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 OVEREXPRESSSION OF CONSTANS* (*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).

March-Díaz et al. 2007). Interaction data suggest that these proteins form complexes similar to SWR1 and Paflc (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 Paflc-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 ecotype C24 (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-polination, were early flowering and morphologically wild-type. When one of the early flowering plants was back-crossed to C24, the F1 progeny flowered at the same time as C24. In the F2 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...
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). T2 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.

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 gFLX::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.

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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, Scortecce 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 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 Pat1-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 Ler parent (Fig. 3B); nor did we find any difference in FLX expression levels between Col and a late flowering ColFRI 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 ColFRI 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 earl (Col, Ler) to intermediate (C24) and very late (Sf2, Pitza) 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 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).

![Figure 4](https://academic.oup.com/pcp/article-abstract/49/2/191/1923104/The-FLX-Gene-of-Arabidopsis-is-Required-for-FRI/fig4.png)
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 \( Arabidopsis \) PaflC-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 \( FLX \) 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 immunoprecipitation (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 T1 generation (data not shown). T2 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 T2 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 T1 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 *ColFRI* 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 Paflc-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 Paflc-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 pSK1015 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 T3 or T4, 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 emerged.

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 Ler background was obtained from Caroline Dean.

**Identification of FLX**

Genomic DNA isolated from the *flx* mutant was analyzed by Southern blot using a HindIII–EcoRI fragment from the LB of pSK1015 as a probe. LB TAIL-PCR using primers SKI1L, 1, 2 and 3 (5' ACAGCGATCCTATAATGTCG, 5' TTCAATTATGTAACGCCTCGGG and 5' GTTTTCTTTTTCCTCATATGTGCC, respectively) as described in Liu et al. (1995), and RB plasmid rescue with restriction enzymes EcoRI and HindIII 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 F2 individuals from an *flx x C24* backcross population were genotyped using primers ca144 (AGATGTCGAACAAATACAGC and ca145 (TTTAAACGATGAAATCAGTGC) to identify the wild-type allele, and SKC12 (Weigel et al. 2000) plus ca145 for the mutant. The implied ~40kb 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 ca145 (ATCTCTTGCTGCAGAATCCTGC, within At2g30210), and ca145 (between At2g30220 and At2g30230) and SKC12 (LB of pSK1015, 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 (CTTGTTACCACTTATTBACAACTTGTGG) and ca152 (GAGGGATCCACTTTTCCTTCAACGTGAAAC) in three independent amplification reactions, to obviate the need to verify sequence fidelity. These fragments were cloned as a Km–BanHI fragment into a binary vector, hyg200 [pPZP200 (Hadjukiewicz et al. 1994), modified to contain a 3SS-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). T2 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 (CGCAAAACCATTAGGCCGACAGATCGTTATATGACC) and ca191 (AGACCATGGGTACCTCATGAGTACCATTAGCC) 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 FLCal7 was replaced by ca218 (CTTGTAAGATCTGTGATAT); *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* (GAGGTCCTGCCCATG CTCC + TCTAATTGGGGAAGCAGG).

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 *ATS51030* were generated by PCR amplification from C24 first-strand cDNA using the following primer pairs: FLCT7 (tttttgacgegtaataagcactataagTTCACACAGAATTAGTCC) and ATS51030T7 (tttttgacgegtaataagcactataagTCAATAAGCCCGGTTATCGCCGCTCGGCT) and FLcomp (GATCAAATGTCAAATATGTGAGTATCG); and ATS51030 comp (CTTGGAGAAGTGGTATGCTCGACCAC) and ATS51030T comp (CATGTTAGAGTTTACTGCGACCAC). The *U6* riboprobe plasmid template was as described in Sheldon et al. (1999).

Real-time PCR analysis was carried out using FL primers (CGGTCCTCATGAGAAGCTC and CCACAAGCTTGCTAT CCACA) and FLX primers (TCGAGCTTACGGGTAAATTA TTGGGA and GAGCAGCCCCTCCTTCTTACGAG) normalized to formaldehyde dehydrogenase, At5g43900 (TGGGAAACCCAT TTTACACTTA and CAGGCAATCCAAACGTGCAG) or At4g264010 (GACGGTAGGCTTTCTCATGAC and GGTCCT CACATACCCCGG).

**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 (GACCTTCGCACTCTTTCCCAAGTGAAC) and ca191 (AGACCCAGATTCATCCTAGTATGAGTACAC) and PhiTurbo DNA Polymerase (Stratagene). This fragment was cloned as a *Pst*I–*Nco*I fragment into pA7-GFP (pA7-GFP is a Pst*I–Nco*I fragment into pA7-GFP [pA7-GFP is a pUC19 derivative with the GFP-containing cassette from the pJH-GFP (J. Harper, personal communication) from a pUC19 derivative with the GFP-containing cassette from the pJH-GFP].

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 FL2C, ChUFCpC, At5g10130 SAM and (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**


Regulation of flowering time through FLX


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