miR156-Regulated SPL Transcription Factors Define an Endogenous Flowering Pathway in Arabidopsis thaliana

Jia-Wei Wang,¹ Benjamin Czech,¹,² and Detlef Weigel¹,*

¹Department of Molecular Biology, Max Planck Institute for Developmental Biology, D-72076 Tübingen, Germany
²Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
*Correspondence: weigel@weigelworld.org
DOI 10.1016/j.cell.2009.06.014

SUMMARY

The FT gene integrates several external and endogenous cues controlling flowering, including information on day length. A complex of the mobile FT protein and the bZIP transcription factor FD in turn has a central role in activating genes that execute the switch from vegetative to reproductive development. Here we reveal that microRNA156-targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes not only act downstream of FT/FD, but also define a separate endogenous flowering pathway. High levels of miR156 in young plants prevent precocious flowering. A subsequent day length-independent decline in miR156 abundance provides a permissive environment for flowering and is paralleled by a rise in SPL levels. At the shoot apex, FT/FD and SPLs converge on an overlapping set of targets, with SPLs directly activating flower-promoting MADS box genes, providing a molecular substrate for both the redundant activities and the feed-forward action of the miR156/SPL and FT/FD modules in flowering control.

INTRODUCTION

The onset of flowering is a major developmental transition that is critical to the reproductive success of plants. In Arabidopsis thaliana, the MADS domain protein APETALA1 (AP1) and the plant-specific transcription factor LEAFY (LFY) are master regulators that control the fate of flowers produced at the shoot apex. In the absence of AP1 and LFY, flowers are replaced by shoots or by flowers that have shoot-like characteristics (reviewed in Lohmann and Weigel, 2002). The activation of LFY and AP1 in response to floral inductive signals involves several MADS domain proteins, including SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), FRUITFULL (FUL), and AGAMOUS-LIKE 24 (AGL24) (Lee et al., 2008; Li et al., 2008; Liu et al., 2007, 2008; Michaels et al., 2003; Yu et al., 2002). Genetic and expression studies have shown that SOC1, FUL, and AGL24 all function downstream of CONSTANS (CO), the output of the photoperiod pathway, which accelerates flowering of A. thaliana in long days (Borner et al., 2000; Lee et al., 2000; Melzer et al., 2008; Samach et al., 2000; Schmid et al., 2003; Yu et al., 2002). CO is connected to the MADS box genes through activation of FT, which integrates information from several positively and negatively acting signaling pathways. An overwhelming amount of circumstantial evidence indicates that the small FT protein is a long-distance signal that moves directly from leaves to the shoot apex (reviewed in Baurle and Dean, 2006; Kobayashi and Weigel, 2007; Turck et al., 2008). FT executes its role through interaction with the bZIP transcription factor FD, which is expressed specifically at the shoot apex, where the FT/FD complex activates several MADS box genes (Aben et al., 2005; Searle et al., 2006; Wigge et al., 2005). Like FT in leaves, FD at the shoot apex is a direct target of the potent repressor FLOWERING LOCUS C (FLC), which mediates the effects of winter-like exposure to cold temperature, vernalization (Searle et al., 2006). FLC forms a complex with another MADS domain protein, SHORT VEGETATIVE PHASE (SVP), and together they repress not only FT but also the FT target SOC1 (Lee et al., 2007; Li et al., 2008).

Another set of transcriptional regulators that is expressed before LFY and AP1 are activated at the shoot apex comprises several microRNA156-regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) factors (Cardon et al., 1997; Schmid et al., 2003). This class of proteins was discovered in snapdragon, from which the founding member was isolated based on its ability to bind a conserved motif in the promoter of the AP1 ortholog SQUAMOSA (Klein et al., 1996). In A. thaliana, there are two broad classes of miR156 targets: SPL3, SPL4, and SPL5 encode small proteins that consist mostly of the SBP DNA binding domain, whereas the other eight miR156 targets in A. thaliana encode much larger proteins (Cardon et al., 1999; Yang et al., 2008). SPL3, SPL4, and SPL5 appear to function mostly in the control of flowering time and phase change, whereas SPL9 and its paralog SPL15, which are members of the second group, also have strong effects on leaf initiation rate. Consistent with both groups of SPL factors being important for flowering, overexpression of SPL3 and SPL9 accelerates flowering, whereas reduction in SPL activity through miR156 overexpression delays the onset of flowering (Cardon et al., 1997; Gandikota et al., 2007; Schwab et al., 2005; Schwarz et al., 2008; Wang et al., 2008; Wu and Poethig, 2006).
Previous expression studies have suggested that the SPL genes affect flowering as downstream targets of CO and FT at the shoot apex (Schmid et al., 2003). Here, we show that SPL genes have an important FT-independent role in regulating flowering. SPLs and the FT/FD complex share several direct targets, including AP1, that act in multiple feed-forward loops. We conclude that the miR156/SPL module represents a major regulatory axis for the promotion of flowering in the absence of photoperiodic cues.

RESULTS

Expression Pattern of miR156 and SPL Genes

We used small RNA blots to complement previous studies on the expression pattern of miR156 during the A. thaliana life cycle (Gandikota et al., 2007; Wang et al., 2008; Wu and Poethig, 2006). In short days, miR156 levels were highest in young seedlings and subsequently declined over several weeks (Figure 1A; Wu and Poethig, 2006). We observed a similar pattern for pri-MIR156a by using reverse transcription followed by quantitative real-time PCR (qRT-PCR) (Figure 1B), suggesting that the primary regulation of miR156 is at the transcriptional level. The temporal expression pattern of SPL3 is the inverse of miR156, with low levels in short-day grown seedlings and a gradual increase during vegetative development (Wu and Poethig, 2006). We observed a similar profile for SPL9 (Figure 1C). The AtGenExpress expression atlas (Schmid et al., 2005) indicated similar trends for several additional miR156 targets (Figure S1 available online).

Inactivation or overexpression of the flowering regulators FLC, CO, FT, or SOC1 had no obvious effects on miR156 levels in whole seedlings. This was also true for plants treated with plant hormones, including the gibberellin GA3, which accelerates flowering (Langridge, 1957; Michaels and Amasino, 1999; Sheldon et al., 1999; Wilson et al., 1992), auxin (IAA), or cytokinin (Figure 1D). Similarly, SPL9 transcript levels in seedlings were not affected by increased expression of the floral repressor FLC in plants that have a functional copy of the FLC activator FRIGIDA (FRI) (Michaels and Amasino, 1999), nor did they respond to vernalization, to GA3 treatment, or to elimination of endogenous gibberellins in the ga1-3 mutant (Figure 1E). Vernalization or GA treatment accelerated the flowering of p35S:MIR156 plants, but less than that of wild-type (Table S1). The flowering responses together with the absence of major effects of vernalization and GA treatment on miR156 and SPL levels suggest that the

Figure 1. Expression of miR156, SPL3, and SPL9

(A) RNA blot analysis of mature miR156 in short-day-grown plants.

(B) qRT-PCR analysis of MIR156A primary transcripts, normalized to that of β-TUBULIN-2. Error bars indicate range of replicates.

(C) qRT-PCR analysis of SPL9 expression in short days, with floral marker AP1 as control.

(D) RNA blot analysis of miR156 in different genetic backgrounds and in response to 6 hr treatments with 100 μM GA3, IAA, or the cytokinin t-zeatin (CK). Seedlings were 1 week old.

(E) qRT-PCR analysis of SPL9 expression in different genetic backgrounds and in response to vernalization or GA3 treatment. Nonvernalized seedlings were 2 weeks old. For vernalization, 1-week-old seedlings were transferred to 4°C for the indicated time. For GA treatment, seedlings were sprayed with 100 μM GA3 at 1 and 2 weeks of age.

(F and G) In situ hybridization to detect SPL3 (F) and SPL9 (G) in shoot apices. Days refer to time after transfer of 30-day-old plants from short to long days. b, cryptic bract; f, flower primordium; l, leaf primordium. Arrows point to provascular strands.

(H) SPL9 expression in apices of ft-10 tsf-1 double mutants. Plants were grown in long days and harvested continuously to obtain apices during the vegetative phase, at the beginning, during, and after the transition to flowering (from left to right). Scale bars represent 50 μm.
miR156/SPL module promotes flowering in parallel with, or upstream of, these two pathways, which was further supported by the finding that SPL3 overexpression from a p35S:rSPL3 construct (see below) could accelerate the flowering of FRI/FLC or ga1-3 plants (Table S1).

At the shoot apex, upregulation of SPL3 and SPL9 is readily detectable by microarrays within 3 days after transfer of vegetative plants from short days to inductive long days, and this induction is much reduced in co and ft mutants (Schmid et al., 2003). Much higher levels of expression in floral induced apices compared to vegetative apices were observed for most miR156 targets (Figure S1). Transfer to long days did not cause an obvious change in miR156 levels at the shoot apex (Figure S2), suggesting that photoperiod-dependent upregulation of SPLs is not due to a release of miR156 inhibition.

In situ hybridization did not reveal a distinct pattern of SPL3 expression in vegetative apices, whereas SPL9 transcripts were strongest in leaf primordia and the provascular strands of young leaves (Figures 1F and 1G; Wang et al., 2008). After transfer to long days, SPL3 and SPL9 levels increased in both existing leaves and newly formed primordia, with SPL9 being also expressed in provascular strands below the shoot apical meristem. SPL9 was transiently upregulated in floral anlagen and very early floral primordia, but declined again by stage 2 of flower development. Expression of SPL3 in newly formed primordia was strongest in cryptic bracts subtending flowers (Long and Barton, 2000). miR156 overexpression reduced SPL3 and SPL9 transcripts to background levels (Figures 1F and 1G).

p35S:MIR156 plants had a reduced response to transient exposure to long days, with 5 instead of 2 days required for quantitative induction of flowering (Schwarz et al., 2008; data not shown). This was not caused by attenuated FT induction, as determined by transiently exposing short-day-grown p35S:MIR156 plants to 2 long days (Figure S3A), nor was it due to reduced expression of the FT partner FD before photoperiodic induction (Figure S3B).

Finally, in ft-10 tsf-1 plants, which lack activity of both FT and its paralog TWIN SISTER OF FT (TSF) (Michaels et al., 2005; Yama-guchi et al., 2005), SPL9 expression in shoot apices increased from the vegetative phase until and through the transition of flowering (Figure 1H), confirming a photoperiod-independent but age-dependent rise in SPL9 expression. Taken together, these data indicate that SPL levels increase throughout the plant with increasing age, but that in addition, acute induction of flowering through the photoperiod pathway can cause an immediate and strong upregulation of SPL genes at the shoot apex.

**Tissue-Specific Effects of SPLs on Flowering Time**

The effects of miR156 and its SPL targets on leaf initiation rate, which is accelerated in plants with attenuated SPL activity but reduced in plants with increased SPL activity (Schwarz et al., 2008; Wang et al., 2008), confounds the measurement of flowering time, which is often expressed as number of leaves produced before the first flower is made. Nevertheless, when taking both leaf number and chronological time into account, it is clear that overexpression of miR156, which reduces SPL expression, moderately delays flowering, whereas inhibition of miR156 activity, either by mutating its target site in SPL3 (p35S:rSPL3) or through a miR156 target mimic (p35S:MIM156), accelerates flowering (Franco-Zorrilla et al., 2007; Gandikota et al., 2007; Schwab et al., 2005; Wang et al., 2008; Wu and Poethig, 2006). In general, the flowering time effects were more pronounced in short than in long days (Table 1).

To explore the tissue requirement of SPLs, we made use of plants that misexpress miR156 from the SUC2 promoter in phloem companion cells, the site of FT action (An et al., 2004; Takada and Goto, 2003), or in FD-expressing cells at the shoot apex. pFD:MIR156 plants (in which plastochron length is unaffected; Wang et al., 2008) flowered even later than p35S:MIR156 plants. This was slightly enhanced when we introduced in addition a pSUC2:MIR156 transgene, which on its own had a lesser effect on flowering (Table 1). Consistent with SPLs being able to act both in the phloem and at the shoot apex, expression of the nontargeted version of SPL3, rSPL3, from either the SUC2 and FD promoter led to early flowering (Table 1; Wang et al., 2008). Thus, although the shoot apex has a higher requirement for SPL activity than does the phloem, SPL genes act at both sites, similar to SOC1 and FLC (Searle et al., 2006), pFD:rSPL3 ft-10 plants were intermediate between the parents, but the ft-10 mutation suppressed the effects of rSPL3 expression in the phloem (Table 1). Similar results were obtained with misexpression of rSPL9 (Wang et al., 2008). Thus, SPLs may act through FT in the phloem.

**Identification of MADS Box Genes as SPL Targets**

To identify SPL targets, we analyzed the transcriptome of argonaute1 (ago1-27) and serrate (se-1) plants, which both overexpress SPL genes resulting from reduced miR156 accumulation (Ronemus et al., 2006; Wang et al., 2008). We compared these two genotypes to p35S:MIR156 se-1, in which SPL transcripts are reduced, and to wild-type. Several MADS box genes, including FUL and SOC1, which have redundant roles in promoting flowering (Melzer et al., 2008), and the SOC1 paralog AGL42 showed a profile across the four genotypes similar to that of the SPLs (Figure S4A). qRT-PCR confirmed that irrespective of photoperiod, FUL, SOC1, and AGL42 levels were increased in p35S:MIM156 seedlings, which have elevated SPL levels because of expression of a miR156 target mimic (Franco-Zorrilla et al., 2007), but decreased in p35S:MIR156 plants, which have reduced SPL levels (Figure 2A; Figure S4B). In contrast to FUL, SOC1, and AGL42, the expression levels of the SOC1 partner AGL24 or of the AGL24 homolog SVP do not substantially increase at the shoot apex in conjunction with the transition to flowering (Figure S1; Hartmann et al., 2000; Michaels et al., 2003; Yu et al., 2002). AGL24 was unaffected in p35S:MIR156 or p35S:MIM156 plants (data not shown). There appears to be some differentiation in SPL function, as indicated by the fact that p35S:rSPL3 strongly induced FUL but had much weaker or no effects on SOC1 and AGL42, whereas pSPL9:rSPL9 had similar effects on all three targets, especially in short days (Figure 2A; Figure S4B).

We used in situ hybridization to compare the expression patterns of SOC1 and FUL as well as the floral marker AP1 at the shoot apex with those of SPL3 and SPL9. No distinct patterns of SOC1 and FUL RNA were detected in vegetative shoot apices, but discrete sites of expression became detectable.
within a day after transfer of plants from short to long days, which was 2 days earlier than for AP1 (Figures 3A, 3C, and 3E; Borner et al., 2000; Hempel et al., 1997; Samach et al., 2000; Schmid et al., 2003). Similar to SPL3 and SPL9 (Figures 1F and 1G), strong expression of SOC1 and FUL in floral anlagen was transient, and both were also upregulated in the provascular strains of existing leaf primordia. Activation of SOC1 and FUL at the shoot apex was delayed in p35S:MIR156 plants, in a manner similar to that in fd-2 plants (Figures 3B and 3D).

To confirm that MADS box genes are early targets of SPLs, we expressed a translational fusion of the hormone-binding domain of rat glucocorticoid receptor (GR) to SPL9 under the control of SPL9 genomic sequences. Treatment with the GR ligand dexamethasone (DEX) induced phenotypes mimicking those of pSPL9:rSPL9 plants (Wang et al., 2008). FUL RNA levels were increased 4-fold after 6 hr in DEX-treated plants and up to 10-fold after 12 hr (Figure 2B). We also generated a translational fusion of β-glucuronidase (GUS) that included 2.5 kb promoter and first intron of FUL in vitro (Figure 2D; Birkenbihl et al., 2005; Klein et al., 1996; Kro- pat et al., 2005; Liang et al., 2008). A fifth region, in the last exon and lacking a GTAC motif, was included as a control. Chromatin extracted from 1-week-old seedlings was treated with either HA or Myc antibody, and the precipitated DNA was subjected to qPCR. There was no apparent enrichment of any of the fragments in wild-type samples, while regions I to IV were readily extracted from p35S:MIR156 plants, which were indistinguishable from p35S:rSPL3 plants. We focused on four regions of the FUL promoter and first intron that contained GTAC boxes, which have been identified as the core binding motif of SPLs in vitro (Figure 2D; Birkenbihl et al., 2005; Klein et al., 1996; Kro-pat et al., 2005; Liang et al., 2008). A fifth region, in the last exon and lacking a GTAC motif, was included as a control. Chromatin extracted from 1-week-old seedlings was treated with either HA or Myc antibody, and the precipitated DNA was subjected to qPCR. There was no apparent enrichment of any of the fragments in wild-type samples, while regions I to IV were readily amplified from p35S:rSPL3-HA samples after immunoprecipitation with HA, but not with Myc, antibody (Figures 2E and 2F).

We used pSPL9:GFP-rSPL9 plants, which express a translational fusion of SPL9 to green fluorescent protein (GFP) and have phenotypes similar to those of pSPL9:rSPL9, to determine whether SPL9 might bind SOC1 and AGL42, since both were strongly induced in short-day-grown pSPL9:rSPL9 plants (Figure 2A; Figure S4B). The genomic regions of SOC1 and AGL42 also contain several GTAC boxes in their promoters and first introns (Figure 2D; Figure S4C), and several fragments spanning the GTAC motifs were efficiently enriched after ChIP with GFP antibody, whether SPL9 or Myc antibody, and the precipitated DNA was subjected to qPCR. There was no apparent enrichment of any of the fragments in wild-type samples, while regions I to IV were readily amplified from p35S:rSPL3-HA samples after immunoprecipitation with HA, but not with Myc, antibody (Figures 2E and 2F).

### Table 1. Effects of Reduced and Increased SPL Activity on Flowering

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Leaves</th>
<th>Flowering Time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Range</td>
</tr>
<tr>
<td>Wild-type</td>
<td>13.4 ± 0.5</td>
<td>13–14</td>
</tr>
<tr>
<td>p35S:MIR156</td>
<td>23.4 ± 0.5</td>
<td>19–21</td>
</tr>
<tr>
<td>p35S:MIM156</td>
<td>5.0 ± 0.6</td>
<td>4–6</td>
</tr>
<tr>
<td>p35S:rSPL3</td>
<td>6.7 ± 0.5</td>
<td>6–7</td>
</tr>
<tr>
<td>p35S:rSPL3 ful</td>
<td>21.1 ± 1.2</td>
<td>19–23</td>
</tr>
<tr>
<td>pSUC2:MIR156</td>
<td>20.6 ± 0.7</td>
<td>20–22</td>
</tr>
<tr>
<td>pFD:MIR156 pSUC2:MIR156</td>
<td>26.9 ± 1.1</td>
<td>26–29</td>
</tr>
<tr>
<td>pFD:rSPL3</td>
<td>9.57 ± 0.5</td>
<td>9–10</td>
</tr>
<tr>
<td>pSUC2:rSPL3</td>
<td>9.36 ± 0.5</td>
<td>9–10</td>
</tr>
<tr>
<td>pFD:rSPL3 pSUC2:rSPL3</td>
<td>8.43 ± 0.7</td>
<td>7–9</td>
</tr>
<tr>
<td>ft-10</td>
<td>40.1 ± 0.5</td>
<td>39–41</td>
</tr>
<tr>
<td>pFD:rSPL3 ft-10</td>
<td>24.8 ± 0.9</td>
<td>24–26</td>
</tr>
<tr>
<td>pSUC2:rSPL3 ft-10</td>
<td>39.9 ± 0.6</td>
<td>39–41</td>
</tr>
</tbody>
</table>

Standard deviation is given. n = 14 for all genotypes.

*Note that plastochron length is unaffected by pFD:MIR156 (Wang et al., 2008) and that leaf number is therefore a good indicator of flowering time.*

### Tissue-Specific Effects of FUL on Flowering Time

Because SPL3 affected primarily FUL expression, we examined the genetic interaction of SPL3 with FUL. p35S:rSPL3 ful-7

---

**Cell** 138, 738–749, August 21, 2009 ©2009 Elsevier Inc.
plants were phenotypically intermediate between the parents, and both flowering time in days and total leaf number were similar to that of wild-type (Table 2; Figure S5A). Conversely, in p35S:FUL plants, which flowered early (Ferrándiz et al., 2000b) and were similar to p35S:rSPL3 plants in both leaf number and days to flowering, the p35S:MIR156 phenotype was strongly suppressed (Table 2; Figure S5B).

Since FUL is expressed in both leaves and at the shoot apex, and since reducing activity of the SPL upstream regulators in either tissue affects flowering time, we determined whether FUL could promote flowering in both tissues as well. In long days, misexpression of FUL from either the phloem-specific SUC2 promoter or from the shoot-apex-specific FD promoter caused early flowering (Table 2), similar to what has been reported for SOC1 (Searle et al., 2006). The ft-10 mutation completely suppressed the early-flowering phenotype of pSUC2:FUL, suggesting that FUL can accelerate flowering through FT, again similar to SOC1 (Searle et al., 2006) and to SPL3. In contrast, pFD:FUL ft-10 plants flowered earlier than nontransgenic ft-10 plants, indicating that FUL acts at the shoot apex downstream of or in parallel with the FT/FD complex.

In a complementary set of experiments, we tested the requirement for FUL activity in leaves and at the shoot apex by reducing its expression with an artificial miRNA (amiR-FUL) (Schwab et al.,

---

**Figure 2. Identification of MADS Box Genes as Direct SPL Targets**

(A) qRT-PCR analysis of FUL and SOC1 expression in 7-day-old long-day-grown and 10-day-old short-day-grown seedlings.

(B) qRT-PCR analysis of FUL expression in pSPL9:rSPL9-GR seedlings after DEX induction. Inset shows mock-treated (left) and DEX-treated pSPL9:rSPL9-GR seedlings.

(C) pFUL:GUS activity in 1-week-old seedlings. Scale bar represents 1 cm.

(D) Diagram of FUL and SOC1 genomic regions. Triangles indicate GTAC boxes in promoter and introns. The first exon of SOC1, indicated in light gray, is entirely noncoding and is not included in all transcripts from this locus.

(E) ChiP analysis of FUL genomic fragments in wild-type and p35S:rSPL3-HA seedlings.

(F) ChiP followed by qPCR of FUL genomic fragments in wild-type and p35S:rSPL3-HA seedlings. Relative enrichment of fragments was calculated by comparing samples treated with HA and Myc antibodies.

(G) ChiP followed by qPCR of SOC1 genomic fragments in wild-type and pSPL9:GFP-rSPL9 seedlings, with Myc antibodies as control.
Inactivation of FUL causes only a small delay in flowering, but it substantially enhances the late-flowering defects of soc1 mutants in both long and short days (Ferrándiz et al., 2000a; Melzer et al., 2008), and double mutants are similar to ft-10 null mutants (Table S2). In addition, double mutants showed a disproportionate increase in the number of cauline leaves (Table S3), indicating a partial uncoupling of stem elongation, one of the responses to floral induction, and specification of floral meristem identity. Because a ful mutation on its own does not strongly delay flowering, we introduced pSUC2:amiR-FUL and pFD:amiR-FUL constructs into the soc1-6 strain. pSUC2:amiR-FUL had only a minor effect on the flowering of soc1-6, but pFD:amiR-FUL soc1-6 plants flowered almost as late as the soc1-6 ful-7 double mutants (Table 2). We conclude that, at least in long days, there is a higher requirement of FUL at the shoot apex than in the phloem, but that similar to SPLs and SOC1, FUL functions in both tissues.

Requirement of FD and SPLs for Floral Meristem Identity

Several previous reports have indicated that MADS box genes controlling the onset of flowering and flower initiation, such as SOC1, FUL, and AP1, act downstream of FD (Abe et al., 2005; Searle et al., 2006; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005). We confirmed that induction of FUL and SOC1 at the shoot apex in response to long days was delayed in ft-2 mutants (Figures 3B and 3D). Conversely, FUL and SOC1 were elevated in p3SS:FD plants (Figure S6). The hypothesis that FD and SPLs act in parallel is consistent with the observation that FD overexpression could accelerate the flowering of p3SS:MIR156 plants and that p3SS:rSPL3 ft-2 plants flowered earlier than ft-2 plants (Table 2). Similarly, abrogating SPL expression in the FD domain through pFD:MIR156 further considerably increased the leaf number of p3SS:amiR-FT/TSF plants (Mathieu et al., 2007), which mimic the phenotype of ft tsf double mutants (Table 2; Mathieu et al., 2007).

Surprisingly, pFD:MIR156 ft-2 plants had a novel inflorescence phenotype, reminiscent of ft lfy, ft lfy, and lfy ap1 double mutants (Figure 4; Abe et al., 2005; Ruiz-García et al., 1997; Wigge et al., 2005). Wild-type plants normally have two or three cauline leaves, in the axis of which side shoots develop, whereas flowers of A. thaliana, in contrast to many other species, are not subtended by leaf-like bracts. The first few flowers of pFD:MIR156 plants were associated with bracts, but the overall number of cauline leaves with side shoots was not substantially increased in ft-2 or pFD:MIR156. In contrast, pFD:MIR156 ft-2 inflorescences produced a large number of cauline leaves from which side shoots arose that repeated the pattern of the main inflorescence with many leaves (Figures 4A and 4B; Figures S7A and S7B). The few flowers that eventually appeared on the inflorescence were subtended by bracts, indicating that complete floral identity was never attained in these plants (Figure S7B).

Because of the similarity of the pFD:MIR156 ft-2 and ft-2 lfy-12 phenotypes (Figure 4B), we tested whether the miR156-regulated SPLs act primarily through LFY. Shoot-apex-specific reduction of SPL activity with pFD:MIR156 enhanced the lfy-12 phenotype, and the inflorescence defects were similar to those of ft-2 lfy-12 (Figure 4B). In addition, we found that some of the severe floral defects in ft-2 lfy-12 plants were rescued after introduction of pSPL9:rSPL9 into these plants (Figure 4B), further confirming that miR156-regulated SPLs do not act merely through LFY.

To understand the origin of the severe inflorescence defects in pFD:MIR156 ft-2 plants, we analyzed the expression of LFY and AP1 as well as TERMINAL FLOWER1 (TFL1), which suppresses floral identity in the inflorescence shoot and has a mutually antagonistic relationship with LFY and AP1 (Liljegren et al., 1999; Parcy et al., 2002; Ratcliffe et al., 1999). LFY and AP1 levels in pFD:MIR156 ft-2 inflorescences were much lower than in wild-type inflorescences and resembled those in vegetative apices of wild-type (Figure 4C). Consistent with the shoot character of the lateral primordia produced by pFD:MIR156 ft-2 inflorescences, these strongly expressed TFL1, whereas...
**DISCUSSION**

In this work, we have revealed a molecular pathway that can induce flowering in the absence of the FT/FD complex, thought to be the major integrator of positive and negative signals controlling the onset of flowering. The importance of the miR156/SPL pathway in flowering at least partially explains why inactivation of FT and its close paralog TWIN SISTER OF FT (TSF) does not prevent flowering of A. thaliana (Michaels et al., 2005; Yamaguchi et al., 2005). Conversely, that FT/FD activity can largely compensate for a lack of SPL function.
explains that despite their discovery more than 10 years ago (Klein et al., 1996), the exact role of SPLs in flowering control has been unclear until now.

In young seedlings, miR156 levels are high and SPL levels are low. Even without photoperiodic induction of flowering, miR156 levels decline over several weeks, with a concomitant rise in SPL levels (this work; Schwarz et al., 2008; Wu and Poethig, 2006). This change in miR156/SPL levels appears to be independent of several known floral regulators, such as flower-promoting gibberellins, which are probably produced in leaves and transported to the shoot apex (Eriksson et al., 2006), or FLC, which is the target of vernalization and can repress flowering in both the phloem and at the shoot apex (Searle et al., 2006). miRNAs were first discovered through their role in controlling developmental timing in C. elegans (Pasquinelli and Ruvkun, 2002). Similar to miR156, C. elegans miRNAs lin-4 and let-7 change in abundance as development progresses. An important outcome of their study during the last decade has been that there is not a simple mechanism that controls the expression levels of these miRNAs, but that likely a series of switches of feedback loops are encoded in the expression of miR156 and the closely related miR157, which continues to rise. Even without photoperiodic induction of flowering, miR156 continues to rise.

In the early part of the flowering network, the FLC/SVP complex represses not only FT and SOC1 in phloem companion cells of leaves, but also SOC1 and FD at the shoot apex (Hepworth et al., 2002; Lee et al., 2007; Liu et al., 2008; Searle et al., 2006), similar to the dual action of SPLs in both tissues. Although the roles of SOC1 and FUL in leaves are not fully understood, one possibility is that they compete with the FLC/SVP complex for access to the FT promoter and thus counteract the repressive activity of FLC/SVP (Hepworth et al., 2002; Li et al., 2008; Liu et al., 2008). Since both SOC1 and FUL proteins can interact with SVP, this might even involve action in a common, large complex of MADS domain proteins (de Folter et al., 2005).

At the shoot apex, both FUL and SOC1 are targets of SPLs and the FT/FD complex. A heterodimeric complex of SOC1 and AGL24 in turn promotes flowering by activating LFY and AP1, with the latter being directly regulated by SPLs and FT/FD as well (this work; Lee et al., 2008; Liu et al., 2008; Wigge et al., 2005). These factors thus form an interlocking set of feed-forward loops (Figure 5). We propose that during early development, high miR156 levels reduce the ability of FT/FD to induce flowering by repressing SPL activity. With decreasing miR156 activity, flowering can be promoted through the photoperiod-dependent FT/FD pathway, and eventually plants can flower without FT/FD activity, as SPL levels continue to rise.

Our findings add to the growing evidence for an intricate web of feed-forward and feedback loops that ensure a sharp and rapid transition from vegetative to reproductive development (Figure 5). It is intriguing that the SPLs, in addition to being posttranscriptionally regulated by miR156, are also targets of the FT/FD transcription factor complex.
**EXPERIMENTAL PROCEDURES**

Sequences of oligonucleotide primers are given in Table S4.

**Plant Material**

Plants were grown at 23°C in long days (16 hr light/8 hr dark) or short days (8 hr light/16 hr dark). p35S::MIR156, pSUC2::MIR156, pFD::MIR156, p3SS::FDR, p3SS::SPL3, p3SS::amiR-FT/TSF, co-9, ft-10, ft-10 tsf-1, ft-2, ify-12, ify-12 tsf-2, and FRI FLC have been described (Balasubramanian et al., 2006; Mathieu et al., 2007; Michaels and Amasino, 1999; Wang et al., 2008; Wigge et al., 2005; Yoo et al., 2005). ful-7 (SALK_033647) and soc1-6 (SALK_138131) are T-DNA insertion lines (Alonso et al., 2003). Double mutants were identified in the F2 generation by PCR-based genotyping. Wild-type was Columbia (Col-0) except for ga7-3, which is in the Ler background (Wilson et al., 1992).

**Transgenic Plants**

Genomic fragments of SPL3 and FUL were amplified by PCR using fusion DNA polymerase (New England Biolabs, Ipswich, MA), cloned into a Gateway entry vector, and subsequently recombined into destination vectors that harbor 3SS, SUC2, or FD promoters, using Gateway LR clonase II Enzyme mix (Invitrogen, Carlsbad, CA). For pSPL9::SPL9-GR, an SPL9 genomic fragment (Wang et al., 2008) was PCR amplified and fused to a fragment encoding the rat glucocorticoid receptor binding domain (Lloyd et al., 1994). At least 50 T1 seedlings were analyzed for each construct.

**RNA Analyses**

Total RNA was extracted from seedlings or vegetative shoot apices with the Plant RNeasy Mini kit (QIAGEN, Hilden, Germany) or with Trizol reagent (Invitrogen).

For qRT-PCR, 1 μg of total RNA was DNase I treated and used for cDNA synthesis with oligo(dt) primer and Superscript reverse transcriptase (Invitrogen). PCR was performed with SYBR-Green PCR Mastermix (Invitrogen) and amplification was monitored on an MJR Opticon Continuous Fluorescence Detection System (Bio-Rad, Hercules, CA). Expression was normalized against β-TUBULIN-2. An amplification efficiency of 1.8 per cycle was assumed. At least two biological replicates were performed, with two technical replicates for each. Results from one biological replicate are shown. For small RNA blots, locked nucleic acid (LNA; Exiqon, Vedbaek, Denmark) oligonucleotide probes were used. GUS staining was performed as described (Blázquez et al., 1998). Probe synthesis and hybridization for analyses with Affymetrix (Santa Clara, CA) ATH1 arrays were performed as described (Schmid et al., 2003). Array data were normalized using gcRMA implemented in GeneSpring 5.1 (Agilent Technologies, Santa Clara, CA). In situ hybridization was carried out as described (Wang et al., 2008).

**Chromatin Immunoprecipitation**

ChIP was performed as described (Wigge et al., 2005). Crude chromatin extract was split into three parts. One part was saved for the input control. The other two were used for immunoprecipitation with Myc, HA, or GFP antibodies (Santa Cruz). After several washes, chromatin crosslinking was reversed, and DNA was purified with the PCR purification kit (QIAGEN). 1 μg of DNA solution was used for quantitative PCR analyses as described above. Amplification was monitored on an MJR Opticon Continuous Fluorescence Detection System (Bio-Rad, Hercules, CA). Expression was normalized against β-TUBULIN-2. An amplification efficiency of 1.8 per cycle was assumed. At least two biological replicates were performed, with two technical replicates for each. Results from one biological replicate are shown. For small RNA blots, locked nucleic acid (LNA; Exiqon, Vedbaek, Denmark) oligonucleotide probes were used. GUS staining was performed as described (Blázquez et al., 1998). Probe synthesis and hybridization for analyses with Affymetrix (Santa Clara, CA) ATH1 arrays were performed as described (Schmid et al., 2003). Array data were normalized using gcRMA implemented in GeneSpring 5.1 (Agilent Technologies, Santa Clara, CA). In situ hybridization was carried out as described (Wang et al., 2008).

**ACCESSION NUMBERS**

Arabidopsis Genome Initiative gene identifiers are as follows: SPL3 (At2g33810); SPL9 (At2g42200); SUC2 (At1g22710); FD (At4g35800); FLC (At5g10140); CO (At5g15840); BETA-TUBULIN-2 (At5g61800); LFY (At5g61850); FT (At5g65480); SOC1 (At2g45660); FUL (At5g60910); AGL42 (At5g62165); TFL1 (At5g30840); AP1 (At1g69120); MIR156a (At2g35095). NCBI-GE accession numbers for microarrays are GSM402170 to GSM402177, series GSE16061.

**SUPPLEMENTAL DATA**

Supplemental Data include eight figures and four tables and can be found with this article online at http://www.cell.com/cell/supplemental/ 5092-8674(09)00713-2.
ACKNOWLEDGMENTS

We thank the European Arabidopsis Stock Centre for seeds; Eunyoung Chae, Yasushi Kobayashi, François Parcy, Patrice Salomé, Markus Schmid, the members of Weigelworld Team miRNA, and Gang Wu and Scott Poethig for discussion; and Ayako Yamanuchi and Doris Wagner for the exchange of unpublished information. This work was supported by an EMBO Long-Term Fellowship to J.-W.W. (ALTF 274-2006), by a DFG-SFB 446 grant, and by European Community FP6 IP SIROCCO (contract LSHG-CT-2006-037900).

Received: February 6, 2009  
Revised: April 24, 2009  
Accepted: June 2, 2009  
Published: August 20, 2009

REFERENCES


