miR824-Regulated AGAMOUS-LIKE16 Contributes to Flowering Time Repression in Arabidopsis

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The timing of flowering is pivotal for maximizing reproductive success under fluctuating environmental conditions. Flowering time is tightly controlled by complex genetic networks that integrate endogenous and exogenous cues, such as light, temperature, photoperiod, and hormones. Here, we show that AGAMOUS-LIKE16 (AGL16) and its negative regulator microRNA824 (miR824) control flowering time in Arabidopsis thaliana. Knockout of AGL16 effectively accelerates flowering in nonvernalized Col-FRI, in which the floral inhibitor FLOWERING LOCUS C (FLC) is strongly expressed, but shows no effect if plants are vernalized or grown in short days. Alteration of AGL16 expression levels by manipulating miR824 abundance influences the timing of flowering quantitatively, depending on the expression level and number of functional FLC alleles. The effect of AGL16 is fully dependent on the presence of FLOWERING LOCUS T (FT). Further experiments show that AGL16 can interact directly with SHORT VEGETATIVE PHASE and indirectly with FLC, two proteins that form a complex to repress expression of FT. Our data reveal that miR824 and AGL16 modulate the extent of flowering time repression in a long-day photoperiod.

INTRODUCTION

An appropriate timing of flowering is essential for plants to maximize reproductive success and adapt to changing environmental conditions. Molecular genetic pathways controlling the switch from vegetative to reproductive growth have been well characterized, especially in the model plant Arabidopsis thaliana. Various endogenous and exogenous cues, including age, circadian clock, temperature, photoperiod, and hormones, have been reported to be involved in the control of flowering time (Simpson and Dean, 2002; Ausín et al., 2005; Bäurle and Dean, 2006; Dennis and Peacock, 2007; Andrés and Coupland, 2012).

Photoperiod changes are perceived in leaves and transduced to regulate the accumulation of CONSTANS, which encodes a transcription factor that activates the transcription of FLOWERING LOCUS T (FT) in the vascular tissues of the leaves (Samach et al., 2000; An et al., 2004; Kobayashi and Weigel, 2007). The FT protein moves to the shoot apical meristem, where it activates the expression of several floral integrators like APETALA1, SUPPRESSOR OF OVEREXPRESSIO
miR824/AGL16 as well as their respective targets are found in many plant families and thus are believed to be ancient modules that regulate developmental timing in plants (Lauter et al., 2005; Chuck et al., 2007; Zhu et al., 2009; Nair et al., 2010; Cho et al., 2012). However, novel regulatory modules seem to evolve frequently in plant lineages, and many miRNA/target gene interactions are family or species specific (Cuperus et al., 2011).

miR824 is a Brassicaceae-specific miRNA (Rajagopalan et al., 2006; Fahlgren et al., 2007; Kutter et al., 2007). miR824 is proposed to have evolved through partial duplication of its target gene, AGAMOUS-LIKE16 (AGL16), which encodes a MADS box transcription factor (Fahlgren et al., 2007; de Meaux et al., 2008). The miR824 precursor displays two alleles and was hypothesized to evolve under balancing selection in Arabidopsis (de Meaux et al., 2008). miR824 is expressed in many tissues including rosette and cauline leaves, shoots, inflorescences, and roots (Kutter et al., 2007). The expression of AGL16, the only confirmed target for miR824, is also detected in these tissues as well as in guard cells, trichomes, and developing siliques (Alvarez-Buylla et al., 2000). In agreement with its expression in guard cells, the miR824/AGL16 module was shown to regulate the development of higher-order stomata complexes by promoting additional divisions of satellite meristemoid cells (Kutter et al., 2007).

However, AGL16 is also expressed in the shoot apex and the root and at a level that is sensitive to light change and environmental stress (https://www.genevestigator.com/gv/plant.jsp). In addition, genome-wide profiling of FLC and SVP binding sites revealed that AGL16 is one of the strongest FLC targets (Deng et al., 2011) and a weak target for SVP (Gregis et al., 2013). These data prompted us to hypothesize that AGL16 might be involved in the control of flowering time. Here, we show that the miR824/AGL16 module participates in the timing of floral transition in Arabidopsis by interacting closely with the FRI/FLC-SVP pathways.

RESULTS

Loss of Function of AGL16 Accelerates Flowering of Nonvernalized Col-FRI in Long-Day Conditions Only

Since AGL16 was reported as one of the major targets of FLC, it was necessary to evaluate its role on flowering time in a background expressing high levels of FLC. We crossed the loss-of-function mutant agl16-1 in the Col-0 background, with Col-FRI, which expresses FLC at high levels due to the introgression of a functional FRI from the accession SF-2 into Col-0 (Michaels and Amasino, 1999). Under long-day (LD) conditions without vernalization, wild-type Col-FRI flowered at around 62 total leaves, whereas the AGL16 loss-of-function mutant (agl16-1; Col-FRI) flowered much earlier at around 43 leaves (Student’s t test, P < 1E-16; Figures 1A and 1C). This experiment was replicated in three further trials. Although the total number of leaves at flowering fluctuated across experiments, AGL16 loss-of-function mutants always had ∼30% (25 to 35%) fewer leaves upon flowering than the wild type (Table 1; Supplemental Data Set 1). To test the implication of AGL16 function in vernalization, agl16-1;Col-FRI was vernalized together with Col-FRI for 4 weeks. Both lines exhibited a typical vernalization response, indicating that AGL16 is not essential for this response (Figures 1B and 1C).

To test if the effect of AGL16 was dependent on a functional copy of FLC, we generated an F2 population segregating for AGL16/agl16-1; FRI/fri, and FLC/flc-3 by crossing the agl16-1 mutant with flc-3, which carries a nonfunctional FLC allele in the Col-FRI background (Michaels and Amasino, 1999). Under inductive LD conditions (16 h light/8 h dark) all plants homozygous

![Figure 1](image1.png)

**Figure 1.** Loss of Function of AGL16 Partially Suppressed the Delayed Flowering Conferred by FRI Introggression into the Col-0 Background under LD Conditions but Did Not Influence the Vernalization Response.

(A) and (B) Comparisons between wild-type Col-FRI (left) and the loss-of-function mutant agl16-1;Col-FRI (right) upon flowering under LD conditions without vernalization treatment (A) or with 4 weeks of vernalization at 4°C (B).

(C) The flowering time behavior of loss of function of AGL16 in the Col-FRI background (agl16-1;Col-FRI) grown under LD conditions. Total leaf number including rosette and cauline leaves at flowering was monitored. Note that under nonvernalized LD conditions, agl16-1;Col-FRI showed strongly accelerated flowering compared with Col-FRI (**P < 0.001, two-tailed Student’s t test; black and white bars, respectively), while under vernalized LD conditions, agl16-1;Col-FRI flowered at the same time as Col-FRI (P = 0.9; yellow and blue bars, respectively). Error bars indicate ± (see Supplemental Data Set 1 for number of plants tested).
for the flc-3 allele flowered after forming ~10 leaves independent on the presence or absence of either a functional FRI or AGL16 (Figure 2A; Supplemental Data Set 1). A small, but reproducible acceleration of flowering was detected in the absence of FRI for both agl16-1 and flc-3 homozygous single mutants in comparison to Col-0 (see below).

The partial suppression of late flowering in Col-FRI by agl16-1 could only be observed when flowering was accelerated by an inductive photoperiod (Figure 2; Supplemental Data Set 1). Under short-day (SD) conditions, irrespective of the presence of FLC, agl16-1 plants flowered at the same time as the corresponding AGL16 siblings (both at around 130 leaves; Figure 2B). Under 12- to 12-h mid-day light cycles conditions, agl16-1;Col-FRI flowered earlier than Col-FRI, but the effect was reduced compared with LD (Figure 2C). In the absence of FRI, the presence or absence of AGL16 did not alter flowering time in SD or mid-day conditions, whereas a small acceleration of flowering was observed in both conditions in the flc-3 background when a functional FRI was present (Figures 2B and 2C). This might suggest that FRI can affect flowering to a limited extent via AGL16 in a FLC-independent manner under noninductive photoperiod conditions.

Taken together, these experiments show that the magnitude of the effect caused by the loss of AGL16 on flowering is not a function of the total time to flowering, but is primarily observed in the presence of strong FLC activity and a positive photoperiod.

### Table 1. Summary of the Flowering Behavior of Mutants under LD Conditions

<table>
<thead>
<tr>
<th>Line Comparisons</th>
<th>No. of Independent Trials</th>
<th>Cumulated No. of Individuals (Mutant vs. Wild Type)</th>
<th>Differences in Mean Leaf No.</th>
<th>Percentage Difference in Mean Leaf No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>agl16-1:Col-FRI vs. Col-FRI</td>
<td>4</td>
<td>73 vs. 94</td>
<td>8.5–19</td>
<td>65.2–75.3%</td>
</tr>
<tr>
<td>agl16-1 vs. Col-0</td>
<td>10</td>
<td>306 vs. 295</td>
<td>0.7–2.2</td>
<td>82.5–90.5%</td>
</tr>
<tr>
<td>m3 vs. Col-0</td>
<td>4</td>
<td>127 vs. 120</td>
<td>0.9–2.3</td>
<td>83.5–92.2%</td>
</tr>
<tr>
<td>mIR824s vs. Col-0 (vector)</td>
<td>3</td>
<td>266 vs. 67</td>
<td>1.4–3.4</td>
<td>111–126%</td>
</tr>
</tbody>
</table>

See Supplemental Data Set 1 for detailed information. Note that $P < 0.01$ (two-tailed Student’s $t$ test) for all comparisons except for the comparison agl16-1 versus Col-0, where one trial (trial 4 in Supplemental Data Set 1) showed $P < 0.05$. Differences in mean leaf number reflect the raw observed difference. The total leaf number at flowering varies with the genetic background in which miR824/AGL16 mutations were assessed. The percentage difference in mean leaf number presents the phenotype of the mutant relative to the wild type.

**Figure 2.** Effects of the Loss of Function of AGL16 on Flowering Time Depend on the Photoperiod.

RLNs at flowering are shown for each line grown under LD (A), SD (B), and equal light-night (MD; C) conditions. Below the graph, the genotypes of each bar are marked for the three genes: AGL16, FRI, and FLC, with a plus sign indicating wild type or functional allele and a minus sign indicating loss of function. The mean values of RLN for each line together with the so are shown. Statistical analyses were performed with two-tailed Student’s $t$ test with Bonferroni correction (shown here) and confirmed with a Wilcoxon test. **$P < 0.001$, ***$P < 0.01$, and *$P < 0.05$. Note that in the flc-3 background, the absence of AGL16 can accelerate the flowering time under both MD (C) and SD (B) conditions.
stimulus. Vernalization is epistatic to a loss of AGL16 function, indicating that this gene does not participate in this response.

Flowering Time Is Sensitive to the Allelic Dosage of AGL16 and FLC

To further evaluate how sensitive the flowering response was to the dosage of FLC and AGL16, we scored flowering time in a segregating F2 population of the cross between agl16-1 (in Col-0) and flc-3 (in Col-FRI). We grew 451 F2 individuals under LD conditions and scored the flowering phenotype by counting the rosette leaf number at flowering (Figure 3A; Supplemental Data Set 2). All three genes showed a Mendelian inheritance (108:223:116, 132:212:102, and 113:224:113 for wild type; heterozygote:mutant for AGL16, FRI, and FLC, respectively; Fisher’s exact test, P = 1.0 for all three comparisons). As expected, the functionality of FRI (P = 0.008) strongly affected the flowering time in this population, and agl16-1 caused strong changes in flowering time when FRI was functional (P < 10E-10).

Figure 3. Flowering Time Is Sensitive to the Quantitative Balance of FLC and AGL16.

(A) RLNs upon flowering are shown for plants of a segregating F2 population of 451 individuals grown under LD conditions. The RLN for each genotype is shown by box plots. Each box encloses the median 50% of the distribution, with the horizontal line marking the median. The lines extending from each box mark the minimum (5%) and maximum (95%) values of the data set. Circles marked the outliers (outside of the 5 to 95% distribution). FRI genotypes are grouped into functional and nonfunctional, as functional FRI is dominant. The allelic numbers of both FLC and AGL16 are coded as 0, 1, and 2, indicating homozygote for knockout allele, heterozygote, and wild-type homozygote, respectively. Numbers at the bottom give the number of plants featuring each multilocus genotype. The AGL16 genotypes alone (P = 0.023) and in interaction with FRI/FLC allelic combinations (P = 0.035) had a significant influence on the flowering time (see main text for details).

(B) Flowering time of plants overexpressing miR824 in both Col-0 (m3) and Col-FRI (m3;Col-FRI) backgrounds. Mean values of rosette leaf number upon flowering for each line together with the SD around the mean were plotted. Differences between lines were evaluated with two-tailed Student’s t test with Bonferroni correction (Supplemental Table 1). ***P < 0.001; *P < 0.05.
The number of functional FLC alleles showed a significant influence on flowering time in general (P < 10E-4), but particularly in the FRI functional background (P < 2E-16). Importantly, AGL16 also displayed an allelic dosage effect and significantly influenced the timing of floral transition in general (P = 0.023) and especially in the functional FRI background (P = 4E-12; Figure 3A). In contrast to the previous experiment (Figure 2A), the flowering time was consistently delayed under LD conditions (−1.7 to 11) to 26% leaves more than Col-0 transformed with an empty vector; Wilcoxon test, P = 2E-11; Figure 4B, Table 1; Supplemental Figure 2 and Supplemental Data Set 1; three independent trials), suggesting that enhancing the expression of AGL16 by around 2-fold can delay flowering.

The miR824/AGL16 Module Regulates Flowering Time by Altering FT Expression

Since the miR824/AGL16 module regulates flowering time in a photoperiod-dependent manner, we wondered whether this

**miR824 Mimicry Causes Later Flowering in Col-0**

We further made use of miR824 target mimic lines (MIM824) (Franco-Zorrilla et al., 2007; Todesco et al., 2010), which were created by overexpressing an artificial noncleavable target mimic for miR824, to assess the role of miR824 in regulating flowering. In these lines, a reduced activity of miR824 increases the expression of AGL16 specifically in the cells where AGL16 is naturally expressed. The phenotypic modifications displayed by MIM824 should thus reveal the native function of the miR824/AGL16 module. The expression of the target mimic in seven independent MIM824 lines consistently doubled the level of AGL16 expression (Figure 4A; two-tailed Student’s t test, all P < 0.01). In all seven lines (Figure 4A), the flowering time was consistently delayed under LD conditions (−1.7 to 11) to 26% leaves more than Col-0 transformed with an empty vector; Wilcoxon test, P = 2E-11; Figure 4B, Table 1; Supplemental Figure 2 and Supplemental Data Set 1; three independent trials), suggesting that enhancing the expression of AGL16 by around 2-fold can delay flowering.

**Figure 4.** Moderately Increasing the Expression of AGL16 in MIM824 Lines Can Delay Flowering Time under LD Conditions.

**A** Relative expression level of AGL16 in Col-0 transformed with an empty vector (open bar) and seven independent MIM824 transgenic lines (filled bars in different shades of gray; T3 lines with homozygous single insertions; the same for **B**) in 4-week-old rosette leaves. Expression values are reported as the mean of two biological replicates (each with three technical replicates) examined by real-time quantitative PCR (normalized to PP2A; significant levels tested with Student’s t test after Bonferroni correction. *P < 0.05; **P < 0.01; ***P < 0.001; the same for **B**). AGL16 expression levels in the aerial part of 10-d-old seedlings gave the same pattern (data not shown).

**B** Rosette leaf production upon flowering for the MIM824 lines (filled bars) and Col-0 transformed with empty vector (open bar). Mean leaf numbers to flowering together with s.e. are given. The flowering time behavior of a second independent experiment is shown in Supplemental Figure 2.
Figure 5. The miR824/AGL16 Module Represses the Expression of FT.

(A) Schematic representation of the miR824 locus. Exons (open bars), introns (horizontal lines), transcription starting site (+1), and positions of miR824 (black arrowhead) and miR824* (gray arrowhead) are indicated. The gray filled bar indicates the last exon of AT4G24400. The long arrow in the dashed line marks the region used as promoter of miR824. The scheme is not drawn to scale.

(B) GUS staining of the first rosette leaf of 2-week-old Col-0 plants transformed with promoter-miR824:GUS. Note the blue stain of the vasculature.

(C) GUS staining of the first rosette leaf of 2-week-old Col-0 plants (negative control).

(D) and (E) Real-time PCR following reverse transcription was used to quantify the level of FT expression, normalized to the expression level of the control gene PP2A, and shown on the y axis. All experiments were performed under LD conditions. Bars indicated ± standard deviation of three technical replicates of the reactions. Experiments were replicated at least twice and all showed similar patterns (Supplemental Figure 5). Significant levels (Student’s t test with Bonferroni correction): *P < 0.05; **P < 0.01; ***P < 0.001.

(D) and (E) The relative expression of FT was quantified following different zeitgeber time points (from ZT0 to ZT24) with 9-d-old seedlings grown on agar plates. From ZT0 to ZT16 is daytime (open bars), while from ZT16 till ZT24 is nighttime (closed bars) as shown beneath the x axis. Note that at ZT16, the FT mRNA levels in both mutants (agl16-1 [D]; and agl16-1;Col-FRI [E]; solid lines) are higher than their corresponding wild types (dotted lines, P < 0.001).
module could regulate the expression of FT, the key factor controlling flowering time under LD conditions (Kardailsky et al., 1999; Samach et al., 2000). FT is the main target of FLC in leaves and is detected in the phloem companion cells of the leaf vasculature (Takada and Goto, 2003; Adrian et al., 2010). We first evaluated whether miR824 and FT expression overlap using promoter-miR824:GUS lines in the Col-0 background. A 4.7-kb fragment comprising 3.093-kb sequences upstream and 1.614 kb downstream of the transcription start site (TSS) was taken as the promoter of miR824 and fused to a reporter gene encoding β-glucuronidase (GUS) (Figure 5A). In T1 transgenic plants, the strongest GUS activity was detected in vascular tissues of rosette leaves (Figure 5B) as well as in guard cells (Supplemental Figure 3); therefore, the expression of miR824 and FT can colocalize. We further evaluated the role of FT in the process of miR824/AGL16-mediated flowering time regulation. We performed real-time quantitative PCR to quantify the expression of FT in agl16-1 mutants in both the Col-0 and Col-FRI backgrounds.

In a 24-h time-course experiment performed with Col-0 plants (9 d after germination), FT expression started to increase strongly between zeitgeber time 12 (ZT12) and 20 (ZT20) (Figure 5D), as expected (Kobayashi et al., 1999). In agl16-1 plants (Supplemental Figure 4), FT mRNA abundance showed a similar diurnal pattern but accumulated to higher levels at ZT16 (Student’s t test, P < 0.001 for two independent trials; Figure 5D; Supplemental Figure 5A). In the Col-FRI background, the diurnal expression of FT was similar to that in Col-0, although the expression levels were more than 10 times reduced (Figure 5E; Supplemental Figure 5B). As in the Col-0 background, the agl16-1 mutation caused a higher expression of FT at ZT16 in Col-FRI (Student’s t test, P < 0.001 for two independent trials; Figure 5E; Supplemental Figures 4 and 5B). We also monitored the FT mRNA level in the Col-FRI background following development from 8 to 12 d after germination at ZT16. For all stages checked, FT levels were consistently higher in the agl16-1 background compared with Col-FRI and the values increased during development (Figure 5F; Supplemental Figure 5C).

Although the loss of function of AGL16 correlated well with elevated expression of FT in both Col-0 and Col-FRI backgrounds, it was unclear if the observed mild differences were sufficient to explain the altered flowering time in these backgrounds. Therefore, we examined whether the early flowering caused by a loss of AGL16 function requires a functional FT, which depends on the molecular interaction of the fusion proteins, was observed in the nucleus of the cells coinfected with 35S:AGL16-nYFP/35S:FLC-cYFP and 35S:FLC-cYFP/35S:SVP-nYFP but not with 35S::AGL16-nYFP/35S::LHP1-cYFP (Figure 6C). The results demonstrate that AGL16 can interact with FLC in the nucleus, thus supporting the idea that AGL16 might control flowering time as a partner of FLC in a repressive MADS factor complex. As FLC and SVP are known to form a repressive

AGL16 Is a Potential Partner of Flowering Repressor Complexes Targeting FT

FLC has been shown to directly target the promoter of FT, thereby repressing the response of FT to photoperiod (Seagle et al., 2006). As a consequence, a loss of function of FLC massively increases the expression of its direct target FT (Seagle et al., 2006). FLC has also been shown to bind to the promoter of AGL16 (Deng et al., 2011), but its loss of function only slightly changes AGL16 expression (Figure 5A; Supplemental Figure 4; Deng et al., 2011). AGL16 could be required for full functionality of FRI/FLC by increasing the expression of FLC in leaves. However, comparing FLC expression levels between agl16-1 Col-FRI and Col-FRI over a developmental window from 8 to 11 d after germination did not reveal massive differences in FLC expression (Figure 6B) with similar pattern observed in the Col-0 background (Supplemental Figure 6B). These results suggest that the genetic interaction between AGL16 and FLC does not act primarily at the transcription level.

We therefore investigated whether the interaction between AGL16 and FLC may take place at the posttranscriptional level. To this end, we examined whether AGL16 could physically interact with FLC with the bimolecular fluorescence complementation (BiFC) technique (Hu and Kerppola, 2003). We fused the C-terminal half of yellow fluorescent protein (cYFP) with the C terminus of AGL16 (AGL16-cYFP) and the N-terminal half of YFP (nYFP) with the C terminus of FLC (nYFP-FLC). AGL16 was located in the nucleus (Figure 6C). As a negative control, the C terminus of LHP1 (a component of the polycomb complex known to be localized in the nucleus) (Zemach et al., 2006) was fused to nYFP (nYFP-LHP1). As a positive control (Fujii et al., 2008; Li et al., 2008), the C termini of SVP and FLC proteins were joined together with nYFP (SVF-nYFP) and cYFP (FLC-cYFP), respectively. Each construct was coinfected into epidermal cells of Nicotiana benthamiana leaves for transient expression (Voinnet et al., 2003). The fluorescent signal of YFP, which depends on the molecular interaction of the fusion proteins, was observed in the nucleus of the cells coinfected with 35S::AGL16-nYFP/35S::FLC-cYFP and 35S::FLC-cYFP/35S::SVP-nYFP but not with 35S::AGL16-nYFP/35S::LHP1-cYFP (Figure 6C). The results demonstrate that AGL16 can interact with FLC in the nucleus, thus supporting the idea that AGL16 might control flowering time as a partner of FLC in a repressive MADS factor complex. As FLC and SVP are known to form a repressive
Figure 6. AGL16 Can Participate in the Repressor Protein Complexes Formed by FLC/SVP.

(A) Loss of function of FLC (flc-3) moderately altered the expression of AGL16 in the Col-FRI background (P < 0.05). Relative accumulation of AGL16 in the flc-3 mutant was monitored by real-time RT-PCR (normalized to PP2A) in different tissues of the aerial parts of 12-d-old seedlings grown under LD conditions. Error bars indicate SE of three biological replicates. Note that AGL16 expression in the flc-3 mutant was slightly enhanced compared with Col-FRI (P = 0.02, indicated by one asterisk; two-tailed Student’s t test). The same pattern was observed in a second independent trial (Supplemental Figure 6A).

(B) Loss of function of AGL16 (agl16-1 Col-FRI) mildly altered the expression of FLC in the Col-FRI background. Relative accumulation of FLC in the agl16-1 Col-FRI mutant was monitored by real-time RT-PCR (normalized to TUB2) along developmental stages from 8 to 11 d after germination on soil.
under LD conditions. Error bars indicate so of three technical replicates. The experiment was also performed in the Col-0 background and gave the same pattern (Supplemental Figure 6B).

(C) BIFC assay shows that AGL16 can form a heterodimer with both FLC and SVP in N. benthamiana leaf epidermis. From left to right, top panel, (1) AGL16 is localized in the nucleus, (2) no signal, showing that AGL16 and LHP1 do not interact, (3) positive signal, showing that FLC and SVP interact, and (4) enlarged image of (3). From left to right, bottom panel, (1) positive signal showing that FLC and AGL16 interact, (2) enlargement of (1), (3) positive signal showing that SVP and AGL16 interact, and (4) enlargement of (3). Bars = 10 µm.

(D) Yeast two-hybrid assay confirms the direct interaction between AGL16 and SVP but not between AGL16 and FLC. Each protein was fused to either the activation domain (AD) as prey or DNA binding domain (BD) as bait. Serial dilutions (from $10^{-1}$ to $10^{-4}$) of J69-4A cells containing different construct combinations indicated on the right were grown on selective medium. The FLC/SVP combination and the protein/empty vector combinations provide positive and negative controls, respectively.

(E) and (F) Genetic analysis reveals that SVP is epistatic to AGL16. The rosette (filled bars) and cauline (open bars) leaves upon flowering (LD growing) are shown for svp-41, agl16-1 svp-41, agl16-1, and Col-0 wild type. Error bars indicate so of means. Significance level was tested with the Wilcoxon test (**P < 0.001; “P < 0.01).

(G) and (H) Expression of either AGL16 (G) or SVP (H) is not significantly changed in the loss-of-function mutant svp-41 (G) or agl16-1 (H) compared with Col-0 wild type (normalized to PP2A). Error bars indicate so of means for three biological replicates.
flowing time in *Arabidopsis*. The magnitude of this role depends on the genetic background and the environmental conditions.

AGL16 belongs to the MADS box family of transcription factors, which are often implicated in controlling structural development and/or transitional timing (Theissen et al., 2000; Becker and Theissen, 2003). Under LD conditions, loss of function of AGL16 in Col-FRI backgrounds counteracted the delayed flowering caused by FRI introgression and the associated strong expression of the floral repressor FLC (Michaels and Amasino, 1999). In contrast to FLC, which delays flowering in both LD and SD conditions, the effect of AGL16 was more specific to the LD photoperiod. This might be due to the repressive action of AGL16 on FT, the major regulator of flowering under LD conditions (Kardailsky et al., 1999; Samach et al., 2000). Interestingly, AGL16 expression seemed to be decreased in plants grown in 24-h light and increased in dark-grown plants (https://www.genevestigator.com/gv/plant.jsp).

Our data show that AGL16 and FLC act additively, in a manner directly proportional to the allelic dosage at the two loci (Figure 3). Our study of protein interactions in yeast and tobacco reveals AGL16 may participate in the repressive complexes formed by SVP and/or FLC (Figure 6), thereby providing a molecular model for the genetic interaction between AGL16 and FLC. AGL16 can directly interact with SVP and potentially form heterodimers with FLC in tobacco leaves, but since the interaction is not detected in yeast, the two proteins might not be in direct contact. SVP could be one partner mediating the interaction between AGL16 and FLC as SVP can directly interact with FLC (Li et al., 2008); FLM could also mediate this interaction as FLM can interact physically with both FLC and SVP (Gu et al., 2013; Lee et al., 2013; Posé et al., 2013). FLC might indeed participate in a large protein complex with a molecular weight exceeding several hundreds of kilodaltons (Hellwell et al., 2006), much larger than if it contained only one FLC protein forming a dimer with SVP (Li et al., 2008) or some other members of the FLC clade (Gu et al., 2013). Therefore, we propose that the miR824/AGL16 module may act as a new trans-factor of FT to regulate the timing of floral transition via interaction with the protein complexes formed by AGL16 and other MADS box proteins.

This model will have to be validated in future studies. Indeed, it is also possible that the activity of AGL16 is regulated at the transcriptional level. AGL16 is one of the major targets bound by FLC (Deng et al., 2011) and a weak target for SVP (Gregis et al., 2013). In loss-of-function mutants for both FLC (flc-3) and SVP (svp-41), the expression of AGL16 was increased (Figure 6; Deng et al., 2011; Gregis et al., 2013). However, this increase was modest, probably due to the negative regulation of AGL16 mRNA abundance by miR824. Multiple negative feedbacks might therefore participate in the regulation of AGL16 activity.

The miR824/AGL16 module was initially reported to promote the development of higher-order stomata complexes, by increasing the number of additional divisions in meristemoid cell lineages (Kutter et al., 2007). The function of AGL16 that we describe here might in fact be the most important for plant fitness. Indeed, the effect of AGL16 is particularly pronounced in a background with high FLC activity, and flowering time has been shown to have a strong impact on the number of branches and plant lifetime fruit production (Fournier-Level et al., 2013). Instead, the effect of agl16-1 on meristemoid division and differentiation did not seem to translate into a macroscopic phenotype such as altered stomatal density. When AGL16 was strongly overexpressed, stomatal density was increased, but these plants also displayed severe growth defects, with leaves that were not fully expanded (Kutter et al., 2007). Nevertheless, it remains possible that AGL16 is involved in additional functions, especially since it is expressed in the root as well (Gan et al., 2010).

Like FLC (Sheldon et al., 2000) and SVP (Hartmann et al., 2000), which control flowering in a quantitative manner, AGL16 controls flowering depending on allelic dosage (Figure 3). The repressive action of AGL16 on flowering time can therefore be modulated by mutations altering the level of AGL16 mRNA, the amount of AGL16 protein, or perhaps the abundance of the putative AGL16-SVP/FLC repressor complexes. FLC, FRI, FLM, and SVP were all reported to control natural variation of flowering time (Johanson et al., 2000; Michaels et al., 2003; Werner et al., 2005; Mendez-Vigo et al., 2013). We reexamined the significant allelic association of AGL16 genotypes with flowering time variation reported in the field (Brachi et al., 2010). Closer inspection suggests that this effect requires the presence of a functional FRI allele and depends on allelic variation at the FLC locus. Therefore, allelic combinations of SVP, FLC, and miR824/AGL16 variants could contribute to natural variation of flowering time in *Arabidopsis*. AGL16 shows no amino acid polymorphism but two SNPs located in introns. These two SNPs do not have obvious consequences on mRNA maturation because they are not in the splicing elements, but an effect on regulation cannot be excluded. Future GWAS studies including larger sets of genotypes will be useful to validate this effect and investigate the role of AGL16 regulation in adaptive evolution.

**METHODS**

**Plant Materials, Growth Conditions, and Flowering Time Scoring**

*Arabidopsis thaliana* plants including wild-type Col-0, agl16-1, and m3 have been described (Kutter et al., 2007). The knockout mutant of FT (ft-10) was reported by Yoo et al. (2005). The flc-3 mutant in the Col-0 background with the St-2 FRI introgression was reported by Michaels and Amasino (1999). To test the effect of modifying AGL16 activity in the Col-FRI background, Col-FRI flc-3 was crossed to Col-0 agl16-1 and Col-0 m3, respectively. Homozygote double mutants at FRI, FLC, or AGL16 were screened by PCR using gene-specific primers (Supplemental Table 2). The F2 population used in Figure 2 was produced by crossing Col-0 agl16-1 with Col-FRI flc-3. The seeds from self-pollinated F1 plants were sown to soil. After all the individuals had bolted, a young leaf was harvested for each plant for rapid genomic DNA isolation using a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M NaCl, and 10% (w/v) sucrose as extraction buffer. The extract was not purified and directly used for genotyping (see Supplemental Table 2 for primer sequences).

The T1 lines of the miR824 target mimicry lines were described by Todesco et al. (2010). The progeny of seven lines with 3:1 segregation were treated with BASTA reagents to screen for the homozygote single insertion of the mimics. T3 seeds were used for scoring flowering phenotypes using the Col-0 line transformed with an empty binary vector as control (Todesco et al., 2010). To test the genetic relationship between AGL16 and FT, agl16-1 was crossed to the ft-10 mutant and the
homozygote double mutant was obtained by screening the segregating F2 population using agl16-1 and ft-10–specific primers (Supplemental Table 2). For investigating the relationship between AGL16 and SVP, agl16-1 was crossed to the svp–41 mutant (Hartmann et al., 2000) with the resulting homozygote double mutant screened by gene-specific primers (Supplemental Table 2).

For promoter-miR824:GUS transgenic lines, the 4.7-kb fragment comprising the 3.093-kb sequences upstream of the TSS (+1) and 1.61-kb segment downstream of TSS was cloned into the Gateway pDONR207 vector (Invitrogen) and then fused with the GUS reporter gene in pGREEN-GW:GUS via Gateway technology (Invitrogen). In this vector, the selection marker BAR is driven by a NOS promoter (Adrian et al., 2010). After sequencing the inserted fragment to confirm the absence of mutations caused by PCR, independent transgenic lines were generated in Col-0 via floral dipping (Clough and Bent, 1998). The first rosette leaf of at least five independent 2-week-old T1 plants was used for GUS staining.

Arabidopsis seeds were stratified in distilled water at 4°C for 72 h and sown in soil and grown under LD (16-h light at 21°C and 8-h night at 18°C) or SD (8-h light at 21°C and 16-h night at 18°C) conditions either in glass houses or growth chambers. Pots and trays were randomized every 2 d to minimize the positional effect on flowering time. For time-course expression of FT, mutant seeds and their corresponding wild-type lines were sterilized with bleach and stratified at 4°C for 72 h. These seeds were then sown on Murashige and Skoog medium plates containing 1% sucrose and grown under LD conditions in growth chambers. For the assays of FT expression across developmental stages and tissues, seeds were sown in soil and grown under LD conditions.

Flowering time was scored for each plant by the RLN or total leaf number (including rosette leaves and cauline leaves), when the first flower was visible. In a few trials, the number of days until the appearance of the first visible flower (days to flowering) was scored in addition to the RLN. Vernalization treatment was applied to LD-grown 10-d-old seedlings in a growth chamber with the temperature set at 4°C under LD conditions. All the experiments were repeated at least two, and up to 10, independent trials, in glass houses and/ or growth chambers. See Supplemental Data Set 1 for detailed information.

**BiFC Assay**

To reveal the cellular localization of AGL16, full-length cDNA without the stop codon of AGL16 was cloned via Gateway technology (Invitrogen) into pEarleyGate101 containing a 35S promoter and YFP-HA tags after the Gateway cassette (Earley et al., 2006). The resulting construct was used to transform the Col-0 agl16-1 mutant. The cellular localization of AGL16 was examined in T1 plants under a LSM 700 confocal laser scanning microscope (Carl Zeiss). For the BiFC assay, PCR fragments amplified with the specific primers for AGL16, FLC, and LHP1 (see Supplemental Table 2 for primer information) were subcloned into the pDONR221 entry vector (Invitrogen). LHP1 was used as a negative control. The resulting plasmids were inserted by Gateway cloning (LR reaction) into the split YFP vectors RIA-sYFP-N:pBatTL-L and RIA-sYFP-C:pBatTL-B. Agrobacterium tumefaciens transformant strains carrying plasmids for BiFC and p19 were grown overnight at 28°C in 10 mL YEP medium plus selective antibiotics, collected by centrifugation, and resuspended in infiltration medium (10 mM MgCl₂, 150 µg/mL acetosyringone, and 10 mM MES-NaOH, pH 5.6) (Voinnet et al., 2003). Cells were kept at 28°C in the infiltration solution for 3 h in darkness and infiltrated into the abaxial surface of 3-week-old Nicotiana benthamiana plants. The fluorescence signal of YFP was observed and recorded using an LSM 700 confocal laser scanning microscope.

**Yeast Two-Hybrid Assay**

To test the ability of AGL16 to interact with FLC and SVP, full-length cDNAs excluding the stop codons for these genes were amplified from an Arabidopsis (Col-0) cDNA pool (see Supplemental Table 2 for primer sequences) and cloned into the pDONR201 or pDONR207 vectors to generate pENTRY plasmids. The bait constructs pDEST32-AGL16 and pDEST32-SVP were generated via the LR reaction between the entry plasmids and pDEST32 (Invitrogen), respectively. Similarly, the prey constructs pDEST22-FLC and pDEST22-SVP were produced by the LR reaction between entry plasmids and pDEST22 (Invitrogen), respectively. Bait plasmids and prey plasmids or the blank pDEST22 or pDEST32 were cotransformed into yeast strain J69–4A (James et al., 1996), respectively. Medium lacking SD-Leu-Trp-His was used for selection.

**Comparison of Phenotypes**

Statistical difference in flowering time in various lines was performed using both Student’s t test (two tailed with two-sample assuming unequal variance) with Bonferroni correction in Microsoft Excel and Wilcoxon rank sum test in R.

Genetic interactions between genotypes in the F2 population were analyzed with the general linear model (glm function) in R following the following model: flowering time = genotype AGL16 × genotype FRI × genotype FLC + tray number + error, in which AGL16 and FLC genotypes were classified into 0 (knockout homozygote), 1 (heterozygote), or 2 (wild-type homozygote); FRI genotypes were classified into functional or non-functional as the functional FRI allele was dominant. Because Poisson distributions are well suited for count data, our data poorly fit to a Poisson distribution and showed clear signs of overdispersion. Therefore, the dispersion parameter of a quasi-Poisson distribution was fitted to the data in the glm function in R, following recommendation by Crawley (2005). Best-fit models were detected by removing nonsignificant interaction items stepwise from the most complex interactions to simpler ones. ANOVA analysis with the Chisq method was used to test whether reducing an interaction item would improve the fit of the model to the data. To test whether the segregation of AGL16, FRI, and FLC follows a Mendelian inheritance, a Fisher’s exact test was performed in R.

**GUS Staining**

For GUS staining, seedlings were incubated for 30 min in 90% (v/v) acetonitrile, rinsed with 50 mM sodium phosphate buffer, pH 7.0, and incubated overnight at 37°C in staining solution (0.5 mg/mL X-Gluc [5-bromo-4-chloro-3-indolyl-β-D-glucuronide], 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% [v/v] Triton X-100). After staining, samples were washed with 50 mM sodium phosphate buffer, pH 7.0, and cleared in 70% (v/v) ethanol. The GUS histochemical staining was visualized under a light stereomicroscope (MZ 16 FA; Leica). The first rosette leaves of at least five 2-week-old T1 transgenic plants were stained. A typical GUS pattern is shown in Figures 5B and 5C.

**RNA Isolation and Real-Time Quantitative RT-PCR Assays**

Total RNA was extracted with TRIzol reagent (Invitrogen). For time-course monitoring of gene expression, both whole seedlings and the aerial parts of the 9-d-old seedlings were used. Tissues were first harvested just before dawn and then collected every 4 h for 24 h. To minimize the duration of sampling at each time point, only one pool of 10 to 15 seedlings was constituted in each of two independent trials. For monitoring tissue-specific expression, the leaf was separated with a forceps from other parts (including the petioles, emerging young rosette leaves, and meristems as well as the hypocotyl without the root) of the 10–12-d-old seedlings and collected separately. For each sampling, three pools (biological replicates) were made with the tissues of 10 to 15 seedlings in each of at least two independent trials, in LD conditions in glass houses or growth chambers. A similar protocol was followed for monitoring gene expression in leaves during development.
Statistical Analysis of Natural Variation at the AGL16 Locus

The 250K SNP array contained two SNPs mapping in gene are listed in Supplemental Table 2. mRNA levels are given in Figures 4A, 6G, and 6H. Primers used for each CFX384 (Eppendorf) or a 12 of 14 The Plant Cell expression of test whether natural variation at the SYBR Green Supermix (Bio-Rad) on either a Mastercycler Realplex2 (Eppendorf) or a CFX384 Touch real-time PCR detection system (Bio-Rad). The relative expression of FT and AGL16 was normalized to the expression of PP2A and/or Tubulin2. The mRNA levels relative to PP2A mRNA levels are given in Figures 4A, 6G, and 6H. Primers used for each gene are listed in Supplemental Table 2.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: miR824 (At4g24415), AGL16 (At3g57230), FLC (At5g10140), PP2A (At1g13320), FRI (At4g00650), PP2A (AT1G13320), LHP1 (AT5G17690), and TUB2 (AT5G62690).

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure 1. AGL16 Expression Was Repressed in m3. Supplemental Figure 2. miR824 Target Mimics Flowered Later under Long-Day Conditions (Supplementary to Figure 4). Supplemental Figure 3. Promoter-miR824:GUS Staining Reveals the Expression of miR824 in Guard Cells. Supplemental Figure 4. The Expression of AGL16 in the Wild Type and Mutants in Both Col-0 and Col-FRI Backgrounds. Supplemental Figure 5. The Relative FT Expression in a Second Independent Trial Confirming the Pattern Shown in Figure 5. Supplemental Figure 6. The Relative Expression in an Independent Trial Confirming the Expression Pattern for AGL16 Observed in Figure 6A in the fcl-3 Mutant and for FLC in the Col-0 Background (Supplemental for Figure 6B). Supplemental Figure 7. Polymorphisms at AGL16 Are Associated with Flowering Time Variation in Common Garden Conditions. Supplemental Table 1. Statistical Significance for Pairwise Mean Flowering Time Differences among Lines Presented in Figure 3B. Supplemental Table 2. Primers Used in This Study.

Supplemental Table 3. Statistical Test of AGL16 Allele Association with Flowering Time in Field Conditions (Data from Brachi et al. [2011]).

Supplemental Table 4. Wilcoxon Rank Sum Test of Pairwise Mean Flowering Comparison for Various Allelic Comparisons.

Supplemental Data Set 1. Summary of All Flowering Assays.

Supplemental Data Set 2. Statistical Analysis of the F2 Segregating Population Presented in Figure 3A.

Supplemental Data Set 3. AGL16 Genotypes for 171 Accessions Used for Reanalyzing the Brachi Flowering Time Data.

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AUTHOR CONTRIBUTIONS


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miR824/AGL16 Controls Flowering Time


