The microRNA156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 Module Regulates Ambient Temperature-Responsive Flowering via FLOWERING LOCUS T in Arabidopsis1[C][W][OA]

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The flowering time of plants is affected by modest changes in ambient temperature. However, little is known about the regulation of ambient temperature-responsive flowering by small RNAs. In this study, we show that the microRNA156 (miR156)-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 (SPL3) module directly regulates FLOWERING LOCUS T (FT) expression in the leaf to control ambient temperature-responsive flowering. Overexpression of miR156 led to more delayed flowering at a lower ambient temperature (16°C), which was associated with down-regulation of FT and FRUITFULL expression. Among miR156 target genes, SPL3 mRNA levels were mainly reduced, probably because miR156-mediated cleavage of SPL3 mRNA was higher at 16°C. Overexpression of miR156-resistant SPL3 [SPL3(−)] caused early flowering, regardless of the ambient temperature, which was associated with up-regulation of FT and FRUITFULL expression. Reduction of miR156 activity by target mimicry led to a phenotype similar to that of SUC2::rSPL3 plants. FT up-regulation was observed after dexamethasone treatment in GVG-rSPL3 plants. Misexpression and artificial microRNA-mediated suppression of FT in the leaf dramatically altered the ambient temperature-responsive flowering of plants overexpressing miR156 and SPL3(−). Chromatin immunoprecipitation assay showed that the SPL3 protein directly binds to GTAC motifs within the FT promoter. Lesions in TERMINAL FLOWER1, SHORT VEGETATIVE PHASE, and EARLY FLOWERING3 did not alter the expression of miR156 and SPL3. Taken together, our data suggest that the interaction between the miR156-SPL3 module and FT is part of the regulatory mechanism controlling flowering time in response to ambient temperature.

Flowering, which is a major developmental transition to the reproductive phase, is affected by various environmental stimuli (Simpson and Dean, 2000). Temperature is one of the most common environmental stimuli affecting plant development. To survive and complete their life cycle, plants continuously adjust their growth and development in response to changing temperature conditions (Penfield, 2008). Although plants generally experience only modest variations in temperature during most of their life cycle, genetic analyses have focused on the processes that modulate flowering under severe temperature conditions, such as vernalization and cold/heat stress (Sheldon et al., 2000; Panchuk et al., 2002).

Changes in ambient growth temperature significantly affect plant flowering time (Fitter and Fitter, 2002) and ultimately the ecological distribution of plant species (Lenoir et al., 2008). To elucidate the molecular mechanisms underlying ambient temperature signaling in plants, genetic screens were performed (Blázquez et al., 2003; Balasubramanian et al., 2006; Lee et al., 2007), which revealed the thermosensory pathway mediating ambient temperature responses (Lee et al., 2008; Fornara et al., 2010). FCA, FVE, SHORT VEGETATIVE PHASE (SVP), EARLY FLOWERING3 (ELF3), and TERMINAL FLOWER1 (TFL1) genes are involved in this pathway (Blázquez et al., 2003; Lee et al., 2007; Strasser et al., 2009). H2A.Z-containing nucleosomes have recently been shown to provide thermosensory information by regulating the ambient temperature transcriptome (Kumar and Wigge, 2010). In addition, SVP has been shown to act as a link in small RNA-mediated flowering in response to different ambient temperatures (Lee et al., 2010). It has also been reported that the microRNA399-PHOSPHATE2 module plays a role in the regulation of ambient temperature-responsive flowering (Kim et al., 2011).

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Taken together, these findings suggest a potential role for microRNAs (miRNAs) in ambient temperature-responsive flowering.

Plant miRNAs are an important class of regulatory molecules affecting diverse aspects of plant growth and development (Carrington and Ambros, 2003). They commonly target mRNAs of specific transcription factors, thereby forming so-called miRNA transcription factor regulatory modules (Dugas and Bartel, 2004; Mallory and Vaucheret, 2006). Examples of such modules in the Arabidopsis (Arabidopsis thaliana) and other plant species include miRNA156 (miR156) and its targets, namely SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes. These miR156-SPL regulatory modules are known to play a central role in the regulation of diverse developmental processes (Schwarz et al., 2008; Wang et al., 2008; Nodine and Bartel, 2010; Xing et al., 2010; Yu et al., 2010; Gou et al., 2011; Yang et al., 2011). The miR156-SPL3 module has been identified as part of a regulatory mechanism that can induce flowering in the absence of photoperiodic cues (Wang et al., 2009). The expression of FRUITFULL (FUL), ÁGAMOUS-LIKE42, and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) is regulated by this module. SPL3 directly activates the expression of LEAFY (LFY), FUL, and APETALA1 (API) to promote floral meristem identity during floral transition (Yamaguchi et al., 2009). Although miR156 was recently identified as an ambient temperature-responsive miRNA (Lee et al., 2010), little is known about its involvement in the molecular mechanism underlying ambient temperature-responsive flowering.

In this study, the miR156-SPL3 module is shown to play an important role in regulating flowering time in response to different ambient temperatures. Expression of miR156, miR156-resistant SPL3, or a target mimic of miR156 affected ambient temperature-responsive flowering and induced changes in FLOWERING LOCUS T (FT) and FUL expression. Genetic analyses indicated that FT, but not FUL, is a major output of the miR156-SPL3 module. The SPL3 protein directly binds to a sequence carrying GTAC motifs within the FT locus in vivo. Our results suggest a model in which the miR156-SPL3 module directly regulates FT expression in the leaf to modulate ambient temperature-responsive flowering in Arabidopsis.

RESULTS

MiR156 Overexpression Prolongs the Delay in Flowering at a Low Ambient Temperature

To determine whether miR156 regulates flowering time in response to ambient temperature, the phenotype of transgenic plants overexpressing miR156 (35S::MIR156b) was analyzed at 23°C and 16°C. Transgenic plants showing strong expression of miR156 at both temperatures were selected (Supplemental Fig. S1). Because overexpression of miR156 is known to increase the leaf initiation rate at the normal temperature (23°C) with a modest delay in flowering (Schwab et al., 2005; Wu and Poethig, 2006), both the plastochron length and the total leaf number were scored in long-day (LD) conditions to measure flowering time. LD conditions were used because, under short-day conditions, total leaf numbers of wild-type plants grown at 23°C were almost indistinguishable from those grown at 16°C, which indicates that low ambient temperature affects the photoperiodic response (Strasser et al., 2009; Supplemental Fig. S2). 35S::MIR156b plants showed moderate late flowering at 23°C in LD conditions (25.6 leaves; Supplemental Table S1 to find detailed information on flowering time of plants used in this study; Fig. 1A). Interestingly, flowering at 16°C was even more delayed (61.4 leaves). Thus, the leaf number ratio of 35S::MIR156b plants (16°C/23°C, see “Materials and Methods”) was 2.4 (compare wild-type plants = 1.9; Fig. 1A). Also, the bolting time of 35S::MIR156b plants was slightly later than that of wild-type plants at both 23°C and 16°C (Supplemental Fig. S3). As observed at 23°C, the rate of leaf production (the total number of leaves/bolting day) of 35S::MIR156b plants was also faster than that of wild-type plants at 16°C (Supplemental Table S1), which indicates that the decreased plastochron length (or increased leaf initiation rates) of 35S::MIR156b plants occurs regardless of ambient temperature. The juvenile leaf number of 35S::MIR156 plants was approximately 14.5 leaves (23°C) and 37 leaves (16°C), indicating that the phase transition in 35S::MIR156 plants was more delayed at 16°C than at 23°C (Fig. 1B). These results suggest that miR156 overexpression led to ambient temperature-sensitive flowering.

Down-Regulation of FT and FUL in 35S::MIR156b Plants

We analyzed the expression of flowering time genes in both the leaf and shoot apical region of 35S::MIR156b plants because (1) miR156 is expressed in the leaf and shoot apical region at 23°C and 16°C (Supplemental Fig. S4), and (2) a recent report showed that miR156 is probably functional in both samples (Wang et al., 2009). To validate all leaf/shoot apex sample preparations used in this study, we first confirmed the preferential enrichment of RbcS (Yamakawa et al., 2004) and SHOOT MERISTEMLESS (Endrizzi et al., 1996) in these samples (Supplemental Fig. S5). In the leaf of 8-d-old 35S::MIR156b plants, FUL (Ferrándiz et al., 2000) expression was down-regulated at both temperatures, whereas FT (Kardailsky et al., 1999; Kobayashi et al., 1999) expression was not obviously altered (Fig. 1C), consistent with results reported previously (Wang et al., 2009; Jung et al., 2011). In the shoot apical region, FUL expression was also low at both temperatures and FT expression was absent. However, the expression levels of TWIN SISTER OF FT (TSF; Yamaguchi et al., 2005) and SOC1 (Lee et al., 2000; Samach et al., 2000), which are putative outputs within the thermosensory pathway (Lee et al., 2007), were not dramatically altered (Supplemental Fig.
Notably, the down-regulation of SPL3 (Wu and Poethig, 2006; Gandikota et al., 2007) was more apparent in the leaf than in the shoot apical region (Fig. 1C), which suggests that the leaf may be the primary site of action of miR156 for the regulation of flowering time.

Due to the shortened plastochron length of miR156-overexpressing plants (Fig. 1B; Supplemental Table S1), the degree of shoot maturation of these plants may differ from that of wild-type plants of the same age, thereby preventing a direct comparison of the expression...
levels of flowering time genes. As a consequence, we also analyzed the expression levels of the flowering time genes at a morphologically defined growth stage 1.02 (DS1.02; Boyes et al., 2001). At growth stage DS1.02, the down-regulation of FT was more apparent in the leaf than in the shoot apical region at both temperatures (Fig. 1D). There was a similar down-regulation of FUL. At DS1.02, there was once again a more significant decrease in the expression of SPL3 in the leaf than in the shoot apical region. These results indicate that although the overexpression of miR156 altered plastochron length at 23°C and 16°C, it consistently down-regulated FT and FUL, which are potential floral activators, at both temperatures.

Down-Regulation of SPL3 via Enhanced Cleavage by miR156 at 16°C

The effect of ambient temperature on the expression levels of SPL genes was examined. The expression of SPL genes was generally lower at 16°C, in contrast to miR156 expression, which was higher at 16°C (Fig. 2A). In particular, SPL3 mRNA levels were dramatically lower at 16°C than at 23°C. However, the expression of CUP-SHAPED COTYLEDON2 (CUC2; Larue et al., 2009) and TCP FAMILY TRANSCRIPTION FACTOR4 (TCP4; Palatnik et al., 2003), which are target genes of nonambient temperature-responsive miRNAs (Lee et al., 2010), was not altered. These results suggest that the elevated miR156 expression at 16°C can enhance SPL3 cleavage, although we cannot exclude the possibility of the translational inhibition of other SPL genes by miR156 at 16°C.

We then examined whether the down-regulation of SPL3 at 16°C was associated with enhanced cleavage of their mRNAs by miR156. No difference in the DNA methylation pattern at the SPL3 locus was observed at 16°C, excluding a change in DNA methylation as an explanation of the down-regulation of SPL3 at 16°C (Supplemental Fig. S7). A gene-specific RNA ligase-mediated 5’ rapid amplification of cDNA ends (RLM 5’-RACE) assay identified cleavage products of SPL genes at 23°C and 16°C (Fig. 2B). Considerably more cleavage products were produced from SPL3 at 16°C (2.7-fold increase). In contrast, the levels of RACE products of CUC2 and TCP4 were similar at 23°C and 16°C. These results suggest that the elevated miR156 expression at 16°C can enhance SPL3 cleavage, although the possibility cannot be excluded that miR156 also inhibits the translation of other SPL genes at 16°C. RLM 5’-RACE products obtained were sequenced to map the cleavage sites. In SPL3-derived transcripts, a major cleavage site was identified between +10 and +11 (relative to the 5’ end of miR156; Fig. 2C) with a few minor, alternative cleavage sites. Collectively, the results obtained by quantitative reverse transcription

**Figure 2.** Expression levels and cleavage sites of SPL genes at 23°C and 16°C. A, Relative expression levels of miR156 and SPL genes in 10-d-old wild-type (WT) plants grown at 23°C and 16°C. CUC2 and TCP4 were used as controls. Error bars indicate the sd. B, Semiquantitative measurement of the level of RLM 5’-RACE products of SPL genes in 10-d-old wild-type plants. RACE products were hybridized with a 5’-RACE adaptor sequence and their relative band intensity is shown. CUC2 and TCP4 were used as controls. C, Map of cleavage sites identified in SPL3 by RLM 5’-RACE. A partial sequence of SPL3 is shown to highlight the miR156a-SPL3 duplex. A period indicates a G-U pair.
(qRT)-PCR and RLM 5'-RACE revealed that SPL3 levels were anticorrelated with the level of miR156 at 16°C.

Overexpression of miR156-Resistant SPL3 Causes Accelerated Flowering at a Low Ambient Temperature

The available spl3 mutants (FLAG_173C12, Wassilewskija [Ws] background) exhibited unexpected early flowering with an increased leaf number ratio (1.8; compare wild-type plants = 1.6) and were found to be a leaky allele (Supplemental Fig. S8), suggesting that these mutants are not suitable for inferring the function of SPL3 in ambient temperature-responsive flowering. Thus, to investigate whether SPL3 is involved in ambient temperature-responsive flowering, the phenotype of transgenic plants overexpressing SPL3 either as a miR156-sensitive version, which has an intact miR156 response element in its 3'-untranslated regions [hereafter, 35S::SPL3(+)], or as a miR156-resistant version with the miR156 response element mutated [35S::SPL3(-)] was analyzed. SPL3 mRNA levels were greatly increased in 35S::SPL3(-) plants, but showed a less-pronounced increase in 35S::SPL3(+) plants (Supplemental Fig. S9A). Based on reports of the translational inhibition of the target mRNA by plant miRNAs (Chen, 2004), the accumulation of the SPL3 protein in 35S::SPL3(-) plants was confirmed (Supplemental Fig. S9B).

35S::SPL3(-) plants exhibited early flowering with similar leaf numbers (with fewer cauline leaves) at both temperatures (5.8 and 7.7 leaves) in LD conditions (leaf number ratio = 1.3; Fig. 3A; Supplemental Fig. S9C). This indicated that the flowering of 35S::SPL3(-) plants was almost insensitive to differences in ambient temperature. Unlike 35S::SPL3(-) plants, 35S::SPL3(+) plants produced more leaves at 16°C (23.7 leaves) than at 23°C (14.1 leaves; leaf number ratio = 1.7). Thus, the flowering of 35S::SPL3(+) plants was more ambient temperature sensitive, which was consistent with the diminished SPL3 expression in these plants (Supplemental Fig. S9A). Less juvenile leaves were produced in 35S::SPL3(-) plants (3.0 and 5.0 leaves at 23°C and 16°C, respectively; Fig. 3B). Adult leaf numbers were also greatly reduced in 35S::SPL3(-) plants. However, the juvenile leaf number of 35S::SPL3(+) plants (7.0 and 11.5 leaves at 23°C and 16°C, respectively) was similar to that of wild-type plants (6.0 and 11.5 leaves at 23°C and 16°C, respectively). These results suggest that SPL3 modulates ambient temperature-responsive flowering.

Up-Regulation of FT and FUL in 35S::SPL3(-) Plants

qRT-PCR analysis revealed strong FUL expression in both the leaf and the shoot apical region of 8-d-old 35S::SPL3(-) plants at both ambient temperatures (Fig. 3C), as well as increased FT expression in the leaf. However, there was no clear change in the expression of TF and SOC1 in these plants at both temperatures (Supplemental Fig. S6B). The expression of FT and FUL was also analyzed at DS1.02, and again FUL expression was found to have increased in both the leaf and the shoot apical region at both ambient temperatures (Fig. 3D). FT expression was also increased in the leaf at DS1.02 at both temperatures (by 4- and 3-fold at 23°C and 16°C, respectively). A slightly reduced expression level of FT at 16°C in 35S::SPL3(-) plants suggest that a weak temperature response of FT still remained. The weak temperature response seen in 35S::SPL3(-) can be explained by the differential expression of FT at different temperature. These data indicated that increased SPL3(-) mRNA expression led to the up-regulation of FT and FUL in the leaf and the shoot apex, which is consistent with their down-regulation in miR156-overexpressing plants (Fig. 1, C and D). It was thus concluded that FT and FUL are likely to be the major downstream genes of the miR156-SPL3 module.

The requirement of SPL3 activity in different tissues was investigated through the misexpression of miR156-resistant SPL3 in the shoot apex (using the FD promoter) and the phloem (using the SUC2 promoter; Wang et al., 2009). The possibility that FD and SUC2 expression may be regulated by ambient temperature was excluded (Supplemental Fig. S10). SUC2::rSPL3 plants, a miR156-resistant version without the miR156 response element, exhibited moderate early flowering, which was intermediate to that of wild-type plants and 35S::SPL3(-) plants, at both temperatures (Fig. 3E). In contrast, flowering of FD::rSPL3 plants was largely indistinguishable from that of wild-type plants at both temperatures. The leaf number ratio of SUC2::rSPL3 plants was 1.6, whereas that of FD::SPL3 plants was 2.0, which indicates that SUC2::rSPL3 plants had reduced temperature sensitivity. These results suggest that modulations in SPL3 activity in the leaf affect ambient temperature-sensitive flowering.

qRT-PCR analysis of the expression levels of FT and FUL in SUC2::rSPL3 and FD::rSPL3 plants revealed that FT and FUL expression increased (by at least 2-fold) in the leaf of 8-d-old SUC2::rSPL3 plants at both temperatures (Fig. 3F). This up-regulation of FT and FUL expression in the leaf of SUC2::rSPL3 plants was more apparent at DS1.02, i.e. at least 3-fold, at both temperatures. In the shoot apical region of 8-d-old seedlings of FD::rSPL3 plants and at DS1.02, FUL expression was increased at both temperatures (Fig. 3G); however, FUL up-regulation was less apparent than in SUC2::rSPL3 plants. Although the expression of FUL was increased in the shoot apical region, this increase seemed to be insufficient to accelerate flowering in FD::rSPL3 plants (Fig. 3E). The results of these expression analyses demonstrate that the flowering of SUC2::rSPL3 plants, which showed stronger up-regulation of FT and FUL in the leaf, was less sensitive to changes in ambient temperature. Thus, together with the down-regulation of SPL3 in the leaf of 35S::miR156b plants (Fig. 1), these results suggest that the regulation of FT and FUL by SPL3 in the leaf is important for ambient temperature-responsive flowering.
35S::MIM156 Plants Show Ambient Temperature-Insensitive Flowering Similar to SUC2::rSPL3 Plants

Analyzing a loss-of-function allele of miR156 is a prerequisite to study miR156’s function, but obtaining a complete knockout allele of miR156 is very difficult because miR156 is generated from eight loci in the Arabidopsis genome. Thus, we analyzed the flowering phenotype of 35S::MIM156 plants (Franco-Zorrilla et al., 2007) in which miR156 activity is reduced via target mimicry. 35S::MIM156 plants were early flowering at both 23°C and 16°C (8.0 and 12.8 leaves, respectively; Fig. 4A). The leaf number ratio of 35S::MIM156 plants was 1.6 (compare wild-type plants = 2.0), indicating that...
Figure 4. Flowering of 35S::MIM156 plants was less ambient temperature sensitive in LD conditions. A, Accelerated flowering of 35S::MIM156 plants at 16°C in LD conditions. Photographs were taken when 35S::MIM156 plants flowered at each temperature. B, The leaf morphologies of 35S::MIM156 plants. An inverted triangle indicates the juvenile-to-adult transition point based on the appearance of abaxial trichomes. C and D, Relative expression levels of SPL genes in 35S::MIM156 plants grown...
the flowering of 35S::MIM156 plants was less sensitive to changes in ambient temperatures, as seen with SUC2::rSPL3 plants (Fig. 3E). The bolting time of 35S::MIM156 plants at 23°C (24.7 d) and 16°C (48.5 d) was similar to that of wild-type plants (24 and 49.7 d at 23°C and 16°C, respectively; Supplemental Fig. S3), indicating that leaf initiation rates were reduced in 35S::MIM156 plants regardless of the ambient temperature. Fewer juvenile leaves were produced in 35S::MIM156 plants (4.8 and 6.3 leaves at 23°C and 16°C, respectively; Fig. 4B), implying that the reduction in miR156 activity accelerated phase transition, which was also seen in 35S::SPL3(−) plants (Fig. 3B).

In 35S::MIM156 plants grown for 8 d and at DS1.02, a general up-regulation of SPL genes was observed at both 23°C and 16°C (Fig. 4, C and D). In particular, the increase in SPL3 expression was more obvious than that of the other SPL genes at both temperatures, which is consistent with the notion that SPL3 is a major target of miR156 in plant responses to ambient temperature changes. The expression levels of FT and FUL were also analyzed in 8-d-old-seedlings of 35S::MIM156 plants. FUL expression was up-regulated at both temperatures, whereas FT expression was not obviously altered (Fig. 4E), consistent with the reduction in leaf initiation rate observed in 35S::MIM156 plants. However, expression analysis of seedlings at DS1.02 revealed that FT and FUL expression levels were apparently up-regulated (by at least 1.7-fold; Fig. 4F). These results together with the up-regulation of SPL3 in 35S::MIM156 plants support the concept that alterations in FT and FUL expression by the miR156-SPL3 module affect ambient temperature-responsive flowering.

Because 35S::MIM156 plants were less insensitive to changes in ambient temperature than 35S::SPL3(−) plants (Figs. 3A and 4A), we analyzed the differences in SPL3 up-regulation in the transgenic plants used in this study. SPL3 expression was lower in 35S::MIM156 plants than in SUC2::rSPL3 plants (Fig. 4G), indicating that the expression level of SPL3 in each transgenic line was largely consistent with the respective ambient temperature-insensitive flowering phenotype. Although SPL3 up-regulation in 35S::MIM156 plants was lower than that in SUC2::rSPL3 plants, flowering times were similar in both, suggesting the possibility that other SPL genes that have different functions were also derepressed and contributed to the phenotype of 35S::MIM156 plants. Taken together, these results suggest that a reduction in miR156 activity via target mimicry affects flowering time in response to the ambient temperature.

The Limited Role of FUL in Ambient Temperature-Responsive Flowering

Because loss-of-function mutants of AP1 and LFY, the direct targets of SPL3 protein (Yamaguchi et al., 2009), showed ambient temperature-responsive flowering (Lee et al., 2007) and FUL expression was significantly altered in 35S::MIR156b, 35S::SPL3(−), and 35S::MIM156 plants (Figs. 1, 3, and 4), the hypothesis that FUL functions in ambient temperature-responsive flowering was tested by analyzing the phenotypes of the gain- and loss-of-FUL function alleles. Flowering of 35S::FUL plants was delayed at 16°C (leaf number ratio = 1.7; Fig. 5A), which was in sharp contrast to the almost identical leaf numbers produced at both temperatures in 35S::FT plants (leaf number ratio = 1.1). Flowering of ful-8, an RNA-null allele newly identified in this study (Supplemental Fig. S11), and ful-2 mutants was normally delayed at 16°C (leaf number ratio = 1.9 and 2.0, respectively), indicating that ful mutants normally responded to ambient temperature changes.

Leaf numbers of plants that misexpressed FUL in the phloem or in the shoot apex were also measured. SUC2::FUL plants showed slightly earlier flowering than wild-type plants at both temperatures (Fig. 5A). The leaf number ratio of SUC2::FUL plants (1.8) was similar to that of wild-type plants (1.9). In contrast, SUC2::FT plants produced almost identical numbers of leaves at both temperatures (leaf number ratio = 1.2), which suggests that the misexpression of FT in the phloem is sufficient to cause ambient temperature-insensitive flowering. Flowering of FD::FUL plants was normally delayed at 16°C (leaf number ratio = 2.2). The leaf number ratio of FD::FT plants was slightly decreased (1.5), which suggests that FT misexpression in the shoot apex is insufficient to cause ambient temperature-insensitive flowering. These results indicated that gain- or loss-of FUL function mutations or those of its mistargeting alleles did not result in an ambient temperature-insensitive flowering phenotype, which suggests that FUL does not play a major role in ambient temperature-responsive flowering.

A ful mutation was introduced into 35S::SPL3(−) plants to test whether the loss of FUL activity alters the ambient temperature-insensitive flowering phenotype seen in 35S::SPL3(−) plants. The leaf number ratio of 35S::SPL3(−) ful-8 plants was slightly higher than that of 35S::SPL3(−) plants (1.5 versus 1.3; Fig. 5B), which indicates that the ful mutation did not mask the ambient temperature-insensitive flowering phenotype of 35S::SPL3(−) plants. Expression analysis to test the effect of the ful mutation on FT up-regulation in 35S::SPL3(−) ful-8 plants revealed that the up-regulation of FT was not altered in the leaves of 35S::SPL3(−) ful-8.

Figure 4. (Continued.) for 8 d (C) and at DS1.02 (D) determined via qRT-PCR. Expression levels of each SPL gene at 23°C were set to one. E and F, Expression of FT and FUL in whole seedlings of 35S::MIM156 plants grown for 8 d (E) and at DS1.02 (F). G, Expression of the SPL3 gene in 8-d-old wild-type (WT), 35S::MIM156, SUC2::rSPL3, and 35S::SPL3(−) plants. Error bars indicate the sd. [See online article for color version of this figure.]
plants at both temperatures (Fig. 5, C and D). The observation that a lesion in FUL did not greatly affect the temperature-responsive flowering of 35S::SPL3(−) plants suggests that FUL has only a limited role in ambient temperature-insensitive flowering.

**FT Acts Downstream of miR156 and SPL3**

We then tested the hypothesis that FT functions downstream of the miR156-SPL3 module. miR156 levels were found to be unaffected in both 35S::FT and ft-10 (Fig. 6A) and 35S::SPL3(+) and 35S::SPL3(−) plants (Fig. 6B) at both temperatures. SPL3 expression levels were similar in 35S::FT and ft-10 plants (Fig. 6C). However, the vasculature-specific expression of FT was notably increased in the cotyledons and distal regions of true leaves of 10- and 12-d-old 35S::SPL3(−) plants (Fig. 6D). In contrast, FT::GUS expression was greatly reduced in the cotyledons and leaves of 35S::MIR156b plants. The altered expression levels of FT::GUS were confirmed by using the 4-methylumbelliferyl glucuronide assay (Supplemental Fig. S12).

To determine the induction pattern of FT and FUL, we analyzed GVG-rSPL3 plants in which rSPL3 transcription was under the control of a dexamethasone (DEX)-inducible promoter (Aoyama and Chua, 1997). Treatment with DEX induced an early flowering phenotype at 23°C (6.4 leaves; Fig. 6E), similar to that seen in 35S::SPL3(−) plants, suggesting that the DEX-induced rSPL3 gene is functional. qRT-PCR analysis using two independent GVG-rSPL3 lines (numbers 8 and 11) showed that the induction of FT and FUL expression began 5 h after the DEX treatment (Supplemental Fig. S13) and that their levels had increased by 2- to 3-fold 1 d after DEX treatment (Fig. 6E), indicating that induction pattern of FT was similar to that of FUL. These induction patterns of FT and FUL suggest that SPL3 regulates both FT and FUL.

**Genetic Relationship of miR156, SPL3, and FT**

To analyze genetic epistasis between miR156 and FT, 35S::MIR156b plants were crossed with 35S::FT plants. FT overexpression almost completely suppressed the late flowering phenotype of miR156-overexpressing plants (Fig. 7A). Moreover, 35S::MIR156b 35S::FT plants flowered with similar leaf numbers at both 23°C and 16°C (leaf number ratio = 1.0). This indicated that FT overexpression fully suppressed ambient temperature-sensitive flowering in 35S::MIR156b plants. A significant decrease in miR156 or FT expression was not found in these plants, excluding the possibility that gene silencing had occurred (Supplemental Fig. S14A). We next explored whether the mistargeting of FT expression in both the leaf and the shoot apex suppresses the effect of miR156 on flowering. The 35S::MIR156b SUC2::FT plants flowered with similar leaf numbers as SUC2::FT plants (leaf number ratio = 1.3 versus 1.2; Fig. 7A). However, although 35S::MIR156b FD::FT plants showed early flowering similar to FD::FT plants, their leaf number ratio was 1.9, which indicated that their flowering at 16°C was normally delayed. These analyses indicated that FT misexpression in the phloem in 35S::MIR156b plants.
more efficiently led to ambient temperature-insensitive flowering than did FT misexpression in the shoot apex. These data suggest that the action of FT in ambient temperature-responsive flowering lies downstream of miR156 in the leaf.

The effect of the inhibition of FT mRNA expression on the ambient temperature-insensitive flowering phenotype caused by SPL3 (−) was then assessed by using an artificial miRNA (amiR-FT) expressed in the leaf or the shoot apex. The 35S::SPL3 (−) SUC2::amiR-FT plants flowered later than 35S::SPL3 (−) plants at both 23°C and 16°C (Fig. 7B), which indicates that amiR-FT expression driven by the SUC2 promoter partially suppressed the early flowering of the 35S::SPL3 (−) plants. Importantly, the leaf number ratio of 35S::SPL3 (−) SUC2::amiR-FT plants was similar to that of SUC2::amiR-FT plants, which indicates that amiR-FT misexpression to the phloem suppressed the effect of SPL3 (−). This suppressive effect was also observed in 35S::SPL3 (−) ft-10 plants (Fig. 7B). Collectively, the results of the genetic analysis suggest that FT is a major output of the miR156-SPL3 module in the leaf associated with ambient temperature-responsive flowering.

Direct Binding of SPL3 Protein to the FT Locus in Vivo

SQUAMOSA PROMOTER BINDING PROTEIN box transcription factors are DNA-binding proteins that recognize the GTAC core motif in their target genes (Birkenbihl et al., 2005; Liang et al., 2008; Yamasaki et al., 2009). To test the possibility that SPL3 protein directly regulates FT expression, chromatin immunoprecipitation (ChIP) experiments were performed using 35S::rSPL3-cMyc plants and anticMyc antibody, because our SPL3 antibodies were not suitable for ChIP (data not shown). The 35S::rSPL3-cMyc plants flowered with similar leaf numbers at 23°C and 16°C (leaf number ratio = 1.3; Fig. 8A), a phenotype similar to that of 35S::SPL3 (−) plants, suggesting that the cMyc-tagged rSPL3 protein is functional. Western-blot analysis confirmed the overproduction of the cMyc-tagged rSPL3 protein in 35S::rSPL3-cMyc plants (Fig. 8B).

Five regions (the upstream promoter region [I, II, and III], the second intron [V], and the 3′ region [VII]) containing GTAC motifs, the putative binding sites for SPL3 proteins, of the FT locus were explored (Fig. 8C). A region (IV) within the first intron and lacking a GTAC motif was used as a negative control. The SPL3 protein was strongly enriched in region III (Fig. 8D). Weak SPL3 enrichment was observed in regions II and V. However, significant SPL3 protein enrichment was not observed in region I, which is distally located, or in regions IV and VI. These results suggest that FT is a direct target of the SPL3 protein.

Because the ectopic expression of SPL3 driven by the 35S promoter may cause potential artifacts, we generated and analyzed 35S::rSPL3-cMyc plants. Most of the 35S::rSPL3-cMyc plants flowered much earlier than
lates genomic loci. Collectively, they suggest that SPL3 protein preferentially bound to region III in the cMyc plants (Fig. 8, D and E) indicate that the SPL3 qPCR analyses using the different SPL3 protein levels. The results of ChIP-plants, suggesting that these differences may be due to SPL3 in region II. The relative binding strength was weaker SPL3-binding site. Weak SPL3 enrichment was observed S15), indicating that the wild-type plants in the T1 generation (Supplemental Fig. FT Is a Main Output of the miR156-SPL3 Module in the Leaf

SPL3 regulates the expression of FUL and SOC1 in the leaf and the shoot apex independently of the FT/ FD complex (Wang et al., 2009). However, the activity of SPL3 appears to be predominant in the leaf, as SPL3

DISCUSSION

Although periodic temperature changes, both diurnal and seasonal, provide important information for the optimal timing of flowering, little is known about the regulation of flowering time by small RNAs in response to changes in ambient temperature. In this study, we show that ambient temperature-responsive flowering in Arabidopsis is also mediated by the miR156-SPL3-FT genetic circuitry.

Role of miR156-SPL3-FT Genetic Circuitry

Because miR172 is another ambient temperature-responsive miRNA and its overexpression leads to ambient temperature-insensitive flowering through the up-regulation of FT (Lee et al., 2010), the genetic interaction between miR172 and the miR156-SPL3 module was investigated. Late flowering of 35S::MIR156b plants was strongly, but not completely, suppressed by miR172 overexpression (Fig. 9A). 35S::MIR156b 35S::MIR172a plants flowered with 8.4 and 13.4 leaves at 23°C and 16°C, respectively. The leaf number ratio of 35S::MIR156b 35S::MIR172a plants was greater than that of 35S::MIR172a plants (1.6 versus 1.1). Gene silencing was not observed in 35S::MIR156b 35S::MIR172a plants (Supplemental Fig. S14B). The number of leaves produced in 35S::SPL3(−) 35S::MIR172a plants (3.8 and 5.3 leaves at 23°C and 16°C, respectively) was lower than the number of leaves produced by their parental lines (Fig. 9A) but the leaf number ratio was similar to that of their parental lines (1.4 versus 1.3). These genetic data suggest that the miR156-SPL3 module acts, at least partially, in parallel with the miR172 pathway in the regulation of ambient temperature-responsive flowering.

It was reported that SVP, TFL1, and ELF3 play roles in the flowering response to changes in ambient temperature (Lee et al., 2000; Strasser et al., 2009). To test whether the expression of miR156 and SPL3 is regulated by these genes, we analyzed miR156 and SPL3 expression levels in svp, tfi1-20, and elf3-1 mutants. No dramatic alteration in miR156 and SPL3 expression was observed in these mutants (Figs. 9, B and C). These results suggest that the miR156-SPL3 module may act independently of other components in ambient temperature-responsive flowering.

Genetic Interactions between the miR156-SPL3 Module and Other Components Involved in Ambient Temperature-Responsive Flowering

Even though these motifs do not correspond to the perfect GTAC motif, they do contain GTAC motifs in the FT genomic loci for the regulation of ambient temperature-responsive flowering.

Figure 7. Flowering phenotypes of various alleles generated by using FT misexpressing lines and 35S::amiR-FT lines. Total leaf numbers (A and B) of mutants generated by crossing various FT alleles with 35S::SPL3(−) or 35S::amiR156b plants. Total leaf numbers of F1 progeny grown at 23°C and 16°C in LD conditions are presented. Numbers listed above the genotypes denote the leaf number ratio. A plus sign (+) indicates a wild-type (WT) background. Error bars indicate SD.
mRNA is barely detected in vegetative shoot apices but is strongly induced in leaves (Wang et al., 2009). This study provides evidence that SPL3 functions as a direct upstream activator of FT to modulate ambient temperature-responsive flowering. This conclusion is based on results showing the up-regulation of FT in the leaves of 35S::rSPL3 plants (Fig. 3), the early up-regulation of FT in GVG-rSPL3 plants (Fig. 6), the epistatic interaction between SPL3 and FT (Fig. 7), and the direct binding of the SPL3 protein to the FT locus (Fig. 8). Our conclusion is consistent with the finding that the loss of FT function completely masks the early flowering phenotype of plants misexpressing SPL3 in the phloem (Wang et al., 2009).

FD protein has been recently reported to bind to the G-box motifs in the SPL genomic loci (Jung et al., 2012), suggesting that the FT-FD module regulates SPL genes in the shoot apex in the control of flowering time. This hypothesis is supported by our observation that SPL3 expression was increased in the shoot apex regions of FD::FT plants, but remained unchanged in the leaves of SUC2::FT plants (Supplemental Fig. S16). However, FD::rSPL3 and FD::FT plants still showed ambient temperature-responsive flowering (Figs. 3E and 7A) compared with SUC2::rSPL3 and SUC2::FT plants. Also, SPL3 expression was increased in the shoot apex regions of FD::FT plants only at 23°C (Supplemental Fig. S16). Thus, it is likely that the regulation of SPL3
Role of miR156-SPL3-FT Genetic Circuitry

The Effect of Low Temperature on Flowering Caused by miR156 Overexpression at 23°C May Be Attenuated by the Relatively Low Cleavage of SPL3 via miR156 at 23°C

Because the miR156-SPL3-FT module also serves as a regulatory mechanism involved in the control of ambient temperature-responsive flowering, an important question that needs to be answered is why 35S::MIR156b and 35S::MIM156 plants showed contrasting temperature responses (Figs. 1A and 4A). Similar to the temperature response of gain and loss of function of SPL3 (Figs. 1A and 4A), the ambient temperature response of gain and loss of function of 35S::SPL3(−) plants (Fig. 5). These findings suggested that the effects of ambient temperature on flowering via the miR156-SPL3 module are mediated primarily by FT action.

Because both FT and FUL act downstream of SPL3, two possible interaction mechanisms can be considered (Fig. 10). The first possibility is that SPL3 controls two separate signaling pathways, namely the control of ambient temperature-responsive flowering by FT in the leaf, and the control of age-dependent flowering by FUL at the shoot apex. In this case, targets of FT other than FUL are likely to be relevant in ambient temperature-responsive flowering. A second possibility is that FUL acts downstream of FT, and the regulation of ambient temperature-responsive flowering by SPL3 is at least partially mediated by FUL. The role of FT upstream of FUL is consistent with the previous observation that the accumulation of FUL transcripts in the leaf is dependent on FT and FD (Teper-Bamnolker and Samach, 2005). Nevertheless, we cannot exclude the possibility that FT and FUL cross-regulate one another in the leaf based both on our findings that 35S::SPL3(−) ful-8 plants were only weakly temperature responsive (Fig. 5) and the report of Wang et al. (2009) that the early flowering phenotype of SUC2::FUL plants is completely suppressed by the ft-10 mutation.

Because FUL expression was more dramatically affected by the miR156-SPL3 module than FT (Figs. 1 and 3) and FUL represents another known direct target of the SPL3 protein (Wang et al., 2009; Yamaguchi et al., 2009), an important question is whether FUL is a major factor in ambient temperature-responsive flowering. Several lines of evidence in this study suggest that, in contrast to FT, FUL is not important. First, mutants with altered FUL activity or misexpression of FUL retained ambient temperature-sensitive flowering, whereas plants constitutively expressing FT or misexpressing FT in the phloem exhibited ambient temperature-insensitive flowering (Fig. 5). Second, early flowering of 35S::SPL3 (−) plants was inhibited by amiR-K FT misexpression to the phloem (Fig. 7), consistent with the observation that the early flowering of SUC2::rSPL3 plants was suppressed by the ft-10 mutation (Wang et al., 2009). Third, the ful mutation failed to suppress the ambient temperature-insensitive flowering of 35S::SPL3(−) plants (Fig. 5). These findings suggested that the effects of ambient temperature on flowering via the miR156-SPL3 module are mediated primarily by FT action.

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Figure 10. A model of flowering time regulation in response to different ambient temperatures. Changes in ambient temperature cause alterations in the expression of miR156, which negatively regulates SPL3. The SPL3 protein directly binds to FT to regulate ambient temperature-responsive flowering. Although FUL is another direct target of SPL3 (Wang et al., 2009; Yamaguchi et al., 2009), it is unlikely to play an important role in ambient temperature-responsive flowering but is possibly important in age-dependent flowering. SPL9 may act redundantly with SPL3 in the regulation of ambient temperature-responsive flowering (see “Discussion”). The miR156-SPL3 module and the miR172 pathway may act in parallel, although the genetic relationship between the miR156-SPL3 module and the target genes of miR172 is not clear. Arrows represent promotion effects, whereas T-bars indicate repression effects. Dotted lines indicate unclear interactions.

Other SPL Genes May Act Redundantly with SPL3 in the Regulation of Ambient Temperature-Responsive Flowering

If SPL3 were to be the sole regulator of ambient temperature-responsive flowering, it would be expected that the ambient temperature response would either disappear or be reduced in spl3 mutants. However, the spl3 mutants (Ws background) that we tested still retained ambient temperature-responsive flowering. This is an apparent contradiction; however, we suggest that the phenotype of the spl3 mutants should be interpreted with caution because they are not RNA and protein null and did show unexpected early flowering (Supplemental Fig. S8), which is contrary to its proposed function as a floral activator. This uncorrelated flowering phenotype may be due to its different genetic background. It is therefore difficult to infer SPL3’s function from the allele. However, if the phenotype of the spl3 mutants were indeed to be a reflection of its function, one possible explanation is that there may be a redundant player in ambient temperature-responsive flowering. One potential candidate is SPL5. Like SPL3, the SPL5 gene is much smaller than other SPL genes and it encodes primarily the DNA-binding domain (Wu and Poethig, 2006; Guo et al., 2008). Although we have demonstrated that SPL5 expression was greatly reduced at a low temperature (Fig. 2A) and that the cleavage products of SPL5 at this low temperature were also increased (Fig. 2B), we do not suggest that SPL5 actually plays a role in ambient temperature-responsive flowering because the leaf number ratios of 35S::SPL5(+/-) plants (1.7–1.8) were similar to that of wild-type plants (2.0; Supplemental Fig. S17).

Another potential candidate redundant player in ambient temperature-responsive flowering is SPL9. SPL9 controls flowering by directly regulating the expression of SOC1 (Wang et al., 2009), a putative target within the thermosensory pathway (Lee et al., 2007). SPL9 expression was down-regulated and cleavage products of SPL9 were enriched at 16°C (Fig. 2). The relationship between SPL3 and SPL9 is reminiscent of that between FT and SOC1, the potential outputs within the thermosensory pathway. Although ft and soc1 single mutants showed ambient temperature-responsive flowering, ft soc1 double mutants showed an additive reduction in temperature sensitivity (Lee et al., 2007). Considering that SPL3 and SPL9 regulate FT and SOC1, respectively, it is possible that SPL3 and SPL9 act redundantly in ambient temperature-responsive flowering. Further investigation on whether the miR156-SPL9-SOC1 regulatory module also acts in ambient temperature-responsive flowering would provide a better understanding of flowering behavior of Arabidopsis at different ambient temperatures.

Possible Connections between the miR156-SPL3 Module and the Thermosensory Pathway

FCA, FVE, and SVP are known to play important roles within the thermosensory pathway (Blázquez et al., 2003; Lee et al., 2007, 2008; Fornara et al., 2010). ELF3 and TFL1 also function in ambient temperature signaling (Strasser et al., 2009). We recently showed that the loss of SVP activity modulates the expression level of miR172 and its target genes and that the overexpression of miR172 causes ambient temperature-insensitive flowering (Lee et al., 2010). This suggests that SVP acts as a link between small RNA-mediated flowering control and the thermosensory pathway. However, the miR156-SPL3 module is unlikely to be regulated by SVP because the loss of SVP function
does not alter the expression of miR156 (Lee et al., 2010) and SPL genes (Supplemental Fig. S18). This reasoning is further supported by the observation that 35S::SPL3(−) plants showed a greater leaf number ratio value than smp mutants (Lee et al., 2007). Furthermore, miR156 and SPL3 expression was not significantly altered in elf3 and tfi1 mutants (Fig. 9). To further examine the genetic relationship between the miR156-SPL3 module and SVP/ELF3/TFL1, we are currently performing genetic interaction studies. Based on these results, we propose that the miR156-SPL3-FT genetic circuitry plays a role in fine tuning ambient temperature-responsive flowering independently of SVP, ELF3, and TFL1 function.

Whether the miR156-SPL3 module is integrated into the SVP-miR172 regulatory circuit has yet to be determined. The possibility of this integration is supported by data showing the regulation of miR172 expression by miR156 in the control of developmental timing (Wu and Poethig, 2006; Wang et al., 2008) and the strong anticorrelation in expression patterns of miR156 and miR172 at 23°C and 16°C (Lee et al., 2010). In this study, 35S::MIR156b 35S::MIR172a plants showed ambient temperature-responsive flowering (Fig. 9), although the early flowering phenotype of 35S::SPL3(−) 35S::MIR172a plants was additive. These results suggest that the miR156-SPL3 module and the miR172 pathway act in parallel in the regulation of ambient temperature-responsive flowering, although it was recently shown that the distinct role of miR156 and miR172 on the developmental transition is mediated by SPL3/4/5 genes (Jung et al., 2011). However, we cannot dismiss the possibility that the miR156-SPL3 module may be affected by a subset of miR172 target genes because SPL3 expression was increased in toc1 toc2 double mutants (Wu et al., 2009). Thus, further investigation is required to elucidate the mechanisms of interaction between the miR156-SPL3 module, miR172 targets, and the SVP-miR172 regulatory pathway before they converge on FT.

In summary, we have shown that the miR156-SPL3 module controls FT expression to regulate ambient temperature-responsive flowering. Vernalization is distinct from other temperature-dependent flowering responses in that it is controlled by a pathway that requires FLOWERING LOCUS C, which appears to be crucifer specific (Amasino and Michaels, 2010). However, in evolutionary terms, miR156 is a highly conserved miRNA, and its interaction with SQUAMOSA PROMOTER BINDING PROTEIN box genes has an ancient origin in land plants (Arazi et al., 2005; Riese et al., 2007; Willmann and Poethig, 2007; Guo et al., 2008; Wu et al., 2009; Gou et al., 2011). Thus, it is possible that the miR156-SPL3-FT genetic circuitry functions in a diverse array of flowering plants. It will be informative and challenging to determine whether the function of the miR156-SPL3-FT genetic circuitry in ambient temperature-responsive flowering is widely conserved.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All of the mutants used in this study were in the Arabidopsis (Arabidopsis thaliana; Columbia) background, except for spl3 (Ws). 35S::SPL3(−) 35S::SPL3 (+), 35S::FT, FT::GUS, ful-2, p9-1, tfi1, soc1-2, and 35S::MIR172a have been described previously (Ferrándiz et al., 2000; Takada and Goto, 2003; Yoo et al., 2005; Gandikota et al., 2007; Lee et al., 2010). The SUC2::SPL3, FDrSPL3, 35S::MIR156b, 35S::FUL, SUC2::FUL, FT::FUL, and 35S::MIM156 seeds (Schwab et al., 2005; Franco-Zorrilla et al., 2007; Wang et al., 2008, 2009) were kindly provided by Dr. Weigel (Max Planck Institute). FDr::FT, SUC2::FT, MIR172b::amiRFT, SUC2::amiRFT, and FDr::amiR-FT (Matieu et al., 2009) were kindly gifts from Dr. Schmid (Max Planck Institute). SAIL_726_E08 (ful-5) was obtained from the Arabidopsis Biological Resource Center (McElver et al., 2001). Plants were grown in soil or Murashige and Skoog medium at 23°C or 16°C in LD conditions (16-h light/8-h dark) at a light intensity of 120 μmol m⁻² s⁻¹.

Flowering data was measured by scoring either total leaf number (at least 10 plants) or bolting days, which was recorded when the primary inflorescence had reached a height of 0.5 cm. The leaf number ratio (16°C/23°C) was used as an indicator of ambient temperature-sensitive flowering (Blázquez et al., 2003; Lee et al., 2007; i.e. a completely ambient temperature-insensitive plant produces an identical total number of leaves at both 23°C and 16°C; thus, its leaf number ratio is 1.0). Because 35S::MIR156b, 35S::SPL3(−), and 35S::MIM156 plants exhibited high or low leaf initiation rates, with altered flowering time at 16°C, we used the leaf number ratio to describe their temperature responses.

Transgenic Plants

To generate 35S::SPL3-cMyc and GVC::SPL3, the coding region of SPL3 was amplified by PCR and cloned into a vector that contained the 35S promoter and a cMyc tag and into a pTA7002 vector, respectively. The pTA7002 vector used in this study is a transcriptional activation system of the target gene, in which an artificial transcription factor (GAL4-VF16-GR) induced by DEX transcriptionally activates the target gene (Aoyama and Chua, 1997; Xie et al., 2000; Desvoyes et al., 2006). To construct SPL3::SPL3-cMyc, we replaced the 35S promoter in 35S::SPL3-cMyc construct with the endogenous 2.4-kb SPL3 promoter. Oligonucleotide primers used for cloning are listed in Supplemental Table S2. Plants were transformed using the floral-dip method with minor modifications (Weigel and Glazebrook, 2002) and transformants were selected for kanamycin, hygromycin, or BASTA resistance. At least 30 T1 seedlings were analyzed for each construct.

Expression Analysis

To determine gene expression levels via qRT-PCR, total RNA was isolated from transgenic lines at DSI.02 (Boyes et al., 2001), unless otherwise noted, at which wild-type plants remained in the vegetative phase. Seedlings at this morphologically defined growth stage were used to compare gene expression levels due to the possibility that the degree of maturation of these plants may differ at different ambient temperatures based on their altered plastochron length (Supplemental Table S1). RNA quality was determined by using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies) and only qualified RNA samples (A260/A230 > 2.0 and A260/A280 > 1.8) were used for subsequent qRT-PCR experiments. To remove possible DNA contamination, RNA samples were treated with DNase (NEB) for 60 min at 37°C. A sample of 1 μg of RNA was used for cDNA synthesis using the transcript-first strand cDNA synthesis kit (Roche Diagnostics). The qRT-PCR primers were designed using SciTools at Integrated DNA Technologies (http://www.idtdna.com) with the criteria of a Tm of 62°C ± 0.5°C. Specific amplification was confirmed by running PCR products in a 12% polyacrylamide gel. The qRT-PCR analysis was carried out in 38-well plates with a LightCycler 480 (Roche Applied Science) using SYBR green. qRT-PCR experiments were carried out using KAPA SYBR green master mixture (KAPA Biosystems Inc.). The following program was used for amplification: pre-denaturation for 3 min at 94°C, followed by 40 cycles of denaturation for 10 s at 94°C, annealing for 10 s at 60°C, and elongation for 10 s at 72°C. Melting curve analysis was performed from 65°C to 97°C to assess the specificity of the qRT-PCR products. For qRT-PCR analysis, the 11 golden rules for qRT-PCR were followed (Livak et al., 2008) to ensure reproducible and accurate measurement of transcript levels. Samples for qRT-PCR were harvested at Zeitgeber time 8, unless otherwise noted. Two reference genes (either AT1G3320/AT1G28390 or AT1G3320/AT1G27960) that are stably expressed at 23°C and 16°C (Hong et al., 2010) were used to normalize the expression data.
were used for quantification. All qRT-PCR experiments were carried out in two or three biological replicates (independently harvested samples on different days) with three technical triplicates each with similar results. The results from a biological replicate are shown and the results from other biological replicates are shown in Supplemental Figure S19. Oligonucleotide primers used in this study are listed in Supplemental Table S2.

For western-blot analysis, anti-SPL3 antibodies were raised against a synthetic peptide corresponding to residues 39 to 52 of SPL3 (LDKQKCKAVSSS), which showed low (14%) similarity to the corresponding regions of SPL4 and SPL5 proteins. Anti-SPL3 antisera were purified using an affinity column immobilized with SPL3 peptides. Total protein extracts were prepared from 10-d-old seedlings and western-blot analysis was performed as described previously (Sambrook et al., 1989). The miRNA northern blots were processed as described previously (Lee et al., 2010). GUS staining was carried out according to standard procedures using 10-d-old seedlings grown on soil (Lee et al., 2007). The 4-methyl umbelliferyl glucuronide assay (Blázquez et al., 1997) was used to quantify GUS activity. This assay was carried out in triplicate.

Determination of the Relative Abundance of Transcripts

Our detailed procedure has been published (Hong et al., 2010). Threshold cycle (Ct) and PCR efficiency of the primers used were calculated using LinRegPCR (Ramakers et al., 2003). The relative abundance of the transcripts was calculated by the statistical formula from the geNorm. From three technical replicates, the coefficient of variation (Cv) was calculated according to the following formula: \( \text{Cv} = 100 \times (\text{SD of Ct}/\text{average of Ct}) \). The Ct and Cv values of each sample were then examined. If a Cv value in a sample was >2.0%, which indicated that there was a reaction that deviated most significantly from the mean in three technical replicates, it was considered an outlier and was thus excluded from further analyses. The gene expression level of wild-type plants at each temperature was set to one to show the effect of a mutation at different ambient temperatures. A >2-fold down-regulation was considered significant.

RLM 5'-RACE

A modified procedure for RLM 5'-RACE was performed as described previously (Llave et al., 2002). Total RNA was prepared from 10-d-old seedlings using a Nucleospin RNA extraction kit (Marchery Nargel). RNA was ligated to the RNA oligo-adaptor with T4 RNA ligase. The oligo(dT) primer was used to amplify the cDNA with Taq DNA polymerase using a Nucleospin RNA extraction kit (Marchery Nargel). RNA was ligated to the RNA oligo-adaptor with T4 RNA ligase. The oligo(dT) primer was used to amplify the cDNA with Taq DNA polymerase. Two rounds of nested PCR were done using two sets of RACE primers used in this study are listed in Supplemental Table S2.

ChiP

One gram of 10-d-old 35S::SPL3-cMyC or SPL3::SPL3-cMyC seedlings grown on soil was cross-linked in 1% formaldehyde solution on ice using vacuum infiltration. Nuclear extracts were isolated and an immunoprecipitation assay was conducted as described previously (Saleh et al., 2008). After shearing chromatin via sonication, mouse anti-cMyC or anti-HA polyclonal antibodies (about 5 μg; Santa Cruz Biotechnology) were used to immunoprecipitate genomic DNA fragments. DNA (1 μL) recovered from immunoprecipitation or 10% input DNA was used for qRT-PCR. The relative enrichment of each fragment was calculated by the ΔΔCt method as described previously (Livak and Schmittgen, 2001). ChiP experiments were performed in biological triplicates and results from one biological replicate were presented. The results from other biological replicates are shown in Supplemental Figure S19.

Arabidopsis Genome Initiative gene identifiers were as follows: API (AT1G69120); CUIC (AT5G53950); FD (AT1G59000); FT (AT1G54800); FULL (AT5G60100); LFY (AT1G61850); PP2A43 (AT1G13320); SAND family protein (AT2G28390); SOC1 (AT2G36600); SPL3 (AT1G43270); SPL5 (AT1G38310); SPL7 (AT2G42080); SPL9 (AT2G42200); SPL10 (AT1G27370); SPL11 (AT1G27640); SPL12 (AT3G56070); SLP15 (AT3G59200); SUC2 (AT1G22710); small nuclear RNA U6-1 (AT3G14735); TCP4 (AT3G15030); TSF (AT2G20370); miR156b (AT4G30972); miR172a (AT2G28056); and UBC9 (AT4G22960).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Confirmation of the overexpression of miR156 in transgenic plants.

Supplemental Figure S2. Total leaf numbers of wild-type plants grown in short days.

Supplemental Figure S3. Plastochron length of 35S::MIR156b, 35S::SPL3 (+/−), and 35S::MIM156 plants.

Supplemental Figure S4. Expression of mature miR156 in the leaves and the shoot apices.

Supplemental Figure S5. Validation of sample preparation.

Supplemental Figure S6. Expression of TSF and SOCI in 8-d-old seedlings.

Supplemental Figure S7. Unaltered cytosine methylation patterns at the SPL3 locus.

Supplemental Figure S8. Characterization of spl3 mutant plants.

Supplemental Figure S9. Characterization of 35S::SPL3(+/−) plants.

Supplemental Figure S10. FD and SUC2 expression in wild-type plants.

Supplemental Figure S11. Characterization of ful-8 allele.

Supplemental Figure S12. Measurement of GUS activity.

Supplemental Figure S13. Expression analysis of FT and FUL in GVG-SPL3 plants.

Supplemental Figure S14. Expression analysis in double mutants.

Supplemental Figure S15. Distribution of total leaf numbers of wild-type and SPL3::SPL3-cMyC plants.

Supplemental Figure S16. Expression of SPL3 gene in SUC2::FT and FD::FT plants.

Supplemental Figure S17. Ambient temperature-sensitive flowering of 35S::SPL5(+/−) plants.

Supplemental Figure S18. Expression of SPL genes in 8-d-old spy-32 plants.

Supplemental Figure S19. Results of biological replicates in the text.

Supplemental Table S1. Flowering time of plants.

Supplemental Table S2. List of oligonucleotide primers.

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