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The Sequential Action of miR156 and miR172 Regulates Developmental Timing in *Arabidopsis*

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SUMMARY

The transition from the juvenile to the adult phase of shoot development in plants is accompanied by changes in vegetative morphology and an increase in reproductive potential. Here, we describe the regulatory mechanism of this transition. We show that miR156 is necessary and sufficient for the expression of the juvenile phase, and regulates the timing of the juvenile-to-adult transition by coordinating the expression of several pathways that control different aspects of this process. miR156 acts by repressing the expression of functionally distinct SPL transcription factors. miR172 acts downstream of miR156 to promote adult epidermal identity. miR156 regulates the expression of miR172 via SPL9 which, redundantly with SPL10, directly promotes the transcription of *miR172b*. Thus, like the larval-to-adult transition in *Caenorhabditis elegans*, the juvenile-to-adult transition in *Arabidopsis* is mediated by sequentially operating miRNAs. miR156 and miR172 are positively regulated by the transcription factors they target, suggesting that negative feedback loops contribute to the stability of the juvenile and adult phases.

INTRODUCTION

Genetic analyses of developmental maturation in *Caenorhabditis elegans* (Moss, 2007; Rougvie, 2005) and plants (Bäurle and Dean, 2006; Chuck and Hake, 2005; Poethig, 2003) have revealed that these phenomena involve several independently regulated processes that must be temporally coordinated for normal development. An important example of this is the coordination between somatic and reproductive maturation, variation in which is the basis for many examples of morphological evolution (Gould, 1977). Each of these maturation processes itself consists of a variety of independently-regulated events that must be temporally coordinated. How this coordination is achieved is a major problem in developmental biology.

In *C. elegans*, transitions between stages of larval development are mediated by an increase in the expression of two sequentially expressed miRNAs, *lin-4* and *let-7* (reviewed in Moss, 2007; Pasquinelli and Ruvkun, 2002; Rougvie, 2005). These were the first miRNAs to be discovered, and they have since served as paradigms for the function of this class of regulatory molecules in animals (Lee et al., 1993; Reinhart et al., 2000). Remarkably, miRNAs have a similar function in plants. As a plant grows, it undergoes a transition from a juvenile to an adult stage of vegetative development (vegetative phase change) and then enters a reproductive phase (reproductive phase change or floral induction), during which it produces flowers or other types of reproductive structures. In *Arabidopsis*, vegetative phase change is marked by changes in the production of trichomes on the abaxial (lower) surface of the leaf, an increase in the length/width (L/W) ratio of the leaf blade, an increase in the degree of serration of the leaf margin and a decrease in cell size (Telfer et al., 1997; Tsukaya et al., 2000; Usami et al., 2009). Recent studies suggest that miR156, and possibly miR172, play pivotal roles in these transitions. In both *Arabidopsis* and maize, miR156 is highly expressed early in shoot development and decreases with time, while miR172 has the opposite expression pattern (Aukerman and Sakai, 2003; Chuck et al., 2007a; Jung et al., 2007; Lauter et al., 2005; Wu and Poethig, 2006). Overexpression of miR156 in both *Arabidopsis* and maize prolongs the expression of juvenile vegetative traits and delays flowering (Chuck et al., 2007a; Wu and Poethig, 2006), whereas overexpression of miR172 in *Arabidopsis* accelerates flowering (Aukerman and Sakai, 2003; Chen, 2004; Jung et al., 2007). These observations suggest that these miRNAs have related, but opposite, functions in shoot maturation.

Although the targets of miR156 and miR172 have been identified, the functions of these targets are still poorly characterized. In *Arabidopsis*, miR156 targets 10 members of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) family of transcription factors (*SPL2, SPL3, SPL4, SPL5, SPL6, SPL9, SPL10, SPL11, SPL13, SPL15*), while miR172 targets 6 *APETALA2-LIKE* (*AP2-like*) transcription factors (*AP2, TOE1, TOE2, TOE3, SMZ, SNZ*). The gain-of-function or loss-of-function phenotype of single gene mutations reveals a high degree of functional redundancy within these families. Loss-of-function mutations of *SPL3* have no obvious phenotype, but constitutive expression of this gene or its closely related paralogs *SPL4* and *SPL5* produces an early flowering phenotype, accelerates the

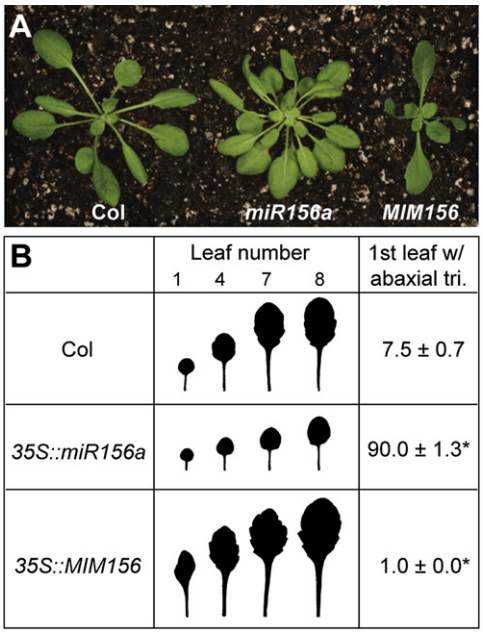


Figure 1. miR156 Is Necessary and Sufficient for the Juvenile Vegetative Phase

(A) 25-day-old wild-type, *35S::miR156a* and *35S::MIM156* plants grown in short days.

(B) The shape and the abaxial trichome phenotypes of fully expanded rosette leaves of wild-type, *35S::miR156a* and *35S::MIM156* plants. *35S::miR156a* prolongs the duration of the juvenile phase and *35S::MIM156* eliminates this phase.

Asterisks indicate significant difference from wild-type ($p < 0.01$, $n = 18$, ± SD).

production of trichomes on the abaxial surface of the leaf (an adult trait) (Gandikota et al., 2007; Wu and Poethig, 2006), and produces changes in cell size and cell number typical of adult leaves (Usami et al., 2009). Overexpression of *SPL9* reduces the rate of leaf initiation and increases leaf size (Wang et al., 2008), and a similar phenotype is observed in a gain-of-function mutant of *SPL15* (Usami et al., 2009). Loss-of-function mutations in either *SPL9* or *SPL15* have minor effects on development. Plants doubly mutant for these related genes have a stronger phenotype than the single mutants, which reveals that they promote both vegetative phase change and flowering (Schwarz et al., 2008; Wang et al., 2008). The targets of miR172 have an opposite effect on phase change. Plants lacking *TOE1* and *TOE2* are early flowering, whereas plants overexpressing *TOE1*, *TOE2*, *SNZ*, or *SMZ* are late flowering (Aukerman and Sakai, 2003; Jung et al., 2007; Schmid et al., 2003). Although there is still no evidence that these *AP2*-like genes contribute to vegetative phase change in *Arabidopsis*, their maize homolog *Glossy15 (Gl15)* promotes juvenile epidermal identity (Evans et al., 1994; Moose and Sisco, 1994, 1996), suggesting that one or more of the *Arabidopsis* homologs may do so as well. Whether these *SPL* and *AP2*-like genes operate in the same or different pathways is unknown.

We undertook a genetic and molecular analysis of miR156, miR172 and their targets to define their roles in vegetative phase change. Our results indicate that miR156 is both necessary and sufficient for the expression of the juvenile phase, and that it functions as a master regulator of this phase. The targets of miR156 act in several pathways that control both flowering time and different aspects of vegetative development. One of these pathways includes *miR172b*. We show that miR156 regulates the expression of *miR172b* via SPL9, which acts as a direct transcriptional regulator of *miR172b*. Our results suggest a model for the temporal coordination of vegetative phase change and floral induction.

RESULTS

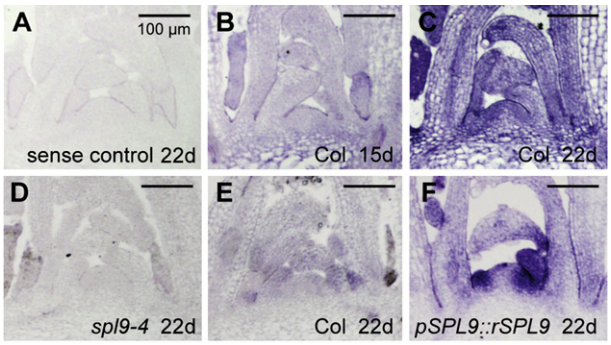
miR156 Is a Master Regulator of the Juvenile Phase

In *Arabidopsis*, floral induction affects the development of unexpanded rosette leaves in ways that can make it difficult to observe the juvenile-to-adult transition. This is particularly problematic in genotypes that flower very early in long days, which applies to many of the stocks used in this study. For this reason, all of the experiments reported here were conducted with plants grown in short days to delay flowering.

Under short day conditions, plants expressing miR156a under the regulation of the constitutive 35S promoter produced approximately 90 leaves that resembled juvenile leaves in size, shape, and their lack of abaxial trichomes (Figures 1A and 1B). In contrast, all leaves produced by plants in which the activity of miR156 was suppressed by constitutively expressing a transcript with non-cleavable miR156 target site (a target-site mimic, MIM156) (Franco-Zorrilla et al., 2007) resembled adult leaves. The effect of *35S::MIM156* on leaf development was particularly striking in the case of the first two rosette leaves. In *35S::MIM156* plants, the first two rosette leaves were unusually large and elongated, and possessed serrated leaf margins and abaxial trichomes–features of adult leaves. Later-formed leaves were larger, but nearly identical in shape to these first two rosette leaves. Thus, miR156 promotes the expression of all juvenile leaf traits, and is both necessary and sufficient for the expression of these traits.

SPL Genes Have Different Roles in Vegetative Phase Change

The *SPL* genes targeted by miR156 can be grouped into four major clades: *SPL3/SPL4/SPL5, SPL2/SPL10/SPL11, SPL9/SPL15, SPL6/SPL13* (Guo et al., 2008). *SPL3*, *SPL9* and *SPL10* are representative members from three of these clades. To investigate the function of *SPL3*, *SPL9* and *SPL10*, we first examined their spatial expression pattern in vegetative shoot apices by RNA in situ hybridization. *SPL3* was present uniformly throughout the shoot apex and in expanding leaf primordia and increased in abundance between 15 and 22 days after planting (Figures 2A–2C). *SPL9* was expressed at a much lower level than *SPL3*, and was barely visible in young leaf primordia in 22-day-old shoots (Figures 2D and 2E). To confirm this expression pattern, we examined plants transformed with *pSPL9::rSPL9*, a construct that expresses a miR156-resistant *SPL9* transcript under the control of its native promoter. Consistent with its wild-type expression pattern, transgenic plants expressed *SPL9* in both pre-emergent and expanding leaf primordia (Figure 2F). These results are consistent with previous suggestions (Wang et al.,



H

	Leaf number 1 4 7 8	1st leaf w/ abaxial tri.	Leaf 7 length/width
Col		7.0 ± 0.8	1.7 ± 0.1
spl9-4		9.8 ± 1.3*	1.5 ± 0.1*
35S::rSPL3		5.5 ± 0.7*	1.7 ± 0.1
pSPL9::rSPL9		1.0 ± 0.0*	2.8 ± 0.3*
pSPL10::rSPL10		1.2 ± 0.5*	2.1 ± 0.1*

Figure 2. *SPL3*, *SPL9*, and *SPL10* Have Diverse Roles in Vegetative Development

(A–C) In situ expression pattern of *SPL3*. (A) 22-day-old vegetative shoot apex hybridized with a sense strand control. (B) 15-day-old vegetative shoot apex hybridized with an antisense probe. (C) 22-day-old vegetative shoot apex hybridized with an antisense probe. The abundance of *SPL3* mRNA increases with time. (D–F) In situ expression pattern of *SPL9*; all samples hybridized with an antisense probe. (D) 22-day-old vegetative shoot apex of an RNA null allele of *SPL9*. (E) 22-day-old wild-type vegetative shoot apex. (F) 22-day-old vegetative shoot apex from a plant expressing a miR156-insensitive *SPL9* genomic sequence under the control of the *SPL9* promoter. *SPL9* is expressed in young leaf primordia.

(G) Four-week-old rosettes of wild-type, mutant and transgenic lines of *Arabidopsis* grown in short days. *rSPL3* = *35S::rSPL3*, *rSPL9* = *pSPL9::rSPL9*, *rSPL10* = *pSPL10::rSPL10*.

(H) The shape and abaxial trichome phenotypes of fully expanded leaves of wild-type, *spl9-4*, and transgenic lines expressing miR156-resistant forms of *SPL3*, *SPL9* and *SPL10*. These genes promote different adult traits. Asterisks indicate significant difference from wild-type ($p < 0.01$, $n = 18$, ± SD).

2008) that miR156 regulates the abundance of *SPL9* transcripts, but not their spatial expression pattern. *SPL10* transcripts were undetectable in wild-type shoot apices.

To define the roles of these genes in vegetative phase change, we characterized their loss- and gain-of-function phenotypes. *spl9-4* delayed abaxial trichome production by 2.8 plastochrons and caused the leaf blade to become rounder (Figures 2G and 2H). *SPL15* is the closest paralog of *SPL9*. *spl15-1* mutants had no obvious effect on abaxial trichome production or leaf shape. Plants doubly mutant for *spl9-4* and *spl15-1* produced abaxial trichomes 1.6 plastochrons later than *spl9-4* but had same leaf shape as *spl9-4* (L/W = 1.48 ± 0.08 for *spl9 spl15* versus 1.50 ± 0.11 for *spl9*). These results imply that *SPL15* has overlapping functions with *SPL9*, but has a less important role in leaf morphogenesis than *SPL9*. Loss-of-function mutations in *SPL3* and *SPL10* had no obvious vegetative phenotype, presumably because their function overlaps with other *SPL* genes. Transgenes expressing miR156-resistant versions of *SPL3*, *SPL9* and *SPL10* affected leaf development in different ways. *35S::rSPL3* accelerated abaxial tricome production by 1.5 plastochrons, but had no significant effect on leaf shape (Figures 2G and 2H). *pSPL9::rSPL9* and *pSPL10::rSPL10* accelerated the expression of all adult-specific leaf traits, producing leaves with an elongated leaf blade, serrated leaf margin, and abaxial trichomes (Figures 2G and 2H). However, the leaves of *rSPL10* plants were flatter, rounder and more serrated than those of *rSPL9,* and also had a more distinct petiole (Figures 2G and 2H). As expected from their high degree of sequence similarity, the phenotype of *pSPL11::rSPL11* was similar to that of *pSPL10::rSPL10* (data not shown). Thus, *SPL3*, *SPL9* and *SPL10/SPL11* have overlapping, but distinct functions in vegetative development.

miR172 Promotes Adult Epidermal Identity via *TOE1* and *TOE2*

miR172 promotes flowering when overexpressed (Aukerman and Sakai, 2003; Chen, 2004; Jung et al., 2007), but whether it plays a role in *Arabidopsis* vegetative phase change is unknown. To address this question we examined the vegetative phenotype of plants transformed with a genomic fragment containing the *miR172b* precursor under the control of the 35S promoter (*35S::miR172b*). The leaves of plants expressing *35S::miR172b* produced abaxial trichomes two plastochrons earlier than normal, but these leaves were otherwise morphologically normal (Table 1). *35S::miR172a* had essentially the same phenotype as *35S::miR172b;* furthermore, a T-DNA insertion in *miR172a* (SALK_045787) delayed abaxial trichome production by about three plastochrons while having no obvious effect on leaf morphology (Table 1). Thus, miR172 promotes adult epidermal identity, but has little, if any, role in the regulation of leaf shape.

miR172 targets 6 *AP2*-like genes in *Arabidopsis*, including *TOE1* and *TOE2* (Aukerman and Sakai, 2003; Schmid et al., 2003). Gain- and loss-of-function mutations in *TOE1* and *TOE2*

Table 1. The Effect of miR172, *TOE1*, and *TOE2* on Abaxial Trichome Production and Leaf Shape

Genotype	1st Leaf with Abaxial Trichomes	Leaf Length/Width (Leaf 7)
Wild-type	7.0 ± 0.5	1.64 ± 0.09
35S::miR172b	5.0 ± 0.9[a]	1.63 ± 0.09
miR172a-1 (SALK_045787)	9.6 ± 1.0[a]	1.61 ± 0.10
Wild-type	6.6 ± 0.5	1.64 ± 0.09
toe1-2	5.6 ± 0.6[a]	1.65 ± 0.11
toe2-1	5.5 ± 0.6[a]	1.66 ± 0.14
toe1-2, toe2-1	3.2 ± 0.4[a]	1.68 ± 0.13
35S::TOE1	10.4 ± 0.9[a]	1.51 ± 0.07[c]
Wild-type	7.2 ± 1.0	
toe2-1	5.5 ± 0.5	
35S::miR156a	90.0 ± 1.6	
toe2-1, 35S::miR156a	9.5 ± 1.4[b]	
Wild-type	7.2 ± 0.7	
spl9-4	9.8 ± 1.3	
toe1-2, toe2-1	3.0 ± 0.2	
spl9-4, toe1-2, toe2-1	3.7 ± 0.7[b]	

[a] Significantly different from wild-type ($p < 0.01$, $n = 24$, ± SD).
[b] Significantly different from all other genotypes ($p < 0.01$, $n = 24$, ± SD).
[c] Significantly different from wild-type ($p < 0.01$, $n = 10$, ± SD).

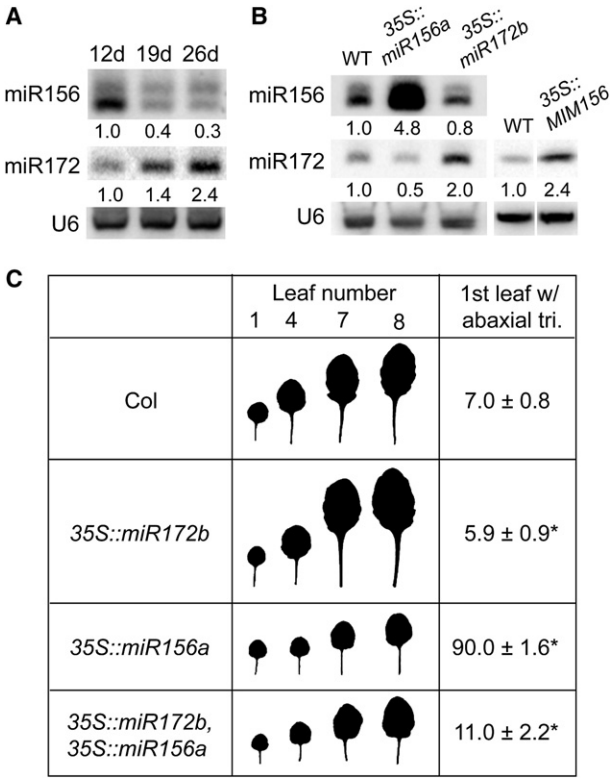


Figure 3. miR172 Acts Downstream of miR156

(A) Blot of small RNA from the shoot apex of wild-type plants of different ages hybridized sequentially with probes to miR156 and miR172. The levels of these miRNAs change in a complementary fashion. U6 served as a loading control.

(B) Blots of small RNA from *35S::miR156a*, *35S::miR172b* (14-day-old) and *35S::MIM156* (20-day-old) plants hybridized sequentially with probes to miR156 and miR172. miR156 represses miR172. U6 was used as loading control.

(C) Leaf shape and abaxial trichome phenotypes of fully expanded rosette leaves of wild-type, *35S::miR156a*, *35S::miR172b* and *35S::miR156a*, *35S::miR172b* double transgenic plants. *35S::miR172b* partially rescues the *35S::miR156a* overexpression phenotype.

Numbers indicate fold change relative to wild-type. Asterisks indicate significant difference from wild-type ($p < 0.01$, $n = 18$ plants, ± SD).

have been shown to affect flowering time (Aukerman and Sakai, 2003; Jung et al., 2007), but their effect on vegetative development is unknown. Abaxial trichome production was accelerated by one plastochron in plants homozygous for *toe1-2* or *toe2-1* (hereafter referred to as *toe1* and *toe2*), and by three plastochrons in *toe1 toe2* double mutants. Neither the single mutants or the double mutant had an effect on leaf shape (Table 1). In contrast, constitutive expression of *TOE1* (*35S::TOE1*) delayed abaxial trichome production by 4 plastochrons and caused the leaf blade to become slightly rounder than normal (Table 1). We conclude that *TOE1* and *TOE2* act primarily to promote juvenile epidermal identity, and probably mediate the effect of mR172 on vegetative development.

miR172 Acts Downstream of miR156

Previous studies have shown that miR156 decreases during shoot development in *Arabidopsis* (Wu and Poethig, 2006), whereas miR172 increases (Aukerman and Sakai, 2003; Jung et al., 2007); however, the expression of these miRNAs has not been directly compared in the same material. For this purpose, RNA blots of shoot apices harvested 12, 19 and 26 days after planting were hybridized sequentially with probes to these miRNAs. miR156 and miR172 were expressed in inverse patterns: miR156 declined between 12 and 19 days after planting, whereas miR172 increased during this same period (Figure 3A). To determine if these changes are causally related, we examined the level of miR156 and miR172 in plants overexpressing these miRNAs. Plants transformed with *35S::miR156a* had half the normal amount of miR172, whereas plants transformed with *35S::MIM156* had over twice the normal amount of miR172 (Figure 3B). By contrast, *35S::miR172b* had little or no effect on miR156. Thus, miR156 regulates the expression of miR172, but not the reverse. To determine if this effect is functionally significant, we examined the phenotype of plants homozygous for both *35S::miR156a* and *35S::miR172b.* These double transgenic plants had leaves that were the size and shape of *35S::miR156a* leaves, but initiated abaxial trichome production earlier than *35S::miR156a* plants (Figure 3C). Indeed, their pattern of abaxial trichome production was much closer to that of *35S::miR172b* than to *35S::miR156a*. This result supports the conclusion that miR172 acts downstream of miR156, and provides additional evidence that miR172 primarily regulates epidermal differentiation.

If miR172 mediates the effect of miR156 on epidermal identity by repressing the expression of *TOE1* and *TOE2*, then the early abaxial trichome phenotype of *toe1* and/or *toe2* should be

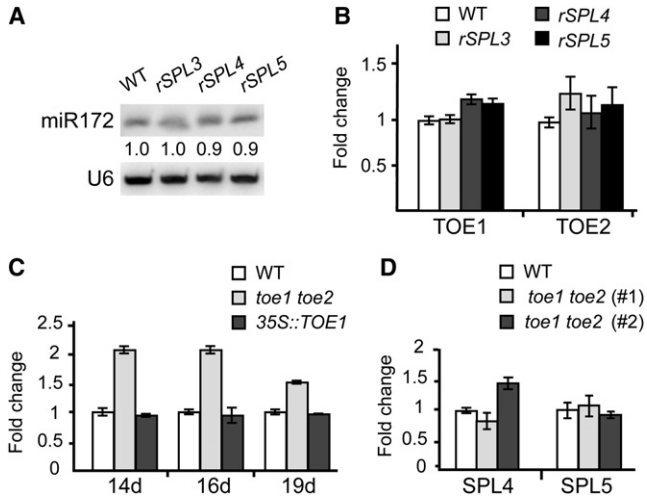


Figure 4. *SPL3*, *SPL4*, and *SPL5* Do Not Regulate miR172 and *TOE1*, *TOE2*

(A) RNA blots of small RNA from 20-day-old wild-type, *35S::rSPL3, 35S::rSPL4* and *35S::rSPL5* rosettes hybridized with a probe to miR172 reveals that these transgenes have no effect on the expression of miR172. U6 served as a loading control. Numbers indicate fold change relative to wild-type.

(B) qRT-PCR analysis of *TOE1* and *TOE2* mRNA from 14-day-old wild-type, *35S::rSPL3*, *35S::rSPL4* and *35S::rSPL5* rosettes indicates that these transgenes have no effect on the expression of *TOE1* and *TOE2*.

(C) qRT-PCR analysis of *SPL3* mRNA in wild-type, *toe1 toe2*, and *35S::TOE1* rosettes reveals that the expression of *SPL3* is increased by *toe1 toe2*, but unaffected by *35S::TOE1*.

(D) qRT-PCR analysis of *SPL4* and *SPL5* mRNA in 2-week-old wild-type and *toe1 toe2* rosettes reveals no consistent change in the expression of these genes; the results of two experiments are shown.

qRT-PCR data represent the average of three technical replicates; samples were normalized to wild-type at each time point; ± SD.

epistatic to the late abaxial trichome phenotype of *35S::miR156*. Consistent with this prediction, *toe2* nearly completely rescued the abaxial trichome phenotype of *35S::miR156a* in double mutants (Table 1). We were unable to examine the genetic interaction between *toe1* and *35S::miR156a* because the *miR156a* transgene was silenced in *toe1-2 35S::miR156a* plants, probably because *toe1-2* is a T-DNA induced mutation and shares sequences with the *35S::miR56a* construct (Daxinger et al., 2008). Although *toe2-1* is also a T-DNA induced mutation, it does not silence *35S::miR56a*.

SPL9 and SPL10 Promote the Transcription of miR172

miR156 represses 10 members of the *SPL* gene family (Rhoades et al., 2002; Schwab et al., 2005). To identify the *SPL* genes that mediate the effect of miR156 on miR172, we analyzed the expression of miR172 in plants expressing miR156-resistant versions of *SPL3, SPL4, SPL5, SPL9* and *SPL10*. Although the phenotypes of *35S::rSPL3*, *35S::rSPL4* and *35S::rSPL5* are similar to that of *35S::miR172b* and *toe1 toe2* (Table 1), these transgenes had no effect on the abundance of miR172 (Figure 4A) or the abundance of the *TOE1* and *TOE2* transcripts (Figure 4B). To determine if *SPL3*, *SPL4* and *SPL5* act downstream of *TOE1* and *TOE2* we examined the their expression in *toe1 toe2* mutants and *35S::TOE1* plants. *SPL3* was slightly (1.5 to 2-fold) but consistently overexpressed in *toe1 toe2*, but was either slightly downregulated or unaffected by *35S::TOE1* (Figure 4C). *SPL4* and *SPL5* were expressed much more variably than *SPL3* in *toe1 toe2*. Although in some cases we observed a slight increase in their expression, in other experiments there was no significant difference between their expression level in *toe1 toe2* and wild-type plants (Figure 4D). These results suggest the effect of *toe1 toe2* on *SPL3*, *SPL4* and *SPL5* expression is indirect.

In contrast to *35S::rSPL3*, *35S::rSPL4* and *35S::rSPL5*, plants expressing *pSPL9::rSPL9* and *pSPL10::rSPL10* had more than 4-fold higher levels of miR172 (Figure 5A). Furthermore, qRT-PCR revealed that the primary transcript of *miR172b*—one of 5 loci encoding miR172—is expressed at uniformly high level in *pSPL9::rSPL9* and *pSPL10::rSPL10* throughout shoot development (Figure 5B). To determine if *miR172b* is a direct target of SPL9, we took advantage of an inducible expression system based on the posttranscriptional activation of the rat glucocorticoid receptor (GR) (Lloyd et al., 1994). *GR* was fused to the 5′ end of *rSPL9*, and this fusion gene was expressed in transgenic plants under the regulation of the 35S promoter. Transgenic seeds were plated on MS medium, and treated with the synthetic ligand dexamethasone (DEX) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) for 4 hr; RNA was then extracted and the abundance of *miR172b* was assessed by qRT-PCR. CHX was used to block the translation of mRNAs regulated by SPL9, and thus prevent secondary effects. In the presence of DEX, this line has a phenotype similar to that of the *pSPL9::rSPL9* line illustrated in Figure 2G. The *miR172b* primary transcript was elevated about 3-fold in samples treated with DEX and with DEX+CHX, strongly suggesting that *miR172b* is a direct transcriptional target of SPL9 (Figure 5C). We then tested if SPL9 binds to *miR172b* by examining the chromatin fragments that immunoprecipitate with a FLAG-tagged SPL9 protein; this epitope-tagged protein was expressed in transgenic plants under the regulation of the *SPL9* promoter and produced the phenotype illustrated in Figure 2G, demonstrating that the protein is functional. As a control, we used plants expressing cMyc-tagged SPL9 under the regulation of the same promoter. The abundance of several 200-bp regions containing the core SPL binding sequence GTAC (Figure 5D) was measured in immunoprecipitated material using qPCR (Figure 5E). One site (S1) located approximately 1.2 kb upstream of the transcriptional start site of *miR172b* was enriched approximately 5-fold in FLAG-SPL9 plants compared to SPL9-cMyc controls; no significant enrichment was observed for the other sites we examined (Figure 5E). To determine if SPL9 is required for the transcription of *miR172b* we examined the effect of *spl9-4* and *spl9-4 spl15-1* on the abundance of the *miR172b* primary transcript. Although *spl9-4* had no significant effect on the *miR172b* transcript, the level of this transcript was slightly reduced in *spl9 spl15* double mutants (Figure 5F), consistent with the observation that this double mutant has a more severe phenotype than either single mutant. These results indicate that SPL9 directly promotes the transcription of *miR172b*.

If SPL9 promotes adult epidermal identity by upregulating the transcription of *miR172b* and thereby repressing *TOE1* and *TOE2*, these *AP2*-like genes should be required for the

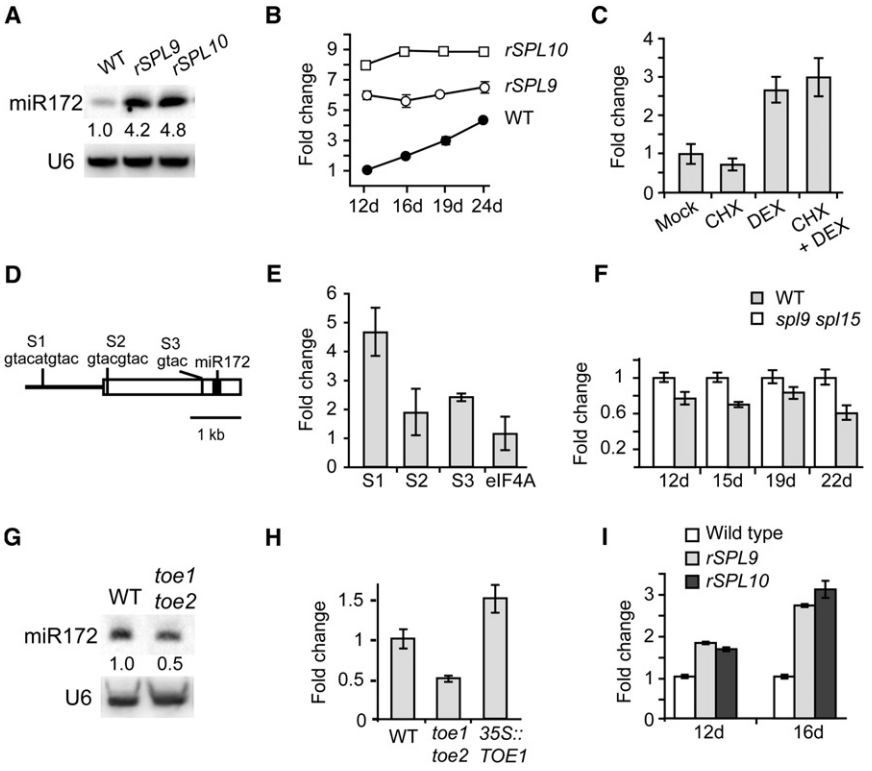


Figure 5. Regulation of *miR172b* and *miR156a* by SPL9, SPL10, TOE1, and TOE2

(A) Northern blot of small RNA from 20-day-old wild-type, *pSPL9::rSPL9* and *pSPL10::rSPL10* rosettes. U6 was used as a loading control. Numbers indicate the fold change relative to wild-type. *pSPL9::rSPL9* and *pSPL10::rSPL10* increase the expression of miR172.

(B) qRT-PCR analysis of the *miR17b* precursor in 12-, 16-, 19-, and 24-day-old wild-type, *pSPL9::rSPL9*, *pSPL10::rSPL10* rosettes. The fold change relative to the 12-day-old wild-type sample is shown; SD bars are obscured by symbols. These transgenes increase the expression of *miR172b* and eliminate its temporal expression pattern.

(C) qRT-PCR analysis of the *miR17b* precursor in 20-day-old *35S::GR-rSPL9* seedlings treated with DEX in the absence or presence of CHX. GR-SPL9 promotes the expression of *miR172b* in the absence of protein synthesis.

(D) The location of three putative SPL9 binding sites in the *miR172b* locus that were tested by ChIP analysis. Open box indicates the *miR172b* transcript.

(E) qPCR analysis of putative SPL9 binding sites in the chromatin of 14-day-old *pSPL9::rSPL9-cMyc* and *pSPL9::3XFLAG-rSPL9* rosettes immunoprecipitated with an antibody to FLAG. The immunoprecipitated values were first normalized to the input values then divided by the *pSPL9::rSPL9-cMyc* value to get a fold enrichment. The numbers represent the fold difference relative to *pSPL9::SPL9r-cMyc* sample. Values are the average of two biological replicates. *eIF4A* was used as a negative control.

(F) qRT-PCR analysis of the *miR172b* precursor in wild-type and *spl9-4 spl15-1* rosettes at different stages of vegetative development. *miR172b* is slightly reduced at all of these stages.

(G) Blot of small RNA from the rosette of 14-day-old wild-type and *toe1 toe2* plants hybridized with a probe to miR172. U6 served as a loading control. Numbers indicate the fold change relative to the wild-type sample.

(H) qRT-PCR analysis of the *miR172b* precursor in wild-type, *toe1 toe2* and *35S::TOE1* rosettes.

(I) qRT-PCR analysis of the *miR156a* precursor in 12- and 16-day-old *pSPL9::rSPL9* and *pSPL10::rSPL10* plants.

qRT-PCR data represent the average of three technical replicates; samples were normalized to wild-type at each time point; ± SD.

juvenilized epidermal phenotype of *spl9-4*. To test this prediction, we examined the timing of abaxial trichome production in *spl9-4*, *toe1 toe2*, and the *spl9-4 toe1 toe2* triple mutant. The timing of abaxial trichome production in the triple mutant was slightly but significantly different from *toe1 toe2* (Table 1). This result suggests that *TOE1* and *TOE2* contribute to the epidermal phenotype of *spl9-4*, but are not solely responsible for this phenotype.

Feedback loops in which a miRNA-regulated transcription factor regulates the transcription of its cognate miRNA have been described in a number of animals (Fazi et al., 2005; Johnson et al., 2005; Kim et al., 2007; Varghese and Cohen, 2007). To determine if miR172 is regulated in this fashion, we examined the effect of *TOE1* and *TOE2* on mature miR172 and the *miR172b* precursor. Both of these molecules were reduced about 50% in *toe1 toe2* double mutants; conversely, the *miR172b* precursor was slightly elevated in plants overexpressing *TOE1* (Figures 5G and 5H). We then examined if *SPL* genes have a similar effect on the expression of the *miR156a* precursor. This transcript was elevated about 1.5 fold in 12-day-old, and 2.5-to-3 fold in 16-day-old *pSPL9::rSPL9* and *pSPL10::rSPL10* plants (Figure 5I). Plants overexpressing *SPL3*, *SPL4* and *SPL5* also had elevated levels of *miR156a*, but this effect was only observed around 2 weeks after planting (data not shown). The evidence that *miR172b* and *miR156a* are positively regulated by the transcription factors they target suggests that the expression of these targets is modulated by a negative feedback loop that buffers against small changes in the level of their mRNA.

DISCUSSION

Shoot maturation depends on the coordinated activity of multiple interacting pathways. The pathways that mediate the transformation of an adult vegetative shoot into a flower-bearing shoot (floral induction) have been intensively studied, and are now well understood (Bäurle and Dean, 2006; Parcy, 2005). Although much less is known about the regulation of vegetative phase change, decades of research on woody plants (Hackett and Murray, 1997), and more recent studies of maize and *Arabidopsis* (Chuck and Hake, 2005; Kerstetter and Poethig, 1998; Poethig, 2003) suggest that this transition also involves the activity of multiple pathways. The results presented here provide new insight into the structure of these pathways in *Arabidopsis*,

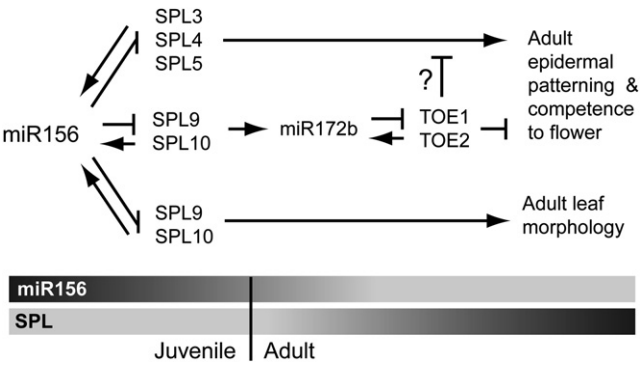


Figure 6. A Model for the Regulation of Vegetative Phase Change by miR156 and miR172

Temporal changes in the level of miR156 and SPL proteins are illustrated by the shaded bars; time increases from left to right. We propose that miR156 coordinates the expression of several pathways by repressing the expression of *SPL* genes that act in these pathways. Each of these pathways controls different phase-specific traits, but have components in common (e.g., *SPL9, SPL10*) and may also share downstream targets. The relationship between *TOE1* and *TOE2* and *SPL3*, *SPL4*, *SPL5* is unclear.

and suggest the mechanism by which their expression is temporally coordinated.

Our results indicate that miR156 is necessary and sufficient for the expression of the juvenile phase and demonstrate that it operates by repressing the expression of *SPL* genes that act in pathways with different developmental functions (Figure 6). These results are consistent with a recent study indicating that the precocious phase change phenotype of the *squint* mutation in *Arabidopsis* is attributable to a defect in the activity of miR156 (Smith et al., 2009), and with previous descriptions of the phenotype of plants expressing miR156-resistant versions of *SPL* genes (Gandikota et al., 2007; Usami et al., 2009; Wang et al., 2008; Wu and Poethig, 2006). Remarkably, overexpression of miR156 in maize produces a phenotype similar to that produced by overexpression of miR156 in *Arabidopsis* (Chuck et al., 2007a). Along with the evidence that miR156 is one of the most highly conserved miRNAs in the plant kingdom (Axtell and Bowman, 2008), these results suggest that miR156 is a master regulator of the juvenile phase in plants.

The identity of all the *SPL* genes that mediate the effect of miR156 on vegetative phase change is difficult to establish because of the high degree of functional redundancy within this family; furthermore, the genomic organization of some of these genes makes it difficult to generate the combination of mutations necessary for this analysis. The genes described in this paper are important components of this mechanism, however. The loss- and gain-of-function phenotypes of *SPL9* demonstrate that it promotes most, if not all, of the traits associated with the adult phase. *SPL10* also regulates all aspects of vegetative phase change, but appears to have different functions than *SPL9* because its overexpression phenotype differs in several respects from that of *SPL9*. *SPL3*, *SPL4*, and *SPL5* have more limited roles in leaf development, acting primarily, although perhaps not exclusively (Usami et al., 2009; Wu and Poethig, 2006), to promote adult patterns of epidermal differentiation. In addition to their roles in vegetative development, all of these genes promote flowering under long day conditions (Cardon et al., 1997; Schwarz et al., 2008; Wu and Poethig, 2006). Indeed, the early flowering phenotype of plants overexpressing *SPL3*, *SPL4*, and *SPL5* raises questions about the previously reported effects of these genes on leaf shape (Wu and Poethig, 2006) and cell size and number (Usami et al., 2009) because floral induction has major effects on leaf development and these previous studies were conducted under floral inductive conditions.

miR172 has been implicated in the regulation of flowering time and floral organ identity in both maize and *Arabidopsis* (Aukerman and Sakai, 2003; Chen, 2004; Chuck et al., 2007b; Zhao et al., 2007). In maize, miR172 targets *Gl15*, a gene that promotes juvenile epidermal identity (Lauter et al., 2005). These genes have complementary expression patterns, so it is reasonable to hypothesize that miR172 plays a role in vegetative phase change. However, there is still no evidence that miR172 is actually important for this process. For example, mutations in *ts4*, which encodes miR172e, have no effect on vegetative phase change in maize (Chuck et al., 2007b). Our results indicate that in *Arabidopsis* miR172 promotes adult epidermal identity, and that this is its primary function during vegetative development. This function is mediated by two of its six targets, *TOE1* and *TOE2*, as demonstrated by the observation that loss-of-function mutations in these genes actually have a more severe effect on abaxial trichome production than the *35S::miR172b* transgene used in these studies. The difference in the severity of these phenotypes can probably be attributed to the relatively small increase of miR172 in this transgenic line. The observation that miR172 levels are affected by changes in the level of miR156, as well as the observation that *35S::miR172b* and *toe2* nearly completely correct the epidermal phenotype of *35S::miR156a*, provide convincing evidence that miR172 acts downstream of miR156, and mediates the effect of miR156 on epidermal identity.

We were intrigued by the possibility that *SPL3*, *SPL4* and *SPL5* might mediate the interaction between *miR156* and *miR172* because overexpression of these *SPL* genes produces a vegetative phenotype very similar to that of *toe1 toe2* mutants or plants overexpressing miR172 (Wu and Poethig, 2006). Furthermore, all of these genotypes are early flowering under long days (Aukerman and Sakai, 2003; Cardon et al., 1997; Chen, 2004; Gandikota et al., 2007; Wu and Poethig, 2006). However, overexpression of *SPL3*, *SPL4* or *SPL5* had no effect on the abundance of miR172, indicating that these genes cannot be responsible for the effect of *miR156* on *miR172*. An alternative possibility is that these *SPL* genes act downstream of *TOE1* and *TOE2*. Although *SPL3* transcripts are elevated in *toe1 toe2*, overexpressing *TOE1* did not produce a corresponding decrease in *SPL3* mRNA; furthermore, *SPL4* and *SPL5* were largely unaffected in *toe1 toe2*. Consequently, we suspect that the effect of *toe1 toe2* on *SPL3* expression is indirect. Our results are more consistent with the hypothesis that *SPL3*, *SPL4*, *SPL5* regulate the same downstream targets as *TOE1* and *TOE2*, but operate largely independently of these genes (Figure 6).

How does miR156 regulate the expression of miR172? Our results indicate that SPL9 is a direct transcriptional activator of *miR172b*, and probably acts redundantly in this process with

SPL10 and several other *SPL* genes. The obvious candidates are *SPL15*–the closest homolog of *SPL9* in *Arabidopsis*–and *SPL11*, the closest homolog of *SPL10*. Indeed, the observation that *spl9 spl15* double mutants have slightly reduced levels of *miR172b* supports the conclusion that SPL15 cooperates with SPL9 in the regulation of this miRNA. *SPL11* is adjacent to *SPL10* in the genome and is nearly identical in sequence to *SPL10*, so it reasonable to assume that these genes have overlapping, if not identical, functions. This conclusion is supported by the observation that T-DNA insertions in these genes have no obvious phenotype (G.W. and R.S.P., unpublished data). A rigorous test of the role of various *SPL* genes in the regulation of *miR172b* will require generating plants lacking combinations of *SPL* genes, and this is particularly difficult in the case of *SPL10* and *SPL11* because of their proximity.

Although SPL9 promotes the transcription of *miR172b*, this is not the only way by which it regulates epidermal identity. This conclusion is supported by the observation that *spl9-4* produces a small but significant delay in abaxial trichome production without having an obvious effect on the abundance of miR172. That is, the effect of *spl9-4* on epidermal identity cannot be explained by a decrease in the level of miR172. Moreover, *pSPL9::rSPL9* has a stronger effect on abaxial trichome production than *35S::miR172*. The simplest interpretation of these observations is that SPL9 has multiple targets involved in epidermal differentiation.

The genes that mediate the effects of *SPL9* and *SPL10* on leaf development are unknown. Many leaf shape mutations have been identified in *Arabidopsis*. However, to our knowledge, the only mutations that have major effects on phase-specific traits are mutations in genes required for the expression or function of miR156, miR172 and their direct targets. The morphological differences between juvenile and adult leaves are relatively subtle in *Arabidopsis*, so genes that act downstream of specific *SPL* genes or *TOE1* and *TOE2* may have been missed in mutant screens. It is also possible that phase-specific aspects of leaf morphology are regulated by genes that individually have only a small effect on these traits. Identifying these downstream genes is an important goal for future research.

There is growing evidence from animal systems that miRNA-regulated transcription factors frequently regulate the transcription of their cognate miRNAs (Fazi et al., 2005; Johnson et al., 2005; Kim et al., 2007; Varghese and Cohen, 2007). A genome-wide survey identified 23 such feedback loops in *C. elegans* (Martinez et al., 2008). The evidence that TOE1 and TOE2 positively regulate their repressor, *miR172b*, suggests that the expression of TOE1 and TOE2 may be modulated by a negative feedback loop involving miR172, and our data suggest that miR156 and its targets have a similar relationship. Interestingly, the miR172 target *AP2* negatively regulates its own expression (Schwab et al., 2005), and may represent another example of this phenomenon. Negative feedback loops typically act to buffer small changes in the expression of proteins with important regulatory functions (Martinez et al., 2008). Negative feedback regulation is an attractive mechanism for stabilizing the expression of genes involved in vegetative phase change, and may be responsible for the remarkable stability of the juvenile and adult phases in some species (Hackett, 1985).

Finally, it is important to emphasize the potential significance of these results for understanding the mechanism of floral induction. Although it has long been known that floral induction depends on the transition to the adult vegetative phase (Hackett, 1985; Zimmerman et al., 1985) the relationship between vegetative phase change and floral induction is still unclear because many factors that affect flowering time (e.g., photoperiod, flowering time mutations) do not affect the timing of vegetative phase change, or have a relatively modest effect on this transition (Telfer et al., 1997). This raises the question of how these processes interact. Several of the targets of miR156 affect flowering time as well as vegetative phase change (Cardon et al., 1997; Schwarz et al., 2008; Wu and Poethig, 2006), and it is reasonable to propose that these genes act as licensing factors for the transition to flowering. If so, this will provide a solution to the long-standing question of how changes in the vegetative morphology of the shoot are coordinated with changes in its reproductive potential.

EXPERIMENTAL PROCEDURES

Genetic Stocks and Growth Conditions

All of the genetic stocks used in this paper were in a Columbia background. *35S::rSPL3*, *35S::miR156a*, *pSPL9::rSPL9*, *pSPL10::rSPL10* and *35S::MIM156* have been described previously (Franco-Zorrilla et al., 2007; Wang et al., 2008; Wu and Poethig, 2006). Additional *pSPL9::rSPL9* and *pSPL10::rSPL10* lines generated in our laboratory were also used for some experiments. Milo Aukerman (DuPont) provided *toe1-2 toe2-1*. *miR172a-1* (SALK_045787), *spl9-4* (SAIL_150_B05) and *spl15-1* (SALK_074426) were obtained from the *Arabidopsis* Biological Resource Center. Seeds were grown on Metromix 200 (Scotts) or Fafard #2 soil and left at 4°C for 2 days. Plant age was measured from the time seeds were transferred to the growth chamber. For phenotypic analysis, plants were grown in Conviron E7/2 chambers in short days (10 hr light:14 hr dark, 23°C), under a 3:1 combination of cool white (F032/841/Eco, Sylvania) and wide spectrum (Gro Lite WS, Interlectric Corp.) fluorescent lights, at light intensity of 300 μmol/m^2/sec. Abaxial trichomes were scored 2-3 weeks after planting with a stereomicroscope. For leaf shape analysis, fully expanded leaves were removed, attached to cardboard with double-sided tape and flattened with transparent tape, and then scanned in a digital scanner. Rips in the leaf blade produced during this process were filled in using Photoshop.

Transgenic Plants

The *TOE1* and *SPL9* coding sequence and a 1018 bp genomic sequence harboring the precursor of *miR172b* were PCR-amplified with pfu TURBO using cDNA (*TOE1*, *SPL9*) or genomic DNA (*miR172b*) as a template. The glucocorticoid receptor (*GR*) sequence was amplified from pBIΔGR and fused to a miR156-insensitive *SPL9* cDNA. All of these constructs were cloned downstream of the CaMV 35S promoter in pEZR-CL. The intergenic regions containing the *SPL9* or *SPL10* promoter and open reading frame were PCR-amplified and mutations were introduced into the miR156 binding sequence. These sequences were cloned into pEG302 and pEG303 gateway vectors with an FLAG and cMyc epitope tag, respectively (Earley et al., 2006). The whole *SPL9* intergenic region was also PCR-amplified and 3 copies of the FLAG epitope tag were fused to the N terminus of SPL9 protein. The fused sequence was then cloned into the *Sma*I and *Nco*I sites in pCambia3301. Plants were transformed using the floral dip method, and transformants were selected on Kanamycin or BASTA. Lines containing single insertions were selected on the basis of the segregation ratio of the resistant or susceptible plants to Kanamycin or BASTA in the progeny of these primary transformants, and homozygous stocks were established from these lines.

GR Induction and RNA Quantitation

35S::GR-rSPL9 seeds were plated onto half strength MS medium containing 50 mg/L Kanamycin. Plates were moved to short days after 2 days a 4°C.

On day 20, the plates were flooded with 0.1% ethanol (mock), 10 μM DEX in 0.1% ethanol, 10 μM CHX in 0.1% ethanol, and 10 μM DEX plus 10 μM CHX. After 4 hr, seedlings were harvested, frozen in liquid nitrogen, and stored at −80°C.

RNA blots were processed as described previously (Wu and Poethig, 2006). Total RNA was isolated using Trizol (Invitrogen), purified with QIAGEN RNeasy, and treated with RNase-free DNAase (QIAGEN). qRT-PCR was performed using SuperScript II reverse transcriptase and Power SYBR Green PCR master mix (Applied Biosystems), and normalized using eIF4A as a standard. The primers used for qRT-PCR are described in Table S1, available with this article online.

Chromatin Immunoprecipitation

pSPL9::rSPL9-cMyc and *pSPL9::3XFLAG-rSPL9* transgenic seedlings were harvested in 1x PBS and cross-linked with 1% formaldehyde in 1× PBS for 12 min using vacuum infiltration. The cross-linking was stopped in 0.1M glycine. Nuclear extracts were prepared and immunoprecipitation was performed as described (William et al., 2004). After chromatin shearing, about 5-μl of anti-FLAG polyclonal anibody (Sigma, F7425) was added to the samples and incubated at 4°C overnight. Beads were then washed and eluted with the lysis buffer (0.1M $NaHCO_3$, 1%SDS). After reversing the cross-linking, DNA was purified using the QIAquick PCR purification Kit (QIAGEN), and resuspended in 80 μl water. 3 μl of diluted DNA was used for real-time qPCR. The sequence of the primers used to amplify different regions in the promoter and coding sequence of *miR172b* are listed in Table S1. PCR conditions were 42 cycles at 94°C for 10 s, 57°C for 20 s, and 72°C for 30 s. Values for the FLAG ChIP samples were first normalized to the input and then were divided by the normalized cMyc signal to obtain a fold enrichment.

In Situ Hybridization

In situ hybridization was performed using a protocol obtained from Jeff Long (www.its.caltech.edu/~plantlab/protocols/insitu.pdf), with the following minor modifications: the probe was hybridized to slides at a temperature of 60-65°C overnight, and the blocking and antibody dilution solutions were produced using maleic acid instead of Tris-HCl. *SPL3* and *SPL9* probes were amplified and transcribed using the primers listed in Table S1.

SUPPLEMENTAL DATA

Supplemental Data include one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00778-8.

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