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Role of *miR156-SPL3-FT* genetic circuitry

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The *miR156-SPL3* Module Regulates Ambient Temperature-Responsive Flowering via *FT* in *Arabidopsis thaliana*

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ABSTRACT

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 the present study, we show that the *microRNA156* (*miR156*)-*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* (*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 (16ºC), which was associated with downregulation of *FT* and *FRUITFULL* (*FUL*) expression. Among miR156 target genes, *SPL3* mRNA levels were mainly reduced, probably because miR156-mediated cleavage of *SPL3* mRNA was higher at 16ºC. Overexpression of miR156-resistant *SPL3* [*SPL3*(-)] caused early flowering, regardless of the ambient temperature, which was associated with upregulation of *FT* and *FUL* expression. Reduction of miR156 activity by target mimicry led to a phenotype similar to that of *SUC2::rSPL3* plants. *FT* upregulation was observed after dexamethasone treatment in *GVG-rSPL3* plants. Misexpression and artificial miRNA-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 *TFL1, SVP,* and *ELF3* 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.

INTRODUCTION

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 (Blazquez 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 FLOWERING 3* (*ELF3*), and *TERMINAL FLOWER 1* (*TFL1*) genes are involved in this pathway (Blazquez 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 *miR399-PHO2* module plays a role in the regulation of ambient temperature-responsive flowering (Kim et al., 2011). 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 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 (Yang et al.; Schwarz et al., 2008; Wang et al., 2008; Nodine and Bartel, 2010; Yu et al., 2010; Gou et al., 2011; Xing 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*), *AGAMOUS-LIKE 42* (*AGL42*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) is regulated by this module. *SPL3* directly activates the expression of *LEAFY* (*LFY*), *FUL*, and *APETALA1* (*AP1*) 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 the present 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 23ºC and 16ºC. 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 (23ºC) 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 23ºC were almost indistinguishable from those grown at 16ºC, 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 23ºC in long-day conditions (25.6 leaves) (Supplemental Table 1 to find detailed information on flowering time of plants used in this study) (Fig. 1A). Interestingly, flowering at 16ºC was even more delayed (61.4 leaves). Thus, the leaf number ratio of *35S::MIR156b* plants (16ºC/23ºC, see methods) was 2.4 (c.f. 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 23ºC and 16ºC (Supplemental Fig. S3). As observed at 23ºC, 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 16ºC (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 (23ºC) and 37 leaves (16ºC), indicating that the phase transition in *35S::MIR156* plants was more delayed at 16ºC than at 23ºC (Fig. 1B). These results suggest that miR156 overexpression led to ambient temperature-sensitive flowering.

Downregulation 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 23ºC and 16ºC (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* (*STM*) (Endrizzi et al., 1996) in these samples (Supplemental Fig. S5). In the leaf of 8-day-old *35S::MIR156b* plants, *FUL* (Ferrandiz et al., 2000) expression was downregulated 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. S6A). Notably, the downregulation 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 1), 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. Consequently, 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 downregulation of *FT* was more apparent in the leaf than in the shoot apical region at both temperatures (Fig. 1D). There was a similar downregulation 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 23ºC and 16ºC, it consistently downregulated *FT* and *FUL*, which are potent floral activators, at both temperatures.

Downregulation of *SPL3* via Enhanced Cleavage by miR156 at 16ºC

The effect of ambient temperature on the expression levels of *SPL* genes was examined. The expression of *SPL* genes was generally lower at 16ºC, in contrast to miR156 expression, which was higher at 16ºC (Fig. 2A). In particular, *SPL3* mRNA levels were dramatically lower at 16ºC than at 23ºC. However, the expression of *CUP-SHAPED COTYLEDON 2* (*CUC2*) (Larue et al., 2009) and *TCP FAMILY TRANSCRIPTION FACTOR 4* (*TCP4*) (Palatnik et al., 2003), which are target genes of non-ambient temperature-responsive miRNAs (Lee et al., 2010), was not altered. These results suggest that the elevated miR156 expression at 16ºC can enhance *SPL3* cleavage, although we cannot exclude the possibility of the translational inhibition of other *SPL* genes by miR156 at 16ºC.

We then examined whether the downregulation of *SPL3* at 16ºC was associated with enhanced cleavage of their mRNAs by miR156. No difference in the DNA methylation pattern at the *SPL3* locus was observed at 16ºC, excluding a change in DNA methylation as an explanation of the downregulation of *SPL3* at 16ºC (Supplemental Fig. S7). A gene-specific RNA ligase-mediated amplification of cDNA ends (RLM 5'-RACE) assay identified cleavage products of *SPL* genes at 23ºC and 16ºC (Fig. 2B). Considerably more cleavage products were produced from *SPL3* at 16ºC (2.7-fold increase). In contrast, the levels of RACE products of *CUC2* and *TCP4* were similar at 23ºC and 16ºC. These results suggest that the elevated miR156 expression at 16ºC can enhance *SPL3* cleavage, although the possibility cannot be excluded that miR156 also inhibits the translation of other *SPL* genes at 16ºC. 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 (qRT)-PCR and RLM 5'-RACE revealed that *SPL3* levels were anti-correlated with the level of miR156 at 16ºC.

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

The available *spl3* mutants (FLAG_173C12, Ws background) exhibited unexpected early flowering with an increased leaf number ratio (1.8) (c.f. 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 region (UTRs) [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 long-day 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 16ºC (23.7 leaves) than at 23ºC (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 23ºC and 16ºC, 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 23ºC and 16ºC, respectively) was similar to that of wild-type plants (6.0 and 11.5 leaves at 23ºC and 16ºC, respectively). These results suggest that *SPL3* modulates ambient temperature-responsive flowering.

Upregulation 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-day-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 *TSF* 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 23ºC and 16ºC, respectively). A slightly reduced expression level of *FT* at 16ºC 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 upregulation of *FT* and *FUL* in the leaf and the shoot apex, which is consistent with their downregulation 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::rSPL3* 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-day-old *SUC2::rSPL3* plants at both temperatures (Fig. 3F). This upregulation of *FT* and *FUL* expression in the leaf of *SUC2::rSPL3* plants was more apparent at DS 1.02, i.e., at least 3-fold, at both temperatures. In the shoot apical region of 8-day-old seedlings of *FD::rSPL3* plants and at DS 1.02, *FUL* expression was increased at both temperatures (Fig. 3G); however, *FUL* upregulation 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 analysis

demonstrate that the flowering of *SUC2::rSPL3* plants, which showed stronger upregulation of *FT* and *FUL* in the leaf, was less sensitive to changes in ambient temperature. Thus, together with the downregulation 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 knock-out 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 23ºC and 16ºC (8.0 and 12.8 leaves, respectively) (Fig. 4A). The leaf number ratio of *35S::MIM156* plants was 1.6 (c.f. wild-type plants = 2.0), indicating that 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 23ºC (24.7 days) and 16ºC (48.5 days) was similar to that of wild-type plants (24 and 49.7 days at 23ºC and 16ºC, 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 23ºC and 16ºC, respectively) (Fig. 4B), implying that the reduction in miR156 activity accelerated phase transition, which was also seen in *35S::SPL3*(-) plants (Fig. 3B).

In 8-day-old *35S::MIM156* plants and at DS 1.02, a general upregulation of *SPL* genes at both 23ºC and 16ºC (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-day-old-seedlings of *35S::MIM156* plants. *FUL* expression was upregulated 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 upregulated (by at least 1.7-fold) (Fig. 4F). These results together with the upregulation 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* upregulation 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* upregulation 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 de-repressed 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 16ºC (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 16ºC (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 16ºC (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* upregulation in *35S::SPL3*(-) *ful-8* plants revealed that the upregulation of *FT* was not altered in the leaves of *35S::SPL3*(-) *ful-8* 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-day-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-methyl umbelliferyl glucuronide (MUG) 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 DEX-inducible promoter (Aoyama and Chua, 1997). Treatment with DEX induced an early flowering phenotype at 23ºC (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 (#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 day 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 23ºC and 16ºC (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 16ºC 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 23ºC and 16ºC (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 (SBP) 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 anti-cMyc antibody, because our SPL3 antibodies were not suitable for ChIP (data not shown). The *35S::rSPL3-cMyc* plants flowered with similar leaf numbers at 23ºC and 16ºC (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 (VI)] containing GTAC motifs, the putative binding sties 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 *SPL3::rSPL3-cMyc* plants. Most of the *SPL3::rSPL3-cMyc* plants flowered much earlier than wild-type plants in the T_1

generation (Supplemental Fig. S15), indicating that the *SPL3::rSPL3-cMyc* construct was indeed functional. Strong enrichment of SPL3 protein was found in region III (Fig. 8E), which contains two consensus SPL3 binding motifs. Because SPL3 prefers cGTAC or GTACg core sequences (Birkenbihl et al., 2005), the first of these motifs likely fits the preferential SPL3 binding site. Weak SPL3 enrichment was observed in region II. The relative binding strength was weaker in *SPL3::rSPL3-cMyc* plants than in *35S::rSPL3-cMyc* plants, suggesting that these differences may be due to the different SPL3 protein levels. The results of ChIP-qPCR analyses using *35S::rSPL3-cMyc* and *SPL3::rSPL3-cMyc* plants (Fig. 8, D and E) indicate that the SPL3 protein preferentially bound to region III in the *FT* genomic loci. Collectively, they suggest that SPL3 regulates *FT* expression via directly binding to the GTAC motifs in the *FT* genomic loci for the regulation of ambient temperature-responsive flowering.

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

Because miR172 is another ambient temperature-responsive miRNA and its overexpression leads to ambient temperature-insensitive flowering through the upregulation 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 23ºC and 16ºC, 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 23ºC and 16ºC, 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-32*, *tfl1-20*, 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.

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.

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* mRNA is barely detected in vegetative shoot apices but is strongly induced in leaves (Wang et al., 2009). The present 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 upregulation of *FT* in the leaves of *35S::SPL3*(-) plants (Fig. 3), the early upregulation 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 23ºC (Supplemental Fig. S16). Thus, it is likely that the regulation of *SPL3* via *FT-FD* modules at the shoot apex region does not affect ambient temperature-responsive flowering.

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-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.

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.* that the early flowering phenotype of *SUC2::FUL* plants is completely suppressed by the *ft-10* mutation (Wang et al., 2009).

The Effect of Low Temperature on Flowering Caused by miR156 Overexpression at 23ºC may be Attenuated by the Relatively Low Cleavage of *SPL3* via miR156 at 23ºC

Since the *miR156-SPL3-FT* module also serves as a regulatory mechanism involved in the control of ambient temperature-reponsive flowering, an important question which needs to be answered is why *35S::MIR156b* and *35::MIM156* plants showed contrasting temperature responses (Fig. 1A and 4A). Similar to the temperature response of gain- and loss-of-function of *FT*, a major output gene within the thermosensory pathway (Fig. 7) (Lee et al., 2007), the ambient temperature response was expected to either disappear or be reduced in its gain- and loss-of-function mutants of miR156. However, *35S::MIR156b* plants showed an increased temperature response due to more delayed flowering at a low temperature, with the delay in flowering being more profound at 16ºC than at 23ºC (Fig. 1A). This result suggests that the effect of low temperature on flowering caused by the overexpression of miR156 at 23ºC may be attenuated by the relatively low cleavage of *SPL3* via miR156 at 23ºC. Our observation that the cleavage of *SPL3* by miR156 was strongly enhanced at 16ºC (Fig. 2) provides support for this notion. The differential *FT* expression seen in *35S::MIR156b* plants at 23ºC and 16ºC provides further support for this concept (Fig. 1D). However, we cannot exclude the possibility that increased or decreased miR156 activity at different ambient temperatures may induce differential responses.

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 downregulated and cleavage products of *SPL9* were enriched at 16ºC (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 (Blazquez et al., 2003; Lee et al., 2007; Lee et al., 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 *svp* mutants (Lee et al., 2007). Furthermore, miR156 and *SPL3* expression was not significantly altered in *elf3* and *tfl1* 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., 2011) and the strong anti-correlation in expression patterns of miR156 and miR172 at 23ºC and 16ºC (Lee et al., 2010). In the present 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 *toe1 toe2* 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* (*FLC)*, which appears to be crucifer-specific (Amasino and Michaels, 2010). However, in evolutionary terms, miR156 is a highly conserved miRNA, and its interaction with SBP-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*) (Col) background, except for *spl3* (Ws). *35S::SPL3*(-), *35S::SPL3*(+), *35S::FT*, *FT::GUS*, *ful-2*, *ft-10*, *tsf-1*, *soc1-2*, and *35S::MIR172a* have been described previously (Ferrandiz et al., 2000; Takada and Goto, 2003; Yoo et al., 2005; Gandikota et al., 2007; Lee et al., 2010). The *SUC2::rSPL3*, *FD::rSPL3*, *35S::MIR156b*, *35S::FUL*, *SUC2::FUL*, *FD::FUL*, and *35S::MIM156* seeds (Schwab et al., 2005; Franco-Zorrilla et al., 2007; Wang et al., 2008; Wang et al., 2009) were kindly provided by Dr Weigel (Max Planck Institute, Germany). *FD::FT*, *SUC2::FT*, *35S::amiR-FT*, *SUC2::amiR-FT*, and *FD::amiR-FT* (Mathieu et al., 2007) were kind gifts from Dr Schmid (Max Planck Institute, Germany). SAIL_726_E08 (*ful-8*) was obtained from the Arabidopsis Biological Resource Center (McElver et al., 2001). Plants were grown in soil or MS medium at 23ºC or 16ºC in long-day conditions (16 h light/8 h dark) at a light intensity of 120 μmol m^{-2} s^{-1}.

Flowering time 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 (16ºC/23ºC) was used as an indicator of ambient temperature-sensitive flowering (Blazquez et al., 2003; Lee et al., 2007) (i.e., a completely ambient temperature-insensitive plant produces an identical total number of leaves at both 23ºC and 16ºC; 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 16ºC, we used the leaf number ratio to describe their temperature responses.

Transgenic Plants

To generate *35S::rSPL3-cMyc* and *GVG-rSPL3*, 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-VP16-GR) induced by DEX transcriptionally activates the target gene (Aoyama and Chua, 1997; Xie et al., 2000; Desvoyes et al., 2006). To construct *SPL3::rSPL3-cMyc*, we replaced the *35S* promoter in *35S::rSPL3-cMyc* construct with the endogenous 2.4 kb *SPL3* promoter. Oligonucleotide primers used for cloning are listed in Supplemental Table 2 (online). 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 T_1 seedlings were analyzed for each construct.

Expression Analysis

To determine gene expression levels via qRT-PCR, total RNA was isolated from transgenic lines at DS 1.02 (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, USA) 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 DNaseI (NEB, USA) for 60 min at 37ºC. A sample of 1 µg of RNA was used for cDNA synthesis using the Transcriptor first-strand cDNA synthesis Kit (Roche Diagnostics, Germany). The qRT-PCR primers were designed using SciTools at Integrated DNA Technologies (IDT) (http://www.idtdna.com) with the criteria of a melting temperature (T_M) of 62±0.5ºC. Specific amplification was confirmed by running PCR products in a 12% polyacrylamide gel. The qRT–PCR analysis was carried out in 384-well plates with a LightCycler 480 (Roche Applied Science, USA) using SYBR green. qRT-PCR experiments were carried out using KAPA SYBR Green Master mixture (KAPA™ Biosystems Inc., USA). The following program was used for amplification: pre-denaturation for 3 min at 94ºC, followed by 40 cycles of denaturation for 10 sec at 94ºC, annealing for 10 sec at 60ºC, and elongation for 10 sec at 72ºC. Melting curve analysis was performed from 65ºC to 97ºC to assess the specificity of the qRT-PCR products. For qRT-PCR analysis, the ‘Eleven Golden Rules for Quantitative RT-PCR’

were followed (Udvardi et al., 2008) to ensure reproducible and accurate measurement of transcript levels. Samples for qRT-PCR were harvested at Zeitgeber time (ZT) 8, unless otherwise noted. Two reference genes (either AT1G13320/AT2G28390 or AT1G13320/AT4G27960) that are stably expressed at 23ºC and 16ºC (Hong et al., 2010) 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 Fig. 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 39–52 of SPL3 (LEKKQKGKATSSSG), 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-day-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). β-Glucuronidase (GUS) staining was carried out according to standard procedures using 10-day-old seedlings grown on soil (Lee et al., 2007). The MUG assay (Blazquez 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: Cv = 100 × (standard deviation of Ct/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 downregulation 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-day-old seedlings using a Nucleospin RNA Extraction kit (Marchery Nargel, Germany). RNA was ligated to the RNA oligo-adaptor with T4 RNA ligase. The oligo-dT primer was used to prime cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, USA). PCR amplification was performed with a GeneRacer 5' primer and a gene-specific 3' primer. Two rounds of nested PCR were done using two sets of RACE adaptors and gene-specific primers. For semi-quantitative measurement, the RLM 5'-RACE products were separated and hybridized with the probes specific to the 5'-RACE adapter sequence for *SPL* genes and *UBQ10*.

ChIP

One gram of 10-day-old *35S::rSPL3-cMyc* or *SPL3::rSPL3-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, USA) 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 $\Delta\Delta C_t$ 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 Fig. S19.

Accession Numbers

Arabidopsis Genome Initiative gene identifiers were as follows: *AP1* (AT1G69120); *CUC2* (AT5G53950); *FD* (AT4G35900); *FT* (AT1G65480); *FUL* (AT5G60910); *LFY* (AT5G61850); *PP2AA3* (AT1G13320); SAND family protein (AT2G28390); *SOC1* (AT2G45660); *SPL2* (AT5G43270); *SPL3* (AT2G33810); *SPL4* (AT1G53160); *SPL5* (AT3G15270); *SPL6* (AT1G69170); *SPL9* (AT2G42200); *SPL10* (AT1G27370); *SPL11* (AT1G27360); *SPL13* (AT5G50670); *SPL15* (AT3G57920); *SUC2* (AT1G22710); small nuclear RNA U6-1 (AT3G14735); *TCP4* (AT3G15030); *TSF* (AT4G20370); miR156b (AT4G30972); miR172a (AT2G28056); and *UBC9* (AT4G27960).

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FIGURE LEGENDS

Figure 1. Overexpression of miR156 Caused Ambient Temperature-Sensitive Flowering in Long-Day Conditions.

(A) Delayed flowering of *35S::MIR156b* plants at 16ºC. Photographs were taken when *35S::MIR156b* plants flowered at each temperature.

(B) The leaf morphologies of *35S::MIR156b* plants. An inverted triangle indicates the juvenile-to-adult transition point based on the appearance of abaxial trichomes.

(C and D) qRT-PCR analysis of *FT*, *FUL*, and *SPL3* expression in the leaves and the shoot apical regions (SA) of 8-day-old seedlings (C) and at DS1.02 (Boyes et al., 2001) (D) of *35S::MIR156b* plants grown at 23ºC and 16ºC. Expression levels were measured at ZT 16, at which point *FT* transcript levels are high (Corbesier et al., 2007). The expression levels of each gene in wild-type (WT) plants at 23ºC were set to one. Error bars indicate the standard deviation (SD).

Figure 2. Expression Levels and Cleavage Sites of *SPL* Genes at 23ºC and 16ºC.

(A) Relative expression levels of miR156 and *SPL* genes in 10-day-old wild-type (WT) plants grown at 23ºC and 16ºC. *CUC2* and *TCP4* were used as controls. Error bars indicate the SD.

(B) Semi-quantitative measurement of the level of RLM 5'-RACE products of *SPL* genes in 10-day-old WT 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.

Figure 3. Overexpression of miR156-Resistant *SPL3* Caused Ambient Temperature-Insensitive Flowering in Long-Day Conditions.

(A) Accelerated flowering of *35S::SPL3*(-) plants at 16ºC. Photographs were taken when the *35S::SPL3*(-) plants flowered at each temperature.

(B) The leaf morphologies of *35S::SPL3*(+) and *35S::SPL3*(-) plants. An inverted triangle indicates the juvenile-to-adult transition point based on the appearance of abaxial trichomes.

(C and D) Expression of *FT* and *FUL* in the leaves and the shoot apical regions (SA) of 8-day-old seedlings (C) and at DS1.02 (D) of *35S::SPL3*(-) plants grown at 23ºC and 16ºC. The expression levels of each gene in wild-type (WT) plants at 23ºC were set to one.

(E) Phenotype and total leaf numbers of *SUC2::rSPL3* and *FD::rSPL3* plants grown at 23ºC and 16ºC. Photographs were taken when the *SUC2::rSPL3* plants flowered at each temperature.

(F) Expression of *FT* and *FUL* in the leaves of 8-day-old seedlings and at DS1.02 of *SUC2::rSPL3* plants.

(G) Expression of *FUL* in the shoot apical regions of 8-day-old seedlings and at DS1.02 of *FD::rSPL3* plants. Error bars indicate the SD.

Figure 4. Flowering of *35S::MIM156* Plants was Less Ambient Temperature-Sensitive in Long-Day Conditions.

(A) Accelerated flowering of *35S::MIM156* plants at 16ºC in long-day 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 for 8 days (C) and at DS1.02 (D) determined via qRT-PCR. Expression levels of each *SPL* gene at 23ºC were set to one.

(E and F) Expression of *FT* and *FUL* in whole seedlings of *35S::MIM156* plants grown for 8 days (E) and at DS1.02 (F).

(G) Expression of the *SPL3* gene in 8-day-old wild-type (WT), *35S::MIM156*, *SUC2::rSPL3*, and *35S::SPL3*(-) plants. Error bars indicate the SD.

Figure 5. *FUL* Plays a Limited Role in Ambient Temperature-Responsive Flowering in Long-Day Conditions.

(A) Total leaf numbers of gain- and loss-of-function alleles of *FUL* grown at 23ºC and 16ºC. *35S::FT*, *SUC2::FT*, and *FD::FT* plants were used as controls. Numbers listed above the genotypes denote the leaf number ratio.

(B) Total leaf numbers of *ful-8*, *35S::SPL3*(-), and *35S::SPL3*(-) *ful-8* plants grown at 23ºC and 16ºC.

(C and D) The effect of the *FUL* mutation on *FT* expression in *35S::SPL3*(-) plants grown for 8 days (C) and at DS1.02 (D). The *FT* expression levels in wild-type (WT) plants at 23ºC were set to one. Error bars indicate SD.

Figure 6. *FT* Acts Downstream of miR156 and *SPL3*.

(A) and (B) Expression of miR156 in *35S::FT* and *ft-10* plants (A) and *35S::SPL3*(+) and *35S::SPL3*(-) plants (B) at DS1.02 grown at 23ºC and 16ºC. U6 RNA served as a loading control in these small RNA blots (Yoo et al., 2011) and the miR156 expression level in wild-type (WT) plants at 23ºC were set to one.

(C) Relative expression levels of *SPL3* in the leaves and the shoot apical regions (SA) of *35S::FT* and *ft-10* plants grown at 23ºC and 16ºC.

(D) *FT::GUS* activity in the cotyledon of 10- and 12-day-old *35S::MIR156b* and *35S::SPL3*(-) plants grown on soil at 23ºC. Inset shows *FT::GUS* staining of the leaf.

(E) Phenotype of *GVG-rSPL3* plants and expression of *FT* and *FUL* of 8-day-old *GVG-rSPL3* seedlings after DEX induction. Mock-treated (left) and 30 μM DEX-treated (right) *GVG-rSPL3* seedlings grown in long-day conditions were photographed. The *FT* and *FUL* expression level was measured 1 day after DEX treatment. Error bars indicate the SD.

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::MIR156b* plants. Total leaf numbers of F_1 progeny grown at 23ºC and 16ºC in long-day 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.

Figure 8. The SPL3 Protein Binds to the Regulatory Region of *FT in vivo*.

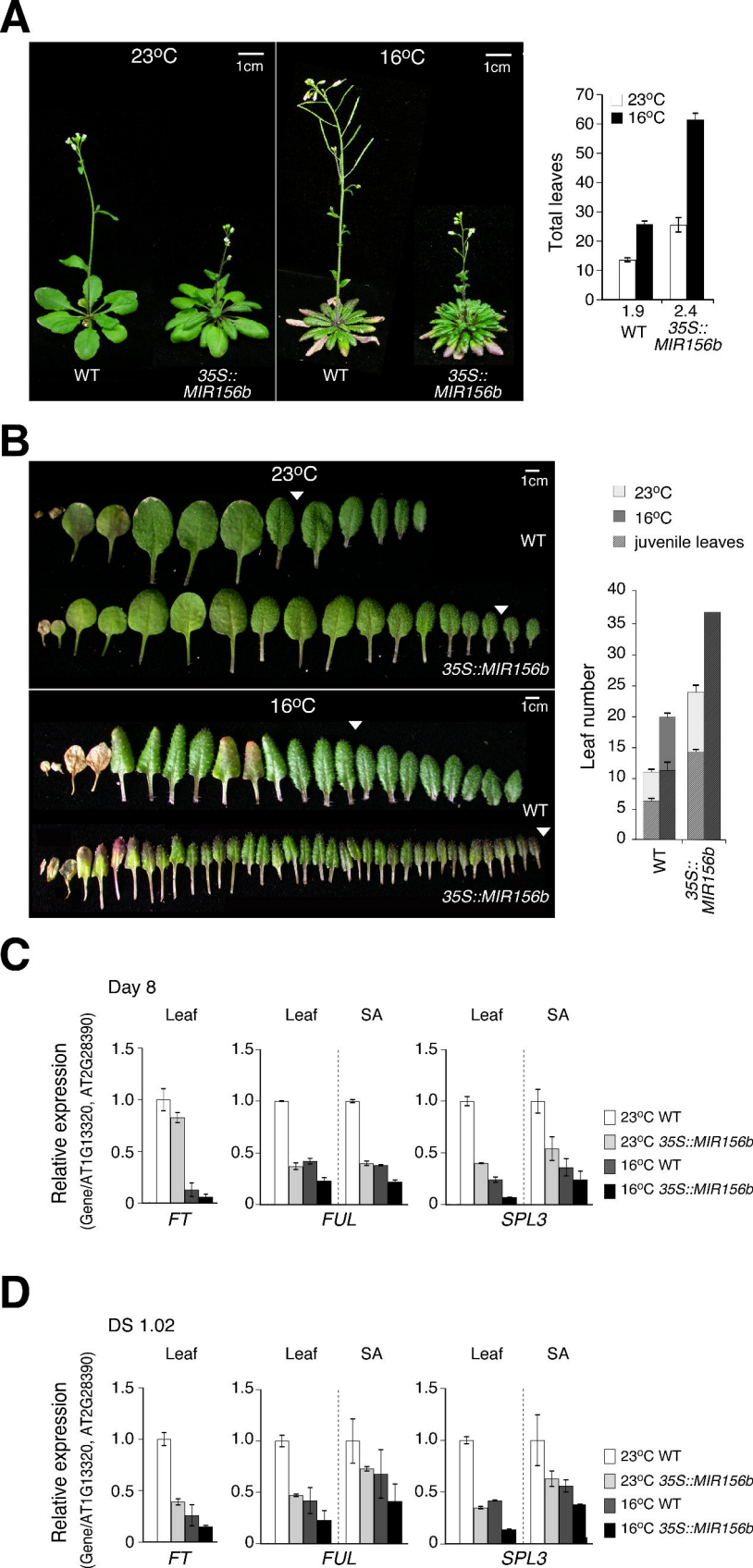
(A) Phenotype and total leaf numbers of *35S::rSPL3-cMyc* plants grown at 23ºC in long-day conditions. Photographs were taken when *35S::rSPL3-cMyc* plants flowered.

(B) SPL3-cMyc protein expression in *35S::rSPL3-cMyc* plants. Anti-cMyc antibody was used to detect SPL3-cMyc protein.

(C) Schematic diagram of the *FT* genomic region. Closed boxes and thin lines represent exons and introns, respectively. Asterisks indicate the presence of a predicted GTAC core recognition sequence. Gray horizontal bars denote the amplified fragments in ChIP-qPCR: Region I (-2876 to -2494, relative to the translational start codon of *FT*); region II (-1874 to -1649); region III (-291 to -2); region IV (+158 to +416); region V (+1196 to +1560); and region VI (+2449 to +2873).
(D) ChIP-qPCR analysis of *FT* genomic fragments in 10-day-old wild-type (WT) and *35S::rSPL3-cMyc* seedlings. Relative enrichment of fragments was calculated by comparing samples immunoprecipitated with HA and cMyc antibodies.
(E) ChIP-qPCR analysis of *FT* genomic fragments in 10-day-old WT and two independent *SPL3::rSPL3-cMyc* seedlings. Error bars indicate SD.

Figure 9. Genetic Interactions Among miR156, *SPL3*, and Other Components Involved in Ambient Temperature-Responsive Flowering.
(A) Total leaf numbers of F_1 progeny grown at 23ºC and 16ºC in long-day conditions are shown. Numbers listed above the genotypes denote the leaf number ratio.
(B and C) Expression of miR156 (B) and *SPL3* (C) in 8-day-old seedlings of *svp-32*, *elf3-1*, and *tfl1-20* mutants grown at 23ºC and 16ºC in long-day conditions. The miR156 expression level in wild-type (WT) plants at 23ºC were set to one. Error bars indicate SD.

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.


A
23ºC
1cm
16ºC
1cm
WT
35S::
MIR156b
WT
35S::
MIR156b
Total leaves
23ºC
16ºC
70
60
50
40
30
20
10
0
1.9
WT
2.4
35S::
MIR156b
B
23ºC
1cm
WT
35S::MIR156b
16ºC
1cm
WT
35S::MIR156b
23ºC
16ºC
juvenile leaves
Leaf number
40
35
30
25
20
15
10
5
0
WT
35S::
MIR156b
C
Day 8
Leaf
Leaf
SA
Leaf
SA
Relative expression
(Gene/AT1G13320, AT2G28390)
1.5
1.0
0.5
0
FT
FUL
SPL3
23ºC WT
23ºC 35S::MIR156b
16ºC WT
16ºC 35S::MIR156b
D
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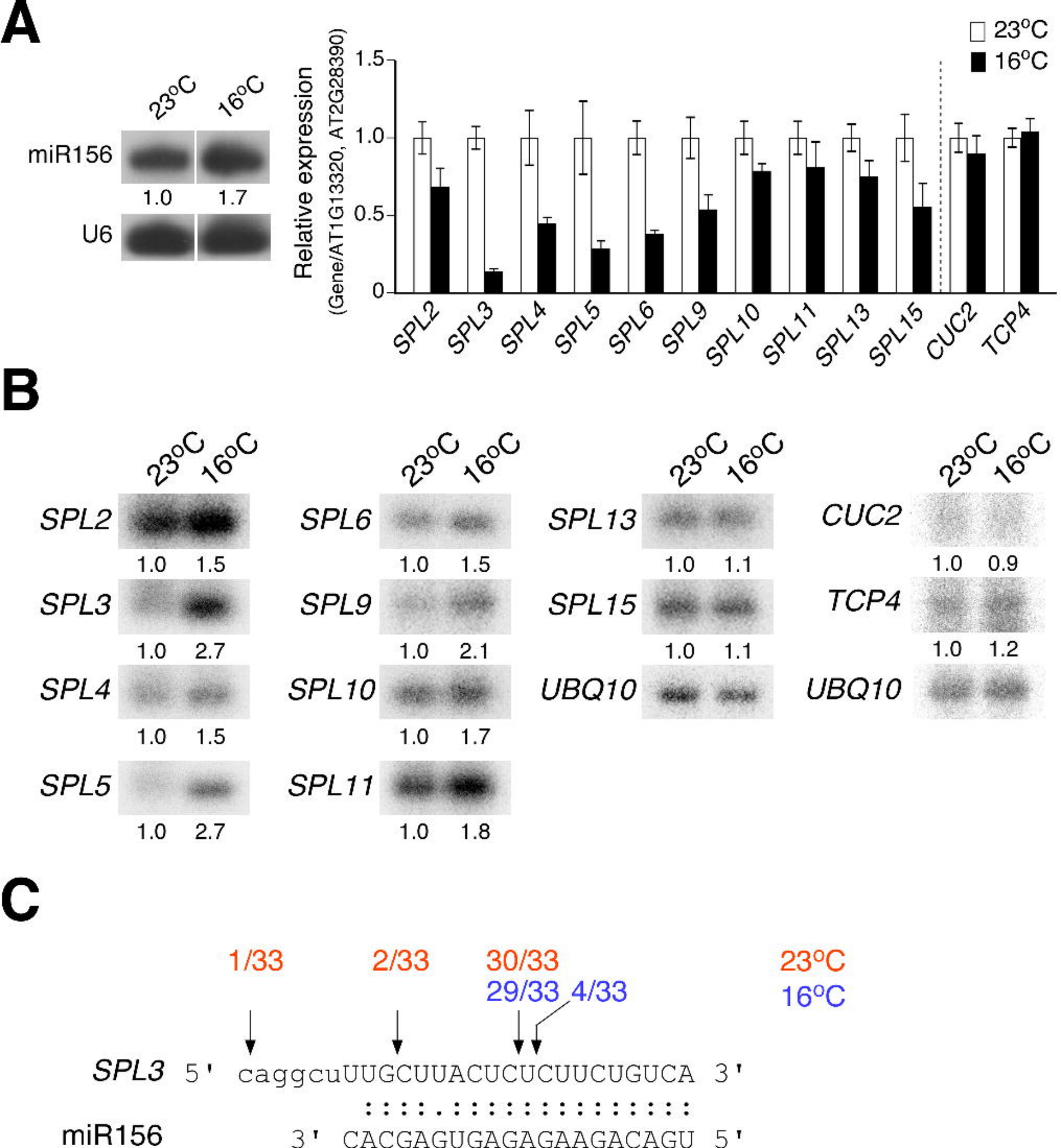
Leaf
Leaf
SA
Leaf
SA
Relative expression
(Gene/AT1G13320, AT2G28390)
1.5
1.0
0.5
0
FT
FUL
SPL3
23ºC WT
23ºC 35S::MIR156b
16ºC WT
16ºC 35S::MIR156b


A
23°C
16°C
miR156
1.0
1.7
U6
Relative expression
(Gene/AT1G13320, AT2G28390)
1.5
1.0
0.5
0
23°C
16°C
SPL2
SPL3
SPL4
SPL5
SPL6
SPL9
SPL10
SPL11
SPL13
SPL15
CUC2
TCP4
B
23°C
16°C
SPL2
1.0
1.5
SPL3
1.0
2.7
SPL4
1.0
1.5
SPL5
1.0
2.7
SPL6
1.0
1.5
SPL9
1.0
2.1
SPL10
1.0
1.7
SPL11
1.0
1.8
SPL13
1.0
1.1
SPL15
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UBQ10
CUC2
1.0
0.9
TCP4
1.0
1.2
UBQ10
C
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30/33
29/33
4/33
23°C
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SPL3 5' caggcuUUGCUUACUCUCUUCUGUCA 3'
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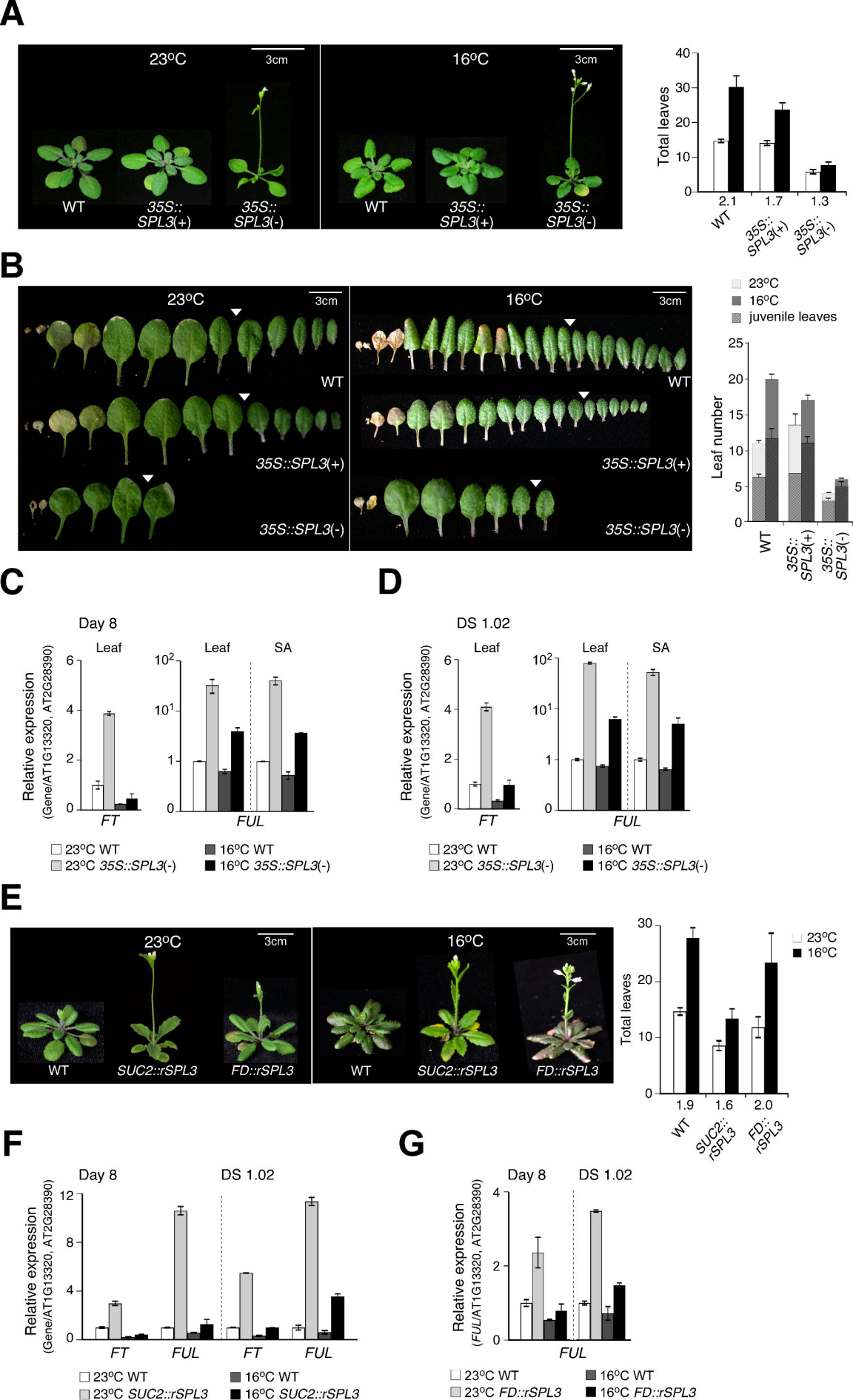
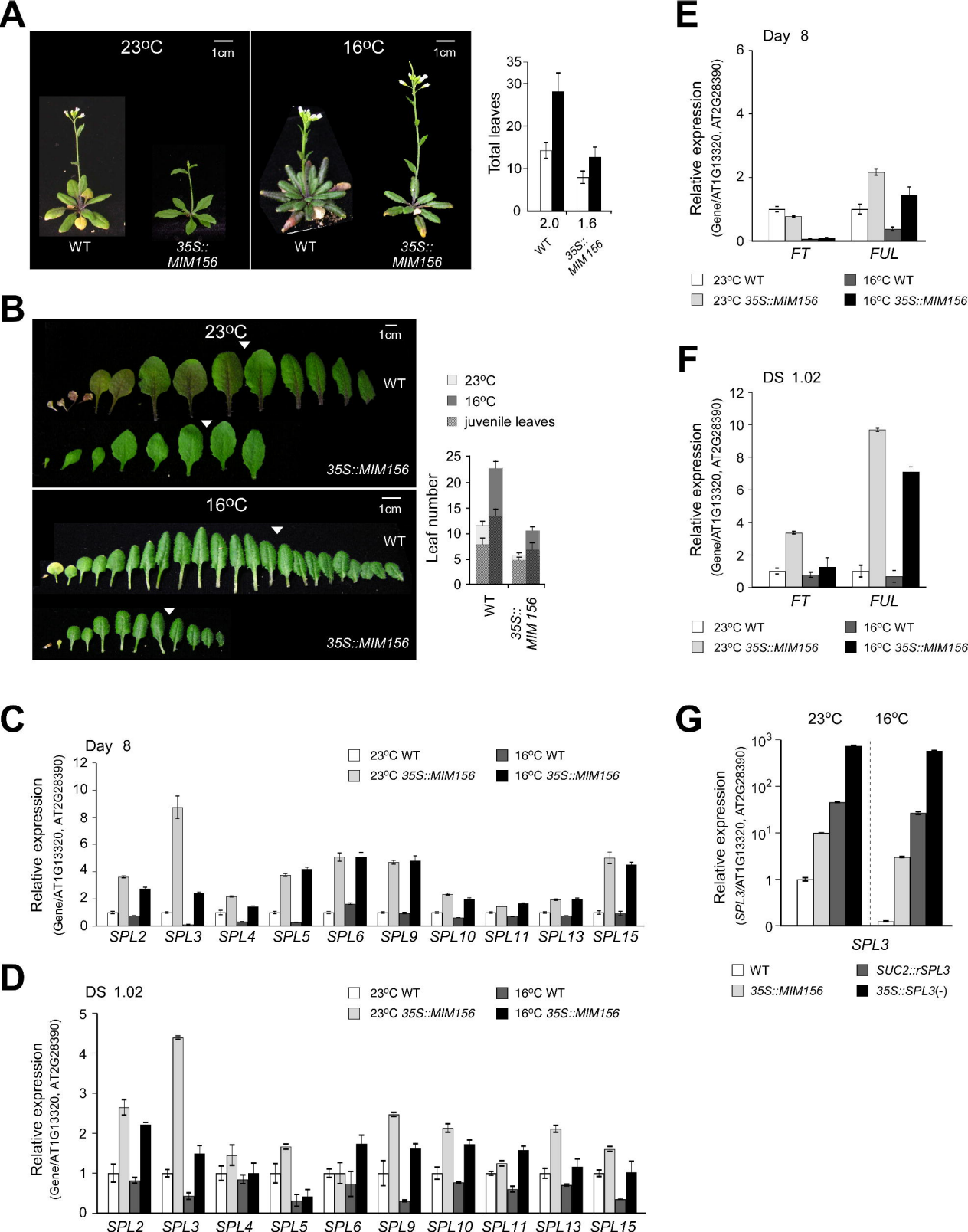
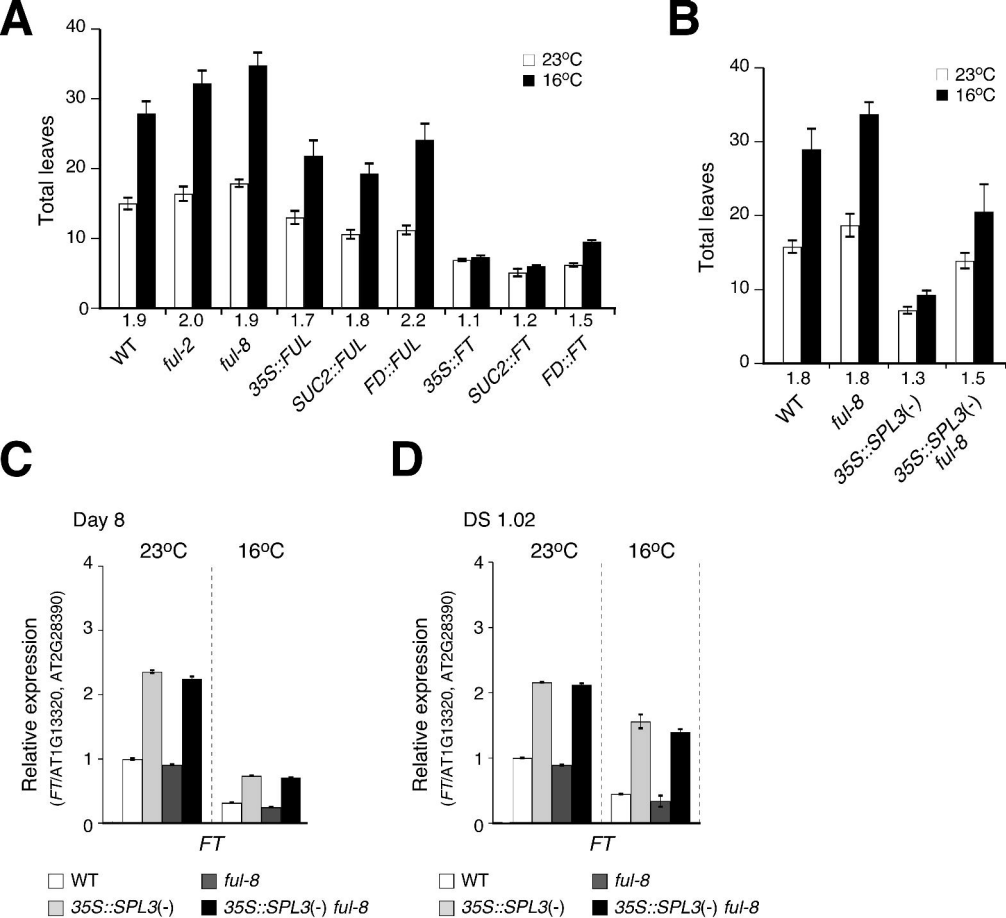
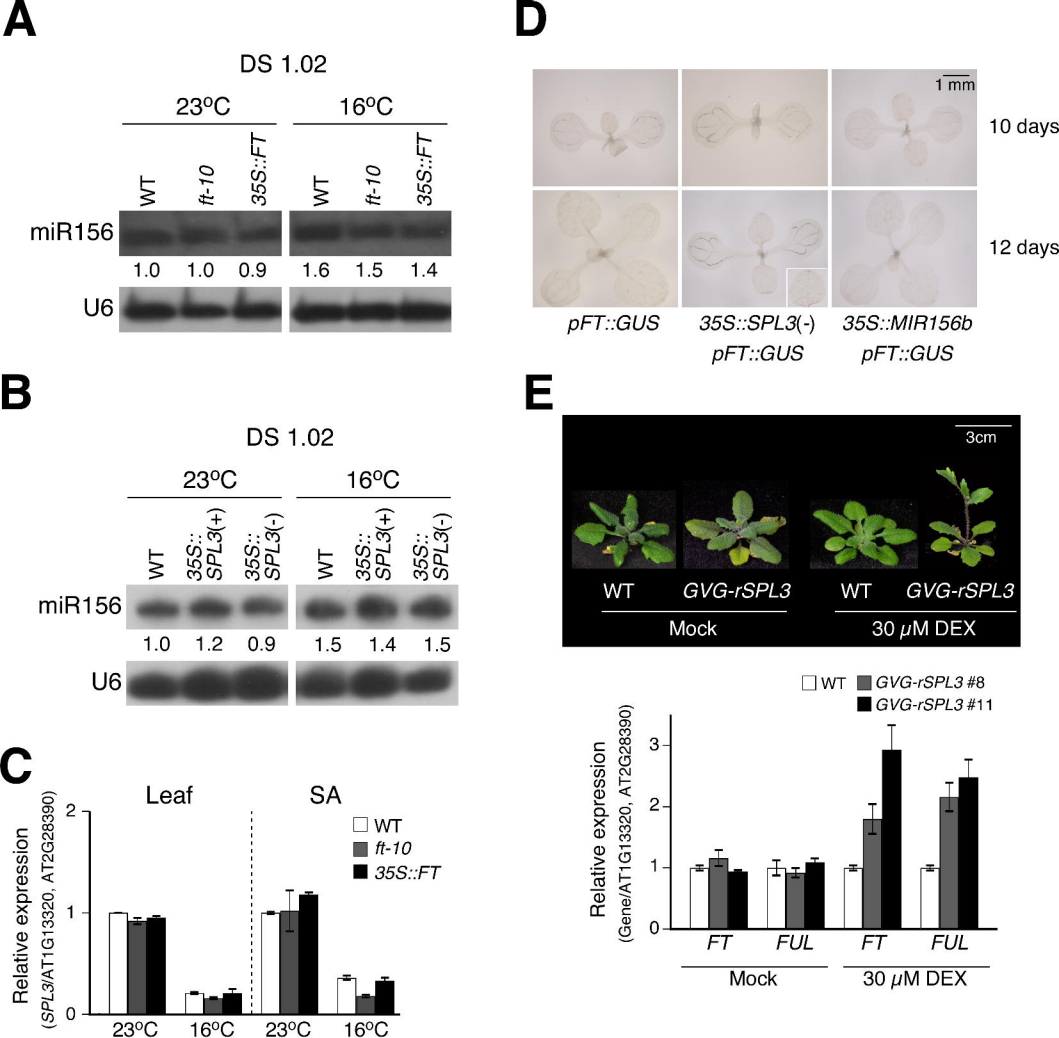
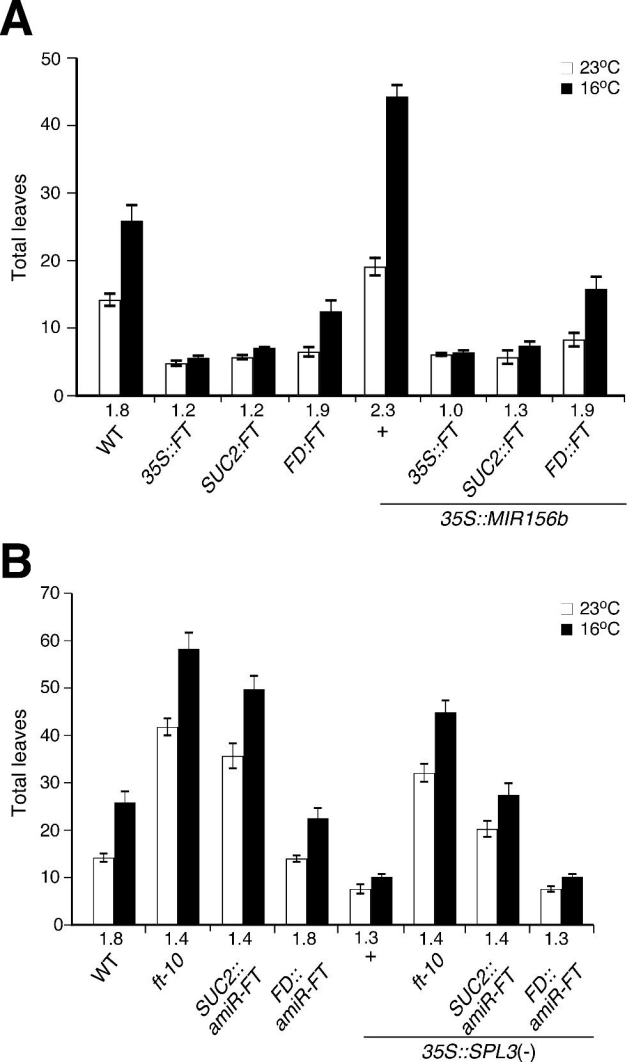
miR156 3' CACGAGUGAGAGAAGACAGU 5'

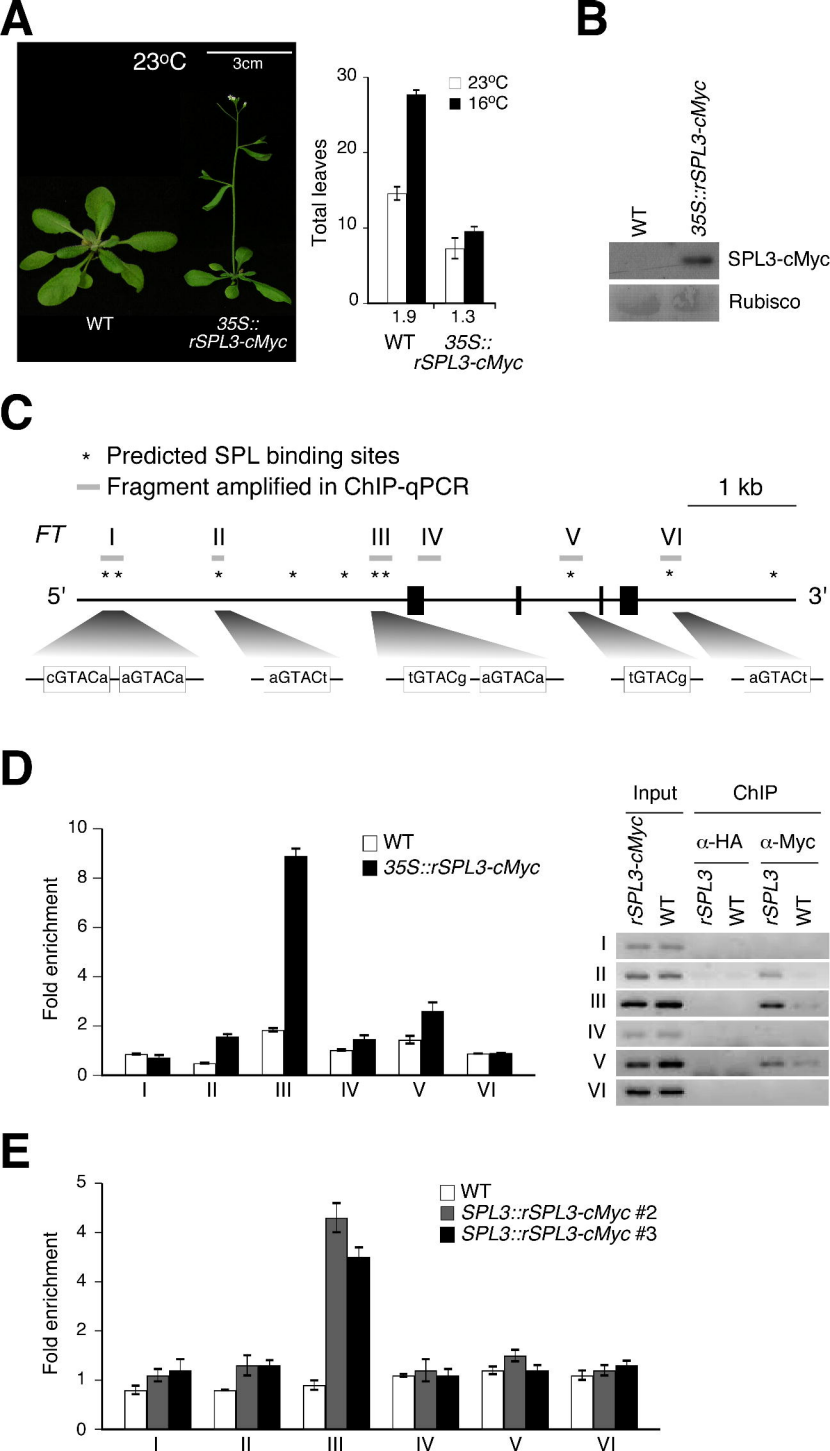

A
23ºC
3cm
16ºC
3cm
WT
35S::SPL3(+)
35S::SPL3(-)
Total leaves
2.1
1.7
1.3
B
23ºC
16ºC
juvenile leaves
Leaf number
C
Day 8
Leaf
SA
Relative expression
(Gene/AT1G13320, AT2G28390)
FT
FUL
23ºC WT
16ºC WT
23ºC 35S::SPL3(-)
16ºC 35S::SPL3(-)
D
DS 1.02
E
SUC2::rSPL3
FD::rSPL3
1.9
1.6
2.0
F
G
(FUL/AT1G13320, AT2G28390)
23ºC FD::rSPL3
16ºC FD::rSPL3

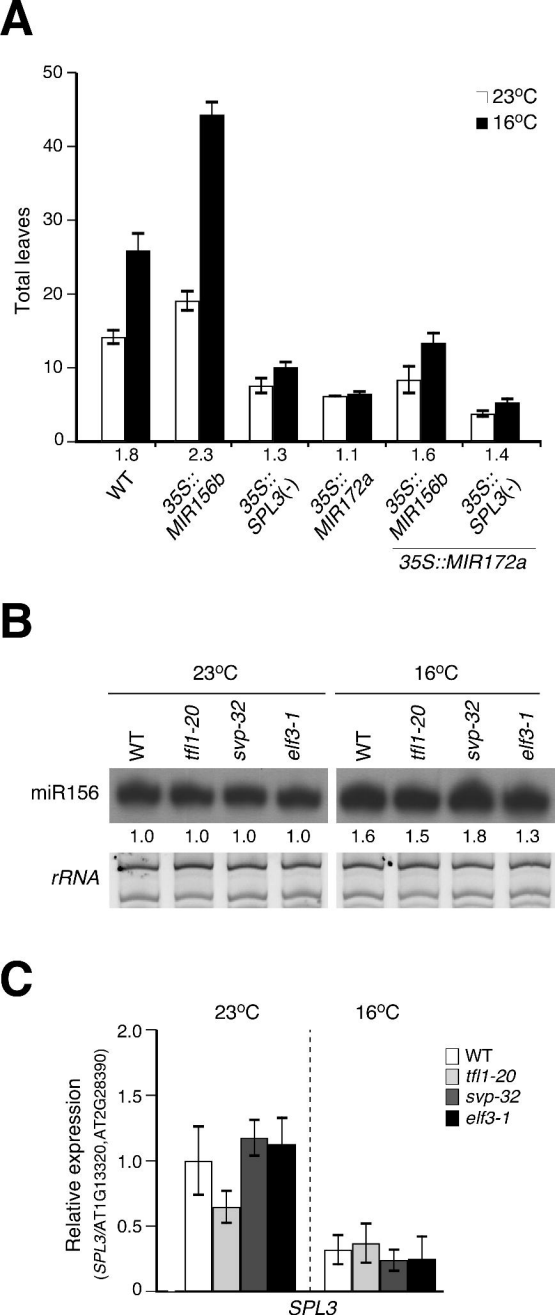

A
23ºC
1cm
16ºC
1cm
WT
35S::
MIM156
WT
35S::
MIM156
Total leaves
35
30
20
10
0
2.0
1.6
WT
35S::
MIM156
B
23ºC
1cm
WT
35S::MIM156
16ºC
1cm
WT
35S::MIM156
23ºC
16ºC
juvenile leaves
Leaf number
25
20
15
10
5
0
WT
35S::
MIM 156
C
Day 8
Relative expression
(Gene/AT1G13320, AT2G28390)
12
10
8
6
4
2
0
23ºC WT
16ºC WT
23ºC 35S::MIM156
16ºC 35S::MIM156
SPL2
SPL3
SPL4
SPL5
SPL6
SPL9
SPL10
SPL11
SPL13
SPL15
D
DS 1.02
Relative expression
(Gene/AT1G13320, AT2G28390)
5
4
3
2
1
0
23ºC WT
16ºC WT
23ºC 35S::MIM156
16ºC 35S::MIM156
SPL2
SPL3
SPL4
SPL5
SPL6
SPL9
SPL10
SPL11
SPL13
SPL15
E
Day 8
Relative expression
(Gene/AT1G13320, AT2G28390)
6
4
2
0
FT
FUL
23ºC WT
16ºC WT
23ºC 35S::MIM156
16ºC 35S::MIM156
F
DS 1.02
Relative expression
(Gene/AT1G13320, AT2G28390)
12
10
8
6
4
2
0
FT
FUL
23ºC WT
16ºC WT
23ºC 35S::MIM156
16ºC 35S::MIM156
G
23ºC
16ºC
Relative expression
(SPL3/AT1G13320, AT2G28390)
10^3
10^2
10^1
1
0
SPL3
WT
35S::MIM156
SUC2::rSPL3
35S::SPL3(-)

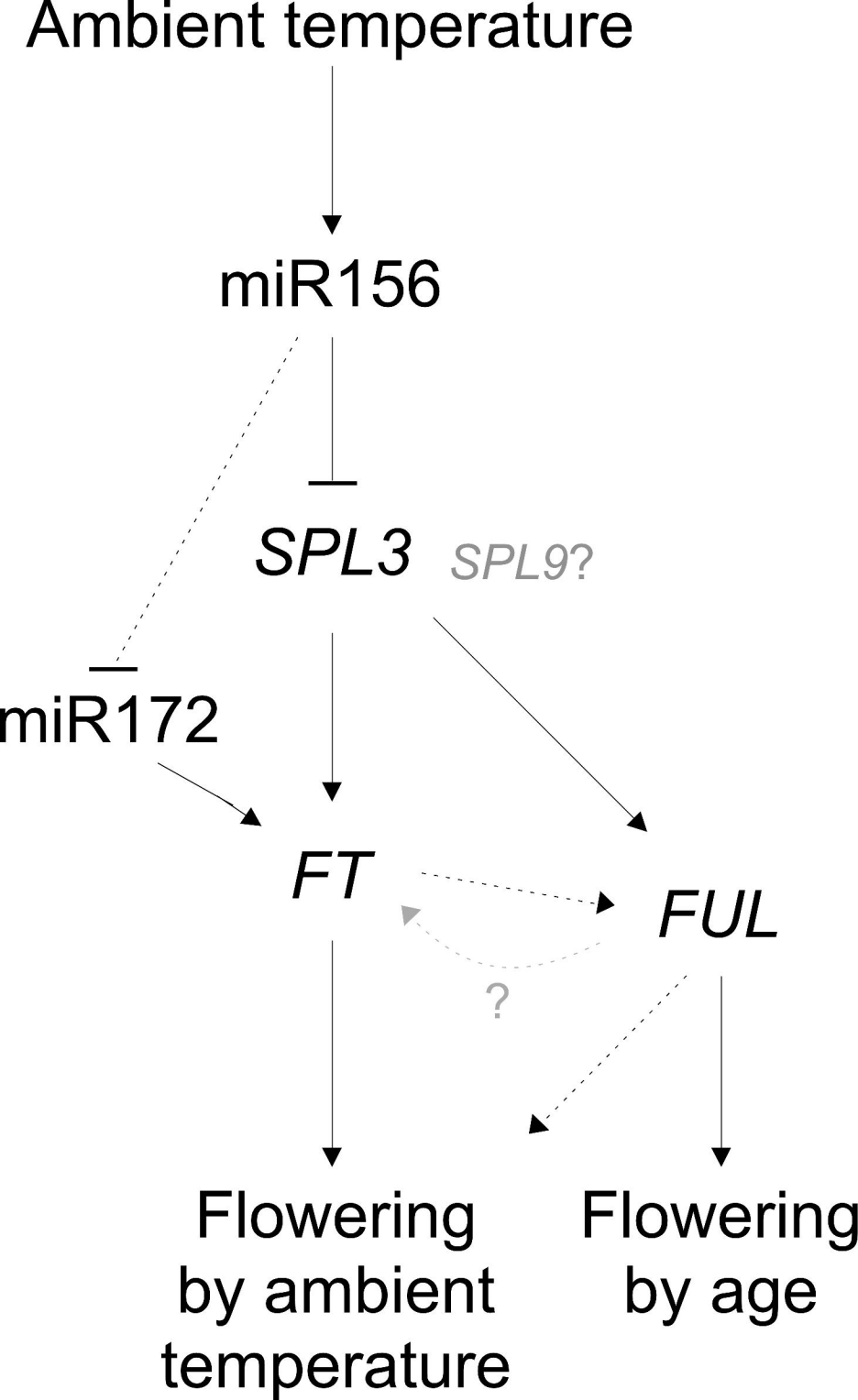

A
Total leaves
23ºC
16ºC
1.9
2.0
1.9
1.7
1.8
2.2
1.1
1.2
1.5
WT
ful-2
ful-8
35S::FUL
SUC2::FUL
FD::FUL
35S::FT
SUC2::FT
FD::FT
B
Total leaves
23ºC
16ºC
1.8
1.8
1.3
1.5
WT
ful-8
35S::SPL3(-)
35S::SPL3(-) ful-8
C
Day 8
23ºC
16ºC
Relative expression
(FT/AT1G13320, AT2G28390)
FT
WT
ful-8
35S::SPL3(-)
35S::SPL3(-) ful-8
D
DS 1.02
23ºC
16ºC
Relative expression
(FT/AT1G13320, AT2G28390)
FT
WT
ful-8
35S::SPL3(-)
35S::SPL3(-) ful-8


A
DS 1.02
23ºC
16ºC
WT
ft-10
35S::FT
miR156
1.0 1.0 0.9 1.6 1.5 1.4
U6
B
DS 1.02
23ºC
16ºC
WT
35S::SPL3(+)
35S::SPL3(-)
miR156
1.0 1.2 0.9 1.5 1.4 1.5
U6
C
Leaf
SA
WT
ft-10
35S::FT
Relative expression
(SPL3/AT1G13320, AT2G28390)
2
1
0
23ºC 16ºC 23ºC 16ºC
D
1 mm
10 days
12 days
pFT::GUS
35S::SPL3(-)
pFT::GUS
35S::MIR156b
pFT::GUS
E
3cm
WT
GVG-rSPL3
WT
GVG-rSPL3
Mock
30 μM DEX
WT
GVG-rSPL3 #8
GVG-rSPL3 #11
Relative expression
(Gene/AT1G13320, AT2G28390)
3
2
1
0
FT
FUL
FT
FUL
Mock
30 μM DEX


A
Total leaves
23ºC
16ºC
1.8
1.2
1.2
1.9
2.3
1.0
1.3
1.9
WT
35S::FT
SUC2:FT
FD:FT
+
35S::FT
SUC2::FT
FD::FT
35S::MIR156b
B
Total leaves
23ºC
16ºC
1.8
1.4
1.4
1.8
1.3
1.4
1.4
1.3
WT
ft-10
SUC2:: amiR-FT
FD:: amiR-FT
+
ft-10
SUC2:: amiR-FT
FD:: amiR-FT
35S::SPL3(-)


A
23ºC
3cm
WT
35S::
rSPL3-cMyc
Total leaves
30
20
10
0
23ºC
16ºC
1.9
1.3
WT
35S::
rSPL3-cMyc
B
WT
35S::rSPL3-cMyc
SPL3-cMyc
Rubisco
C
* Predicted SPL binding sites
Fragment amplified in ChIP-qPCR
1 kb
FT
I
II
III
IV
V
VI
5'
3'
cGTACa
aGTACa
aGTACt
tGTACg
aGTACa
tGTACg
aGTACt
D
Fold enrichment
10
8
6
4
2
0
WT
35S::rSPL3-cMyc
I
II
III
IV
V
VI
Input
ChIP
rSPL3-cMyc
WT
α-HA
α-Myc
rSPL3
WT
rSPL3
WT
E
Fold enrichment
5
4
4
2
1
0
WT
SPL3::rSPL3-cMyc #2
SPL3::rSPL3-cMyc #3
I
II
III
IV
V
VI


A
Total leaves
23ºC
16ºC
50
40
30
20
10
0
1.8
2.3
1.3
1.1
1.6
1.4
WT
35S::MIR156b
35S::SPL3(-)
35S::MIR172a
35S::MIR156b
35S::SPL3(-)
35S::MIR172a
B
23ºC
16ºC
WT
tfl1-20
svp-32
elf3-1
WT
tfl1-20
svp-32
elf3-1
miR156
1.0
1.0
1.0
1.0
1.6
1.5
1.8
1.3
rRNA
C
23ºC
16ºC
Relative expression
(SPL3/AT1G13320,AT2G28390)
2.0
1.5
1.0
0.5
0
WT
tfl1-20
svp-32
elf3-1
SPL3


Ambient temperature
miR156
SPL3
SPL9?
miR172
FT
FUL
?
Flowering by ambient temperature
Flowering by age