The miRNA156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings

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Summary

miRNAs are a class of versatile small RNAs that control gene expression post-transcriptionally, governing many facets of plant cell functions. They interact with their target mRNA at a site of sequence complementarity and modulate their expression levels. Here, we provide evidence, based on transient assays and stable transgenic lines, that the 3′ UTR of the Arabidopsis SBP box gene SPL3 contains a functional miRNA-responsive element (MRE) that is complementary to miR156 and miRNA157. Seedlings of transgenic lines constitutively over-expressing an SPL3 transgene either carrying an unaltered or a disrupted MRE accumulate considerable levels of SPL3 transcripts. However, while the unaltered MRE UTR does not allow the expression of detectable levels of SPL3 protein, the altered MRE does. Translational inhibition thus provides an important mechanism for miRNA-mediated post-transcriptional repression of SPL3. As a consequence of precocious translation of the constitutively expressed SPL3 transgene, due to the absence of a functional MRE, plants exhibit very early flowering in addition to frequent morphological changes.

Keywords: SPL3, SBP box gene, flowering, miR156, microRNA, post-transcriptional regulation.

Introduction

Micro-RNAs (miRNAs) are small endogenous non-coding RNAs of 20–22 nt in length, present in plants and animals, and synthesized from hairpin precursors. They modulate the expression of target mRNAs at regions of complementarity, termed miRNA-responsive elements (MREs). In plants, MREs are found in the coding regions (Bartel, 2004; Jones-Rhoades and Bartel, 2004), the 5′ UTRs (Allen et al., 2005; Chioù et al., 2006; Fujii et al., 2005; Lu et al., 2005; Sunkar and Zhu, 2004) or the 3′ UTRs of the targets (Rhoades et al., 2002; Sunkar and Zhu, 2004).

Irrespective of the location of the MREs, miRNAs recruit effector complexes to direct various modes of post-transcriptional gene silencing at the MRE, such as cleavage and/or translational repression of the target mRNA. RNA cleavage products have been detected in vivo and in vitro by 5′ RACE and Northern blot analysis (Allen et al., 2005; Kasschau et al., 2003; Llave et al., 2002; Tang et al., 2003). In addition, expression arrays have been used to show target level reduction in response to miRNA expression (Palatnik et al., 2003; Schwab et al., 2005). Although a wealth of information exists on miRNA-directed transcript cleavage, to date the miR172–AP2 mRNA interaction remains the only example of miRNA-directed translational inhibition reported in plants (Chen, 2004). In contrast, miRNA-directed control mechanisms acting at translation initiation, or post-initiation steps, such as elongation, termination or the release of stable protein, have been shown to be common in mammals, worms and insects (Humphreys et al., 2005; Olsen and Ambros, 1999; Pillai et al., 2005).

In Arabidopsis, 11 out of 17 SBP box genes, known as SPL genes, have been predicted to be targets of the highly similar miRNAs miR156 and miR157 (Rhoades et al., 2002). SBP box genes represent a family of plant-specific transcription factors sharing a highly conserved DNA-binding domain, the SBP domain (Birkenbihl et al., 2005; Cardon et al., 1999; Klein et al., 1996; Yamasaki et al., 2004). The function in
development of only a few SBP box genes has been determined based on mutant phenotypes. These include the Arabidopsis genes SPL8, affecting fertility (Unte et al., 2003), and SPL14, determining the sensitivity to the fungal toxin fumonisin B (Stone et al., 2005), and the maize SBP-homologous genes LG1 and TGA1 that are involved in leaf and glume development, respectively (Moreno et al., 1997; Wang et al., 2005). Furthermore, the constitutive over-expression of an SPL3 cDNA, notably lacking most of the 3' UTR, results in an early-flowering phenotype, suggesting a role in floral transition (Cardon et al., 1997).

Whereas SPL8 and SPL14 do not carry an MRE to miR156/157, SPL3 does in its 3' UTR. Experimental evidence shows that miR156/miR157 do indeed mediate lower SPL3 transcript levels. SPL3 transcripts levels were found to be decreased in inflorescence apices of Arabidopsis transgenics constitutively over-expressing Mir156b (Schwab et al., 2005). In contrast, higher accumulation of SPL3 transcripts was found in hasty mutants, known to be defective in miRNA biosynthesis (Park et al., 2005). Furthermore, a decrease in the steady-state level of SPL3 mRNA could be observed in plants over-expressing the viral suppressor protein P69, negatively affecting the siRNA pathway but promoting the miRNA pathway (Peragine et al., 2004). Actual degradation of SPL3 mRNA and its cleavage within the miR156/157 MRE was demonstrated by Chen et al. (2004).

While the above examples show the effect of the miR156/157-SPL3 interaction on transcript level, the biological consequences remain unknown. SPL3, like the highly similar genes SPL4 and SPL5, is expressed most strongly in flowering shoots, suggesting a role in the flowering process (Cardon et al., 1997; Schmid et al., 2003; Zimmermann et al., 2004). This is supported by the observation that SPL3 over-expressing transgenics display an early-flowering phenotype (Cardon et al., 1997). However, the molecular genetic mechanisms underlying this behaviour of SPL3 transgenics are not understood, and an SPL3 knockout line with a discernable phenotype has not yet been described.

In the present study, we demonstrate the involvement of miR156/157 in regulating SPL3 expression through translational repression by monitoring SPL3 protein levels. In addition, we describe the phenotypic consequences of disruption of the SPL3 MRE located in the 3' UTR on the flowering behaviour of SPL3 transgenics.

Results

Conservation of the SPL MRE

Eleven of the 17 SPL genes in Arabidopsis contain a target site for miRNAs 156/157. The two genes At5g50570 and At5g50670 identified as miRNA156/157 targets by Rhoades et al. (2002) in fact represent a duplication of SPL3 found in the middle of a near-perfect tandem repeat spanning approximately 66 kb on chromosome V. Because of the perfectly conserved sequence identity, it is impossible to discriminate between transcripts derived from these two SPL3 loci (known as SPL3A and B). The SPL MREs for all of these targets lie downstream of the conserved SBP box and are part of the coding sequence of the last exon, with the exception of SPL3, 4 and 5, where they are located in the 3' UTR (Figure 1a). The alignments shown are the reverse complements of miR156C and miR157C, with MREs of the SPL mRNAs. However, various members of the miR156/miR157 family, like all other plant miRNAs, differ slightly in their sequences. All SPL mRNAs show perfect complementarity to the 5' half of miR156/157 (proximal region), with the exception of the last nucleotide in miR157C (Figure 1b). However, the MREs present in the 3' UTRs differ, with a few mismatches to the 3' end of the miRNA (distal region) (Figure 1b). Despite the fact that the positioning of the MRE relative to the reading frame should not have an effect on its function, all SPL MREs are conserved in the same reading frame, like most plant miRNA targets. The SPL MREs participate in coding the motif ALSLLS embedded in an otherwise non-conserved protein region.

An alignment of presumed SPL3 orthologs from different plant species such as Antirrhinum majus (AmSBP1; Klein et al., 1996) and Betula pendula (BpSPL3; Länneppää et al., 2004) shows that the miR156/157-related MRE in the 3' UTR

![Figure 1](image-url)
is conserved (Figure 1c). Any sequence divergence, as well as a few mismatches to the miR156/157 complementary sequence, remains restricted to the distal half of the MRE. It is notable that, outside the dicots, we have not been able to identify SPL3-like genes that encode small proteins characterized by an acidic domain upstream of the conserved SBP box and contain an MRE in their 3′UTR.

The effect of an SPL3 transgene on flowering time depends on an MRE in its 3′UTR

Because of the role of SPL3 in generating early-flowering phenotypes (Cardon et al., 1997), and with the emergence of the role of miRNAs in development and the possible interaction of miR156/157 with the SPL3 MRE, we have looked for the contribution of the SPL3 MRE to flowering. Hence, we generated mutations disrupting the MRE within the SPL3 3′UTR and brought the gene under the control of the strong constitutive CaMV 35S promoter (Figure 2). 35S::SPL3-UTRΔ2 carries a truncation in its 3′UTR resulting in the loss of 9 nt at the proximal end of its MRE and the lack of all 3′UTR sequences downstream of the MRE. 35S::SPL3-UTRΔ3 and 35S::SPL3-UTRΔ4 were generated with a full-size 3′UTR, and only the predicted miR156/157 recognition element was mutated. 35S::SPL3-UTRΔ3 carries a 10 nt substitution in the proximal half and 35S::SPL3-UTRΔ4 a 10 nt substitution in the distal half of the MRE. In addition, we generated an SPL3 transgene with an unaltered 3′UTR referred to as 35S::SPL3-UTRwt. For convenience, we renamed the transgene previously described by us as 35S::SPL3se (which carries a truncation of its 3′UTR resulting in the loss of 4 nt at the proximal end of its MRE; Cardon et al., 1997) to 35S::SPL3-UTRΔ1. We then looked for the effect on the expression of the SPL3 transgene and the consequences on flowering time in stably transformed lines.

When the SPL3-UTRΔ2 transgene was introduced into Col and Ler backgrounds through Agrobacterium-mediated transformation (C35S::SPL3-UTRΔ2 and L35S::SPL3-UTRΔ2), all T1 transgenic plants flowered earlier than wild-type. In addition they exhibited morphological defects.

Of the 32 C35S::SPL3-UTRΔ2 (T1) plants, three independent Col transgenic lines (C-UTRΔ2-2, -5 and -3), segregating for a single transgenic locus, were made homozygous and subjected to a detailed analysis. Over several generations, these lines were consistently found to flower very early, while the other transgenic lines showed a range of flowering time reduction. An evaluation of flowering time, reflected in the total number of leaves (TLN) formed on the main stem before the first flower appears, is shown in Figure 3 for T5 plants.

Under both long-day (LD) and short-day (SD) conditions, the C35S::SPL3-UTRΔ2 transgenic lines flowered clearly earlier than wild-type (TLN 5 or 6 and TLN 30–36, respectively; Figure 3). The C35S::SPL3-UTRΔ2 transgenics thus remained photoperiod-sensitive as previously reported for

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**SPL3-**

**MRE::miR156 complementarity**

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**Figure 2.** Transgene 3′UTR-MRE modifications.

The miR156c complementary regions in the UTRs of the different SPL3 transgenes are shown above the 20 nt long miR156c sequence. Matches are indicated by ‚,’ G/U wobble base pairing by ‚Æ’. Deviations from SPL3 wild-type sequence are in lower case. Vector-derived nucleotides are in italic.

**Figure 3.** Effect of mutations in the MRE of SPL3 transgenes on flowering time.

(a) Total leaf numbers (TLN) of wild-type and independent transgenic lines carrying various SPL3::UTR transgenes in Ler and Col backgrounds grown under long-day conditions.

(b) TLN of wild-type and independent transgenic lines carrying various SPL3::UTR transgenes in the Col background grown under short-day conditions.

The transgenes and line numbers are indicated below the bars. TLN values are shown on the y-axis. The numbers within the bars indicate the number of plants analyzed per line, with the exception of the C-UTRΔ3 and C-UTRΔ4 transgenes where it represents the number of independent primary transformants. Values are means ± standard deviations.

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the C35S::SPL3-UTRΔ1 transgenics (Cardon et al., 1997). The early-flowering phenotype of SPL3-UTRΔ2 was also confirmed in transgenic lines with a Ler background (Figure 3a).

C35S::SPL3-UTRΔ3 and -UTRΔ4 transgenics also exhibited early flowering, with an average TLN similar to C35S::SPL3-UTRΔ2 lines, thus indicating that the MRE but not the 3′ UTR sequences downstream of the MRE are involved in the effect of the transgene on flowering time (Figure 3a). In contrast, Col T1 transgenics carrying a full-size wild-type 3′ UTR flowered only slightly earlier than wild-type. This suggests that, due to the disruption of the MRE, SPL3 levels increase, with early flowering as a consequence. Three independent transgenic lines, i.e. C-UTRwt-7, -8 and -9, segregating for a single transgenic locus, were subjected to quantitative analysis. Under both LD and SD conditions, the C35S::SPL3-UTRwt transgenic lines showed a modest decrease in TLN compared to Col wild-type (Figure 3b).

Disruption of the MRE in SPL3 transgenics causes anomalies in flower and inflorescence development

Because all proximal mutations of the SPL3 MRE affected flowering time to a similar extent, we used homozygous T5 35S::SPL3-UTRΔ2 transgenic lines for detailed phenotypic analysis.

In both Ler and Col background, these transgenic lines displayed increased vegetative and floral morphological anomalies in correlation with a prolonged photoperiod and a reduction in flowering time. Under LD and continuous light (CL) conditions, the cotyledons and first rosette leaves appeared normal, but the lamina of later rosette and cauline leaves curled upwards (Figure 4a,c, Supplementary Table S1). Furthermore, the early flowers mostly developed normally, whereas later flowers appeared increasingly abnormal, leading to a complex structure of partially fused sepaloid, stamenoid and carpelloid organs and determinate growth of the inflorescence (Figure 4b, Supplementary Table S1). These anomalies, appearing with a higher frequency in the Ler background (Supplementary Table S1), are depicted in Figure 5.

SPL3 transgenics with a functional MRE are prone to translational repression in seedlings

To uncover the molecular basis for the differences in phenotypic expression of the SPL3-UTRwt and SPL3-UTRΔ2 transgenics, we determined the relative SPL3 transcript and SPL3 protein levels in seedlings and inflorescences of the Col transgenics (Figure 6). Three independent single-copy lines used for flowering time evaluations for each of the two transgenics were selected and compared to Col wild-type. Semi-quantitative RT-PCR performed on 1 μg of total RNA within the linear range of amplification, using a primer pair amplifying an SPL3 cDNA fragment upstream of the pre-
miRNA regulation of SPL3

Not only zygosity but also genomic insertion site, plant generation and environment contribute to position effects and post-transcriptional gene silencing, thus influencing transgene expression. To equalize inter-transgenic differences, thereby allowing a better quantitative comparison, we generated pools of T2 progeny of C35S::SPL3-UTRwt, C35S::SPL3-UTRα3 and C35S::SPL3-UTRα4 transgenic lines. Each of the pools represented 25 independent transgenic lines harvested at the seedling stage. As shown in Figure 6(e), and in agreement with the analysis described above, the different transgenic pools contained comparable SPL3 transcript levels as tested by semi-quantitative RT-PCR. Furthermore, a quantitative real-time RT-PCR analysis performed on a different set of pooled T2 transgenic seedlings showed that levels of SPL3 mRNA were increased 50-fold over wild-type levels in UTRwt transgenics. Relative to the UTRwt transgenics, the levels of SPL3 mRNA increased again another three- to fivefold in the UTRα3 and UTRα4 transgenic lines, respectively (Supplementary Table S2). These findings are consistent with data describing miR156 regulation of SPL3 at the transcriptional level (Chen et al., 2004; Schwab et al., 2005).

In the same samples used for transcript analysis, we determined the relative SPL3 protein levels by Western blot analysis using affinity-purified anti-SPL3 antibodies. In wild-type seedlings, the level of endogenous SPL3 protein remained below the level of detection (Figure 6b). Remarkably, none of the C35S::SPL3-UTRwt transgenic seedlings showed any detectable SPL3 protein despite considerably higher SPL3 transcript levels. In contrast, however, C35S::SPL3-UTRα2 seedlings accumulated appreciable amounts of SPL3 protein. Wild-type inflorescences and inflorescences expressing the C35S::SPL3-UTRwt transgene accumulated SPL3 protein to comparable levels, while the C35S::SPL3-UTRα2 lines expressed clearly higher levels of SPL3 protein (Figure 6d). At the protein level, the pooled lines of C35S::SPL3-UTRα3 and C35S::SPL3-UTRα4, like the single lines of C35S::SPL3-UTRα2, contained substantial levels of SPL3 protein. As found for the corresponding single lines, in the pooled lines of C35S::SPL3-UTRwt, the protein levels at the seedling stage remained consistently almost non-detectable (Figure 6f).

Thus, based on the comparative data on mRNA and protein levels in seedlings and inflorescences, we propose that miR156/157-mediated repression of SPL3 primarily occurs at the level of SPL3 protein accumulation in seedlings.

**Figure 5.** SEM analysis of SPL3 transgenic inflorescences and flower development.
(a) Top view of a Ler wild-type inflorescence. A similar appearance with clearly separated and normal developing floral buds was obtained for Col wild-type (not shown).
(b) Top view of an inflorescence of a L35S::SPL3-UTRα2 transgenic plant.
(c) Top view of an inflorescence of a C35S::SPL3-UTRα2 transgenic plant. The inflorescences shown in (b) and (c) terminate in a complex of structurally highly abnormal flowers formed of largely stamenoid and carpelloid organs.
(d) Young Ler wild-type flower before anthesis, displaying typical and unfused sepals.
(e) Flower of a L35S::SPL3-UTRα2 transgenic plant with severely fused first-whorl organs displaying obvious carpelloid features such as stigmatic papillae (arrow) and ectopic ovules (arrowhead). The inner organs cannot be seen.
(f) Flower of a C35S::SPL3-UTRα2 transgenic plant with partly fused sepals bearing stigmatic papillae (arrow) and ovule primordia (arrowhead). Petals seem to be lacking, but stamen and carpels, albeit incompletely fused, are present.

Not only zygosity but also genomic insertion site, plant generation and environment contribute to position effects and post-transcriptional gene silencing, thus influencing
the wild-type 3' UTR (SPL3-UTRwt) or an UTR with an altered MRE at the proximal end (SPL3-UTRΔ1, -UTRΔ2 and -UTRΔ3). These constructs were biolistically co-transformed into leaf epidermal cells of Arabidopsis wild-type and miR156b over-expressing plants (kindly provided by D. Weigel and R. Schwab, Max Planck Institute for Developmental Biology, Tübingen, Germany) (Schwab et al., 2005), and assayed for the effect of the endogenous and elevated miR156 levels on SPL3 expression. The spectrally distinct fluorescent tags in these transient expression assays allowed us to compare in the same cells the SPL3 protein levels obtained in the presence of a wild-type or a mutated MRE. To correct for a possible discrepancy in the strength of the different fluorophores, they were swapped between the constructs, which also enabled us to measure the influence of miR156 on the respective protein levels under exactly the same conditions. The relative intensities of the two fluorophores were determined using digitized fluorescence microscopy images.

The results of these transient co-expression assays are depicted in Figure 7. In Col wild-type, the difference in expression levels of 35S::YFP:SPL3-UTRΔ1, -UTRΔ2 and -UTRΔ3 compared to 35S::CFP:SPL3-UTRwt was not discernable, as deduced from the near 1:1 YFP:CFP fluorescence ratio. The same conclusion could be drawn after swapping the fluorophores (data not shown; in general the relative fluorescence intensity of the YFP translational fusions was slightly higher than those with CFP). However, the fluorescence ratio was found to differ considerably from 1 when the mutated MREs and SPL3-UTRwt transgenes were transiently expressed in cells from miR156 over-expressing plants. Fusion protein derived from the construct with the non-functional MRE accumulated to levels two- to threefold higher than those produced by the wild-type MRE-carrying construct. While in wild-type leaves both constructs resulted in comparable protein levels, in miR156 over-expressing leaves the construct containing the wild-type MRE yielded noticeably lower levels of SPL3 protein.

Figure 6. Effect of mutations in the MRE of SPL3 transgenes on SPL3 mRNA and SPL3 protein levels in seedlings and inflorescences. (a, c) RT-PCR analysis of SPL3 transcript levels in total RNA isolated from seedlings or inflorescences. Three independent transgenic lines carrying either the C35S::SPL3-UTRwt or C35S::SPL3-UTRwt transgene are compared with Col wild-type. The transgenic lines are indicated above the lanes. RAN3 served as loading control. The leftmost lane contains PCR product amplified from genomic DNA with the same set of primers. It is longer than the RNA-derived bands due to the presence of an intron. (b, d) Western blot analysis of SPL3 protein levels (upper part) in the corresponding samples shown in (a) and (c). The lower parts of both panels show the protein loading control using an antibody against GST and the proteosome small subunit beta.

(a, f) SPL3 RNA and SPL3 protein levels in seedlings carrying the C35S::SPL3-UTRΔ3 or C35S::SPL3-UTRΔ4 transgenes. Total RNA and protein were extracted from a pool of 30 independent lines for each transgene and processed as in (a)-(d). SPL3 and the RAN3 control were separately analysed by RT-PCR starting from the same RNA sample master (unlike in (a) and (c) where all primers were in the same mix).

Figure 7. Effect of mutations in the SPL3 MRE on SPL3 protein level in MIR156b-over-expressing plants. 35S::SPL3-UTRΔ1, -UTRΔ2 or -UTRΔ3 and 35S::SPL3-UTRwt, coding for a fusion protein with either YFP or CFP, respectively, were co-bombarded into leaf epidermal cells from Col wild-type and miR156-over-expressing plants. The relative expression levels of either CFP- or YFP-SPL3 was then analysed by fluorescence microscopy as described in Experimental procedures. The bars represent the mean ± standard deviation.
indicating that this construct is under negative control by miR156 while the construct containing the altered MRE escaped repression by miR156.

Discussion

Conservation of the miR156/157 response element across species

miRNAs 156 and 157 have been shown computationally to simultaneously and exclusively target Arabidopsis SBP box genes (Rhoades et al., 2002). Subsequently, the interaction of miRNA156/157 with SPL (including SPL3) was confirmed by in vitro and in vivo experiments (Chen et al., 2004; Kasschau et al., 2003; Parizotto et al., 2004; Schwab et al., 2005). Here, we provide evidence that the SPL3 MRE does not only function at the mRNA level by mediating degradation of the transcript, but also at the protein level by mediating a translational block, and that its function has consequences on the flowering behaviour of Arabidopsis.

To date, the highly conserved miR156/miR157 have been identified in 45 different plant species (Zhang et al., 2006). Co-evolution of the miRNAs 156/157 and particular SBP box genes from mosses and lycopsids to flowering plants has been shown, with the MRE also conserved in the reading frame and encoding the amino acid residues ALLSLLS (Arazi et al., 2005; Axtell and Bartel, 2005). Furthermore, their developmental expression patterns, as analysed in Arabidopsis (Axtell and Bartel, 2005; Reinhart et al., 2002) and Nicotiana (Válocci et al., 2006), suggest that the interactions between miR156/miR157 and SBP box genes play significant roles in conserved biological functions during plant development.

Precocious appearance of SPL3 protein causes early flowering

Disruption of the SPL3 MRE in transgenics resulted in high levels of temporally mis-expressed SPL3 protein, accompanied by pleiotropic developmental effects such as a considerable decrease in flowering time and severe vegetative and floral abnormalities. In contrast, lines constitutively over-expressing SPL3 transcript in the presence of a non-disrupted MRE showed no SPL3 protein expression in seedlings and only to a moderate level in inflorescence tissues, accompanied by a slightly earlier flowering phenotype. This strongly suggests that the severity of the SPL3 transgenic phenotype is associated with the SPL3 protein level.

Remarkably, SPL3-UTRΔ1, carrying a 4 nt mismatch to the 5’ end of miR156 due to truncation of the SPL3 MRE, did not result in such dramatic reduction of flowering time (Cardon et al., 1997) as observed with the other mutants. Studies on pairing requirements between miRNA and its target suggest that mismatches to the 5’ region of the miRNA lower the efficiency of miRNA binding to the target and subsequently its down-regulation (Doench and Sharp, 2004; Kiriakidou et al., 2004; Mallory et al., 2004; Parizotto et al., 2004; Schwab et al., 2005). An explanation of this seeming discrepancy could well be that the SPL3-UTRΔ1-carrying transgene constructed previously by Cardon et al. (1997) was not only based on a slightly different transformation vector, but, probably more importantly, the corresponding transgenic lines were obtained through a tissue culture-dependent transformation protocol that may have selected against more severe phenotypes. This conclusion is further supported by the transient expression studies of SPL3-UTRΔ1, -UTRΔ2 and -UTRΔ3, which show comparable CFP/YFP expression in the Col background (Figure 7). It is interesting to note that Lännenpää et al. (2004) neither observed reduction in flowering time nor floral abnormalities in transgenic Arabidopsis constitutively over-expressing the presumed SPL3 ortholog from birch. This might be due to functional differences between the orthologs, but we consider it to be due to the fact that their construct still contained the intact 3’ UTR MRE.

SPL3 transcript levels rise at the onset of flowering, preceding activation of the floral meristem identity gene AP1. This increase is delayed in under SD conditions (Cardon et al., 1997). Charting the global expression profiles of the Arabidopsis shoot apex identified SPL3 as third among the top 500 genes induced in response to photoperiodic flowering between days 0 and 7 in Col and Ler (Table S4 of Schmid et al., 2003). The photoperiod response was found to be attenuated by mutations of CONSTANS (CO), a gene required for sensing day length, or of its immediate target FLOWERING TIME (FT) (Samach et al., 2000). FT is required for the integration of different flowering pathways (Boss et al., 2004). Based on these observations, we assume that SPL3 acts downstream of the photoperiod-dependent flowering pathway. The present study shows that the constitutive SPL3 over-expressing transgenics exhibit photoperiod-sensitive flowering (Figure 3), and morphological abnormalities (Figures 4 and 5). Furthermore, we found that constitutive over-expression of SPL3 forces the late-flowering co and ft mutants to flower earlier, albeit not as early as when over-expressed in wild-type (Cardon and Huijser, unpublished results). Hence we conclude that SPL3 function is dependent, at least in part, on factors controlled by photoperiod. In this context, it should be noted that the other SPL genes carrying an miR156/157 MRE are also responsive to photoperiodic induction (Figure 10c, Schmid et al., 2003).

Ectopic expression of SPL3 protein causes morphological abnormalities

MiR156/157 levels are most abundant in seedlings (Axtell and Bartel, 2005; Reinhart et al., 2002; Válocci et al., 2006), suggesting that miR156/157 are necessary to repress expression of SPL3 and other SPL genes during early stages.
of Arabidopsis development. Similarly, moderate levels of miR156/157, as found in rosette and cauline leaves (Axtell and Bartel, 2005), may be required for proper leaf development. This interpretation is supported by the observation of a leaf curling phenotype caused by ectopic mis-expression of SPL3 lacking a functional MRE. Active repression of SPL3 thus seems to be required for proper vegetative development, and a failure results in early flowering.

Hence, one could speculate that the function of SPL3 in normal development is to promote reproductive growth, in particular carpel development and determinate growth as deduced from the floral and inflorescence anomalies seen in transgenics (Figures 4 and 5, Supplementary Table S1). As carpelloidty of sepal and determinate growth in SPL3 transgenics could only be observed in the absence of a functional MRE, we assume that in these organs and tissues miR156/157 is required to modulate SPL3 transcript and protein levels. The over-representation of miR156 in ovules and meristematic leaf tissues, as shown by in situ analysis (Váločzi et al., 2006), reflects the significance of miR156 in these tissues in modulating SPL levels, including SPL3. However, in the absence of loss-of-function mutants, the precise role of SPL3 in normal development remains unclear.

miRNA-directed translational repression versus target degradation

Analysis of the influence of miR156/157 on transcript and protein levels in transgenic SPL3-UTRwT seedlings demonstrated the absence of productive translation despite a considerable increase in the SPL3 transcripts. However, in transgenic lines of SPL3-UTRA2 and in pooled lines of SPL3-UTRA3 and -UTR4 with impaired MREs, not only SPL3 mRNA levels but also SPL3 protein levels were high. This suggests that translational repression is the primary mode of post-transcriptional gene silencing of SPL3 in seedlings. In addition, miRNA-directed degradation of the transcript occurs when the MRE is intact, as indicated by the higher mRNA levels accumulated in transgensics carrying mutated versions of the MRE (Figure 6e). The cleavage of SPL3 transcript in planta has been demonstrated by detection of the degradation products (Chen et al., 2004), and this was given further support in miR156 over-expressing lines by use of expression arrays (Schwab et al., 2005). Moreover, earlier studies showed that SPL3 transcript levels were elevated in hasty mutants, which accumulate less miR156 (Park et al., 2005). Elevated mRNA levels were also found in mutants such as sgs3, zip and rdr6, which act in the regulatory pathways promoting transition form the juvenile to adult phase (Peragine et al., 2004), suggesting an miR156/157-dictated control of SPL3 at the transcript level. Further, the reduced levels of miR156 in miRNA biosynthetic mutants such as dcl1, hyl1 and hen1, resulting in the elevation of SPL2 and SPL10 transcript levels (Vazquez et al., 2004), indicates transcript degradation of SBP box gene family members whose MRE is in the ORF. However, our study demonstrates considerable translational repression of the SPL3 protein governed by miR156/miR157 in young Arabidopsis seedlings.

In contrast, the reduced miR172 levels in hen1-1, hen1-2 or dcl1-9 mutants do not affect AP2 mRNA levels, while the AP2 protein levels are increased (Chen, 2004) in agreement with the idea that translational repression is the major form of post-transcriptional gene silencing of miR172-directed AP2 gene regulation. It is also interesting that, in hasty mutants, the levels of miR172 are normal or slightly elevated (Park et al., 2005), perhaps due to the association of miR172 with its target and its export by the mRNA export pathway, like many translational repressors in animals (Dreyfuss et al., 2002). Details on how miRNA mechanistically affects the translational machinery are lacking in plants.

The expressivity of the phenotype of the C35S::SPL3-UTRA2, -UTRA3 and -UTRA4 transgenic plants, accumulating higher levels of SPL3 RNA and SPL3 protein, was evident through a concomitant decrease in flowering time. However, line 5 shows lower levels of SPL3 protein compared with parallel lines (Figure 6b), possibly due to differences in the extent of translational repression that are not yet understood. To circumvent such quantitative individual differences, we analysed per transgene pools of lines with the same outcome (Figure 6b).

In inflorescences, where SPL3 mRNA could be detected in similar amounts as in seedlings, the UTRwT lines also showed appreciable protein levels. The preferential suppression of translation of SPL3-UTRwT-derived transcripts in seedlings correlates with the highest abundance of both miR156 and miRNA157 in seedlings and young leaves, and lowest levels in inflorescences (Axtell and Bartel, 2005; Reinhart et al., 2002; Váločzi et al., 2006). Additionally, cleavage of the SPL2 transcript mediated by miR156/157 was found to be decreased preferentially in inflorescences, while levels of the SPL2 cleavage products were elevated in the seedlings of TuMV-encoded RNA silencing suppressor protein P1/HC-Pro (Kasschau et al., 2003).

Evidence suggests that different SPL genes are transcriptionally active during the course of development and generate high transcript levels in inflorescences and flowers (Schmid et al., 2005; Zimmermann et al., 2004). Releasing the translational block by master regulators miR156/157 would then synchronize and orchestrate different SPL gene functions as required, allowing fine tuning of their expression.

Millar and Waterhouse (2005) suggested that the miRNA-directed transcriptional cleavage and translational repression pathways overlap, as both involve the same ribonucleoprotein complex (miRNP). Binding to the mRNA alone should be sufficient for a translational block, while mRNA is cleaved with more or less efficiency, depending on the extent of sequence complementarity. However, the two
examples in plants, miR172 with AP2 and miR156/157 with SPL3, which have near-perfect sequence homology, prefer translational repression over transcript degradation, thus refuting the hypothesis that the extent of homology is the deciding factor for plant miRNA-mediated regulation of the target. Although, in general, gene suppression by miRNA primarily occurs by RNA cleavage as reported previously, focused studies at the protein level, such as the present one, are nevertheless essential to determine the extent of translational repression.

Future experiments may clarify whether miR172- and miR156/157-guided repression of AP2 and SPL3 protein accumulation, respectively, is based on similar or different mechanisms.

Experimental procedures

Plant material and growth conditions

Arabidopsis plants were grown in plastic trays filled with ready-to-use commercial, pre-fertilized soil mixture (Type ED73, Werkverband EV, Sinntal-Jossa, Germany). For stratification, seeds were kept on wet filter paper for 4 days at 4°C in the dark before transferring to soil. To compare flowering time under long-day (LD) conditions, plants were grown under greenhouse conditions with additional light to obtain a day length of 16 h. To score morphological abnormalities and flowering time under short-day (SD) conditions (8 h light, 16 h dark), plants were cultivated in growth chambers at 22°C, 50% relative humidity and under approximately 150 μE m⁻² sec⁻¹ light (fluorescent Sylvania F72T12 cold white light, 75%, and incandescent Sylvania 100 W lamps, 25%).

Transgene constructs and generation of transgenic plants

The 35S::SPL3-UTRΔ1 transgenic plant in the Col background was generated through root transformation using the vector pG1-35S, with Hpt as a selectable marker (Cardon et al., 1997). The corresponding transgene SPL3-UTRΔ1 carries a distally truncated version of SPL3 cDNA, with the four most proximal nucleotides of the MRE and all downstream sequences of the three UTR deleted (see Figure 2 for all MRE mutations and their deviations from miR156C). In SPL3-UTRΔ2, a similar truncation of the three UTR deleted nine nucleotides of the MRE and all downstream sequences. SPL3-UTRΔ3 carries a ten-nucleotide exchange at the proximal end and SPL3-UTRΔ4 a nine-nucleotide exchange at the distal end of the SPL3 MRE, with the remaining part of the three UTR unchanged as in SPL3-UTRwt. SPL3-UTRΔ2, Δ3 and Δ4, as well as SPL3-UTRwt, were cloned into the XbaI site of the binary vector pBAR35S, a pBIN19 derivative with Bar as a selectable marker and a CaMV 35S promoter/terminator cassette identical to the one present in pG1-35S (Cardon et al., 1997). They were transformed into the Col or Ler background by vacuum infiltration (Bechtold and Pelletier, 1998). Transgenic plants or lines carrying one of the above-mentioned transgenes are either referred to as C35S::SPL3-UTR or L35S::SPL3-UTR.

Transient expression of SPL3 fused to CFP and YFP

For transient expression, SPL3-UTRwt and SPL3-UTRΔ1, -UTRΔ2 and -UTRΔ3 were cloned into the gateway destination vectors pEnSG-CFP and pEnSG-YFP to create C-terminal fusions to CFP and YFP according to protocols provided by Invitrogen (Carlsbad, CA, USA). The constructs were used in the subsequent studies.

For bombardment, miR156b-over-expressing plants (Schwab et al., 2005) and control Col plants were grown under LD conditions for 3 weeks. The leaves were harvested and placed on MS medium, abaxial side up. DNA coating of gold particles and bombardment was done according to the protocol described by Panstruga et al. (2003). To determine the relative fluorescence light intensity of nuclear-localized CFP- or -YFP-SPL3 derived from 35S::SPL3-UTRwt with YFP, or CFP-SPL3 derived from 35S::SPL3-UTRΔ1, -UTRΔ2 or -UTRΔ3, 10–15 single cells were recorded by fluorescence microscopy (Axioskop, Zeiss, Göttingen, Germany, equipped with a KY-F5U 28CCD camera, JVC, Yokohama, Japan) such that all pixel values remained within the dynamic range.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from seedlings and inflorescences of the Col wild-type and transgenic lines using the RNeasy plant mini kits (Qiagen, Hilden, Germany). RT-PCR with equal amounts of RNA was performed using the one-step RT-PCR kit (Qiagen) with the SPL3 adapter-linked specific primer pair GGTCTAGAATTGTTGTGCTAGAGAGAGTACGACGAGGATGTGAC (forward) and CATCTAGATTGTCAGTTGTGCCTTTTCGCCCT (reverse), which amplifies a 240 bp SPL3 fragment spanning the entire SBP box of the SPL3 transcript including the 19 bp adaptor, to yield the final product of 259 bp. Amplification of the SPL3 genomic fragment, including the 91 bp intron and the 19 bp adaptor to yield a final product of 350 bp, served as genomic control to exclude any DNA contamination of the RNA preparations. RT-PCR of the loading control (RAN3; At5g55190) was also performed in the same reaction vial with the primer pair ACCAAGAACGTGAGATTCCCTAG (forward) and ATCCACAAAGTGAAGATCTAGCCT (reverse) to yield 531 bp RAN3 mRNA (genomic RAN3 is expected to yield 1314 bp). The reactions were stopped when they were still in the linear range of amplification.

The linear range of amplification was determined by running an increasing cycle number and analysing the amount of cDNA fragments on 2% agarose gels. RT-PCR analysis for Figure 6(e) was performed separately for SPL3 and the RAN3 control.

Real-time quantitative RT-PCR

To perform quantitative RT-PCR (using the iQ5 real-time PCR detection system, Bio-Rad, Munich, Germany) total RNA was extracted from pooled one-week-old transgenic seedlings using the RNeasy plant mini kit (Qiagen), including an on-column DNase digestion. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) starting with 2 μg of total RNA primed with an oligo(T)12–18 primer (Gibco BRL, Karlsruhe, Germany). SPL3-specific primers, CAAGTACTAGTGAGGAGTGTGTGCTAG (forward) and TTTCGCGCTCTCTCCTGTTGTC (reverse), were designed to generate a PCR product of 239 bp. Quantitative RT-PCR of RAN3 (see above for primer pair) was performed for normalization. The PCR efficiency for SPL3 and RAN3 primers was 93.8% and 93.7%, respectively. Quantifications, in triplicate, were performed using the Brilliant SYBRGreen QPCR kit (Stratagene), according to the manufacturer's protocol, in a final volume of 25 μl. PCR was carried out in 250 μl optical reaction vials (Stratagene, La Jolla, CA, USA) heated for 10 min at 95°C to hot-start the Taq polymerase, followed by 40 cycles of denaturation (30 sec at 95°C), annealing (1 min at 56°C) and extension (1 min at 72°C).
Antibodies and Western blot analysis

Recombinant SPL3 protein, containing an N-terminal DHFRS and a C-terminal His tag, was produced in *Escherichia coli* using expression vector pQE16 (Qiagen). The protein was purified under denaturing conditions via the His tag according to the Qiagen protocol. Before immunization of rabbits, urea was replaced by PBS. SPL3-specific antibodies were purified from the polyclonal serum by affinity chromatography using the recombinant protein. The specificity of the anti-SPL3 antibodies was established by the lack of cross-reactivity on Western blots to closely related over-expressed SPL4 and SPL5 proteins.

For Western blots, total protein extracts from seedlings and inflorescences were prepared from 200 mg of tissue, which was homogenized in lysis buffer (2 × Laemmli SDS sample buffer), ground with a quarter volume of sand for 1 min, boiled for another 2 min, and centrifuged in a table-top centrifuge for 2 min at approximately 16,000 g. Aliquots (15 μl) of the supernatants, containing approximately 30 μg of protein, were separated by reducing SDS–PAGE (15%), and transferred to nitrocellulose membrane. The membranes were blocked for 1 h with 5% non-fat dry milk in PBST buffer, rinsed, the membranes were incubated for 1 h with the first specific antibodies was established by the lack of cross-reactivity on Western blots to closely related over-expressed SPL4 and SPL5 proteins.

Scanning electron microscopy

Samples for SEM analysis were prepared as described by Sommer et al. (1990), and images were taken with a Zeiss DSM 940 electron microscope (Zeiss, Göttingen, Germany).

Remaining techniques and methods

Standard molecular biology techniques were performed as described by Sambrook et al. (1989). Digital photographic images were cropped and assembled using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Frequencies of morphological abnormalities observed in different photoperiods

Table S2 Real-time PCR analysis of SPL3 transcript levels in seedlings of C-UTrw-7, -Δ2, -Δ3 and -Δ4 transgenics compared to non-transgenic Col-0

This material is available as part of the online article from http://www.blackwell-synergy.com

References


Accession numbers: The GenBank accession numbers of *SPL3* and *SPL3*-related genes are as follows: Y09427 (*A. thaliana* cv. Col *SPL3*), X92369 (*Antirrhinum majus* *SPL1*) and AJ558183 (*Betula pendula* *BPSPL1*).