

The effect of the floral repressor *FLC* on the timing and progression of vegetative phase change in *Arabidopsis*

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SUMMARY

Plants undergo two major post-embryonic developmental transitions – the juvenile-to-adult vegetative transition (vegetative phase change) and the adult-to-reproductive transition (flowering). In woody plants, these transitions can be separated by years, but in herbaceous species they are often very close together, making it difficult to differentiate the effects of vegetative phase change and floral induction on vegetative development. To distinguish between these factors, we have compared the vegetative morphology of plants highly expressing the floral repressor *FLC* (*FRI;FLC*) with plants mutant for this gene (*FRI;flc-3*) under both photoinductive (long day, LD and night interruption, NI) and non-photoinductive (short day, SD) conditions. We show that the onset of abaxial trichome production is insensitive to floral induction, but the distribution and overall number of abaxial trichomes, as well as several other leaf traits associated with vegetative change, are strongly influenced by flowering. Most of the major differences in leaf morphology between *FRI;FLC* and *FRI;flc-3* plants grown in LD can be attributed to the early flowering phenotype of *FRI;flc-3*, because these differences are not apparent in plants grown in SD. These include differences in leaf size, hydathode number and the distribution of abaxial trichomes along the length of the leaf. Leaf shape and the total number of abaxial trichomes are affected by *FLC* independently of its effect on flowering. Our results demonstrate that the onset and the progression of vegetative phase change are regulated by different combinations of endogenous and environmental factors, and reveal a role for *FLC* in vegetative development.

KEY WORDS: FLC, Flowering, Shoot development, Vegetative phase change

INTRODUCTION

The sexual life cycle of both plants and animals involves several changes in state during which the organism is transformed from a somatically immature, sexually incompetent individual, to a somatically mature, sexually reproductive adult. In flowering plants, post-embryonic life is characterized by at least three distinct phases of shoot development – a reproductively incompetent juvenile vegetative phase, a reproductively competent adult vegetative phase and a reproductive phase (Poethig, 2003). Each of these stages produces different types of leaves, buds and internodes. In angiosperms, the adult-to-reproductive transition (floral induction) is marked by a change in the architecture of the shoot and the appearance of flowers. The juvenile-to-adult transition (vegetative phase change) usually involves changes in a variety of species-specific traits, which can include leaf shape, the presence of trichomes and thorns, the production of phytochemicals, leaf retention, internode length, and disease and pest resistance (Doorenbos, 1965; Hackett, 1985; Kerstetter and Poethig, 1998; Schaffalitzky de Muckadell, 1954). In addition to these phase-specific characteristics, there are gradual changes that are related to the physiological aging of the shoot, but which are sometimes influenced by the juvenile-to-adult transition (Bond, 2000; Wareing, 1959).

Maize and *Arabidopsis* have proven to be particularly useful genetic models for studying the regulation of vegetative phase change. In maize, the juvenile and adult phases differ in wax and

trichome production, cuticle thickness, cell wall biochemistry, internode length and axillary bud identity (Poethig, 2003). In *Arabidopsis*, juvenile leaves are flat and round to orbicular in shape, with a smooth margin, trichomes restricted to the adaxial surface and a long petiole. Adult leaves are curled, spatulate and serrated, with trichomes on both the adaxial and abaxial surfaces and a shorter petiole (Chien and Sussex, 1996; Röbbelen, 1957; Telfer et al., 1997). The presence of trichomes on the abaxial surface of the leaf is often used as a marker for the adult phase in *Arabidopsis* because it is a qualitative trait that is easy to score. Traits such as the overall number of serrations (Röbbelen, 1957), trichomes (Martinez-Zapater et al., 1995) and hydathodes (Tsukaya et al., 2000), the size of the petiole and leaf blade, the length-to-width ratio of the leaf blade, vascular complexity (Cookson et al., 2007; Steynen et al., 2001) and cell size (Cookson et al., 2007; Usami et al., 2009) vary continuously, but have also been used to study vegetative phase change.

The relationship between vegetative phase change and flowering in *Arabidopsis* is still unresolved. This issue is of fundamental interest, and also has important practical consequences. There is considerable evidence that reproductive competence is associated with the adult vegetative phase of the shoot in woody plants (Zimmerman et al., 1985), and this also appears to be the case in *Arabidopsis* (Telfer et al., 1997; Weigel and Nilsson, 1995). This observation raises the issue of whether genes involved in floral induction play a role in vegetative phase change, as has previously been proposed (Schultz and Haughn, 1993; Steynen et al., 2001). From a practical standpoint, the fact that most of the accessions used in *Arabidopsis* genetics are extremely early flowering suggests that some of the traits used to study vegetative phase change in this species may be influenced by floral induction, and, therefore, reflect the reproductive state of the shoot, not its vegetative phase.

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It has been known for some time that factors that affect flowering time can also affect the expression of phase-specific vegetative traits. This is well known in the case of gibberellin, which delays or accelerates both vegetative phase change and flowering in a number of species (Evans and Poethig, 1995; Zimmerman et al., 1985), including *Arabidopsis* (Chien and Sussex, 1996; Telfer et al., 1997). Photoperiod also has a significant effect on both of these transitions (Chien and Sussex, 1996; Steynen et al., 2001; Telfer et al., 1997). Chien and Sussex observed that the sensitivity of abaxial trichome production and flowering to photoperiod was age dependent and occurred only within a specific window of time (Chien and Sussex, 1996). Interestingly, the window for the photoperiodic induction of abaxial trichomes was 3 days earlier than the window for flowering, suggesting that, although these transitions may be regulated by common factors, their timing is separable (Chien and Sussex, 1996). This is also evident from the phenotypes of three classes of mutations – those affecting the timing of vegetative phase change but not flowering (Berardini et al., 2001; Hunter et al., 2003), those affecting the timing of flowering but not vegetative phase change (Gomez-Mena et al. 2001; Michaels et al., 2003a; Soppe et al., 1999), and those affecting both transitions (Chien and Sussex, 1996; Martinez-Zapater et al., 1995; Telfer et al. 1997). This latter class includes loss-of-function mutations in members of the autonomous (*FPA*, *FCA*, *FVE*), photoperiodic (*CO*, *GI*) and GA pathways (Chien and Sussex, 1996; Martinez-Zapater et al., 1995; Telfer et al., 1997). In addition, it was recently discovered that *miR156* and its targets – which are important regulators of vegetative phase change – also affect flowering time and floral morphogenesis (Cardon et al., 1997; Shikata et al., 2009; Wang et al., 2009; Wu and Poethig, 2006; Yamaguchi et al., 2009).

The transcription factor *FLC* has also been implicated in the control of both flowering time and vegetative phase change. Plants with high *FLC* activity are late flowering because *FLC* directly represses the expression of the floral inducers *FT* and *SOC1* (Helliwell et al., 2006; Hepworth et al., 2002; Searle et al., 2006). *FLC* is positively regulated by *FRI*, and much of the natural variation in flowering time in *Arabidopsis* is attributable to polymorphisms within the genes encoding these proteins (reviewed in Amasino, 2010). Late-flowering accessions often have functional alleles of both genes, whereas early flowering ones typically possess loss-of-function alleles of either *FRI* or *FLC* (Gazzani et al., 2003; Johanson et al., 2000; Lempe et al., 2005; Michaels et al., 2003b; Shindo et al., 2005). For example, Columbia (Col) has a wild-type allele of *FLC*, but is early flowering because it has a loss-of-function mutation in *FRI* (i.e. its genotype is *fri;FLC*). *FRI;FLC* plants can be induced to flower early by prolonged exposure to cold temperature, which leads to epigenetic modifications that block its transcription (reviewed by Sung and Amasino, 2005). In addition to their late flowering phenotype, plants with high levels of *FLC* have been reported to have a prolonged juvenile phase (Lee et al., 2000; Martinez-Zapater et al., 1995; Telfer et al., 1997) and to display enhanced germination at cool, but not warm temperatures (Chiang et al., 2009).

Here, we present a detailed morphological analysis of the growth and development of *FRI-Sf-2;FLC* (hereafter *FRI;FLC*) and a mutant derivative of this line, *FRI-Sf-2;flc-3* (*FRI;flc-3*) (Lee et al., 1993; Michaels and Amasino, 2001), demonstrating the wide-ranging effects of flowering and *FLC* on vegetative development in *Arabidopsis*. In addition to the effect of floral induction on the final size and shape of pre-existing leaves, this event has a

significant effect on trichome production – a key marker of vegetative phase change in *Arabidopsis*. We show that *FLC* has both flowering-dependent, as well as flowering-independent effects on vegetative phase change and leaf morphology, and that some of its effects are dependent on light intensity. These results demonstrate some of the complex interactions that influence the vegetative morphology of *Arabidopsis*, and reveal a new function for *FLC* in vegetative development.

MATERIALS AND METHODS

Plant materials

All seed stocks were in the Columbia (Col) accession. Seeds of *FRI;FLC* (Col with an introgressed *FRI* allele from Sf-2; Lee et al., 1993), *FRI;flc-3*, *35S::FLC* (Michaels and Amasino, 1999), *fpa-7;FLC* and *fpa-7;flc-3* (Michaels and Amasino, 2001) were provided by Rick Amasino (University of Wisconsin, Madison, WI) and Scott Michaels (Indiana University, Bloomington, IN); *ft-1* (Kardailsky et al., 1999; Kobayashi et al., 1999; Moon et al., 2005), *soc1-2* (Lee et al., 2000), *ft-1;soc1-2* and *ft-1;soc1-2;flc-3* (Moon et al., 2005) were obtained from Ilha Lee (Seoul University, Seoul, South Korea); *LFY::GUS* (Blázquez et al., 1997) was obtained from Detlef Weigel (Max-Planck Institute, Germany); and *ft-10* (GabiKat-290E08) (Yoo et al., 2005) was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). The hydathode GFP marker E325 (<http://enhancertraps.bio.upenn.edu>) and *LFY::GUS* were introduced into *FRI;FLC* and *FRI;flc-3* by crossing.

Plant growth conditions

Plants were sown in Fafard #2 soil in 96-well flats. The flats were placed at 4°C for 2 days before being put in a Conviron growth chamber at a constant 23°C under a one-to-one ratio of T8 Sylvania Octron 4100K Ecologic and GroLite WS fluorescent lamps (Interlectric). Plant age was measured from the date flats were transferred to the growth chamber. The photoperiods and light intensities used for the various experiments were as follows: long days=16 hour light (125 $\mu\text{mol m}^{-2} \text{s}^{-1}$):8 hour dark; short days=8 hours light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$):16 hours dark; night interruption=6 hours light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$):8 hours dark:2 hours light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$):8 hours dark; low light intensity=16 hours light (75 $\mu\text{mol m}^{-2} \text{s}^{-1}$):8 hours dark. Low-light conditions were created by using multiple layers of Miracloth to shield the light bank; measurements taken with a LabSpec VNIR 512 spectroradiometer (Analytical Spectral Devices, Boulder, CO) revealed that this treatment had no effect on the light spectrum.

Phenotypic analyses

The onset of abaxial trichome production was measured as the presence of at least one trichome on the abaxial leaf surface; typically, this trichome was located at the base of the midrib. The juvenile-to-adult transition was considered complete when there was at least one trichome within 2 mm of the distal end of the leaf blade. Juvenile leaves were defined as rosette leaves without abaxial trichomes, transition leaves as rosette leaves with abaxial trichomes that did not fully span the proximodistal axis, and adult leaves as rosette leaves with abaxial trichomes to the distal tip. To minimize the effect of stochastic variation in these traits, at least one of the two subsequent leaves on the shoot had to meet the same criterion to be recorded. Hydathodes were observed using the GFP enhancer trap line E325. Leaf shape was recorded by attaching leaves to paper with double-sided tape, flattening them with strips of single-sided tape, and then photocopying the sheet; photocopies were scanned into a computer for subsequent image analysis. Leaf primordia were observed using plants expressing *LFY::GUS* and stained according to Donnelly et al. (Donnelly et al., 1999). To determine the number of visible leaves, only the leaves that could be viewed without the aid of a microscope were counted. To determine the point of full expansion, leaves were measured daily until the average daily increase in total leaf length was less than 1 mm on each of two consecutive days, with the second day called the point of full expansion. The length-to-width ratio of the leaf blade is the ratio of the distance from the blade:petiole junction to the distal leaf tip and the width of the blade at the midpoint of this line. Leaf length is the length of the blade plus the petiole. To determine palisade cell area, the outlines of at

least 60 cells in six water-infiltrated leaf samples were traced using a camera lucida and scanned into the computer. The cross-sectional cell area was measured using Photoshop.

RESULTS

The progression but not the onset of vegetative phase change is delayed by FLC under long-day conditions

Non-vernalized *FRI;FLC* plants grown under long day conditions (LD) are very late flowering (Lee et al., 1993) (Fig. 1A). Previous studies have reported that *FRI;FLC* and other genotypes with elevated *FLC* expression also have delayed vegetative phase

change, as indicated by the onset of abaxial trichome production (Lee et al., 2000; Martinez-Zapater et al., 1995; Telfer et al., 1997). However, we observed no difference in the timing of abaxial trichome production between *FRI;FLC* and *FRI;flc-3* plants (Fig. 1A).

During vegetative phase change, *Arabidopsis* produces a series of transition leaves in which abaxial trichomes do not completely span the proximodistal axis of the leaf but are found in progressively more distal regions. We noticed that, although the onset of abaxial trichome production was identical in *FRI;FLC* and *FRI;flc-3*, their distal progression seemed to be slower in *FRI;FLC*. To characterize this phenotype, we recorded the position of the first

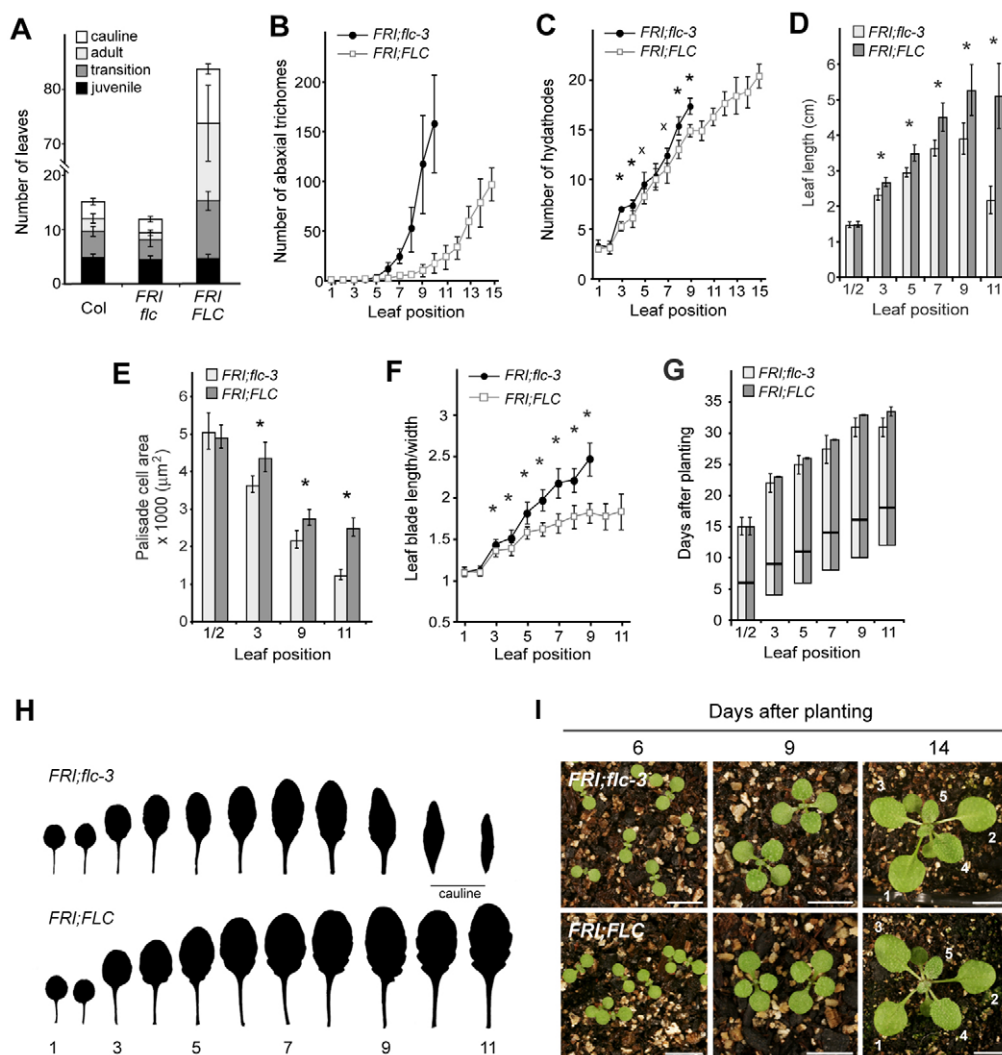


Fig. 1. *FRI;FLC* delays the progression but not the timing of vegetative phase change. All data are from long-day-grown plants; under these conditions, leaves 10–12 in *FRI;flc-3* are cauline leaves. (A) The number of rosette leaves lacking abaxial trichomes (juvenile), partially covered with abaxial trichomes (transition) and completely covered with abaxial trichomes (adult), and the number of cauline leaves in Col (*fri;FLC*), *FRI;FLC* and *FRI;flc-3* plants. There is no significant difference in the number of juvenile leaves in these genotypes. (B) The temporal increase in the total number of abaxial trichomes on rosette leaves is slower in *FRI;FLC*. (C) The temporal increase in the number of hydathodes is slower for *FRI;FLC*. (D) Starting with leaf 3, the length of the leaf blade is shorter in *FRI;flc-3* than in *FRI;FLC*. (E) Starting with leaf 3, palisade cells are larger in *FRI;FLC* than in *FRI;flc-3*. (F) Starting with leaf 3, the rosette leaves of *FRI;FLC* are rounder than those of *FRI;flc-3*. (G) The rate of leaf initiation and the duration of leaf growth do not differ significantly in *FRI;flc-3* and *FRI;FLC*. The bottom of each bar shows the average time from leaf initiation to full expansion. The black line within each bar indicates when the leaf primordium was 1 mm in length. The results are the average of two experiments. (H) The morphology of the first 11 leaves of *FRI;flc-3* and *FRI;FLC*, arranged from left to right in order of initiation. (I) Rosette morphology of *FRI;flc-3* and *FRI;FLC*. Scale bars: 0.5 cm. Significantly different (Student's *t*-test): * $P \leq 0.01$, $^x P \leq 0.05$. Results are mean \pm s.d.

leaf that was completely covered with abaxial trichomes, i.e. the first fully adult leaf, allowing us to calculate the number of transition and adult leaves. We found that *FRI;FLC* makes significantly more transition leaves than *FRI;flc-3* ($P < 0.0001$, Student's *t*-test) (Fig. 1A), suggesting that *FLC* delays the progression of the juvenile-to-adult transition.

Abaxial trichome distribution is only one of several traits that change during vegetative development in *Arabidopsis*. To determine whether *FLC* has a global effect on vegetative phase change, we examined several of these traits in *FRI;FLC* and *FRI;flc-3* plants. The spread of abaxial trichomes towards the distal end of the leaf is associated with an increase in their total number (Chien and Sussex, 1996). The rate of this increase was much lower in *FRI;FLC* than in *FRI;flc-3* (Fig. 1B). The number of hydathodes also increases in successive leaves within the rosette (Hunter et al., 2003; Tsukaya et al., 2000). Hydathode number increased at approximately the same rate in *FRI;FLC* and *FRI;flc-3*; however, *FRI;FLC* leaves had slightly fewer hydathodes than *FRI;flc-3* leaves (Fig. 1C), even though they were larger overall (Fig. 1D,H). Cell size has long been known to decrease in successively higher leaves on the shoot (Ashby, 1948), and this is also true in early flowering genotypes of *Arabidopsis* (Usami et al., 2009). Palisade cell size declined gradually with leaf position in both *FRI;FLC* and *FRI;flc-3*, but – with the exception of leaf 1 – cell size was consistently larger in *FRI;FLC* than *FRI;flc-3* (Fig. 1E). Vegetative phase change is also associated with a change in leaf shape (Fig. 1H), which can be measured by the length-to-width ratio of the leaf blade (Steynen et al., 2001). Although young rosettes of *FRI;flc-3* and *FRI;FLC* look grossly similar to one another (Fig. 1I), the length:width ratio of rosette leaves revealed that all but the first two leaves of *FRI;FLC* are significantly rounder than those of *FRI;flc-3*, and this ratio increases more slowly in *FRI;FLC* than in *FRI;flc-3* (Fig. 1F,H).

We have previously hypothesized that transition leaves arise from the imposition of a new cell fate on incompletely determined organs present on the shoot apex when phase change occurs (Orkiszewski and Poethig, 2000). According to this hypothesis, one way in which *FRI;FLC* could extend the transition zone is by increasing the number of incompletely determined leaf primordia at the shoot apex. This would occur if *FRI;FLC* had a faster rate of leaf initiation than *FRI;flc-3* or if leaf development was prolonged in *FRI;FLC* relative to *FRI;flc-3*. To address this issue, we measured the rate of leaf initiation and the duration of leaf expansion at six different positions on the shoot in plants grown in LD. Leaves 1 and 2 were examined together because they are initiated at the same time in the embryo. We determined when each of these leaves was initiated by crossing the *LFY::GUS* transcriptional reporter into both genotypes (Blázquez et al., 1997). *LFY::GUS* is expressed in very young leaf primordia, disappearing around the time a primordium is 1.0 mm long (Blázquez et al., 1997) (data not shown). By counting leaf primordia with, and without, GUS activity in rosettes harvested at different times, we obtained a rough estimate of the growth rate of these young primordia (Fig. 1G). We also recorded the date at which leaves were 1.0 mm in length, and the date at which they reached full expansion. As shown in Fig. 1G, we found that the rate of leaf initiation and the rate of primordium expansion was the same in both genotypes for all leaf positions considered. Thus, the difference in the length of the juvenile-to-adult transition in *FRI;flc-3* and *FRI;FLC* is not related to the rate of leaf initiation or the growth rate of the primordia in these genotypes.

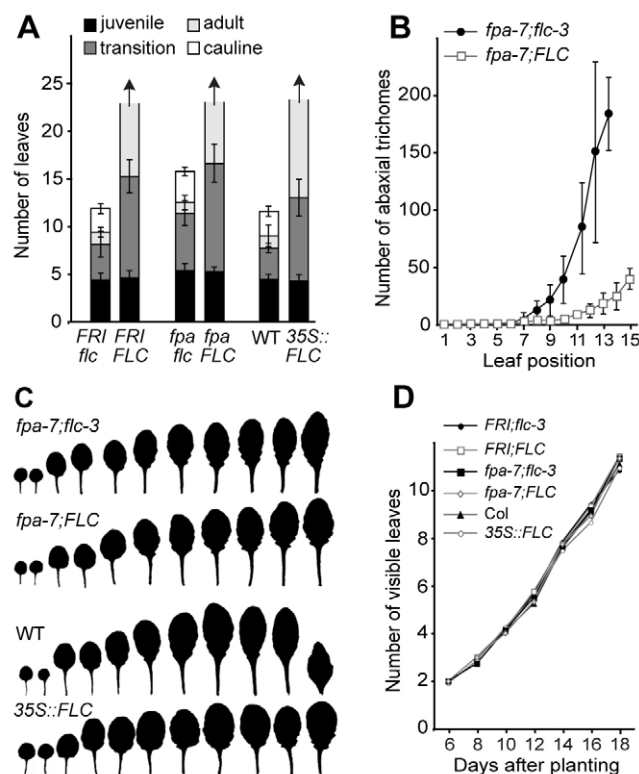


Fig. 2. High *FLC* expression delays the progression of vegetative phase change. (A) The number of rosette leaves lacking abaxial trichomes (juvenile), partially covered with abaxial trichomes (transition) and completely covered with abaxial trichomes (adult), and the number of cauline leaves in genotypes with low (*FRI;flc-3*, *fpa-7;flc-3*, WT) and high (*FRI;FLC*, *fpa-7;FLC*, *35S::FLC*) levels of *FLC*, grown in LD. There is no significant difference in the number of juvenile leaves in these genotypes. (B) The increase in the total number of abaxial trichomes in successive leaves is slower in *fpa-7;FLC* than in *fpa-7;flc-3*. (C) *fpa-7;FLC* and *35S::FLC* have rounder leaves than their respective controls. (D) The rate of leaf emergence was the same in all genotypes. Results are mean \pm s.d.

To further explore the effects of *FLC* on vegetative development, we examined the phenotypes of the late flowering genotype *fpa-7;FLC*, which has elevated levels of *FLC* (Rouse et al., 2002), and Col plants expressing *FLC* under the regulation of the constitutive CaMV 35S promoter (*35S::FLC*). The phenotype of *fpa-7;FLC* was compared with *fpa-7;flc-3* to control for any effects of *fpa-7* that are independent of *FLC*. There was no significant difference in the onset of abaxial trichome production between these high *FLC* genotypes and the low *FLC* controls (Fig. 2A); however, like *FRI;FLC*, both genotypes had an increased number of transition leaves (Fig. 2A), a slower increase in abaxial trichome abundance (Fig. 2B), and rounder leaves (Fig. 2C) than plants with low levels of *FLC*. These differences were not attributable to differences in the rate of leaf initiation between genotypes (Fig. 2D). Thus, *FLC* has a role in vegetative development in addition to its well-known effect on flowering time.

Flowering-dependent and flowering-independent effects of *FLC* on vegetative development

As mentioned above, *FRI;FLC* plants flowered after making over 70 leaves, nearly two months after planting. By contrast, *FRI;flc-3* plants were florally induced by 11 or 12 days after planting, as

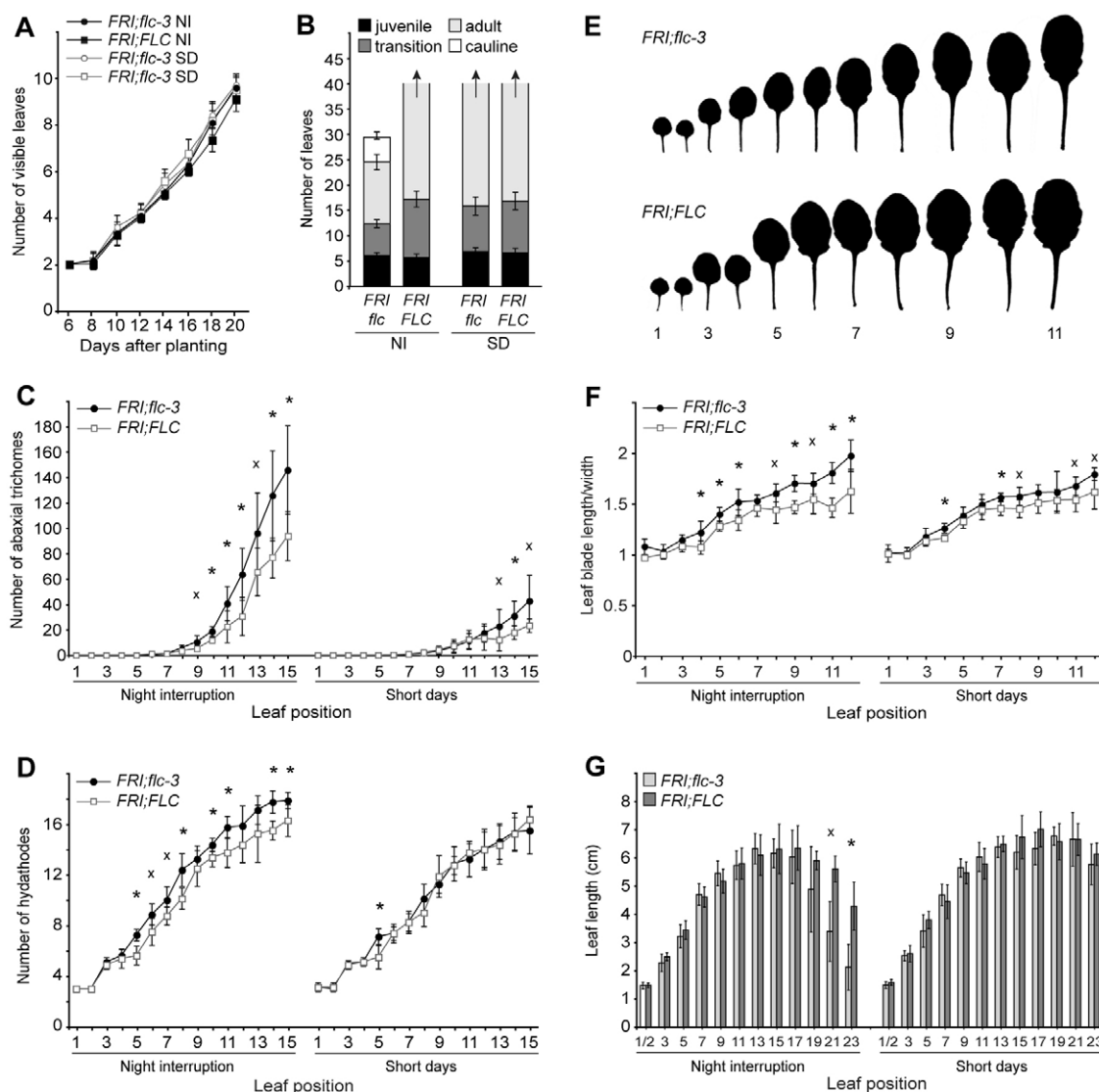


Fig. 3. The phenotypes of *FRI;FLC* and *FRI;flc-3* under floral-inductive (night interruption, NI) and non-floral-inductive (short day, SD) conditions. (A) *FRI;flc-3* and *FRI;FLC* have the same rate of leaf initiation under SD and NI conditions. (B) The number of rosette leaves lacking abaxial trichomes (juvenile), partially covered with abaxial trichomes (transition) and completely covered with abaxial trichomes (adult), and the number of cauline leaves in *FRI;FLC* and *FRI;flc-3* plants. The number of juvenile leaves is increased in SD by one node in both genotypes (Student's *t*-test, $P \leq 0.05$), but there is no significant difference between genotypes in either condition. Arrows designate continued leaf production. (C) SD reduces abaxial trichome production in both *FRI;flc-3* and *FRI;FLC*, but does not eliminate the difference between genotypes. (D) SD eliminates the difference in hydathode numbers between *FRI;flc-3* and *FRI;FLC*. (E) Morphology of the first eleven rosette leaves of *FRI;flc-3* and *FRI;FLC* grown under SD. (F) SD reduces the leaf blade length-to-width ratio in *FRI;flc-3*, but does not eliminate the leaf shape difference between *FRI;flc-3* and *FRI;FLC*. (G) The expansion of the last few rosette leaves of *FRI;flc-3* is inhibited by flowering. Significantly different (Student's *t*-test): * $P \leq 0.01$, $\times P \leq 0.05$. Results are mean \pm s.d.

evident from a change in the morphology of the shoot apical meristem and the fact that they produced no additional leaves after this time (Fig. 1G). At this stage, leaves 7 and 9 (the last rosette leaf) were less than 1.0 mm in length, and none of the other rosette leaves were fully expanded. Thus, all of the leaves of this early flowering genotype completed their development after the shoot had been induced to flower. This observation suggests that many of the differences in the vegetative morphology of *FRI;FLC* and *FLC;flc-3* might arise from the effect of floral induction on leaf development.

To test this hypothesis, we compared the phenotypes of *FRI;FLC* and *FRI;flc-3* plants grown under short days (SD), and SD with a 2-hour night interruption (NI); this relatively brief light

exposure induces flowering in *FRI;flc-3* (albeit less strongly: 24.5 rosette leaves in NI versus 9.4 in LD), without affecting the total amount of light received by plants in these treatments. The phenotypic differences between *FRI;FLC* and *FRI;flc-3* in the NI treatment reflect the direct effects of *FLC* on vegetative development, as well as the effect of the difference in the flowering time of these genotypes. Differences attributable to flowering should disappear in SD because *FRI;FLC* and *FRI;flc-3* are both late flowering under these conditions; differences that remain under SD are controlled by *FLC* independent of its effect on flowering.

The rate of leaf initiation was identical in *FRI;FLC* and *FRI;flc-3* under both SD and NI conditions (Fig. 3A), indicating that all of the differences observed between these genotypes reflect

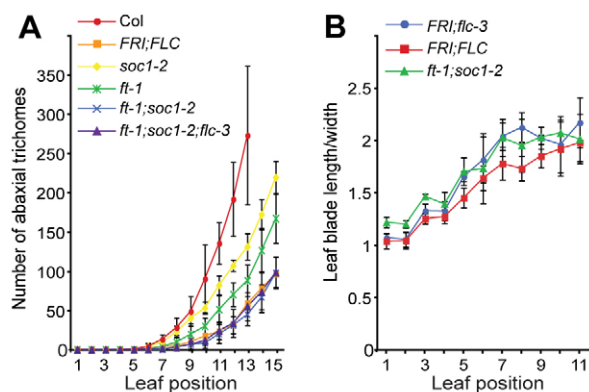


Fig. 4. *FT* and *SOC1* influence total abaxial trichome numbers but not leaf roundness. (A) *FT* and *SOC1* have additive effects on the total number of abaxial trichomes under long days; *ft-1;soc1-2*, *ft-1;soc1-2;flc-3* and *FRI;FLC* have similar numbers of abaxial trichomes. (B) The effect of *FLC* on leaf shape is probably independent of *FT* and *SOC1* because the blade length-to-width ratios of *FRI;flc-3* and *ft-1;soc1-2* are similar. Results are mean \pm s.d.

differences in the timing of these processes. Consistent with the results obtained in LD (Fig. 1A), *FRI;FLC* and *FRI;flc-3* plants grown in NI produced abaxial trichomes at the same leaf position, but the length of the transition zone was significantly longer in *FRI;FLC* than *FRI;flc-3* (Fig. 3B). The onset of abaxial trichome production was delayed by one plastochron in both genotypes in SD (Fig. 3B), but this is probably independent of the photoperiodic effect on flowering because high *FLC* expression produces a stronger delay in flowering than SD alone (Michaels and Amasino, 2001) and yet does not alter the timing of vegetative phase change. Under SD conditions, *FRI;flc-3* plants had significantly more transition leaves than plants grown in NI, and were identical to *FRI;FLC* (Fig. 3B). Thus, the difference in the length of the transition zone in *FRI;FLC* and *FRI;flc-3* in NI appears to be attributable to the difference in their flowering phenotype. Flowering also explains the difference in hydathode number and leaf length in these genotypes under NI because these differences disappeared in SD (Fig. 3D,G). By contrast, the difference in the total number of abaxial trichomes and length:width ratio persisted in SD (Fig. 3C,E,F). These results indicate that *FLC* slightly suppresses abaxial trichome production and enhances the lateral expansion of the leaf blade independent of its effect on flowering time. With the exception of the total number of trichomes, all of these traits were not significantly different in *FRI;FLC* plants grown in NI and SD (see Fig. S1 in the supplementary material), demonstrating that photoperiod does not affect their expression independently of *FLC*. By contrast, the total number of abaxial trichomes was significantly lower in *FRI;FLC* plants grown in SD than in NI (Fig. 3C), implying that photoperiod has an influence on trichome production independent of the effect of flowering on this trait.

FLC is a transcriptional repressor of the floral pathway integrators *FT* and *SOC1* (Helliwell et al., 2006; Hepworth et al., 2002; Searle et al., 2006). To determine whether *FLC* regulates leaf development via these genes, we examined the phenotype of *ft-1* and *soc1-2* single and double mutants, reasoning that the phenotype of these loss-of-function mutations should be identical to *FRI;FLC* if they are solely responsible for its vegetative phenotype. *ft-1* and *soc1-2* single mutants had a reduced number of abaxial trichomes;

the *ft-1;soc1-2* double mutant and the *ft-1;soc1-2;flc-3* triple produced even fewer abaxial trichomes and were indistinguishable from *FRI;FLC* for this trait (Fig. 4A). This result suggests that the effect of *FLC* on abaxial trichome number is mediated by these transcription factors. By contrast, the shape of the leaf blade in *ft-1;soc1-2* was significantly different from *FRI;FLC*, but not significantly different from *FRI;flc-3* (Fig. 4B), implying that other targets of *FLC* control this phenotype.

Light intensity modulates vegetative phase change in *FRI;FLC* but not *FRI;flc-3*

In contrast to the results presented here, previous studies have shown that genotypes with increased *FLC* activity, including *FRI;FLC*, *fpa;FLC* and *fve;FLC*, have delayed trichome production (Lee et al., 2000; Martinez-Zapater et al., 1995; Telfer et al., 1997). We noticed that the timing of abaxial production in *FRI;FLC* varied with the age of the fluorescent bulbs in our growth chambers, suggesting that light intensity might be responsible for the difference between our results and these earlier studies. Consistent with this idea, Lee and colleagues (Lee et al., 2000) reported that the light intensity in their growth chambers was $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, which is lower than the light intensity ($125 \mu\text{mol m}^{-2} \text{s}^{-1}$) in our chambers. Our original studies in which we observed an effect of *FLC* on abaxial trichome production (Telfer et al., 1997) were performed in growth chambers illuminated with VHO fluorescent light bulbs, the light output of which declined from $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ to less than $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ within a few weeks. Although we did not measure the light intensity in these original experiments, it is reasonable to assume that it was closer to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ than to $140 \mu\text{mol m}^{-2} \text{s}^{-1}$.

To test the effect of light intensity on abaxial trichome production, we compared the onset of abaxial trichome production under light intensities of $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ (high light) and $75 \mu\text{mol m}^{-2} \text{s}^{-1}$ (low light) in plants growing in LD. Low-light conditions were produced by shielding the fluorescent light bank with Miracloth, which did not produce a change in light spectrum. Low-light conditions did not affect the onset of abaxial trichomes in *FRI;flc-3*, but produced a significant delay in abaxial trichome production in *FRI;FLC* (Fig. 5A). Other genotypes with low *FLC* levels (Col and *fpa;flc-3*) were also unaffected by low light, whereas genotypes with high *FLC* (*fpa;FLC* and *35S::FLC*) produced abaxial trichomes later under low-light conditions than under high light (Fig. 5A). Leaf initiation was slowed equally in all genotypes by the lower light levels (Fig. 5B). Thus, in the presence of *FLC*, the onset of abaxial trichome production is exquisitely sensitive to light intensity. This sensitivity is probably due to the lower *FT* expression in these genotypes because *ft-1* and *ft-10* were also sensitive to light intensity, whereas *soc1-2* was insensitive to this condition (Fig. 5A).

DISCUSSION

The identity of lateral organs of the shoot of flowering plants is determined by the timing of two key postembryonic developmental transitions – vegetative phase change and flowering. In late-flowering plants, floral induction occurs long after vegetative phase change so that it is relatively easy to distinguish changes that are specific to vegetative phase change from those that are the result of floral induction. However, in early flowering plants, flowering occurs shortly after the juvenile-to-adult transition. This complicates analyses of vegetative phase change because it raises the possibility that changes induced by flowering might overlap with, and potentially modify, the expression of traits associated

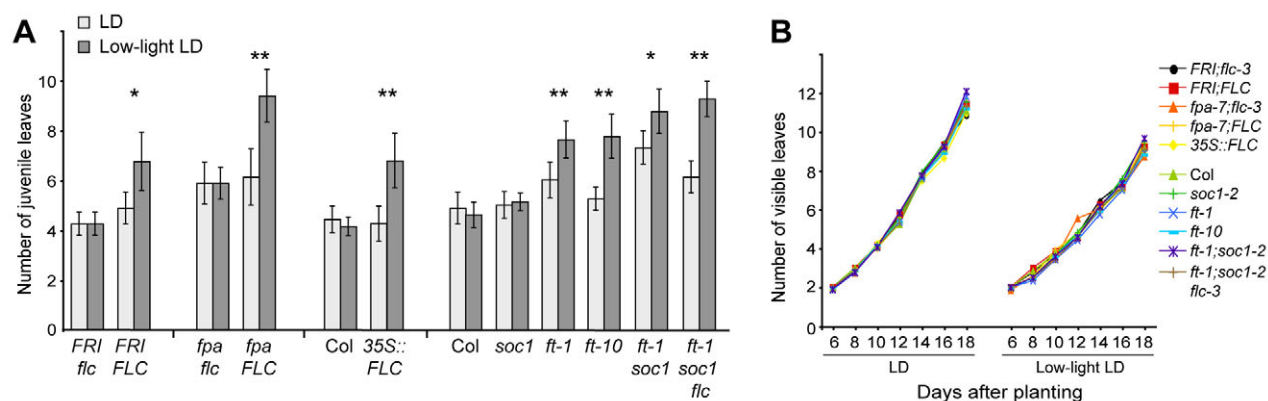


Fig. 5. Low light intensity delays vegetative phase change in genotypes with low *FT*. (A) The onset of abaxial trichomes is delayed in *FRI;FLC*, *fpa-7;FLC*, *35S::FLC*, *ft-1* and *ft-10* mutants under low-light conditions. Bars show the first leaf with abaxial trichomes in plants grown in long days under normal (LD) and low light intensity (low-light LD). Significantly different (Student's *t*-test): * $P \leq 0.01$, ** $P \leq 0.001$. Results are mean \pm s.d. (B) The rate of leaf initiation is identical in the genotypes shown in A under LD and low-light LD, but is slightly slower in low-light LD.

with the juvenile-to-adult transition. To distinguish traits that are components of vegetative phase change from features that are affected by floral induction, we compared the phenotype of the late-flowering genotype *FRI;FLC* with an early-flowering mutant derivative of this line, *FRI;flc-3*. Our results are consistent with the results of Cookson et al. (Cookson et al., 2007), and indicate that floral induction affects some features of leaf morphology and not others, and that most rosette leaves are modified by this event. Importantly, flowering had no effect on the onset of abaxial trichome production, a key marker of vegetative phase change. We conclude that genes involved in floral induction do not regulate the timing of the juvenile-to-adult transition, as has been previously proposed (Haughn et al., 1995; Schultz and Haughn, 1993; Steynen et al., 2001), but instead act after vegetative phase change has occurred to affect the expression of some aspects of this vegetative transition. We also found that *FLC* has effects on leaf morphology that are independent of its effect on flowering time, revealing a novel function for this gene in vegetative development.

FLC, light intensity, and the onset of vegetative phase change

Many traits vary as a function of leaf position in *Arabidopsis*. We focused on abaxial trichome production because this trait has been widely used as a marker of vegetative phase change. Previous studies have shown that increasing the expression of *FLC*, blocking gibberellin synthesis or signaling, and growing plants under non-inductive photoperiods, delay the onset of abaxial trichome production and reduce the number of adaxial trichomes (Chien and Sussex, 1996; Lee et al., 2000; Martinez-Zapater et al., 1995; Telfer et al., 1997). Because all of these conditions affect flowering time, it was not clear whether their effect on trichome production is an indirect result of their effect on flowering time, or a consequence of their involvement in vegetative phase change.

We found that the onset of abaxial trichome production is insensitive to floral induction, indicating that the presence versus absence of abaxial trichomes is a good marker of the vegetative phase of the shoot. Unexpectedly, we also discovered that the onset of abaxial trichome production is sensitive to light intensity, but only in plants with high levels of *FLC*. This phenotype probably reflects the effect of *FLC* on the expression of *FT*. *FLC* directly represses *FT* and *SOC1*, and most of its effects have been attributed to one or both of these genes (reviewed in Amasino, 2010).

Whereas *soc1-2* has no effect on abaxial trichome production under either high or low-light conditions, *ft* mutants are as sensitive to light intensity as *FLC*, and this phenotype is epistatic to the light-insensitive phenotype of *flc-3*; in other words, *FT* is required for the light-insensitive phenotype of *flc-3*. The observation that abaxial trichome production is delayed in the absence of *FT* under low-light conditions (i.e. in *FRI;FLC* and in *ft* mutants), but not in the presence of *FT* (i.e. in *FRI;flc-3*) indicates that *FT* overrides the effect of light intensity on this trait and, more importantly, demonstrates that *FT* directly or indirectly regulates vegetative development.

Loss-of-function mutations of the *FT* ortholog *SFT* have been reported to affect leaf morphology in tomato (Shalit et al., 2009), but this is the first evidence that *FT* regulates vegetative development in *Arabidopsis*. *FT* is transcribed in cotyledons and leaf primordia and is expressed at a low level in the shoot apex of 6- to 7-day-old seedlings in LD (Kardailsky et al., 1999; Kobayashi et al., 1999; Yamaguchi et al., 2005). In addition, *FT* protein is translocated from leaves to the shoot apex during floral induction (Corbesier et al., 2007; Jaeger and Wigge, 2007). Floral induction occurs between 9 and 11 days after planting in LD, at which time the first transition leaves (leaves 5 or 6) are 1 mm or less in length (Fig. 1G). It is unclear whether the effect of *FT* on leaf development is mediated by the *FT* protein produced in leaf primordia or *FT* protein imported into leaf primordia upon floral induction but, in either case, it is present in the shoot apex at the correct time to affect the expression of phase-specific leaf traits. How might it regulate these traits? The mRNA levels of *SPL3*, *SPL9* and several other *SPL* genes are reduced in the shoot apex of *ft* mutants (Schmid et al., 2003), whereas *SPL3* mRNA is elevated in plants over expressing *FT* (Schmid et al., 2003; Wang et al., 2009; Yamaguchi et al., 2009), demonstrating that *SPL* genes are directly or indirectly regulated by *FT*. *SPL* genes regulate various aspects of vegetative phase change, including abaxial trichome production (Schwarz et al., 2008; Usami et al., 2009; Wu et al., 2009; Wu and Poethig, 2006). Consequently, it is reasonable to propose that *FT* regulates abaxial trichome production via its effect on *SPL* gene expression. The observation that light intensity affects abaxial trichome production in the absence of *FT* (i.e. in *FRI;FLC* and *ft* mutants) implies that light acts downstream or in parallel to *FT*. One possibility is that light intensity acts by affecting the abundance of miR156, a negative regulator of *SPL* gene expression, such that miR156 is expressed at higher levels

under low light intensity. In this scenario, FT promotes the transcription of *SPL* genes in leaf primordia, thereby overcoming the increased miR156-mediated repression of these genes under low light conditions. In the absence of FT, *SPL* gene expression would become acutely sensitive to light-dependent changes in miR156 expression.

Flowering speeds the juvenile-to-adult transition and has other effects on leaf development

Although flowering does not affect the onset of vegetative phase change, it has a significant effect on the subsequent progression of this process. *FRI;FLC* plants grown under LD, NI or SD, and *FRI;flc-3* plants grown under SD to prevent flowering, produce significantly more transition leaves than *FRI;flc-3* plants grown in LD or NI. Vegetative phase change is mediated by an increase in the expression of miR156-regulated members of the *SPL* gene family, which occurs in response to a decrease in the expression of miR156 (Chuck et al., 2007; Wu et al., 2009; Wu and Poethig, 2006). In LD, but not in SD, some of these miR156-regulated *SPL* genes undergo a further increase in expression during the adult phase (Cardon et al., 1999; Chuck et al., 2007; Schmid et al., 2003). We suggest that the onset of vegetative phase change is regulated by the increase in *SPL* gene expression that results from a decrease in miR156 levels, and that the rate of this transition is dependent on the level of *SPL* gene transcription, which is influenced by a variety of factors. Our observation that the length of the transition zone is identical in *FRI;FLC* plants grown in LD and SD, and in *FRI;FLC* and *FRI;flc-3* plants grown in SD, suggests that the difference in the expression of *SPL* genes in SD and LD (Cardon et al., 1999) is attributable to floral induction, not photoperiod per se.

In addition to influencing vegetative phase change, flowering has other effects on leaf development. Leaf length, the shape of the leaf blade, the rate of leaf expansion, cell size, cell number and hydathode number all differ in early and late-flowering plants, and these differences are apparent as early as leaf 3 (Cookson et al., 2007). In early-flowering varieties of *Arabidopsis*, floral induction occurs while all of the leaves are still expanding, so it is perhaps not surprising that most rosette leaves are affected by this event. The effect of flowering on leaf development should be taken into account when studying the effects of early flowering genotypes on leaf development and vegetative phase change, to avoid confusing the direct and indirect effects of these genes.

Flowering-independent effects of *FLC* on leaf development

Although most of the effects of *FLC* on leaf morphology are an indirect result of its effect on flowering, *FLC* also has a more direct role in leaf development. This is apparent from the observation that *FRI;FLC* has significantly rounder leaves and fewer abaxial trichomes than *FRI;flc-3* under SD conditions, when both genotypes are late flowering. Along with the evidence that *FLC* regulates seed germination (Chiang et al., 2009), these results indicate that *FLC* has additional functions outside its well-described role in flowering. This might be expected from the fact that *FRI;FLC* plants flower only under certain environmental conditions, usually after a long exposure to cold temperatures (Amasino, 2010). *FRI;FLC* genotypes that fail to flower before the onset of winter must be adapted to cold, and many other biotic and abiotic stresses. Selection for a winter annual flowering habit would necessarily have been accompanied by selection for traits that allow plants to survive winter, and it may be that *FLC* has both of these functions.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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