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EARLY FLOWERING 5 acts as a floral repressor in *Arabidopsis*

Yoo-Sun Noh[1,2,3], Colleen M. Bizzell[1], Bosl Noh[2,3], Fritz M. Schomburg[1] and Richard M. Amasino[1,*]
[1]*Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI 53706-1544, USA,*
[2]*Kumho Life & Environmental Science Laboratory, 1 Oryong-Dong, Puk-Gu, Kwangju 500-712, Korea, and*
[3]*Environmental Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea*

Received 14 January 2004; accepted 10 February 2004.
*For correspondence (fax +1 608 262 3453; e-mail amasino@biochem.wisc.edu).

Summary

***EARLY FLOWERING 5* (*ELF5*) is a single-copy gene involved in flowering time regulation in *Arabidopsis*. *ELF5* encodes a nuclear-targeted protein that is related to the human nuclear protein containing a WW domain (Npw)38-binding protein (NpwBP). Lesions in *ELF5* cause early flowering in both long days and short days. *elf5* mutations partially suppress the late flowering of both autonomous-pathway mutants and *FRIGIDA* (*FRI*)-containing lines by reducing the expression of *FLOWERING LOCUS C (FLC)*, a floral repressor upon which many of the flowering pathways converge. *elf5* mutations also partially suppress photoperiod-pathway mutants, and this, along with the ability of *elf5* mutations to cause early flowering in short days, indicates that *ELF5* also affects flowering independently of *FLC*.**

Keywords: *Arabidopsis*, autonomous, ELF5, FLC, flowering, photoperiod.

Introduction

The transition from vegetative growth to flowering is an important developmental switch in plants. Proper timing of flowering is important to ensure maximum reproductive success; thus, this switch is highly regulated in many species. In rapid-cycling accessions of *Arabidopsis thaliana*, three pathways have been identified that regulate this transition: the photoperiod, autonomous, and gibberellin (GA) pathways (for reviews, see Mouradov *et al.*, 2002; Simpson and Dean, 2002). The photoperiod pathway regulates flowering in response to daylength. *Arabidopsis* is a facultative long-day plant, and thus flowering is promoted in long days. Mutations in photoperiod-pathway genes (e.g. *constans* (*co*), *fd*, *fe*, *cryptochrome2* (*cry2*), *ft*, *fwa*, and *gigantea* (*gi*)) delay flowering in long days but have little effect on flowering in short days; in contrast, autonomous-pathway mutants (*fca*, *flowering locus D* (*fld*), *fpa*, *fve*, *fy*, and *luminidependens* (*ld*)) are delayed in flowering under all daylengths, but still respond to photoperiod by flowering earlier in long days than short days (e.g. Mouradov *et al.*, 2002; Simpson and Dean, 2002). The autonomous pathway may regulate flowering in response to internal signals such as developmental status (Simpson and Dean, 2002) or ambient temperatures (Blazquez *et al.*, 2003). The GA pathway is required for flowering in non-inductive short days. Mutations that reduce GA levels or GA perception (e.g. *ga1* or *gai*) are extremely delayed in flowering in short days (Wilson *et al.*, 1992).

In winter-annual accessions of *Arabidopsis*, an additional pathway, the vernalization pathway, also regulates the transition to flowering. Vernalization confers the competence to flower after an extended cold treatment, thus preventing premature flowering in the fall (Henderson *et al.*, 2003; Michaels and Amasino, 2000). The winter-annual habit is conferred by dominant alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). *FLC* is a MADS box transcription factor that represses flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *FRI* encodes a novel protein with two coiled-coil domains (Johanson *et al.*, 2000). The presence of *FRI* increases *FLC* expression to levels that delay flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Vernalization promotes flowering by overcoming the effect of *FRI* and repressing *FLC* expression.

A major role of the autonomous pathway is to repress *FLC*. In many rapid-cycling accessions of *Arabidopsis* that lack an active *FRI* allele, *FLC* expression is low and flowering

occurs rapidly without vernalization. In the absence of *FRI*, the autonomous pathway is sufficient to repress *FLC*. However, rapid-cycling accessions containing mutations in autonomous-pathway genes assume a winter-annual habit: they have elevated levels of *FLC* expression and delayed flowering that can be overcome by vernalization (Michaels and Amasino, 1999, 2001; Sheldon *et al*., 1999).

The flowering pathways discussed above have been largely defined by mutations that cause late flowering (Mouradov *et al*., 2002; Simpson and Dean, 2002). However, many other mutations that cause early flowering have been described (for review, see Sung *et al*., 2003). Some of these mutations appear to be in genes involved in one of the defined pathways. As circadian rhythms and photoperiod perception are linked, early flowering mutants that disrupt circadian rhythms generally affect the photoperiod pathway (e.g. *elf3*, *elf4*, and *timing of CAB expression 1* (*toc1*)). Recently, a MADS-box gene, *FLOWERING LOCUS M* (*FLM*), was also shown to interact most strongly with the photoperiod pathway (Scortecci *et al*., 2003). Other mutants, such as *early flowering in short days* (*efs*), *early in short days4* (*esd4*), and *photoperiod independent early flowering 1* (*pie1*), define genes involved in the regulation of the autonomous pathway. *PIE1* and *ESD4* are required for high levels of *FLC* expression in autonomous-pathway mutants. However, *pie1* and *esd4* mutations also appear to affect flowering in an *FLC*-independent manner (Noh and Amasino, 2003; Reeves *et al*., 2002).

There are many other early flowering mutations that define genes that do not fall into any of the described pathways. Instead, these genes appear to act more broadly, affecting multiple flowering and developmental pathways (Sung *et al*., 2003). Many of these mutations are in genes whose products are likely to be involved in chromatin modeling (e.g. *LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER 2* (*LHP1/TFL2*), *EMF1*, *EMF2*, *FIE*, *CLF*, and *EBS*; see Sung *et al*., 2003). Lesions in these chromatin-modeling genes often lead to the premature de-repression of genes involved in floral meristem identity and organ identity, such as *APETALA 1* (*AP1*) and *AGAMOUS* (*AG*), and altered expression of floral pathway integrators, such as *FT*. Thus, because these mutations affect genes downstream of the described flowering pathways, it is not surprising that they are epistatic to lesions in multiple flowering pathways.

Here, we report the identification of *EARLY FLOWERING 5* (*ELF5*), a gene that encodes a nuclear-targeted protein that is required for high levels of *FLC* expression. The *elf5* mutation causes early flowering in part through the reduction of *FLC* expression, but the mutation also appears to affect flowering through other pathways in addition to its effect on *FLC*. The predicted ELF5 protein is similar to nuclear protein containing a WW domain (Npw)38-binding protein (NpwBP), a human nuclear protein that is possibly involved in RNA binding.

Results

Identification and characterization of elf5

A population of the Wassilewskija (Ws) accession of *Arabidopsis* that had been mutagenized by *Agrobacterium*-mediated transformation using the pSKI015 vector (Weigel *et al*., 2000) was screened in short days for plants that flowered early. A recessive mutant, termed *elf5*, was identified. The early flowering of *elf5* appears to be caused by a T-DNA insertion because the F_2 generation of a cross to the wild type segregated 3 : 1, wild type to early flowering, and all early flowering plants contained the T-DNA (data not shown).

To identify the gene responsible for the observed phenotype, the insertion site of the T-DNA was determined by thermal asymmetrical interlaced (TAIL)-PCR (see Experimental procedures). The T-DNA was found to be inserted into the 3′ end of a gene on the bottom of chromosome V (At5g62640) that was annotated as an unknown protein (Figure 1a). Transformation with the genomic region of At5g62640 (pNA144 as delineated in Figure 1a) fully rescued the mutant phenotype. Thus, At5g62640 corresponds to the *ELF5* gene. Two additional T-DNA insertion alleles of *elf5* in the Ws background (*elf5-2* and *elf5-3*) were obtained from the Arabidopsis Knockout Facility (http://www.biotech.wisc.edu/Arabidopsis/), and three additional alleles in the Columbia (Col) background were obtained from the Salk Institute Genome Analysis Laboratory (SIGnAL) population: SALK_013050, SALK_025959, and SALK_040343, which are designated *elf5-4*, *elf5-5*, and *elf5-6*, respectively (http://signal.salk.edu). The lesions in these alleles are presented in Figure 1a.

elf5-1 was grown in a variety of daylengths and was found to flower earlier than the wild type in all conditions tested. In different trials, slight variations were seen in the leaf number of both wild-type and *elf5-1* mutants; however, *elf5-1* always flowered earlier than the wild type (Figure 2; Table 1). The most pronounced difference in flowering time between *elf5-1* and the wild type occurred in non-inductive photoperiods (Figure 2; Table 1). *elf5-1* retains a photoperiod response, although the difference in flowering time in inductive versus non-inductive photoperiods is much smaller than that for the wild type. The *elf5-1* phenotype is fully recessive in both long days and short days (data not shown), and no pleiotropic phenotypes were observed in any daylength examined.

elf5-2 and *elf5-3* were also found to flower early in both long days and short days. In short days, *elf5-2* flowered similarly to *elf5-1*, whereas *elf5-3* flowered slightly later (Table 1). The later flowering in *elf5-3* may be because of the more C-terminal site of the T-DNA insertion, which may create a weak allele. Indeed, *elf5-3* had detectable levels of *ELF5* mRNA, although the mRNA levels were greatly

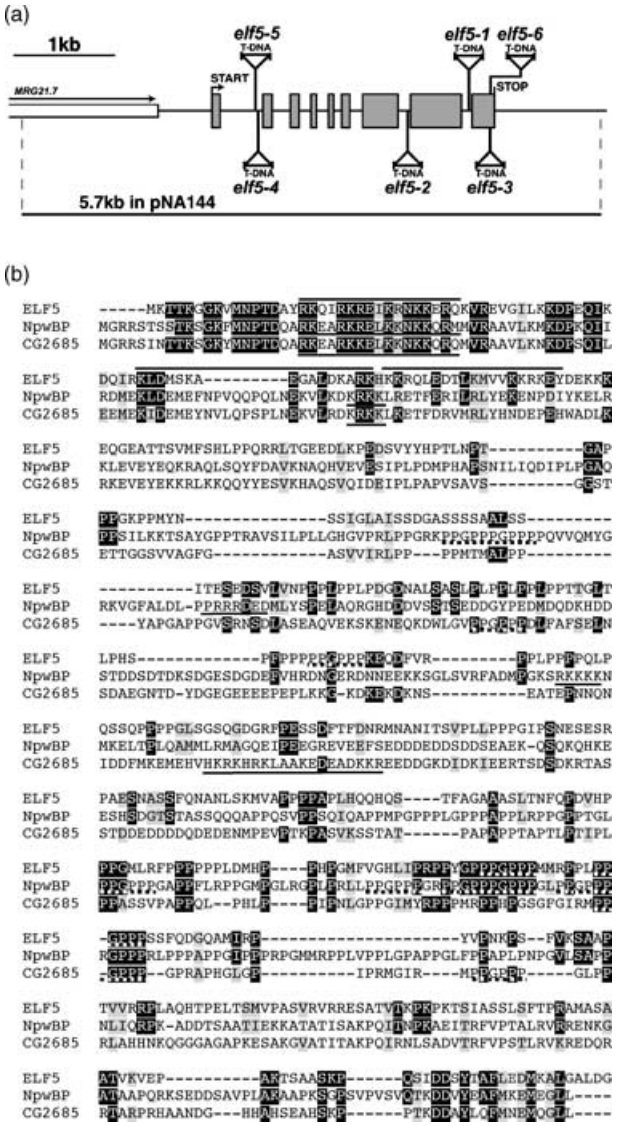

Figure 1. Genomic structure and relatives of ELF5
(a) Genomic arrangement of *ELF5*. The translation start and stop sites and the T-DNA insertion sites in the different *elf5* alleles are indicated. Gray boxes indicate translated exons, and lines indicate introns or intergenic sequences. The 5.7-kb genomic fragment in pNA144 used to rescue the *elf5-1* early flowering phenotype is indicated.
(b) Sequence comparison of ELF5 with human NpwBP and *Drosophila* CG2685. Putative nuclear localization signals are marked by bars, and PPGPPP motifs are marked by dots. GenBank Accession number for NpwBP is AAH01621 and for CG2685 is NP_570023.

reduced as compared to the wild type (data not shown), indicating that some C-terminally truncated protein may be produced in *elf5-3*.

The Col alleles of *elf5* also flowered earlier than the Col wild type in both long days and short days (Table 1; data from two independent trials are presented because the Col leaf number values varied among trials). The Col alleles of *elf5* are not as early as the Ws alleles in terms of percentage reduction in leaf number compared to the wild type. The difference between the behavior of *elf5* in Col and Ws is unlikely to be because of the specific *elf5* alleles that were studied because the strongest alleles in both ecotypes are likely to be null mutations. Instead, the difference may be because of a lesser effect of the loss of *elf5* in the Col accession.

ELF5 shows similarity to NpwBP

EARLY FLOWERING 5 is a single-copy gene with no obvious relatives in the *Arabidopsis* genome. The closest relatives of the predicted protein include an unknown protein from *Drosophila* (CG2685) and the human NpwBP (Figure 1b) as well as several vertebrate homologs of NpwBP. ELF5 and the *Drosophila* and vertebrate relatives have blocks of identity at the N-terminal region, in proline-rich regions in the middle, and in C-terminal parts of the protein. Little is known about the function of these proteins. NpwBP was identified as interacting with Npw38, a WW domain-containing transcriptional activator, and it has been proposed that NpwBP and Npw38 might function in RNA processing (Komuro *et al.*, 1999).

The interaction of NpwBP and Npw38 prompted us to investigate whether there is a homolog of Npw38 in *Arabidopsis* that may interact with ELF5. The *Arabidopsis* genome contains one predicted protein (At2g41020) that exhibits similarity to Npw38. At2g41020 contains a WW domain, which is the region of Npw38 that was found to interact with NpwBP (Komuro *et al.*, 1999). Two T-DNA insertion alleles of *At2g41020* in the Ws background were obtained from the Arabidopsis Knockout Facility (http://www.biotech.wisc/edu/Arabidopsis/). In contrast to the *elf5* mutants, the *At2g41020* mutants flowered identically to the wild type in both long days and short days. Thus, there is no evidence that *At2g41020* is involved in flowering as would be expected if the product of this single-copy gene interacted with ELF5 to regulate flowering in *Arabidopsis*.

Interaction with FRI *and* FLC

As many flowering pathways converge on the regulation of *FLC* expression, it was of interest to determine whether the *elf5* lesion affected *FLC* expression. The three *elf5* mutant alleles in Ws did not have detectable levels of *FLC* mRNA (Figure 3a). However, *FLC* expression in the wild-type Ws background is not very high. Thus, we determined whether *elf5* could suppress *FLC* expression in a background with elevated *FLC* levels. *FRI* is a dominant positive regulator of *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *elf5-1* was introduced into an *FRI* background by crossing the mutant with a Ws line into which an active *FRI* allele had been introgressed (see Experimental procedures). *FRI elf5-1* plants had detectable, but reduced, levels of *FLC*

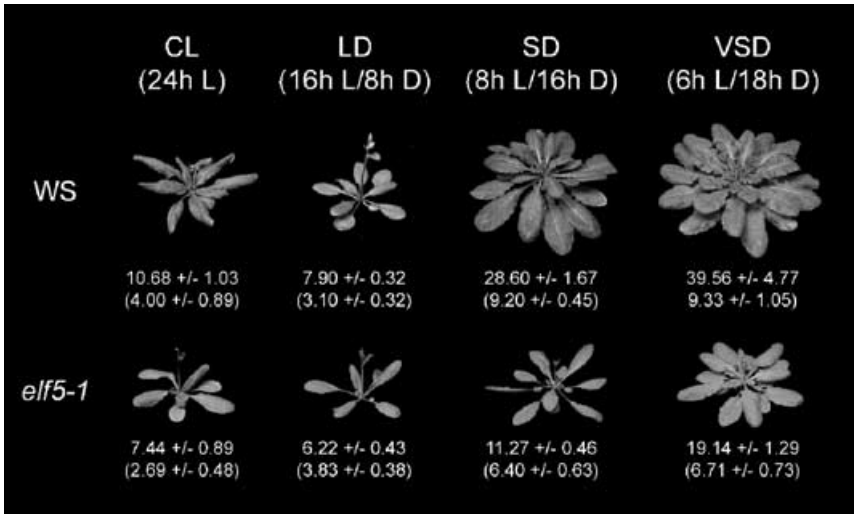

Figure 2. Early flowering of *elf5-1* compared with the wild type.
Wild type (Ws) and *elf5-1* plants were grown under continuous light (CL), long days (LD), short days (SD), or very short days (VSD). Photographs were taken when flowering initiated and the inflorescence stem began to elongate. Flowering behavior is presented as the number of rosette leaves followed by the number of cauline leaves (given in parentheses below). Both are ±1 SD.

expression compared to the control *FRI*-containing line (Figure 3a), indicating that *elf5* only partially reduced the increased expression of *FLC* caused by *FRI*. *FRI elf5-1* plants also flowered intermediate between the early flowering *fri elf5-1* plants and the late flowering *FRI ELF5* plants (Figure 4). This partial suppression of the *FRI* phenotype is consistent with the partial reduction of *FLC* expression levels.

To determine if *ELF5* might be regulated by *FLC*, the level of *ELF5* expression was examined in plants with and without active alleles of *FRI* and *FLC*. The presence of active *FRI* or *FLC* and the level of *FLC* expression had no effect on *ELF5* expression (Figure 3b), indicating *ELF5* is not regulated by *FLC*.

To determine if some component of the early flowering of *elf5* was independent of *FLC*, *elf5* mutants were compared to *flc* null mutants. For comparison with the Ws alleles of *elf5*, *flc-3* was introgressed from Col into the Ws background (see Experimental procedures). In short days, all three alleles of *elf5* flowered earlier than the *flc-3* mutant (Table 1), indicating that *elf5* has an *FLC*-independent component to its early flowering phenotype. The Col alleles of *elf5* however flowered similarly to the *flc* null (Table 1). As discussed above, *elf5* has a weaker phenotype in Col than in Ws; so, this genetic background difference in comparison to the *flc* null is not surprising. It is important to note, however, that the introgressed *flc-3* lesion has almost no effect on the flowering time of Ws, whereas it does have an effect on the flowering time of Col in short days (Table 1).

To further examine the relationship between *FLC* and *ELF5*, double mutants were made between *flc-3* in Col and *elf5-4*. *elf5 flc* double mutants flowered slightly earlier than either single mutant in both long days (Figure 4) and short days (data not shown), indicating that *flc* and *elf5* act additively, and that *elf5* has an *FLC*-independent component to its early flowering.

elf5 *in combination with other mutations affecting flowering time*

To further define the role of *elf5* in flowering, double mutants were made between *elf5-1* and alleles of the

Table 1 Leaf number at flowering of *elf5* mutants

	Long day (16 h light/8 h darkness)	Short day (8 h light/16 h darkness)	
		Experiment 1	Experiment 2
Ws	7.4 ± 0.5 (2.3 ± 0.5)	28.6 ± 1.7 (9.2 ± 0.5)	28.8 ± 1.6 (8.9 ± 0.8)
flc-3 WS	7.8 ± 0.8 (2.0 ± 0.0)	ND	27.1 ± 1.6 (8.8 ± 0.9)
elf5-1	5.6 ± 0.5 (2.8 ± 0.4)	11.3 ± 0.5 (6.4 ± 0.6)	17.3 ± 1.2 (7.0 ± 0.6)
elf5-2	6.1 ± 0.5 (3.2 ± 0.4)	11.7 ± 0.6 (6.8 ± 0.6)	17.3 ± 0.9 (8.3 ± 0.8)
elf5-3	6.1 ± 0.4 (3.0 ± 0.0)	17.5 ± 2.8 (6.9 ± 0.7)	23.8 ± 1.5 (8.5 ± 0.7)
Col	17.3 ± 1.4 (5.0 ± 0.9)	50.4 ± 2.9 (8.6 ± 1.6)	46.0 ± 2.5 (7.1 ± 1.2)
flc-3 Col	12.6 ± 1.3 (2.3 ± 0.5)	45.9 ± 3.6 (9.6 ± 0.9)	36.6 ± 3.2 (6.0 ± 1.1)
elf5-4	13.8 ± 1.2 (3.8 ± 0.6)	44.1 ± 2.5 (11.0 ± 1.1)	ND
elf5-5	11.8 ± 0.8 (3.0 ± 0.4)	ND	33.5 ± 0.7 (6.7 ± 0.6)
elf5-6	14.0 ± 0.8 (3.8 ± 0.5)	ND	35.0 ± 2.6 (8.3 ± 1.2)

Values shown are mean numbers ± 1 SD of rosette leaves and cauline leaves (in parentheses) at flowering. ND, not determined.

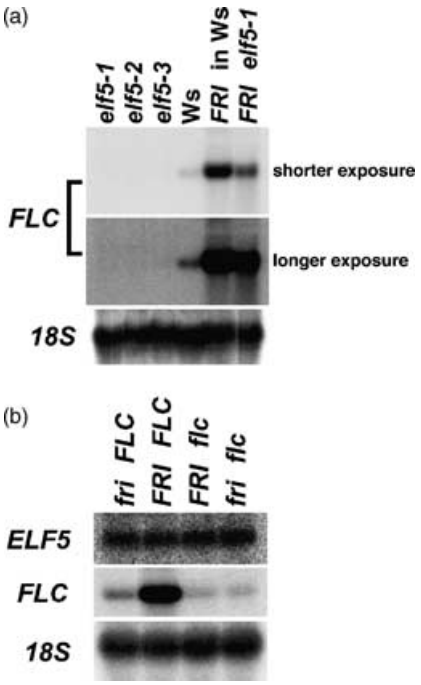

Figure 3. Effect of *elf5* mutation on *FLC* expression.
(a) The steady-state *FLC* mRNA level is reduced by *elf5* mutations. The blot was exposed at two different levels for visualization of *FLC* expression. The blot was probed first with *FLC* and then with 18S ribosomal DNA as a loading control.
(b) *ELF5* mRNA expression is not regulated by *FRI* or *FLC*. The blot was probed first with *ELF5*, then with *FLC*, and then with 18S ribosomal DNA as a loading control.
RNA was isolated from 10-day-old seedlings of each genotype grown under continuous light.

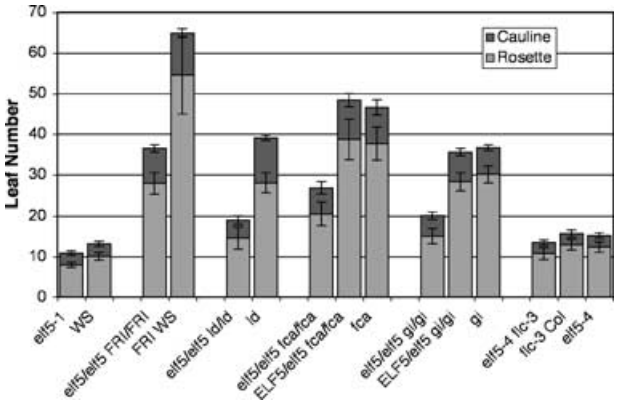

Figure 4. Suppression of flowering time mutants by *elf5*.
elf5-1 partially suppresses the late flowering of both autonomous and photoperiod-pathway mutants as well as *FRI*. *elf5-4 flc-3* double mutants flower earlier than either single mutant. Rosette leaf numbers are indicated in light gray; cauline leaf numbers are in dark gray. Bars represent ±1 SD. *elf5-4*, *flc-3*, and the *elf5-4 flc-3* double are in a Col background; all other mutants are in a Ws background. All plants were grown under long days.

late-flowering mutants *gi*, *ld*, and *fca*, which are in the same Ws genetic background as *elf5-1*. *GI* is in the photoperiod pathway, whereas *LD* and *FCA* are in the autonomous pathway. In order to examine plants that were both homozygous and heterozygous for *elf5-1*, the F_3 progeny of F_2 plants that were homozygous for the late-flowering mutation and heterozygous for *elf5* were scored for flowering time. In all cases, the double mutants flowered with a leaf number intermediate between *elf5* and the late-flowering mutant (Figure 4). Plants heterozygous for *elf5-1* in the *gi-13* and *fca-11* mutant backgrounds were carefully examined, but no heterozygous phenotype was observed, i.e. the *elf5* heterozygotes flowered at the same time as the late-flowering mutant (Figure 4), confirming the fully recessive nature of *elf5*.

elf5 has a phenotype similar to that of *pie1* (Noh and Amasino, 2003); thus, it was of interest to evaluate the phenotype of the *elf5 pie1* double mutant. The *elf5-1 pie1-1* double mutant flowered earlier (11.6 ± 1.6 rosette leaves) than either single mutant (*elf5-1* flowered with 18.3 ± 0.9 rosette leaves and *pie1-1* flowered with 16.6 ± 1.9 rosette leaves in the short-day conditions in which the double mutant flowered with 11.6 leaves), indicating these two genes are likely to act independently in the control of flowering.

Expression pattern and nuclear localization of ELF5

The spatial expression pattern of *ELF5* was examined using a fusion of the *ELF5* upstream and coding region to the reporter gene *β-glucuronidase* (*GUS*; Figure 5a). This construct was capable of rescuing the *elf5* mutant phenotype, indicating that it is functional (data not shown). In seedlings, expression was highest in the shoot apical meristem and root tip (Figure 5b), an expression pattern that is similar to that of *FLC* and other autonomous-pathway genes (e.g. Aukerman *et al.*, 1999; Macknight *et al.*, 2002; Michaels and Amasino, 2001; Schomburg *et al.*, 2001). Furthermore, this functional fusion protein was localized to the nucleus (Figure 5c), which is consistent with the presence of predicted nuclear-localization signals (Figure 1b).

ELF5 *overexpression*

An *ELF5*-overexpression (OE) construct was introduced into the *elf5-1* mutant background. The early flowering phenotype of *elf5-1* was rescued in 14 of 18 first generation transgenic (T_1) lines grown in short days. Three of these rescued lines (*ELF5OE1*, *ELF5OE2*, and *ELF5OE3*) were further characterized. All three lines showed elevated levels of *ELF5* expression as compared to wild-type Ws (Figure 6a). However, the flowering time of these three lines was not significantly different from that of the wild type in either long days (data not shown) or short days

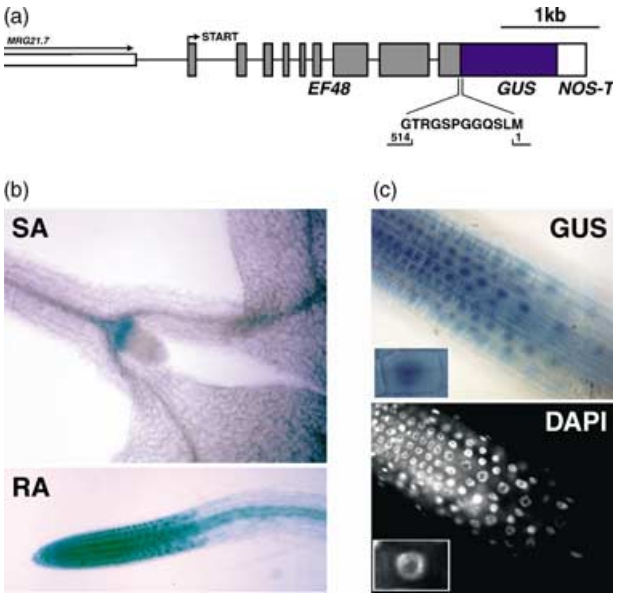

Figure 5. Spatial expression pattern and nuclear localization of ELF5.
(a) Schematic representation of the *ELF5:GUS* translational fusion construct. The translated ELF5 exons are indicated by gray boxes. Lines indicate introns or intergenic sequences. The linker sequence between the last amino acid (G) of ELF5 and the first amino acid (M) of GUS is shown. Numbers indicate amino acid positions in ELF5 or GUS.
(b, c) Histochemical GUS staining of 5-day-old transgenic *Arabidopsis* containing the *ELF5:GUS* fusion grown under continuous light. (b) Preferential expression of the *ELF5:GUS* in the shoot apical meristem region and the root tip. (c) Nuclear localization of the ELF5:GUS in the cells of the root distal elongation region. The root tissue was also stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The tissue was examined with a fluorescence microscope at 400× magnification. Inset images of single cells were further magnified digitally.

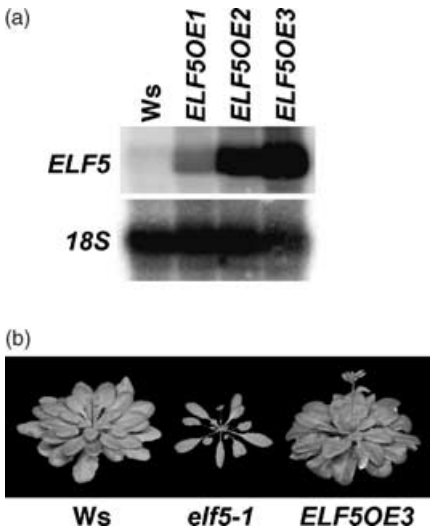

Figure 6. Overexpression of *ELF5* does not delay flowering.
(a) *ELF5* expression in three *ELF5* overexpression lines in the *elf5-1* background (*ELF5OE1*, *ELF5OE2*, and *ELF5OE3*). RNA was isolated from 10-day-old seedlings grown under continuous light. The blot was probed first with *ELF5* and then re-probed with 18S ribosomal DNA as a loading control.
(b) *ELF5* overexpression rescues the *elf5* early flowering phenotype but does not cause late flowering. Representative plants of Ws, *elf5-1*, and *ELF5OE3* in the *elf5-1* background grown in short days are shown. Photographs were taken at the initiation of flowering.

(Figure 6b). Therefore, the overexpression of *ELF5* rescues the *elf5* early flowering phenotype but does not delay flowering. This indicates that *ELF5* expression may not be limiting in wild-type *Arabidopsis*.

Discussion

We have identified *ELF5* as a repressor of flowering. Like several other genes that cause early flowering when mutated, *ELF5* does not fall clearly into any of the established flowering pathways. The *elf5* lesion partially suppresses the late flowering of both autonomous and photoperiod-pathway mutants as well as the late flowering caused by *FRI*. Thus, *elf5* mutations appear to cause a general promotion of flowering. Part of this promotion involves reducing *FLC* expression levels; thus, wild-type *ELF5* gene product appears to be required for high levels of *FLC* expression. In addition, in Ws, *elf5* mutants flower earlier than *flc* null mutants, and double mutants between *elf5* and *flc-3* in Col are earlier flowering than either single mutant, indicating that in both accessions, *elf5* must act, in part, in an *FLC*-independent manner. Moreover, *flc* mutations in Col were shown not to suppress the late flowering of *gi* (Michaels and Amasino, 2001); thus, the partial suppression of *gi* by *elf5* is likely to occur through an *FLC*-independent mechanism.

Mutations in *elf5* cause a stronger promotion of flowering in Ws than in Col. In all conditions tested, *elf5* in Ws flowered earlier than *elf5* in Col based on a percentage reduction of leaf number compared to the wild type. Thus, the pathway that *ELF5* is involved in is more active in repressing flowering in Ws than in Col under the conditions examined. The reason for this difference is not yet known.

Overexpression of *ELF5* does not delay flowering, indicating that ELF5 activity is not limiting for flowering. The lack of an overexpression phenotype is consistent with ELF5 acting as part of a protein complex for which the levels of other components are limiting. ELF5 is similar to the human NpwBP. NpwBP has two proline-rich regions that are capable of binding the WW domain of Npw38 *in vitro* (Komuro *et al.*, 1999). The WW domain preferentially binds to a PPGPPP motif that has a nearby arginine residue (Komuro *et al.*, 1999). To see if these interactions were conserved in *Arabidopsis*, we searched for a homolog of Npw38. A single gene, *At2g41020*, was found to encode a predicted protein that was similar to Npw38 and contained a WW domain. Mutations in this gene, however, did not cause early flowering. Thus, ELF5 probably does not act with At2g41020 to affect flowering. However, there are many other WW domain-containing proteins predicted in

the *Arabidopsis* genome, and it is possible that one of these other proteins may interact with ELF5.

Recently, a protein interaction map was created for *Drosophila* (Giot *et al.*, 2003). The *Drosophila* homolog of ELF5, CG2685, was found to interact *in vitro* with another protein, CG4887, that does not contain a WW domain but may be involved in RNA binding (http://portal.curagen.com/cgi-bin/interaction/flyHome.pl). Thus, another possibility is that ELF5 may interact with a protein like CG4887. Of course, in plants, ELF5 may have evolved a different mode of action that involves unique binding partners. Nevertheless, it is interesting to note that both known partners of ELF5-like proteins in animals, Npw38 and CG4887, have been proposed to be involved in RNA binding. Thus, if ELF5 has a similar activity, it may act in a complex that regulates other flowering-time genes, such as *FLC*, at the RNA level.

Experimental procedures

Plant materials and growth conditions

The three *elf5* alleles in Ws (*elf5-1*, *elf5-2*, and *elf5-3*) and the two *At2g41020* alleles were isolated from the BASTA population of the Arabidopsis Knockout Facility (http://www.biotech.wisc.edu/Arabidopsis/). *elf5* alleles in Col (*elf5-4*, *elf5-5*, and *elf5-6*) were isolated from the Salk collection (http://signal.salk.edu/). *elf5-2*, *elf5-3* and the two *At2g41020* alleles were isolated by a PCR-based reverse genetic approach (Krysan *et al.*, 1996). *FRI* in Ws was obtained by introgressing *FRI* into Ws through six backcrosses from *FRI* in Col (Lee and Amasino, 1995). *flc-3* in Ws was obtained by introgressing *flc-3* into Ws through six backcrosses from *flc-3* in Col (Michaels and Amasino, 1999). *FRI elf5* was generated by crossing *elf5-1* to *FRI* in Ws. The following mutants are in the Ws background: *ld-2* (Lee *et al.*, 1994), *gi-13*, and *fca-11*. All plants were grown under approximately 100 μE m^{-2} sec^{-1} cool-white fluorescent light at 22°C. Long days consisted of 16 h of light followed by 8 h of darkness; short days consisted of 8 h of light followed by 16 h of darkness; very short days consisted of 6 h of light followed by 18 h of darkness.

T-DNA flanking sequence analyses

The sequence flanking the T-DNA of *elf5-1* was obtained by TAIL-PCR (Liu *et al.*, 1995) as described by Schomburg *et al.* (2003). The T-DNA borders of *elf5* alleles 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 web pages listed above for the T-DNA populations.

RNA gel blot analyses

Total RNA was isolated using TRI Reagent (Sigma St Louis, MO, USA) according to the manufacturer's instructions. For RNA gel blot analysis, 40 μg of total RNA was separated by denaturing formaldehyde–agarose gel electrophoresis as described by Sambrook *et al.* (1989). The *FLC* probe was a cDNA fragment lacking the conserved MADS domain sequence. The *ELF5* probe was a 352-bp cDNA fragment obtained by PCR using BAC clone MRG21-2 (5′-CATATGTACCAAACAAACCATCTTTTGTA-3′) and ELF5-3 (5′-AACTTTGTTTGAAGACTACAACTCTAAAGT-3′) as primers. Blots also were probed with 18S rDNA as a control for the quantity of RNA loaded.

Sequence analyses

Genes were predicted with GENSCAN (Burge and Karlin, 1997). Protein sequences were analyzed with SMART (Schultz *et al.*, 1998), PSORT (Nakai and Kanehisa, 1992), and BLAST (Altschul *et al.*, 1997) searches. Protein sequence alignments were generated using CLUSTALW (Thompson *et al.*, 1994). The *ELF5* and *At2g41020* cDNAs were cloned as described previously by Noh and Amasino (2003) using ELF5-5 (5′-GGAGGGAGATTGAAGAACGGTAAACGGAAA-3′) and ELF5-3 (5′-AACTTTGTTTGAAGACTACAACTCTAAAGT-3′) or T3K95END (5′-GGTTGACGCTGAACTAAATAATGGGAGAAG-3′) and T3K9KO-2 (5′-ACAAAGCACAAGAAGACACGGTAACCAACT-3′) as primers, respectively. The sequence was determined with Big-Dye reaction mix (Amersham) using an ABI automated sequencer (Applied Biosystems, Foster City, CA, USA).

Genetic characterization

Double mutants between *elf5-1* and *ld-2* or *FRI* and between *elf5-4* and *flc-3* were selected from F_2 populations using molecular markers for the mutant alleles. Double mutants between *elf5-1* and *gi-13* or *fca-11* were obtained by identifying F_2 plants that were heterozygous for *elf5* (based on molecular markers) and were late flowering and thus homozygous for either *gi-13* or *fca-11*. The F_3 progeny of these F_2 plants provided a population that segregated for *elf5* in the late-flowering background. The relationship between flowering and the *elf5* genotype was determined in the F_3 population by genotyping for *elf5*. Several independent F_3 populations were analyzed for each double mutant. The *ELF5* genotype was determined with the primers MRG21-1 (5′-CTATTGCATTTCTACTTTAGCTTATGGTT-3′) and MRG21-2, which gives a PCR product for wild-type *ELF5* allele and does not amplify the *elf5-1* allele. The presence of the *elf5-1* allele was determined by the primers MRG21-1 and the T-DNA primer JL202 (http://www.biotech.wisc.edu/Arabidopsis/), which gives a PCR product for the mutant allele but not for wild-type allele. The presence of the *elf5-4* allele was determined at the same time as for wild-type allele using the primers 5′-CTGTGAGCAAATCCTAAATCGGTG-3′, 5′-TTGTCCAAAGCACCTTCCGC-3′ and SALK_LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′). The *ld-2* allele was determined as described by Scortecci *et al.* (2003). The *FRI* allele was determined using the primers 5′-AGATTTGCTGGATTTGATAAGG-3′ and 5′-GAAATTCACCGAGTGAGAACAGA-3′, which produces a larger fragment for *FRI* and a smaller fragment for *fri*. The presence of the *flc-3* and *FLC* alleles was determined using the primers FLC24 (5′-CGTATCGTAGGGGAGGAAAGATAG-3′) and FLC33 (5′-CTCATGTATCTATCATGGTCGCAG-3′), which produces a larger PCR product for *FLC* and a smaller one for *flc-3*.

Histochemical GUS assays

The *ELF5:GUS* translational fusion construct was generated by PCR amplification of 4.6 kb of the *ELF5* genomic DNA fragment using MRG21GUS-1 (5′-GGC**GCATGC**GGGGTAGAGTTTGTCAGTTGCTATTCCTAT-3′) and MRG21GUS-2 (5′-CCG**ACTAGT**TCCATCAAGTGCGCCAAGAGCTTTCATGTC-3′) as primers; restriction sites are shown in boldface, and sequences corresponding to

the *ELF5* genomic DNA are underlined. The resulting PCR product was digested with *Sph*I–*Spe*I (*Sph*I sites were blunted with T_4 DNA polymerase (New England Biolabs, Beverly, MA, USA) after digestion) and ligated to pPZP211-GUS (Noh and Amasino, 2003) that had been digested with *Pst*I–*Xba*I (*Pst*I sites were blunted with T_4 DNA polymerase after digestion), resulting in pNA164. *Arabidopsis* (Accession Ws) plants were transformed with pNA164-containing *Agrobacterium tumefaciens* strain ABI by infiltration (Clough and Bent, 1998). Transgenic lines were selected as described by Noh and Amasino (2003). Staining for GUS activity was performed as described by Schomburg *et al*. (2001).

Complementation of elf5

For the molecular complementation of the *elf5* mutant, a 5.7-kb genomic DNA fragment containing the *ELF5* upstream, coding, and downstream region (Figure 1) was amplified by PCR using MRG21C-1 (5′-GGC**GTCGAC**GGGGTAGAGTTTGTCAGTTGCTAT-3′) and MRG21C-2 (5′-CCG**GAGCTC**CAAGATAAAGTTTGTATAT-TATAT-3′) as primers; restriction sites are shown in boldface, and sequences corresponding to the *ELF5* genomic DNA are underlined. The resulting PCR product was digested with *Sac*I–*Sal*I and ligated to pPZP211 (Hajdukiewicz *et al*., 1994), which had been digested with *Sac*I–*Sal*I, resulting in pNA144. *elf5-1* mutant plants were transformed with pNA144-containing *A. tumefaciens* strain ABI by infiltration (Clough and Bent, 1998). Transgenic lines were selected and tested in short days for flowering time.

ELF5 *overexpression*

For the overexpression of *ELF5*, a 3.85-kb genomic DNA fragment containing the *ELF5* coding and downstream regions was amplified by PCR using MRG2135S-1 (5′-GGC**ACTAGT**ATGAAGACGAC-GAAGGGAGGCAAA-3′) and MRG21C-2 (5′-CCG**GAGCTC**CAAGA-TAAAGTTTGTATATTATAT-3′) as primers; restriction sites are shown in boldface, and sequences corresponding to the *ELF5* genomic DNA are underlined. The resulting PCR product was digested with *Sac*I–*Spe*I and ligated to pPZP211-GUS (Noh and Amasino, 2003), which had been digested with *Sac*I–*Xba*I, resulting in pNA143 that has a transcriptional fusion between the cauliflower mosaic virus 35S promoter and *ELF5*. Transformation of *elf5-1* plants with pNA143-containing *Agrobacterium* and selection of transgenic plants were performed as described above.

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