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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; Chiou *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 *SPL13* 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 *SPL13* loci (known as *SPL13A* 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*; Lännenpää *et al.*, 2004) shows that the miR156/157-related MRE in the 3′ UTR

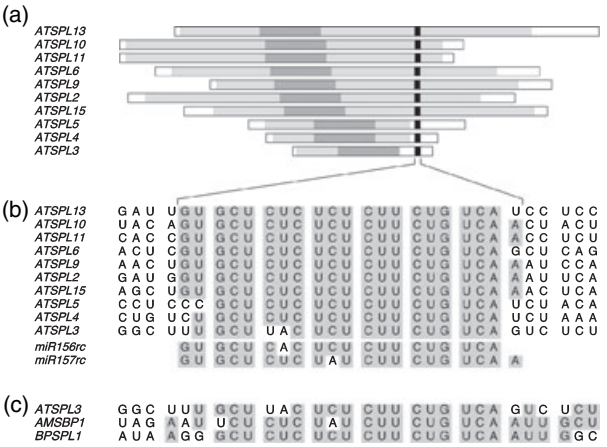

Figure 1. Position and sequence conservation of the predicted MRE within the *SPL* gene family and *SPL3* orthologous SBP box genes.
(a) Graphic representation of the cDNAs of all *SPL* gene family members carrying the predicted MRE (black box). Coding sequences are shaded grey, the conserved SBP box dark grey. Note that in *SPL3*, *4* and *5*, the predicted MRE is located in the non-coding 3′ UTR.
(b) Alignment of the predicted MRE sequences within the *SPL* mRNAs. To allow direct comparison with miR156C and miR157C, the reverse complementary sequences of these miRNAs are included.
(c) Alignment of the predicted MRE sequences within the 3′ UTRs of mRNAs of putative *SPL3* orthologs.
Nucleotides conserved in more than half of the aligned sequences shown in (b) and (c) are shaded. *AT*, *Arabidopsis thaliana*; *AM*, *Antirrhinum majus*; *BP*, *Betula pendula*.

SPL3-	MRE::miR156 complementarity
UTRwt	5'..GCUUUGCUUACUCUCUUCUGUCAGUC.. ::::·::::::::::::::: 3' CACGAGUGAGAGAAGACAGU
UTRΔ1	5'..GCUUUGCUUACUCUCUUCU*cgagGgg*.. ::::·:::::::::::: · 3' CACGAGUGAGAGAAGACAGU
UTRΔ2	5'..GCUUUGCUUACUCU*agagUcgaccUg*.. ::::·::::: : 3' CACGAGUGAGAGAAGACAGU
UTRΔ3	5'..GCUUUGCUUACUC*gaggagacuu*GUC.. ::::·:::: · 3' CACGAGUGAGAGAAGACAGU
UTRΔ4	5'..GCUU*gaagcuugagC*UUCUGUCAGUC.. : · ::::::::: 3' CACGAGUGAGAGAAGACAGU

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 '·'. Deviations from *SPL3* wild-type sequence are in lower case. Vector-derived nucleotides are in italic.

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-UTR*Δ2 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-UTR*Δ3 and *35S::SPL3-UTR*Δ4 were generated with a full-size 3′ UTR, and only the predicted miR156/157 recognition element was mutated. *35S::SPL3-UTR*Δ3 carries a 10 nt substitution in the proximal half and *35S::SPL3-UTR*Δ4 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-UTR*Δ1. 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-UTR*Δ2 transgene was introduced into Col and L*er* backgrounds through *Agrobacterium*-mediated transformation (*C35S::SPL3-UTR*Δ2 and *L35S::SPL3-UTR*Δ2), all T_1 transgenic plants flowered earlier than wild-type. In addition they exhibited morphological defects.

Of the 32 *C35S::SPL3-UTR*Δ2 (T_1) plants, three independent Col transgenic lines (*C-UTR*Δ*2-2*, *-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 T_5 plants.

Under both long-day (LD) and short-day (SD) conditions, the *C35S::SPL3-UTR*Δ2 transgenic lines flowered clearly earlier than wild-type (TLN 5 or 6 and TLN 30–36, respectively; Figure 3). The *C35S::SPL3-UTR*Δ2 transgenics thus remained photoperiod-sensitive as previously reported for

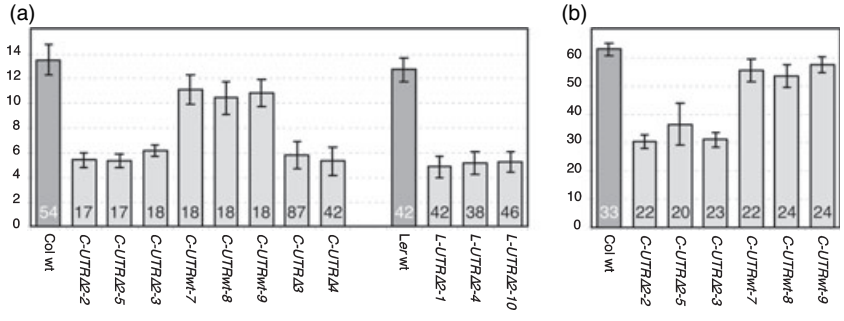

Figure 3. Effect of mutations in the MRE of *SPL3* transgenes on flowering time.
(a) Total leaf numbers (TLN) of wild-type and independent transgenic lines carrying various *SPL3::UTR* transgenes in L*er* and Col backgrounds grown under long-day conditions.
(b) TLN of wild-type and independent transgenic 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-UTR*Δ3 and *C-UTR*Δ4 transgenes where it represents the number of independent primary transformants. Values are means ±standard deviations.

the *C35S::SPL3-UTR*Δ1 transgenics (Cardon *et al.*, 1997). The early-flowering phenotype of *SPL3-UTR*Δ2 was also confirmed in transgenic lines with a L*er* background (Figure 3a).

*C35S::SPL3-UTR*Δ3 and -*UTR*Δ4 transgenics also exhibited early flowering, with an average TLN similar to *C35S::SPL3-UTR*Δ2 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 T_1 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 *transgenes 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 T_5 *35S::SPL3-UTR*Δ2 transgenic lines for detailed phenotypic analysis.

In both L*er* 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 L*er* background (Supplementary Table S1), are depicted in Figure 5.

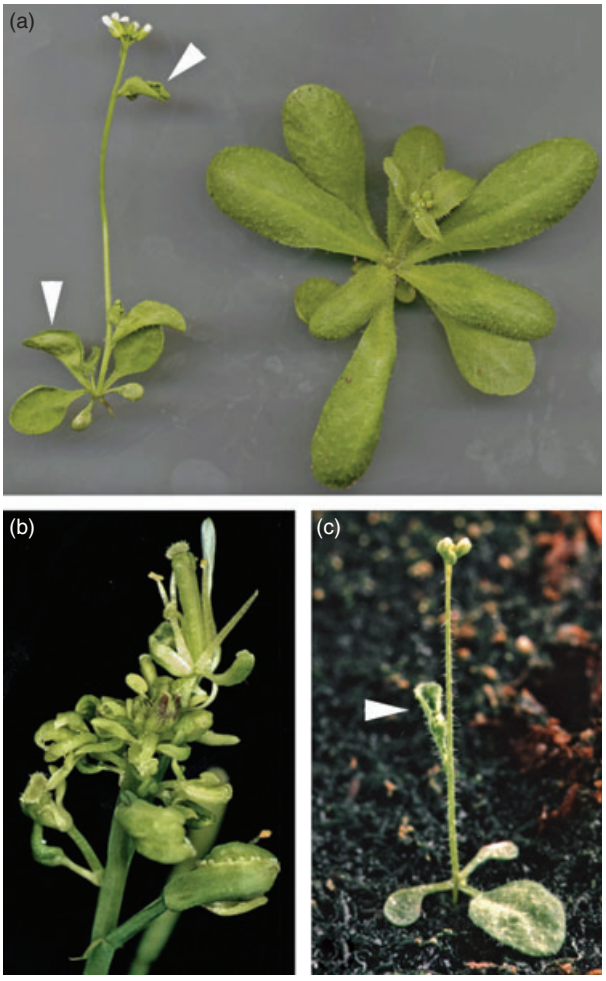

Figure 4. Phenotype of *SPL3* transgenic plants.
(a) L*er* wild-type plant (right) compared with a plant expressing the *L35S::SPL3-UTR*Δ2 transgene (left) after 19 days under long-day conditions. The transgenic plant flowers earlier after having formed fewer leaves.
(b) Inflorescence tip of a *L35S::SPL3-UTR*Δ2 transgenic plant grown under continuous light. Note the complex arrangement of largely abnormal flowers and floral organs terminating further inflorescence development.
(c) A very-early-flowering *C35S::SPL3-UTR*Δ2 transgenic plant grown under continuous light. In addition to the two cotyledons, only one rosette leaf and one cauline leaf were formed before the appearance of the first flower. Arrowheads in (a) and (c) indicate the strongly curled leaves of the transgenic plants.

SPL3 *transgenes 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-UTR*Δ2 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 transgenes 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 presumptive MRE, revealed that the transgenic seedlings contained elevated levels of *SPL3* transcript, with *UTR*Δ2 resulting in higher levels than *UTRwt* (Figure 6a). In inflorescences, the endogenous *SPL3* mRNA levels were higher than in seedlings. Here, however, the *C35S::SPL3-UTRwt* and *C35S::SPL3-UTR*Δ2 transgenic inflorescences showed only a moderately increased *SPL3* transcript level compared to wild-type, with less difference between *UTR*Δ2 and *UTRwt* (Figure 6c). It should be noted that the three *C35S::SPL3-UTR*Δ2 lines (*C-UTR*Δ2-2, -5, -3) represented the progeny of homozygous T_4 plants, whereas the *C35S::SPL3-UTRwt* lines (*C-UTRwt*-7, -8, -9) were T_1 plants.

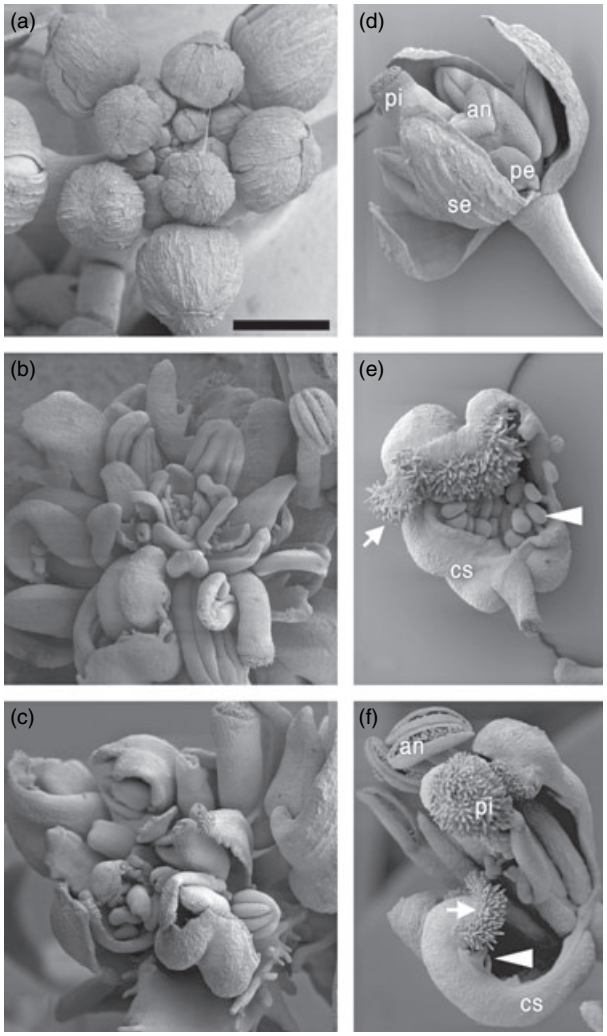

Figure 5. SEM analysis of *SPL3* transgenic inflorescences and flower development.
(a) Top view of a L*er* wild-type inflorescence. A similar appearance with clearly separated and normal developing floral buds was obtained for Col wild-type (not shown).
(b) Top view of an inflorescence of a *L35S::SPL3-UTR*Δ2 transgenic plant.
(c) Top view of an inflorescence of a *C35S::SPL3-UTR*Δ2 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 L*er* wild-type flower before anthesis, displaying typical and unfused sepals.
(e) Flower of a *L35S::SPL3-UTR*Δ2 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-UTR*Δ2 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.
an, anther; cs, carpelloid sepal; pe, petal; pi, pistil; se, sepal. Images are all at the same scale. Bar = 500 μm.

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 T_2 progeny of *C35S::SPL3-UTRwt*, *C35S::SPL3-UTR*Δ3 and *C35S::SPL3-UTR*Δ4 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 T_2 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 *UTR*Δ3 and *UTR*Δ4 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-UTR*Δ2 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-UTR*Δ2 lines expressed clearly higher levels of SPL3 protein (Figure 6d). At the protein level, the pooled lines of *C35S::SPL3-UTR*Δ3 and *C35S::SPL3-UTR*Δ4, like the single lines of *C35:SPL3-UTR*Δ2, 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.

Transient expression analysis confirms the interaction of miR156 with the SPL3 *MRE*

The severe reduction of flowering time coupled with the vegetative and gross floral abnormalities observed suggests that disruption of the MRE at its proximal end in *SPL3-UTR*Δ2 is sufficient to affect the interaction of miRNA156 with *SPL3*. *In* order to obtain more direct evidence of the functional interaction of miR156 with the *SPL3* MRE, we generated CFP- and YFP-SPL3 translational fusion constructs carrying either

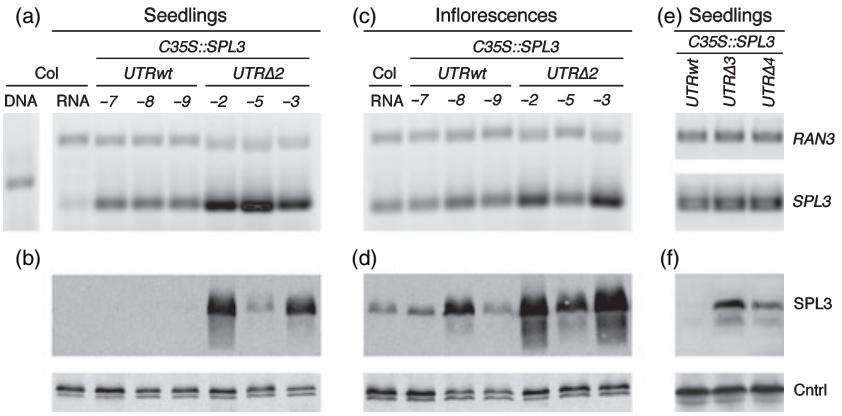

Figure 6. Effect of mutations in the MRE of *SPL3* transgenes on *SPL3* mRNA and SPL3 protein levels in seedlings and inflorescences.
(a, c) RT-PCR analysis of *SPL3* transcript levels in total RNA isolated from seedlings or inflorescences. Three independent transgenic lines carrying either the *C35S::SPL3-UTR*Δ2 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 alpha.
(e, f) *SPL3* RNA and SPL3 protein levels in seedlings carrying the *C35S::SPL3-UTRwt*, *C35S::SPL3-UTR*Δ3 or *C35S::SPL3-UTR*Δ4 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].

the wild-type 3′ UTR (*SPL3-UTRwt*) or an UTR with an altered MRE at the proximal end (*SPL3-UTR*Δ1, *-UTRΔ2 and -UTR*Δ3). 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-UTR*Δ1, *-UTRΔ2 and -UTR*Δ3 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

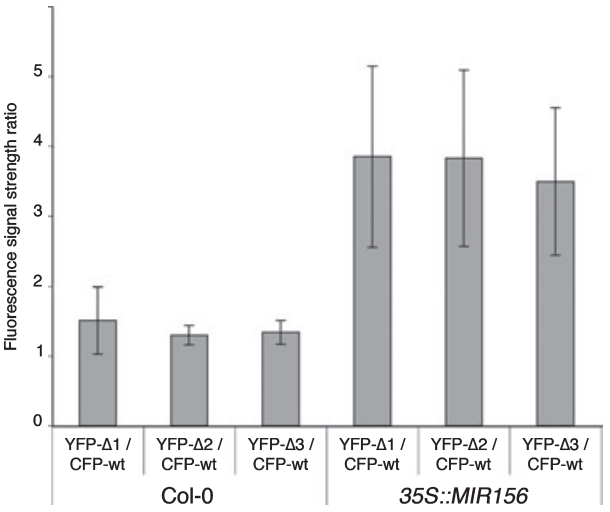

Figure 7. Effect of mutations in the *SPL3* MRE on SPL3 protein level in MIR156b-over-expressing plants.
*35S::SPL3-UTR*Δ1, *-UTR*Δ2 or *-UTR*Δ3 and *35S::SPL3-UTRwt*, coding for a fusion protein with either YFP or CFP, respectively, were co-bombarded into leaf epidermal cells from Col wild-type and 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.

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,

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

miRNAs156 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 lycopods 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* (Válóczi *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-UTR*Δ1, 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-UTR*Δ1-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-UTR*Δ1, *-UTR*Δ*2 and -UTR*Δ3, 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 L*er* (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; Válóczi *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 carpelloidy of sepals 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 (Válóczi *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-UTR*Δ2, and in pooled lines of *SPL3-UTRΔ3 and* -Δ4 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 transgenics 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-UTR*Δ2, *-UTR*Δ3 and *-UTR*Δ4 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; Válóczi *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 $\mu E\ m^{-2}\ sec^{-1}$ 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-UTR*Δ1 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-UTR*Δ1 carries a distally truncated version of *SPL3* cDNA, with the four most proximal nucleotides of the MRE and all downstream sequences of the 3′ UTR deleted (see Figure 2 for all MRE mutations and their deviations from miR156C). In *SPL3-UTR*Δ2, a similar truncation of the 3′ UTR deleted nine nucleotides of the MRE and all downstream sequences. *SPL3-UTR*Δ3 carries a ten-nucleotide exchange at the proximal end and *SPL3-UTR*Δ4 a nine-nucleotide exchange at the distal end of the *SPL3* MRE, with the remaining part of the 3′ UTR unchanged as in *SPL3-UTRwt*. *SPL3-UTR*Δ2, -Δ3 and -Δ4, as well as *SPL3-UTRwt*, were cloned into the *Xba*I 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 L*er* 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-UTR*Δ1, -*UTR*Δ2 and -*UTR*Δ3 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-UTR*Δ1, -*UTR*Δ2 or -*UTR*Δ3, 10–15 single cells were recorded by fluorescence microscopy (Axiophot, 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 GTTCTAGAATGGTTTGTCAGGTCGAGAGTTGTAC (forward) and CATCTAGATTAGTCAGTTGTGCTTTTCCGCCT (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 ACCAGCAAACCGTGGATTACCCTAGC (forward) and ATTCCACAAAGTGAAGATTAGCGTCC (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 RNase H reverse transcriptase (Invitrogen) starting with 2 μg of total RNA primed with an oligo$(T)_{12\text{-}18}$ primer (Gibco BRL, Karlsruhe, Germany). *SPL3*-specific primers, CAAGTAGTAGTGGAGTTTGTCAGGTCG (forward) and TTTCCGCCTTCTCTCGTTGTGTCC (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 83.7%, 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 boiled for 2 min in 200 μl 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 (137 mM $NaCl_2$, 2.7 mM KCl, 10 mM KH_2PO_4, 0.1% Tween-20). After rinsing, the membranes were incubated for 1 h with the first antibody in PBST (affinity-purified anti-SPL3, with affinity-purified anti-GST-proteosome small subunit alpha as loading control). After washing three times for 5 min in PBST, the immune complexes were detected by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham, Buckinghamshire, UK) for 30 min. After extensive washing in PBST, blots were developed with the ECL Plus Western blotting detection system (Amersham) and exposed to X-ray films.

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-UTRwt-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

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Accession numbers: The GenBank accession numbers of *SPL3* and *SPL3*-related genes are as follows: Y09427 (*A. thaliana* cv. Col *SPL3*), X92369 (*Antirrhinum majus SBP1*) and AJ558183 (*Betula pendula BPSPL1*).

