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Temporal regulation of shoot development in *Arabidopsis thaliana* by *miR156* and its target *SPL3*

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SPL3, *SPL4* and *SPL5* (*SPL3/4/5*) are closely related members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* family of transcription factors in *Arabidopsis*, and have a target site for the microRNA *miR156* in their 3′ UTR. The phenotype of *Arabidopsis* plants constitutively expressing *miR156*-sensitive and *miR156*-insensitive forms of *SPL3/4/5* revealed that all three genes promote vegetative phase change and flowering, and are strongly repressed by *miR156*. Constitutive expression of *miR156a* prolonged the expression of juvenile vegetative traits and delayed flowering. This phenotype was largely corrected by constitutive expression of a *miR156*-insensitive form of *SPL3*. The juvenile-to-adult transition is accompanied by a decrease in the level of *miR156* and an increase in the abundance of *SPL3* mRNA. The complementary effect of *hasty* on the *miR156* and *SPL3* transcripts, as well as the *miR156*-dependent temporal expression pattern of a *35S*::*GUS-SPL3* transgene, suggest that the decrease in *miR156* is responsible for the increase in *SPL3* expression during this transition. *SPL3* mRNA is elevated by mutations in *ZIPPY/AGO7*, *RNA DEPENDENT RNA POLYMERASE 6* (*RDR6*) and *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*), indicating that it is directly or indirectly regulated by RNAi. However, our results indicate that RNAi does not contribute to the temporal expression pattern of this gene. We conclude that vegetative phase change in *Arabidopsis* is regulated by an increase in the expression of *SPL3* and probably also *SPL4* and *SPL5,* and that this increase is a consequence of a decrease in the level of *miR156*.

KEY WORDS: miRNA, Heterochrony, Phase change

INTRODUCTION

miRNAs were first identified as regulators of the juvenile-to-adult transition in *Caenorhabditis elegans*. The founding members of this class of regulatory RNAs, *lin-4* (Lee et al., 1993) and *let-7* (Reinhart et al., 2000), are expressed late in larval development and promote adult development by repressing the expression of genes required for the expression of juvenile traits (Ambros, 2000; Pasquinelli and Ruvkun, 2002). Recent studies indicate that miRNAs and other endogenous small RNAs also regulate developmental transitions in plants. In plants, the shoot apex progresses through juvenile and adult phases of vegetative development before undergoing a transition to reproductive development (Baurle and Dean, 2006; Poethig, 1990). Several miRNAs have been shown to affect flowering time when over-expressed in *Arabidopsis* (Achard et al., 2004; Chen, 2004; Schwab et al., 2005), and the temporal expression pattern of one of these, *miR172*, suggests that it may promote both vegetative phase change and floral induction (Aukerman and Sakai, 2003). *miR172* exhibits a similar temporal expression pattern in maize, where it targets *Glossy15*(*Gl15*), a gene required for the expression of juvenile epidermal traits (Evans et al., 1994; Lauter et al., 2005; Moose and Sisco, 1994). Additional evidence for the involvement of small RNAs in vegetative phase change is provided by the observation that mutations in the genes required for the biogenesis of miRNAs (Clarke et al., 1999; Grigg et al., 2005; Park et al., 2005; Ray et al., 1996; Telfer and Poethig, 1998), or for post-transcriptional gene silencing (PTGS, also known as RNAi) (Morel et al., 2002; Peragine et al., 2004), have a precocious vegetative phenotype. However, there is still no conclusive evidence that temporal variation in miRNAs or endogenous siRNAs contribute to vegetative phase change or floral induction.

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To identify genes directly involved in vegetative phase change, we used microarray analysis to search for genes whose mRNA is elevated in *zip-1*, *rdr6-11* and *sgs3-11* (Peragine et al., 2004). These mutations have identical phenotypes, and we reasoned that the genes responsible for this phenotype would be expressed in a similar way in all three mutants. Three such genes were identified: *ETTIN/ARF3*, *ARF4* and *SPL3*. This paper concerns the regulation and function of *SPL3* and the closely related genes, *SPL4* and *SPL5*. These genes are members of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) gene family, members of which share a DNA-binding domain (the SPB domain) first identified in proteins that bind to sequence motif present in the promoter of the *SQUAMOSA* gene in *Antirrhinum majus* (Cardon et al., 1999; Cardon et al., 1997; Klein et al., 1996). Members of this plant-specific structurally diverse family are found in non-vascular (Arazi et al., 2005) and vascular plants, and are required for such processes as ligule (Moreno et al., 1997) and glume (Wang et al., 2005b) development in maize, and leaf (Stone et al., 2005) and flower (Klein et al., 1996; Unte et al., 2003) development in *Arabidopsis* and *Antirrhinum*. Cardon and colleagues (Cardon et al., 1997) originally proposed a role for *SPL3* in floral induction based on the observations that *SPL3* mRNA increases during the transition to flowering in plants grown in long or short days, and that overexpression of *SPL3* produces early flowering. Consistent with this hypothesis, *SPL3* mRNA rapidly increases in plants exposed to a photoinductive stimulus (Schmid et al., 2003). *SPL4* and *SPL5* are closely related to *SPL3*, and have a similar temporal expression pattern (Cardon et al., 1999). The function of these genes is unknown, however, as plants overexpressing *SPL4* and *SPL5* have no apparent phenotype (Cardon et al., 1999). *SPL* genes were among the first miRNA-regulated genes to be identified in *Arabidopsis* (Rhoades et al., 2002). Ten out of the 16 *SPL* genes in *Arabidopsis* – including *SPL3*, *SPL4* and *SPL5* – have target sites for *miR156*. All of these genes are specifically downregulated in plants that

DEVELOPMENT

constitutively overexpress *miR156b* (Schwab et al., 2005), but only two of these transcripts have been shown to be cleaved by this miRNA (Chen et al., 2004; Kasschau et al., 2003).

Here, we show that *SPL3*, *SPL4* and *SPL5* have overlapping functions in the regulation of vegetative phase change and floral induction in *Arabidopsis* and demonstrate that *miR156* is responsible for the temporal change in *SPL3* expression during vegetative development. We also show that *ZIP* and *RDR6* either directly or indirectly repress the expression of *SPL3* during vegetative development, but do not contribute significantly to the temporal expression pattern of this gene.

MATERIALS AND METHODS

Genetic stocks and growth conditions

With one exception, all of the genetic stocks described in this paper were in Columbia background. The T-DNA insertion SALK_035860 was obtained from the *Arabidopsis* Biological Resource Center. The insertion FLAG_173C12 was obtained from the FST project and was characterized in a Ws genetic background. Plants homozygous for these insertions were identified by PCR using allele-specific primers. Seeds were grown on Metromix 200 (Scotts) and left in 4°C cold room for 2 days before transfer to growth chambers. Plant age was measured from the time seeds were transferred to the growth chamber. For phenotypic analysis, plants were grown in 96-well flats under continuous fluorescent light (100 μE/minute/m^2; Sylvania VHO) at 22°C. Abaxial trichomes were scored 2 weeks after planting with a stereomicroscope. Flowering time represents the appearance of the first open flower.

RNA blots

Total RNA was isolated using Trizol (Invitrogen) from shoot apices of plants grown under short-day conditions (10 hours light:14 hours dark) at 22°C. Whole seedlings (including cotyledons) were used for 8- to 14-day-old seedlings, the cotyledons and leaves 1 and 2 were removed from plants harvested between 18-21 days after planting (dap), and the cotyledons and leaves 1-4 were removed from plants harvested between 23-28 dap. The number of leaves present on the shoot apex was determined by comparison with a growth curve generated by dissecting shoot apices grown under the same SD conditions. To measure *SPL3* mRNA, 20 μg of total RNA was run on 1.2% agarose gels, transferred to Hybond N$^+$ nylon membranes (Amersham Pharmacia), and crosslinked under UV light. Hybridizations were performed at 68°C in PerfectHyb plus buffer (Sigma). The *SPL3* probe was PCR amplified using the primers 5′ ACGAGAGAAGGCGGAA-AAGCACAA 3′ and 5′ CGGGATCCCTAAGTCTCAATGCATTTAT 3′ from a *SPL3* cDNA clone and was labeled with ^{32}P-dCTP using Prime-II Random Primer Labeling Kit (Stratagene). Blots were hybridized for 8 hours at 68°C, washed once in 2×SSC and 0.1% SDS solution for 5 minutes at room temperature, twice in 0.5×SSC and 0.1% SDS for 20 minutes at 68°C, and once in 0.1×SSC and 0.1% SDS for 20 minutes at 68°C, and were scanned with a Storm 860 (Molecular Dynamics). To measure *miR156*, 50 μg total RNA was separated on 8 M urea/15% denaturing polyacrylamide gels and electrically transferred to Hybond N$^+$ nylon membranes. Blots were hybridized with a *miR156*-complementary oligonucleotide labeled with ^{32}P-ATP (New England Biolabs) at 40°C in ULTRAhyb-oligo hybridization buffer (Ambion, Austin, TX). Blots were washed twice at 40°C in 2×SSC and 0.5% SDS for 30 minutes before scanning.

5′ and 3′RACE

Total RNA was isolated from leaf or floral tissue as described above. 5′RACE and 3′RACE were carried out using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX). For standard 5′ RACE, 5 μg of total RNA was ligated to the RNA adapter after treatment with calf intestinal phosphatase and tobacco acid pyrophosphatase. For 5′RLM-RACE, RNA was ligated to the RNA oligo adapter without pre-treatment. cDNA was synthesized using the 3′RACE oligo d(T) adapter supplied by the manufacturer. Nested PCR was carried out using the nested adapter primer, and primers specific for *SPL3*, *SPL4* and *SPL5*. RACE products were gel purified and cloned into pGEM T easy vector (Promega) for sequencing.

Transgenic plants

SPL3 (ORF plus 3′UTR) and a sequence lacking the 3′ UTR (*SPL3Δ*) were PCR-amplified with pfu TURBO (Stratagene) using cDNA as template. *SPL3m* was generated by introducing 7 mutations into the predicted *miR156* binding site using recombinant PCR. *GUS-Plus* was amplified from the pCAMBIA1305.1 vector and fused in frame to the 5′ end of these genes to generate GUS-tagged proteins. All of these constructs were cloned downstream of the CaMV 35S promoter in pEZR-CL. Constructs overexpressing putative *miR156* precursors were generated by cloning 0.3-0.8 kb of intergenic genomic sequence containing the precursor in this vector. Plants were transformed using the floral dip method (Bechtold et al., 1993).

GUS activity assays

Visual (i.e. non-quantitative) analyses of GUS expression were conducted by staining plants according to the method of Senecoff et al. (Senecoff et al., 1996). The effect of various mutations on the expression of *35S::GUS-SPL3* constructs was determined as follows. T1 seeds from plants treated with *Agrobacterium* were grown on 1/2 MS medium supplemented with 50 mg/l kanamycin to identify transgenic plants. Resistant plants were transferred to soil in 96-well flats and grown under continuous light at 22°C. Leaves 3 and 4 were harvested when plants had about five fully expanded leaves, and stored at –80°C for subsequent analysis. Upon flowering, adult rosette or cauline leaves were visually assayed for GUS activity to identify plants containing an active transgene. Quantitative GUS assays were carried out on stored leaves from these plants. Leaves were ground to a fine powder in liquid nitrogen in a microfuge tube, and suspended in a buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100. After centrifugation, 10 μl of the supernatant was added to preheated GUS assay buffer (2 mM 4-Methylumbelliferyl β-D-Glucuronide in GUS extraction buffer) at 37°C and incubated for 30 minutes. The reaction was stopped by addition of 900 μl of 0.2 M Na_2CO_3, and fluorescence was measured with LS-50B luminescence spectrometer (PerkinElmer) with emission and excitation filters set at 455 nm and 365 nm, respectively. Total protein was determined using the Bradford Assay (BioRad).

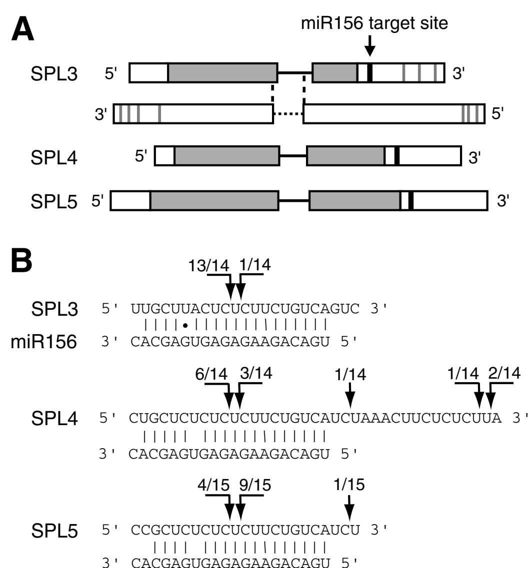

Fig. 1. *SPL3*, *SPL4* and *SPL5* are structurally similar and produce transcripts that are cleaved by *miR156*. (**A**) Genomic structure of *SPL3*, *SPL4* and *SPL5*. White boxes indicate untranslated sequences; grey boxes indicate coding sequences; horizontal lines indicate introns; vertical lines indicate transcription start site or polyadenylation site identified by 5′ or 3′ RACE. (**B**) Cleavage sites identified by 5′RLM-RACE.

DEVELOPMENT

RESULTS

The genomic structure of *SPL3*, *SPL4* and *SPL5*

cDNA sequences deposited in GenBank, as well as data from the Tiling Array Transcriptome Express Tool (Yamada et al., 2003), indicate that *SPL3* (At2g33810) has a complex transcription pattern. To determine the structure of this locus, we performed 5′ and 3′ RACE in both sense and antisense orientations. These experiments revealed that the *SPL3* sense transcript (ST) has a single 5′ end and multiple poly(A) sites in its 3′ UTR (Fig. 1A). The *miR156* target site is located in the 3′ UTR, 50 nucleotides from the stop codon. We also identified several ~950 nucleotide antisense transcripts (AST), which completely encompass the ST and have an intron located in nearly the same position as the intron in the ST (Fig. 1A). Four transcription start sites and 5 poly(A) sites were identified in these AS transcripts.

SPL4 (At1g53160) and *SPL5* (At3g15270) encode, respectively, 174 amino acid and 181 amino acid proteins that are 65% identical (76% similar) to the 131 amino acid protein encoded by *SPL3*. The structure of these genes is also very similar to *SPL3* (Fig. 1A). These genes also possess a *miR156* target site located within their 3′ UTR and 5′RLM-RACE demonstrated that *SPL3/4/5* are all cleaved in the middle of this target site (Fig. 1B).

The regulation and function of *SPL3*

In order to study the role of *miR156* in the regulation of *SPL3*, we produced transgenic plants expressing *miR156*-sensitive and *miR156*-resistant forms of this gene under the regulation of the constitutive CaMV 35S promoter. We chose to use the 35S promoter rather than the endogenous *SPL3* promoter in order to eliminate effects on *SPL3* expression due to transcriptional cross-regulation. The constructs generated for this experiment express an *SPL3* transcript with a normal 3′ UTR (*35S*::*SPL3*), a transcript with seven mutations in the *miR156* target site (*35S::SPL3m*), and a transcript lacking the 3′ UTR (*35S::SPL3Δ*) (Fig. 2A). Constructs in which the *GUS Plus* sequence was fused in frame to the 5′ end of these sequences were also generated in order to evaluate the expression of the SPL3 protein. Plants expressing GUS-tagged versions of SPL3 resembled plants expressing untagged proteins, although their phenotype was slightly weaker. Homozygous stocks containing a single insert were established from transgenic lines, and these stocks were subjected to Northern analysis to ensure that the transgene was overexpressed.

GUS expression was absent in the progeny of a cross between transgenic plants carrying *35S::GUS-SPL3* and plants carrying *35S::miR156a* (Fig. 2B). Progeny from a cross between *35S::GUS-SPL3m* and *35S::miR156a* had high levels of GUS activity, and there was no significant difference between the amount of GUS activity in these plants (35,821±2815 pmol 4-MU/minute·μg protein) and the progeny of a control cross between *35S::GUS-SPL3m* and plants carrying an empty vector (33,599±2109 pmol 4-MU/minute·μg protein). These results demonstrate that the *SPL3* mRNA is a direct target of *miR156* and show that the mutations we introduced in the *miR156* target site completely block the activity of this miRNA.

Transgenic plants carrying *35S::SPL3* were essentially normal; they flowered at nearly the same time and with the same number of leaves as wild-type plants, and had the same pattern of trichome

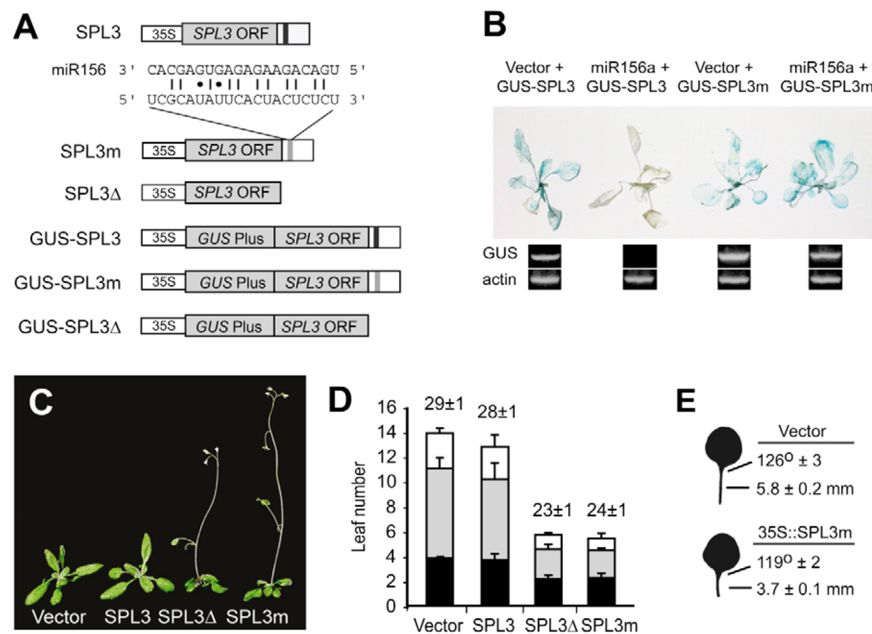

Fig. 2. *SPL3* promotes vegetative phase change and floral induction and is repressed by *miR156*. (**A**) Structure of constructs used in this study. The sequence of the mutated *miR156* target site is illustrated. A wild-type site is indicated by a black line and a mutated site is indicated by a grey line. (**B**) GUS expression (top) and RT-PCR of *GUS* mRNA (bottom) in progeny of crosses between transgenic plants constitutively expressing *miR156a*, and *miR156*-sensitive or insensitive versions of the *GUS-SPL3* mRNA. (**C**) Morphology of transgenic plants expressing *miR156*-sensitive (*SPL3*) and *miR156*-insensitive (*SPL3m, SPL3Δ*) versions of *SPL3* under the regulation of the 35S promoter. (**D**) The number of leaves without abaxial trichomes (black), with abaxial trichomes (grey), cauline leaves (white) and flowering time (days after planting, top of bar) for the genotypes illustrated in C (± s.e.m.). Plants transformed with *35S::SPL3* are not significantly different from control plants. *35S::SPL3m* and *35S::SPL3Δ* have significantly fewer juvenile, adult and cauline leaves than control plants (n>30 for each genotype, P<0.01). (**E**) The effect of *35S::SPL3m* on the morphology of leaves 1 and 2. This transgene produces a significant decrease in the length of the petiole and a slightly more acute leaf base (n=30 for each genotype; P<0.01).

production (Fig. 2C,D). By contrast, plants expressing *SPL3* with mutated *miR156* target site (*35S::SPL3m*) or *SPL3* lacking the 3′ UTR (*35S::SPL3Δ*), flowered early, had a significantly reduced number of juvenile, adult and cauline leaves (Fig. 2C-E). The first two leaves of transgenic plants had a shorter petiole and a more acute leaf base than wild-type leaves (Fig. 2E); these features are typical of adult leaves. These results indicate that *SPL3* promotes both vegetative and reproductive phase change and is normally repressed by *miR156*.

Plants constitutively expressing the a, b, c, d, e and f isoforms of *miR156* were generated by transforming wild-type plants with constructs containing 0.3-0.8 kb of intergenic genomic sequence under the regulation of the 35S promoter (Fig. 3A). All of these transgenic lines had a similar phenotype (Fig. 3B), and resembled the *35S::156b* transgenics described by Schwab et al. (Schwab et al., 2005). Thus, all these loci are capable of producing *miR156*, although whether they are all transcribed in vivo is unknown. A detailed phenotypic analysis was performed on plants overexpressing *miR156a*. In continuous light, transgenic plants had a significantly larger number of leaves without abaxial trichomes (n=24; P<0.01 for all traits) (Fig. 3C). Furthermore, most of the leaves produced by these plants had a round lamina, long petiole and were relatively small in size – all characteristics of juvenile leaves. Transgenic plants also flowered significantly later than vector controls with many additional leaves (Fig. 3C). To determine if downregulation of *SPL3* is responsible for this phenotype, we examined the phenotype of F2 progeny from plants heterozygous for *35S::miR156a* and the *miR156*-insensitive transgene, *35S::SPL3m*. Plants carrying both of these transgenes produced significantly fewer leaves without abaxial trichomes than plants expressing *35S::miR156a* (Fig. 3D). Plants with both transgenes resembled *35S::SPL3m* in producing leaves with short petioles, but the shape of the lamina in double transgenics was more similar to that of *35S::miR156a* than *35S::SPL3m* (Fig. 3E). We conclude that downregulation of *SPL3* accounts for the effect of *35S::miR156a* on abaxial trichome production, petiole length and flowering time, but that the effect of this miRNA on lamina shape is mediated by a different gene or genes. In this respect, *SPL3* resembles *Gl15* in maize, which regulates phase-specific patterns of leaf epidermal differentiation and flowering time, but does not affect leaf shape (Evans et al., 1994; Lauter et al., 2005; Moose and Sisco, 1994).

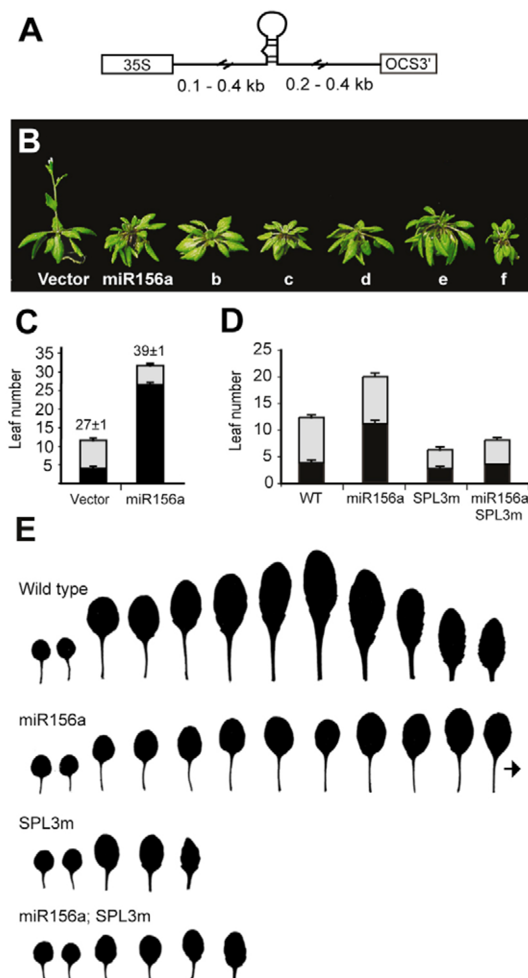

Fig. 3. *miR156* promotes juvenile development. (**A**) Structure of the constructs used to overexpress *miR156*. DNA from the intergenic region containing *miR156* was placed under 35S regulation. (**B**) Morphology of transgenic plants expressing the constructs illustrated in A. (**C**) The number of leaves without abaxial trichomes (black), with abaxial trichomes (grey) and flowering time (dap) in transgenic plants with an empty vector or *35S::miR156a*. (**D**) The number of leaves without abaxial trichomes (black) and with abaxial trichomes (grey) in the F2 progeny of plants hemizygous for *35S::miR156a* and *35S::SPL3m*. (**E**) Successive rosette leaves from representative F2 plants illustrated in B. Only some of the leaves of *35S::miR156a* are illustrated.

SPL4 and *SPL5* have the same function as *SPL3* and are repressed by *miR156*

Plants homozygous for T-DNA insertions that partially (SALK_035860) or completely (FLAG_173C12) block the production of the *SPL3* mRNA (data not shown) displayed a very

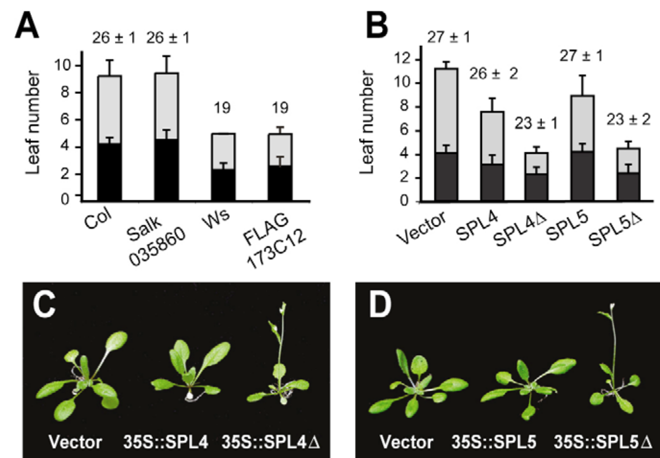

Fig. 4. *SPL4* and *SPL5* promote vegetative phase change and flowering, and are repressed by *miR156*. (**A**) The number of leaves without abaxial trichomes (black), with abaxial trichomes (grey) and flowering time (dap, top of bar) in plants homozygous for T-DNA insertions in *SPL3*; SALK_035860 was isolated in Col, whereas FLAG_173C12 was isolated in Ws. The small delay in abaxial trichome production in FLAG_173C12 is significant (n=22 for each genotype; P<0.05). (**B**) The number of leaves without abaxial trichomes (black), with abaxial trichomes (grey), and flowering time (dap, top of bar) in plants constitutively expressing *SPL4* and *SPL5* transcripts with or without (Δ) a 3′ UTR. Error bars=s.e.m. (**C**,**D**) Morphology of the transgenic lines illustrated in B.

DEVELOPMENT

slight, but statistically significant (*n*=22; *P*<0.05) delay in abaxial trichome production in the case of FLAG_173C12 (Fig. 4A). This result is consistent with the evidence that *SPL3* promotes the adult phase, but indicates that it is functionally redundant. To determine whether *SPL4* and *SPL5* have overlapping functions with *SPL3*, we produced transgenic lines transformed with *35S::SPL4*, *35S::SPL4Δ*, *35S::SPL5* and *35S::SPL5Δ* constructs analogous in structure to the similarly named *SPL3* constructs (Fig. 2A). *35S::SPL4* and *35S::SPL5* reduced the number of adult leaves (*n*=50; *P*<0.01), but had no significant effect on the number of leaves without abaxial trichomes or on flowering time (Fig. 4B-D). By contrast, *35S::SPL4Δ* and *35S::SPL5Δ* significantly accelerated abaxial trichome production and floral induction (*n*=48; *P*<0.01). These results indicate that *SPL4* and *SPL5* have the same function as *SPL3,* and that they are normally repressed by *miR156*.

Temporal regulation of *SPL3* by *miR156*

We studied the level of *SPL3* and *miR156* RNA in plants grown in short days (SD; 10 hours light: 14 hours dark) in order to distinguish changes in the expression of these genes associated with vegetative phase change from those associated with floral induction (Schmid et al., 2003). Under our SD conditions, the first leaf with abaxial trichomes (leaf 8 or 9) is initiated 15-16 dap and flowering occurs approximately 60 dap, after the shoot has produced about 45 leaves (Telfer et al., 1997; Bollman et al., 2003). Thus, changes in gene expression observed during the first 30 days of growth in SD are associated with vegetative phase change, not floral induction.

The expression of *SPL3* and *miR156* was determined by northern analysis of RNA from shoot apices harvested from juvenile (12 dap; 4 leaves), transition (19 dap; 10 leaves) and adult (26 dap; 16 leaves) plants growing in SD. *SPL3* mRNA was nearly undetectable at 12 dap, and was present in increasing amounts in the 19 and 26 dap samples. A probe complementary to *miR156* detected both 20 and 21 nucleotide miRNAs; the 20 nucleotide miRNA is derived from one or more of the *miR156a-f* loci, whereas the 21 nucleotide miRNA is probably derived from *miR157a-d*, which encode a miRNA that is very similar to *miR156* (Reinhart et al., 2002; Xie et al., 2005). The 21 nucleotide miRNA was expressed uniformly, whereas the 20 nucleotide miRNA was expressed at a high level at 12 dap and at uniformly low level thereafter (Fig. 5A). Thus, *SPL3* and *miR156* are expressed in a complementary fashion early in vegetative development.

To determine if *miR156* contributes to the temporal expression pattern of *SPL3*, we examined the expression of *miR156*-sensitive (*35S::GUS-SPL3*) and *miR156*-insensitive (*35S::GUS-SPL3m*) reporter genes under the regulation of the constitutive CaMV 35S promoter. All of the leaves of plants homozygous for *35S*::*SPL3m* had high levels of GUS activity and there was relatively little variation in activity in plants harvested at different times (Fig. 5B,C). By contrast, plants transformed with *35S::GUS-SPL3* expressed GUS only in apical leaves. Lines with strong activity displayed GUS expression earlier in shoot development than did lines with lower activity, and often became completely silenced in subsequent generations. The line illustrated in Fig. 5 displays the pattern of GUS activity characteristic of the most stable lines we obtained. GUS expression in this line was observed starting with leaf 7 or 8 (Fig. 5B), and increased gradually during shoot development (Fig. 5C). Consistent with this expression pattern, *miR156* was more abundant at 8 dap than at the later time points (Fig. 5D). These results demonstrate that temporal variation in the expression *35S::GUS-SPL3* is regulated post-transcriptionally by *miR156*.

ZIP and *RDR6* regulate the amplitude of *SPL3* expression, but not its temporal expression

SPL3 initially came to our attention because it is one of a relatively small number of genes that is over-expressed in *zip*, *rdr6* and *sgs3* mutants. Two other genes that have this expression pattern, *ETTIN/ARF3* and *ARF4*, are targets of *trans*-acting siRNAs (ta-siRNAs) (Allen et al., 2005; Williams et al., 2005). ta-siRNAs are produced by a pathway involving *SGS3*, *RDR6* and *DCL4* from transcripts that are cleaved by a miRNA (Dunoyer et al., 2005; Gasciolli et al., 2005; Yoshikawa et al., 2005). The observation that *SPL3* mRNA is elevated in these three mutants therefore suggests that *SPL3* is either directly or indirectly regulated by this silencing pathway.

To determine the relative importance of *miR156*-directed cleavage and RNAi in the regulation of *SPL3*, we compared the effect of *zip-1*, *rdr6-11* and *hst-6* on the accumulation of *miR156* and *SPL3* RNA in plants grown in SD. We chose *hst-6* because this mutation specifically affects miRNAs, produces a significant decrease in *miR156* (Park et al., 2005) and has a precocious vegetative phenotype (Telfer and Poethig, 1998). *miR156* was present at a reduced but readily detectable level in *hst-6* at 14 dap, and was nearly undetectable in this mutant at 21 dap. Consistent with its effect on *miR156*, *SPL3* was only slightly elevated in *hst-6* at 14 dap,

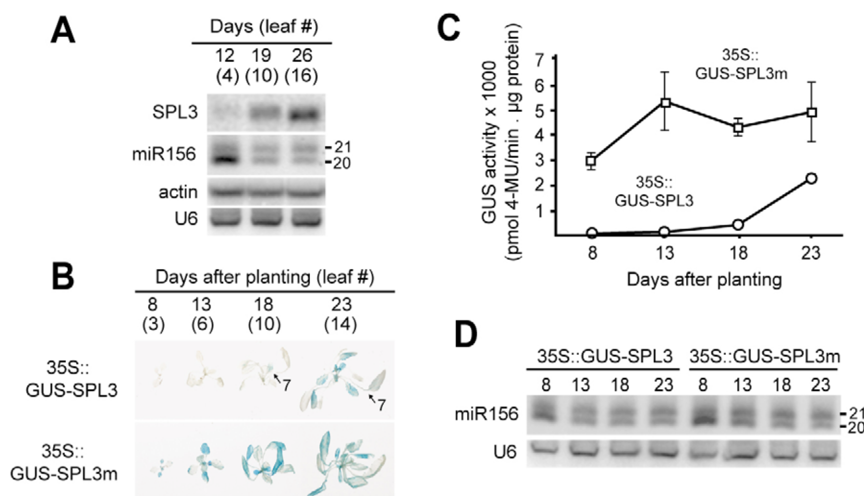

Fig. 5. *miR156* is responsible for the temporal expression of *SPL3*. (**A**) Blots of total RNA from the shoot apex of plants grown for different lengths of time in SD. The approximate number of leaves greater than 100 μm in length present at each time point is indicated in parentheses. Actin was used as a loading control for *SPL3*, and *U6* was used as a loading control for *miR156*. (**B**) GUS expression in transgenic plants of different ages. Leaf number 7 is indicated. The approximate number of leaves greater than 100 μm in length in these samples is indicated in parentheses. (**C**) Quantitative analysis of GUS activity in extracts of the lines illustrated in B. Error bars indicate s.e.m. (**D**) Northern analysis of *miR156* levels in the transgenic lines illustrated in B and C.

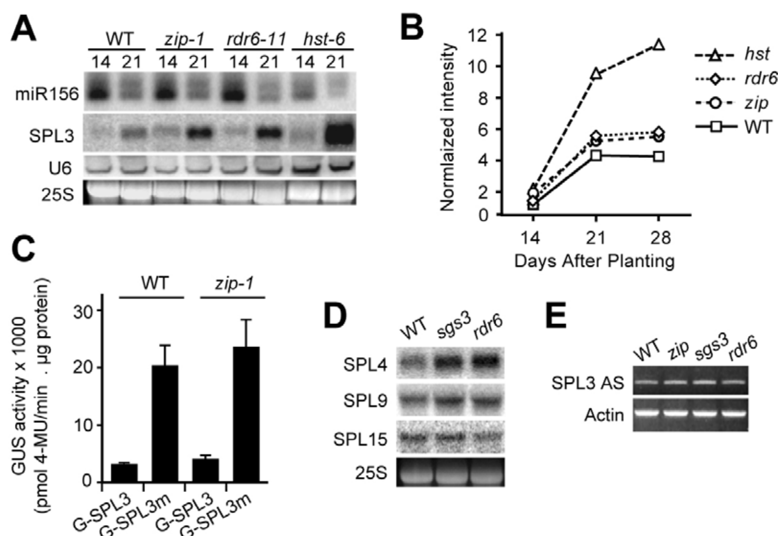

Fig. 6. *zip-1* and *rdr6-11* affect the amplitude of *SPL3* expression, but not its temporal pattern. (**A**) Northern analysis of *SPL3* and *miR156* RNA in SD-grown plants at 14 dap (6 leaves) and 21 dap (12 leaves). *zip-1* and *rdr6-11* produce a modest increase in *SPL3* mRNA without affecting the level of *miR156*. *hst-6* reduces the accumulation of *miR156* and produces a dramatic increase in *SPL3* mRNA at 21 dap. *U6* was used as a loading control for *miR156* and 25S rRNA was the loading control for *SPL3*. (**B**) Northern analysis of *SPL3* mRNA in SD-grown wild-type and mutant plants of different ages. Hybridization intensity was determined for three blots using NIH image, averaged and normalized to the intensity in wild-type plants at 14 dap. (**C**) GUS activity in the 3rd and 4th leaf of primary transgenic plants transformed with *35S::GUS-SPL3* (*G-SPL3*) or *35S::GUS-SPL3m* (*G-SPL3m*). More than 30 plants of each genotype were analyzed. The difference between the expression of these constructs in wild type and *zip-1* is not statistically significant. (**D**) Northern blot of *SPL4*, *SPL9* and *SPL15* mRNA in flowers of wild-type, *sgs3-11* and *rdr6-11* plants. (**E**) RT-PCR analysis of the *SPL3* antisense transcript (*SPL3 AS*).

but was two- to threefold higher than normal at later times in development (Fig. 6A,B). This result supports the conclusion that temporal variation in *miR156* is responsible for changes in the abundance of *SPL3* mRNA during vegetative development,

zip-1 and *rdr6-11* had no effect on the accumulation of *miR156*, and produced a modest and fairly uniform increase in *SPL3* mRNA (Fig. 6A,B). Thus, *ZIP* and *RDR6* regulate the amplitude of *SPL3* expression, but not its temporal pattern. *ZIP/AGO7* encodes one of 10 Argonaute-like proteins in *Arabidopsis*, at least one of which (AGO1) mediates miRNA- and ta-siRNA-directed RNA cleavage (Baumberger and Baulcombe, 2005; Qi et al., 2005). To determine if ZIP is required for *miR156*-directed repression of *SPL3*, we transformed wild-type and *zip-1* plants with *35S::GUS-SPL3* and *35S::GUS-SPL3m*. Quantitative GUS assays were performed on leaf 3 and 4 from 30 or more primary transformants in which these transgenes were expressed in the inflorescence (see Materials and methods); this was carried out to ensure that the transgenes were capable of being expressed. *35S::GUS-SPL3* was expressed at significantly lower levels than *35S::GUS-SPL3m* in the juvenile leaves of both wild-type and *zip-1* plants, and there was no significant difference in amount of GUS activity in these genotypes (Fig. 6C). This result, and observation that *zip-1* does not affect the abundance of *miR156* (Fig. 6A), indicate that the increase in *SPL3* mRNA in this mutant is not mediated by a change in the expression of *miR156* or by a defect in *miR156*-directed repression of *SPL3*.

The effect of *sgs3-11* and *rdr6-11* on the level of *SPL3* mRNA suggests that this gene may be a direct target of RNAi. To test this hypothesis, we searched for siRNAs derived from *SPL3* by hybridizing blots of low molecular weight from wild-type and mutant plants with a probe that spans the *SPL3* ORF and 3′UTR, as well as with oligonucleotide probes to sequences 5′ and 3′ of the *miR156* target site. No siRNAs were detected with any of these probes. Furthermore, a search of the MPSS database (mpss.udel.edu/at/) revealed that no small RNAs have been cloned from *SPL3*. To determine if *SGS3* and *RDR6* play a general role in the expression of genes targeted by *miR156*, we examined the level of *SPL4*, *SPL9* and *SPL15* mRNA in *sgs3-11* and *rdr6-11* mutants; *SPL9* and *SPL15* have a *miR156* target site in their ORF and are downregulated in plants constitutively expressing *miR156b* (Schwab et al., 2005). *SPL4* mRNA was elevated in both mutants, but we observed little or no change in *SPL9* or *SPL15* (Fig. 6D). Semi-quantitative RT-PCR analysis of the expression of the *SPL3* antisense transcript in *zip*, *rdr6-11* and *sgs3-11* demonstrated that these mutations also have no effect on this transcript (Fig. 6E). These results suggest that the increase in *SPL3* mRNA in *sgs3* and *rdr6* is an indirect effect, and does not reflect the involvement of *SGS3* and *RDR6* in *miR156*-initiated transitive silencing of this gene.

DISCUSSION

The discovery of miRNAs in many plant and animal species, and the growing evidence that these molecules regulate the activity of genes involved in a wide range of developmental and physiological processes represent a significant advance in our understanding of the regulatory mechanisms that control gene expression (Carrington and Ambros, 2003; Kidner and Martienssen, 2005). Here, we show that *miR156* regulates the expression of three genes that promote vegetative phase change in *Arabidopsis* – *SPL3*, *SPL4* and *SPL5* – and that temporal variation in the level of this miRNA contributes significantly to the temporal change in *SPL3* activity during vegetative development.

The function of *SPL3*, *SPL4* and *SPL5*

SPL3 was originally identified because of its homology to SBP genes in *Antirrhinum* and was reported to cause early flowering when overexpressed (Cardon et al., 1997). We confirmed this observation, but found that overexpression of *SPL3* only has a major

effect on flowering time if the *miR156* target site in the 3′ UTR is mutated or deleted. The results obtained by Cardon et al. (Cardon et al., 1997) are probably attributable to the fact that the construct used in that study had a truncated *miR156* target site. Overexpression of *SPL3* also accelerated two traits characteristic of adult leaves – abaxial trichome production and short petioles – but had relatively little effect on other leaf traits. This result demonstrates that *SPL3* regulates a subset of traits associated with the adult phase, but is not entirely responsible for vegetative phase change. Plants overexpressing *miR156*-sensitive and *miR156*-insensitive versions of the *SPL4* and *SPL5* mRNAs have essentially the same phenotype as plants expressing the corresponding *SPL3* constructs, indicating that these genes have similar, if not the same functions as *SPL3*. Whether *SPL4* and *SPL5* have unique functions is still unknown because loss-of-function mutations in these genes have not yet been identified. Other genes that affect both vegetative phase change and floral induction include genes involved in gibberellin biosynthesis/signaling, as well as genes involved in the autonomous floral induction pathway (Scott et al., 1999; Silverstone et al., 1997; Telfer et al., 1997). It will be important to determine if *SPL3/4/5* reside in one or the other of these pathways. The observation that *35S::SPL3m* is incapable of rescuing the effect of *35S::miR156a* on lamina shape suggests that other targets of this miRNA also play a role in vegetative phase change.

Temporal regulation of *SPL3* by *miR156*

The level of *SPL3* mRNA increases early in vegetative development (this study), and during the transition to flowering (Cardon et al., 1999; Cardon et al., 1997; Schmid et al., 2003). Because of our interest in vegetative phase change, we focused on the regulation of *SPL3* early in vegetative development, when the expression of this gene and *miR156* change in a complementary fashion. We found that the constitutively transcribed *miR156*-sensitive reporter gene *35S::GUS-SPL3* displayed GUS activity only in apical leaves, whereas the *miR156*-insensitive *35S::GUS-SPL3*m transgene displayed constitutive GUS activity. This result is consistent with the capacity of these transgenes to affect plant morphology, and demonstrates that *SPL3* is repressed post-transcriptionally by *miR156* early in shoot development. Additional support for this conclusion was provided by the observation that the temporal expression pattern of the endogenous *SPL3* transcript is disrupted by *hst-6*, a mutation that strongly reduces the accumulation of *miR156*. Interestingly, the amount of *miR156* present in young plants appears far in excess of the amount actually necessary to block *SPL3* activity. This conclusion is supported by the observation that *35S::SPL3* was unable to overcome the effect of endogenous *miR156*, and the observation that *hst-6* had relatively little effect on *SPL3* mRNA levels early in shoot development, when *hst-6* still has a significant amount of *miR156*.

It should be emphasized that our results do not eliminate the possibility that transcriptional regulation also plays a role in the temporal expression of *SPL3*. *SPL3*/4/5 are transcription factors and may crossregulate each other's expression, as has been observed in the case of the *AP2* family of *miR172*-regulated transcription factors (Chen, 2004; Schmid et al., 2003). Indeed, the dramatic increase in the expression of *SPL3/4/5* during the floral transition is probably regulated at a transcriptional level rather than by *miR156* because *miR156* does not decrease markedly during this transition (G.W. and R.S.P., unpublished).

How is the expression of *miR156* regulated early in vegetative development? *miR172* is expressed in a pattern complementary to that of *miR156*, increasing with time during shoot development (Aukerman and Sakai, 2003). Furthermore, the phenotype of plants overexpressing *miR172* is nearly the exact opposite of plants overexpressing *miR156*, and strongly resembles the phenotype of plants over-expressing *SPL3m* (Aukerman and Sakai, 2003; Chen, 2004). The targets of *miR172* include the *AP2*-like transcription factors, *TOE1*, *TOE2*, *SMZ* and *SNZ* in *Arabidopsis* (Aukerman and Sakai, 2003; Chen, 2004; Schmid et al., 2003), and *Gl15* in maize (Lauter et al., 2005). These genes act as floral repressors and, in the case of *Gl15*, also promote the expression of juvenile vegetative traits. The complementary relationship between the expression pattern and functions of *miR172* and *miR156* is striking and raises the possibility that these two miRNAs and their targets act in sequence in the same regulatory pathway.

The function of RNAi in the regulation of *SPL3*

Transcripts that contain a miRNA target site are frequently silenced in an *RDR6*-dependent fashion when they are expressed as transgenes, and it has been reported that miRNA-directed cleavage sensitizes transcripts to transitive silencing (Parizotto et al., 2004). Consistent with this report, we found that all of our constructs with a functional *miR156* target site were significantly more susceptible to silencing than constructs that lacked this site. This made it difficult to generate reporter lines and to maintain these lines over many generations. It did not interfere with our ability to identify reliable reporter lines, however, because transgenes undergoing post-transcriptional silencing have a different expression pattern than *SPL3*. Whereas the expression of *SPL3* increases with time, transgenes undergoing silencing are either permanently silenced very early in shoot development, or display progressively lower levels of expression during shoot growth (de Carvalho et al., 1992; Glazov et al., 2003; Palauqui et al., 1996; Vaucheret et al., 2004). Although it is clear that transgenes expressing transcripts with miRNA-target sites are often subject to RNAi, whether this is also true for endogenous transcripts is less certain. miRNA-directed transitive silencing is well documented in the case of transcripts that produce *trans*-acting siRNAs (Allen et al., 2005; Yoshikawa et al., 2005). However, most protein-coding transcripts that are cleaved by miRNAs – including the *miR156*-regulated transcripts *SPL9* and *SPL15* – are not affected by *sgs3* or *rdr6*, and are therefore unlikely to be targets of RNAi (Allen et al., 2005; Peragine et al., 2004). The observation that *SPL3* and *SPL4* are elevated in these mutants suggests that these genes either have features that make them unusually susceptible to RNAi (e.g. the presence of the *miR156* target site in their 3′ UTR), or that they are regulated indirectly by this mechanism – for example, by a transcription factor that itself is a target of RNAi. We have no conclusive evidence for either possibility. However, the observation that *zip-1*, *rdr6-11* and *sgs3-11* have nearly identical effects on *SPL3* expression (Fig. 6A) (Peragine et al., 2004) suggests that these genes regulate *SPL3* by the same mechanism. Because ZIP is not generally required for RNAi (Hunter et al., 2003) or for the *miR156*-dependent suppression of *35S::GUS-SPL3* (Fig. 6C), this observation may indicate that these genes regulate *SPL3* expression indirectly.

A large fraction of the genes in *Arabidopsis* have antisense transcripts (Borsani et al., 2005; Jen et al., 2005; Wang et al., 2005a; Yamada et al., 2003). In most cases, these transcript pairs represent neighboring, divergently transcribed protein-coding genes that overlap for a region of their 3′ ends. By contrast, the *SPL3* antisense transcript is nearly the same size as the sense transcript, it completely encompasses the sense transcript and it does have significant coding potential, suggesting that this transcript is dependent on sequences

that are important for the expression of the sense transcript. Whether this transcript plays a role in the expression of the sense transcript remains to be determined.

The results of this and other (Schmid et al., 2003; Schwab et al., 2005) studies indicate that *SPL3* and the related genes, *SPL4* and *SPL5*, are under complex transcriptional and post-transcriptional regulation. We are particularly intrigued by these three genes because they establish a link between miRNAs and vegetative phase change in *Arabidopsis*, and reveal that temporal variation in miRNA expression plays a regulatory role in developmental timing in plants. Further studies will be necessary to determine the precise mechanism by which *SPL3/4/5* expression is controlled during vegetative and reproductive phase change, and the regulatory pathways in which these genes operate.

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DEVELOPMENT

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DEVELOPMENT