stimulus. *Curr. Biol*. 14: 911–916.

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.

Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) The small, versatile *pPZP* family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol*. 25: 989–994.

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.

Helliwell, C.A., Wesley, S.V., Wielopolska, A.J. and Waterhouse, P.M. (2002) High-throughput vectors for efficient gene silencing in plants. *Funct. Plant Biol*. 29: 1217–1225.

Helliwell, C.A., Wood, C.C., Robertson, M., Peacock, W.J. and Dennis, E.S. (2006) The *Arabidopsis* FLC protein interacts directly in vivo with *SOC1* and *FT* chromatin and is part of a high molecular weight complex. *Plant J*. 46: 183–192.

Hepworth, S.R., Valverde, F., Ravenscroft, D., Mouradov, A. and Coupland, G. (2002) Antagonistic regulation of flowering-time gene *SOC1* by CONSTANS and FLC via separate promoter motifs. *EMBO J*. 21: 4327–4337.

Hirano, T., Mitchison, T.J. and Swedlow, J.R. (1995) The SMC family: from chromosome condensation to dosage compensation. *Curr. Opin. Cell Biol*. 7: 329–336.

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.

Johnson, L.M., Cao, X. and Jacobsen, S.E. (2002) Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol*. 12: 1360–1367.

Kim, S., Choi, K., Park, C., Hwang, H.-J. and Lee, I. (2006) *SUPPRESSOR OF FRIGIDA4*, encoding a C2H2-type zinc finger protein, represses flowering by transcriptional activation of *Arabidopsis FLOWERING LOCUS C*. *Plant Cell* 18: 2985–2998.

Kim, S.Y. and Michaels, S.D. (2006) *SUPPRESSOR OF FRI 4* encodes a nuclear-localized protein that is required for delayed flowering in winter-annual *Arabidopsis*. *Development* 133: 4699–4707.

Koiwa, H., Barb, A.W., Xiong, L., Li, F., McCully, M.G., et al. (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate *Arabidopsis thaliana* abiotic stress signaling, growth, and development. *Proc. Natl Acad. Sci. USA* 99: 10893–10898.

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

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.

March-Díaz, R., García-Domínguez, M., Florencio, F.J. and Reyes, J.C. (2007) SEF, a new protein required for flowering repression in Arabidopsis, interacts with PIE1 and ARP6. *Plant Physiol*. 143: 893–901.

Marchler-Bauer, A. and Bryant, S.H. (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Res*. 32: 327–331.

Martin-Trillo, M., Lázaro, A., Poethig, R.S., Gómez-Mena, C., Piñeiro, M.A., Martinez-Zapater, J.M. and Jarillo, J.A. (2006) *EARLY IN SHORT DAYS 1* (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodeling complexes that positively regulates *FLC* accumulation in *Arabidopsis*. *Development* 133: 1241–1252.

Matsuoka, R., Beisel, K.W., Furutani, M., Arai, S. and Takao, A. (1991) Complete sequence of human cardiac alpha-myosin heavy chain gene and amino acid comparison to other myosins based on structural and functional differences. *Am. J. Med. Genet*. 41: 537–547.

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., 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., He, Y.H., 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., 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.

Mirzayan, C., Copeland, C.S. and Snyder, M. (1992) The *NUF1* gene encodes an essential coiled-coil related protein that is a potential component of the yeast nucleoskeleton. *J. Cell Biol*. 116: 1319–1332.

Ng, H.H., Robert, F., Young, R.A. and Struhl, K. (2003) Targeted recruitment of Set1 histone methylase by elongating PolII provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* 11: 709–719.

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.

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.

Pfaffl, M.W. (2002) Quantification strategies in real-time PCR. *In* A–Z of Quantitiative PCR. Edited by Bustin, S.A. pp. 1–23. International University Line, La Jolla, CA.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J. and Riechmann, J.L. (2003) Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. *Plant Cell* 15: 1159–1169.

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.

Sanda, S.L. and Amasino, R.M. (1996) Interaction of *FLC* and Late-Flowering Mutations in Arabidopsis thaliana. *Mol. Gen. Genet*. 251: 69–74.

Schlappi, M.R. (2006) *FRIGIDA LIKE 2* is a functional allele in Landsberg *erecta* and compensates for a nonsense allele of *FRIGIDA LIKE 1*. *Plant Physiol*. 142: 1728–1738.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D. and Lohmann, J.U. (2005) A gene expression map of Arabidopsis development. *Nature Genet*. 37: 501–506.

Schmitz, R.J., Hong, L., Micheals, S. and Amasino, R.A. (2005) *FRIGIDA-ESSENTIAL 1* interacts genetically with *FRIGIDA* and *FRIGIDA-LIKE 1* to promote the winter-annual habit of *Arabidopsis thaliana*. *Development* 132: 5471–5478.

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.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R.A. and Coupland, G. (2006) The transcription factor FLC

confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. *Genes Dev*. 20: 898–912.

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.

Sheldon, C.C., Conn, A.B., Dennis, E.S. and Peacock, W.J. (2002) Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. *Plant Cell* 14: 25–27.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J. and Dennis, E.S. (2000) The molecular basis of vernalization: the central role of *FLOWERING LOCUS C* (*FLC*). *Proc. Natl Acad. Sci. USA* 97: 3753–3758.

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.

Simpson, G.G. and Dean, C. (2002) *Arabidopsis*, the Rosetta stone of flowering time? *Science* 296: 285–289.

Sohlenkamp, C., Wood, C.C., Roeb, G.W. and Udvardi, M.K. (2002) Characterization of Arabidopsis AtAMT2, a high-affinity ammonium transporter of the plasma membrane. *Plant Physiol*. 130: 1788–1796.

Sung, S., Schmitz, R.J. and Amasino, R.A. (2006) A PHD finger protein involved in both the vernalization and photoperiod pathways in *Arabidopsis*. *Genes Dev*. 20: 3244–3248.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., et al. (2000) Activation tagging in Arabidopsis. *Plant Physiol*. 122: 1003–1014.

Zhang, H., Ransom, C., Ludwig, P. and Van Nocker, S. (2003) Genetic analysis of early flowering mutants in Arabidopsis defines a class of pleiotropic developmental regulator required for expression of the flowering-time switch Flowering Locus C. *Genetics* 164: 347–358.

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.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiol*. 136: 2621–2632.

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