LETTER

FLOWERING LOCUS C Influences the Timing of Shoot Maturation in Arabidopsis thaliana

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Many plant species undergo changes in leaf morphology as the vegetative shoot meristem matures (Poethig, 2003; Telfer et al., 1997). In Arabidopsis, juvenile leaves are produced before shoot maturation and are relatively round and lack trichomes on the abaxial side (underside) of the leaves. Adult leaves formed after the shoot meristem matures are more elliptical and have abaxial trichomes. An important difference between the juvenile-vegetative and adult-vegetative phases is that only during the adult-vegetative phase can the meristem become competent to respond to environmental and endogenous signals that promote flowering (Telfer et al., 1997).

Genetic backgrounds and environmental conditions that delay flowering also delay shoot maturation (Telfer et al., 1997). For example, both flowering and shoot maturation occur later in the Columbia accession (Col) than in the Landsberg errecta accession (Ler) (Lee et al., 1993; Telfer et al., 1997). An important difference between these two accessions occurs at the FLC locus. FLC levels are slightly elevated in Col compared to Ler, which has a weak allele of FLC (Michaels et al., 2003). FLC encodes a MADS-domain transcription factor that acts as a repressor of flowering (Michaels and Amasino, 1999a; Sheldon et al., 1999). Flowering is delayed under conditions or in genetic backgrounds in which FLC expression is elevated (Michaels and Amasino, 1999a). The observation that shoot maturation is delayed in Col relative to Ler suggests that FLC influences the rate of shoot maturation. However, there are many genetic differences between Col and Ler making it difficult to determine whether FLC expression is solely responsible for the delay in shoot maturation observed in Col.

To further explore the potential role of FLC in the regulation of shoot maturation, we performed experiments in the Col background using mutations that eliminate FLC function, as well as mutations in known regulators of FLC. This allowed us to create a series of lines containing various levels of FLC expression that could be used to determine if there is a correlation between FLC expression and the timing of shoot maturation. The appearance of abaxial trichomes was used to determine when the transition from juvenile to adult leaves occurred (Telfer et al., 1997). The first leaf with four or more abaxial trichomes was scored as the first adult-vegetative leaf. Leaves were photographed at the time of trichome counting, and those images confirm that the appearance of abaxial trichomes corresponds to leaf shape changes associated with shoot maturation in Arabidopsis (Fig. 1) (Telfer et al., 1997). The number of leaves produced before flowering was also recorded for each genotype and treatment (Table 1).

In the Col background, shoot maturation occurred earlier in an flc null mutant (flc-3) $_{\text{than}}$ in wild type (t-test, $P < 0.0001$) (Table 1, Fig. 2a). Thus, FLC acts to delay shoot maturation, and a reduction in FLC expression is sufficient to promote shoot maturation.

To test whether increased FLC expression would further delay shoot maturation, we examined the timing of shoot maturation in late-flowering autonomous pathway mutants that have elevated levels of FLC expression (Michaels et al., 2003). Previous work in the Ler background has shown that shoot maturation is slightly delayed by autonomous pathway mutants (Telfer et al., 1997). In the Ler background, lacking a strong allele of FLC, autonomous-pathway mutants are known to have little effect on flowering time (Michaels et al., 2003). Therefore, we examined the effect of autonomous-pathway mutants in Col. FCA is part of the autonomous pathway, and loss of function of FCA delays flowering (Sheldon et al., 2000). Shoot maturation was delayed in the presence of an fca null allele (fca-9) relative to wild type (t-test, $P < 0.0001$) (Table 1, Fig. 2a). In the flc-3 background, fca-9 did not influence the

timing of shoot maturation (flc-3, FCA was indistinguishable from flc-3, fca -9) (t-test, P = 0.373) indicating that the delay induced by fca was FLC-dependent. Loss-of-function of a different gene in the autonomous pathway, LUMENIDEPENDENS (LD) did not influence shoot maturation. The null allele ld-4 had no influence on shoot maturation in either the FLC or flc-3 background (Table 1, Fig. 2a). It is interesting that while both fca-9 and ld-4 delay flowering to a similar degree, only fca-9 delays shoot maturation. It is known that FLC is upregulated in both fca and ld backgrounds (Michaels and Amasino, 1999a; Sheldon et al., 2000). Perhaps, FLC upregulation in ld occurs too late in shoot development to influence the transition from juvenile to adult leaves but early enough to influence flowering.

A dominant allele of FRIGIDA (FRI) promotes FLC expression causing a delay in flowering (Michaels and Amasino, 2001). Common laboratory strains of Arabidopsis thaliana are relatively early flowering because they lack strong alleles of FLC or FRI or both (Gazzani et al., 2003; Michaels and Amasino, 2001; Michaels et al., 2003). Shoot maturation was delayed in Col plants that had been introgressed with a late-flowering allele of FRI (t-test, P < 0.0001) (Table 1, Fig. 2b). Vernalization, an extended exposure to cold, is known to promote flowering in many species. One way that vernalization promotes flowering is by inducing epigenetic silencing of FLC (Michaels and Amasino, 2001; Sheldon et al., 2000; Sung and Amasino, 2004). To determine the effect of vernalization on shoot maturation, FLC plants with and without a late-flowering allele of FRI were cold-treated for 0, 20, or 60 days. In the absence of a late-flowering allele of FRI, vernalization did not influence the rate of shoot maturation (Fig. 2b). Shoot maturation occurred earlier following vernalization in plants with a late-flowering allele of FRI and 20 days of cold was sufficient to saturate this response. Following 20 days of vernalization FLC, FRI was indistinguishable from FLC, fri (t test, P = 0.35) (Table 1, Fig. 2b). Interestingly, 20 days of cold was not sufficient to eliminate the effect of FRI on the transition to flowering (Table 1).

In summary, loss of FLC function is associated with early shoot maturation, while some genetic backgrounds (FRI, fca) in which FLC levels are elevated show delayed shoot maturation. Vernalization, which induces silencing of FLC, promotes earlier shoot maturation in a line with elevated FLC expression. Together, these observations support a role for FLC as an inhibitor of shoot matura-tion. The only observation not consistent with this model is that loss of function of LD, known to result in upregulation of FLC, does not delay shoot maturation.

FLC does not represent the first identified regulatory overlap between shoot maturation and flowering. In Arabi-dopsis, plants deficient for biosynthesis of gibberellic acid (GA) have delayed flowering (Michaels and Amasino, 1999b). One such mutant line, ga-1, displays a delay in shoot maturation. Application of exogenous GA acceler-ates shoot maturation (Telfer et al., 1997). Photoperiod influences the timing of flowering and shoot maturation. Arabidopsis is a facultative long-day (LD) plant, and flower-ing is delayed under short-day (SD) conditions. LD photo-periods promote shoot maturation, whereas SD photoper-iods have the opposite effect (Telfer et al., 1997). Together, these observations suggest that considerable regulatory overlap exists between shoot maturation and flowering.

All genotypes examined were in the Col background. The mutant alleles examined were ld-4, fca-9, and flc-3 (Michaels and Amasino, 2001). Col introgressed with the late-flowering allele of FRI from the Sf-2 background was also examined (Lee et al., 1994).

Seeds were germinated on solidified media containing 0.4% Dyna-Gro 7-9-5 fertilizer (Dyna-Gro Co., San Pablo, CA), 0.05% MES (pH 5.8), and 6% phytagel. For vernaliza-tion, seeds were distributed on the surface of the solidi-fied media and allowed to imbibe for 6 h after which they were incubated in the dark at 40C for 20 or 60 days. Non-vernalized controls were incubated at 40C for 48 h to pro-mote synchronous germination. Seeds were allowed to germinate under LD conditions (16 h of light and 8 h of darkness) and were then transplanted to soil (Scotts redi-earth plug and seedling mix) and grown in LD conditions (described above). The first nine leaves were removed and a dissecting microscope was used to count abaxial tri-chomes. At this point, the first nine leaves were photo-graphed. At least five plants

from each test group were allowed to flower, and the number of primary leaves pro-duced before flowering was determined.

All statistical conclusions were determined through t-tests in Minitab 15.1.20 (Minitab Inc., State College, PA, USA).

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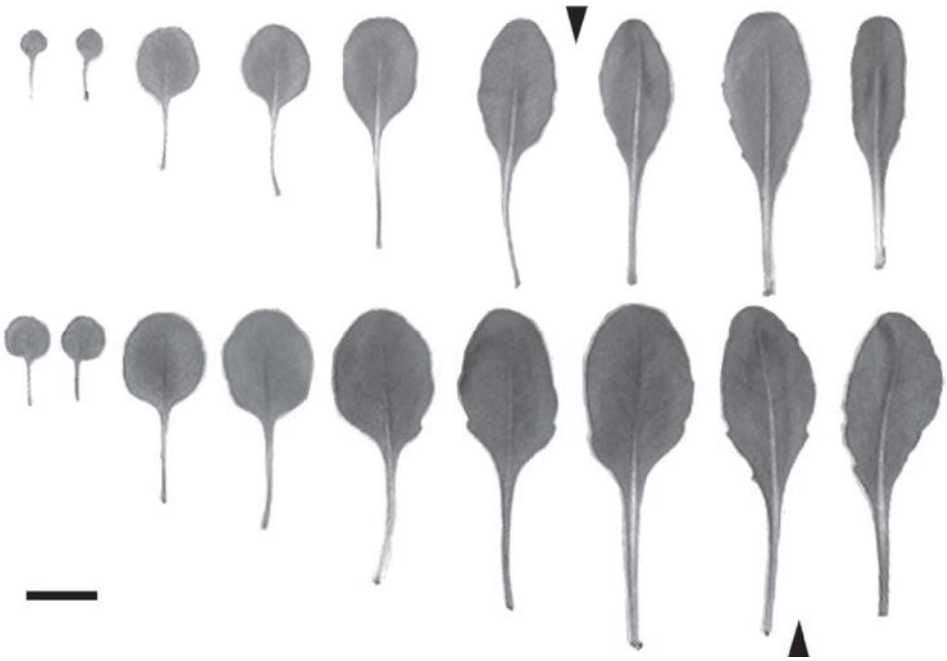

FIG. 1. Juvenile and adult leaves of Arabidopsis. The first nine true leaves of the Col accession of Arabidopsis are shown from two genetic backgrounds (FLC, fri (top row) and FLC, FRI (bottom row)). The arrows indicate the transition from juvenile to adult leaves based on the appearance of >4 abaxial trichomes. The appearance of >4 abaxial trichomes roughly correlates with changes in leaf shape representative of the juvenile and adult vegetative phases. Bar represents 1 cm.

Table 1
Leaf Number at the Vegetative Phase Change and at Flowering

	Vernalization		
	0 Days	20 Days	60 Days
FLC	**8.7 ± 0.8 (36)**		
	13.6 ± 1.0 (11)		
flc-3	**6.8 ± 0.7 (34)**		
	8.9 ± 0.7 (30)		
FLC, fca-9	**10.4 ± 1.5 (22)**		
	>60 (6)		
flc-3, fca-9	**6.9 ± 0.7 (36)**		
	11.0 ± 0.7 (30)		
FLC, ld-4	**8.8 ± 1.0 (26)**		
	>60 (6)		
flc-3, ld-4	**7.1 ± 0.8 (34)**		
	8.9 ± 0.8 (34)		
FLC, fri	**6.9 ± 0.6 (32)**	**7.2 ± 0.6 (32)**	**7.0 ± 0.6 (26)**
	9.3 ± 0.7 (10)	9.4 ± 0.9 (11)	8.2 ± 0.9 (10)
FLC, FRI	**8.4 ± 0.9 (31)**	**7.2 ± 0.7 (36)**	**6.9 ± 0.8 (30)**
	>60 (5)	17.8 ± 6.7 (6)	8.0 ± 3.2 (6)

For each genetic background and vernalization treatment, the mean first leaf with >4 abaxial trichomes is shown in bold accompanied by standard deviation (sample size is shown in parentheses). The mean number of leaves produced prior to flowering is shown in plain text. For extremely late-flowering plants, it was difficult to obtain an accurate leaf count; therefore, leaf number was not determined for plants that produced > 60 leaves prior to flowering. Vernalization treatments involved incubating imbibed seeds at 4°C for 0, 20, or 60 days.

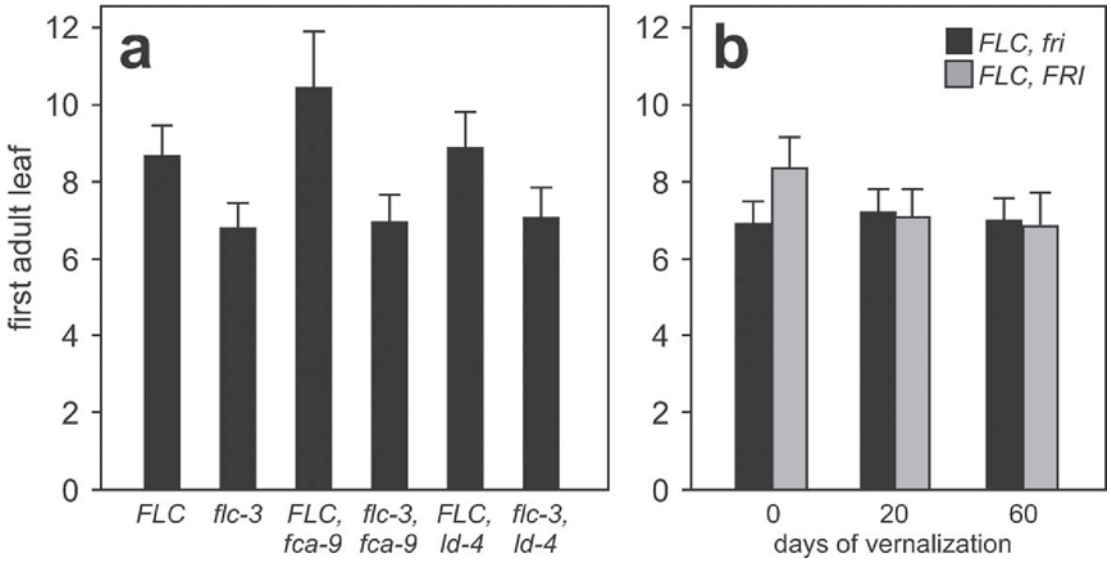

FIG.2. Appearance of first adult leaf. (a) Appearance of the first adult leaf in several genetic backgrounds. (b) Appearance of the first adult leaf in the FLC, fri background (black bars) or FLC, FRI background (grey bars) that had been exposed to 4°C for 0, 20 or 60 days. Error bars represent the standard deviation of the mean. Sample sizes for each genetic background and treatment are shown in Table 1.