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 RNA molecules, 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.

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: ETIN/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
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; Kasselchau 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/m²; Sylvania VHO) at 22°C. Abaxial trichomes were scored 2 weeks after planting with a stereomicroscope. Flowering time represents 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' ACGAGAGAAGGGGAAA-AAGAACAAA 3' and 5' CGGGATCCCTAAGTCTAATGCTTATTT 3' from a SPL3 cDNA clone and was labeled with 32P-dCTP using Prime-II (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' ACGAGAGAAGGGGAAA-AAGAACAAA 3' and 5' CGGGATCCCTAAGTCTAATGCTTATTT 3' from a SPL3 cDNA clone and was labeled with 32P-dCTP using Prime-II Random Primer Labeling Kit (Stratagene). Blots were hybridized for 8 hours at 68°C, washed once in 2X SSC and 0.1% SDS solution for 5 minutes at room temperature, twice in 0.5X SSC and 0.1% SDS for 20 minutes at 68°C, and once in 0.1X 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 32P-ATP (New England Biolabs) at 40°C in ULTRAhyb-oligo hybridization buffer (Ambion, Austin, TX). Blots were washed twice at 40°C in 2X 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 (ORE 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 pCAMBIA1301.5 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 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 Na2CO3, 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.
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 transcript 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

![Image](http://example.com/image.png)

**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 miRNA. (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::SPL3a), 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 \( (n=24; P<0.01 \text{ for all traits}) \) (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).

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...
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., 2005). The 21 nucleotide miRNA was expressed uniformly, whereas the 21 nucleotide miRNA is probably derived from miR156, which encodes a miRNA that is very similar to miR156 is probably derived from miR156, which encodes a miRNA that is very similar to miR156a-f (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 SG53, RDR6 and DCL4 from transcripts that are cleaved by a miRNA (Dunoyer et al., 2005; Gascioli 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 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,
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 abundance of miR156 temporally variation in development (Fig. 6A,B). This result supports the conclusion that zip-1 is not statistically significant. (Fig. 6C). This result, and observation that the increase in SPL3 mRNA in wild type, and zip-1 leaves of both wild-type and rdr6-11 plants, and there was no significant difference in amount of GUS activity in these genotypes 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 introduced 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 (SPL3 AS).

**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
**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-SPL3m 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-translationally 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 GI15 in maize (Lauter et al., 2005). These genes act as floral repressors and, in the case of GI15, 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; Palaquiu 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 SPL3, 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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References


