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The microRNA regulated SBP-box genes *SPL9* and *SPL15* control shoot maturation in Arabidopsis

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Abstract Throughout development the Arabidopsis shoot apical meristem successively undergoes several major phase transitions such as the juvenile-to-adult and floral transitions until, finally, it will produce flowers instead of leaves and shoots. Members of the Arabidopsis SBP-box gene family of transcription factors have been implicated in promoting the floral transition in dependence of miR156 and, accordingly, transgenics constitutively over-expressing this microRNA are delayed in flowering. To elaborate their roles in Arabidopsis shoot development, we analysed two of the 11 miR156 regulated Arabidopsis SBP-box genes, i.e. the likely paralogous genes *SPL9* and *SPL15*. Single and double mutant phenotype analysis showed these genes to act redundantly in controlling the juvenile-to-adult phase transition. In addition, their loss-of-function results in a shortened plastochron during vegetative growth, altered inflorescence architecture and enhanced branching. In these aspects, the double mutant partly phenocopies constitutive *MIR156b* over-expressing transgenic plants and thus a major contribution to the phenotype of these transgenics as a result of the repression of *SPL9* and *SPL15* is strongly suggested.

Keywords Arabidopsis · Juvenile phase · miRNA · Phase change · SBP-box genes · Shoot maturation

Abbreviations

DAS	Days after sowing
GA	Gibberellic acid
LD	Long day
miRNA	microRNA
qRT-PCR	Quantitative real-time PCR
SAM	Shoot apical meristem
SBP	SQUAMOSA PROMOTER BINDING PROTEIN
SD	Short day
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*

Introduction

During maturation, plants pass through several developmentally distinct growth phases in which the shoot gradually gains reproductive competence (Poethig 1990). After the transition from embryonic to postembryonic growth, plants undergo at least two further phase transitions, the vegetative as well as the reproductive phase change. During vegetative growth, rosette leaves are initiated at the flanks of the shoot apical meristem (SAM) with a certain frequency, referred to as plastochron (Erickson and Michelini 1957). After going through the reproductive phase transition, also known as the floral transition, the SAM starts to initiate floral buds instead of leaves. In Arabidopsis, as in many other plants showing day length dependent flowering, the floral transition is preceded by a transition from juvenile to adult growth. This switch, known as the vegetative phase change, is physiologically defined as achieving competence to respond to photoperiodic induction of flowering (Poethig 1990).

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The transition from juvenile to adult growth is gradual and rather subtle but generally can be followed by several morphological markers. In Arabidopsis, for example, leaves produced in the juvenile phase have long petioles, are small, round and lack abaxial trichomes. In contrast, short petioles, elliptical anatomy and the development of trichomes on the abaxial side represent adult traits (Telfer et al. 1997).

Regulation of these developmental transitions is largely dependent on (changes in) environmental cues such as day length, light intensity and temperature, as well as on endogenous factors such as the plant hormone gibberellin (Telfer et al. 1997). Whereas the molecular genetic mechanisms underlying the floral transition are already worked out in increasing detail (Komeda 2004), it has only been recently that we begin to understand the molecular genetic basis of the vegetative phase change. Most genes suggested to play a role in promoting the latter phase change have been identified by the analysis of mutants showing a precocious onset of adult traits and intriguingly, link vegetative phase change to RNA silencing pathways. These genes include the Arabidopsis ortholog of *exportin 5/MSN5*, *HASTY* (*HST*; Telfer and Poethig 1998; Bollman et al. 2003), the zinc-finger-domain protein encoding locus *SERRATE* (*SE*; Clarke et al. 1999) and *ZIPPY* (*ZIP*), an AGO-family member (Hunter et al. 2003). More recently, screens for mutations with *zip*-like phenotypes resulted in alleles of *SUPRESSOR OF GENE SILENCING3* (*SGS3*) and *RNA-DEPENDENT POLYMERASE6* (*RDR6*), both genes required for posttranscriptional gene silencing (PTGS) and acting in the same pathways as *ZIP* and *HST* (Peragine et al. 2004). Furthermore, a precocious vegetative phase change has also been found in *dicer-like 4* (*dcl4*) mutants (Gasciolli et al. 2005; Xie et al. 2005; Yoshikawa et al. 2005).

One explanation for the observed effects could be that target genes of this silencing pathway play a positive role on the vegetative phase change and their down-regulation consequently promotes juvenility. Hence, mutations in genes involved in this silencing pathway, as the ones described above, cause an accelerated vegetative development.

In line with this idea, members of the plant specific SBP-box gene transcription factor family have been implicated in promoting vegetative and floral phase transitions. In particular, overexpression of the Arabidopsis SBP-box gene *SPL3* leads to early flowering and a significant earlier appearance of abaxial trichomes on the rosette leaves (Cardon et al. 1997; Wu and Poethig 2006). Interestingly, together with 10 of 16 other family members, *SPL3* expression is post-transcriptionally controlled by miR156 and probably also by the very closely related miR157 (Rhoades et al. 2002; Schwab et al. 2005; Wu and Poethig 2006; Gandikota et al. 2007). Consistent with its role of down-regulating *SPL3* and related *SPL* target-genes, constitutive overexpression of miR156 encoding loci has been shown to cause the production of a significantly larger number of leaves with juvenile characteristics and a delay in flowering (Schwab et al. 2005; Wu and Poethig 2006).

Although the available data clearly point to a regulatory role for the miRNA regulated *SPL* genes in the temporal development of the Arabidopsis shoot, the contribution of the single genes to the described phenotypes remains to be determined. Therefore, we identified and isolated mutant alleles for single *SPL* genes. In comparison to other miR156 targeted *SPL* genes, available expression data (AtGenExpress; Schmid et al. 2005) show *SPL9* and *SPL15* to be already quite active in the vegetative shoot apex. Accordingly, their mutant phenotypes were found to affect vegetative development. Here we report the mutant analysis of *SPL9* and *SPL15*, two likely paralogous members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factor family (Cardon et al. 1999), and discuss their redundant regulatory role on the vegetative phase change and the temporal initiation of rosette leaves.

Materials and methods

Plant material and plant growth conditions

All of the genetic stocks described in the paper were in Columbia background. The T-DNA insertion lines SALK_006573 (*spl9*-2), SALK_074426 (*spl15*-1) and SALK_138712 (*spl15*-2) were obtained at the Nottingham Arabidopsis Stock Centre (NASC). The T-DNA insertion lines GABI-Kat 544F04 (*spl9*-3) and WiscDsLox 457 (*spl15*-3) were obtained from GABI-Kat and the *Arabidopsis* Biological Research Centre (ABRC), respectively. Insertion mutant information for NASC- or ARBC-lines was obtained from the SIGNAL website at http://signal.salk.edu. Plants homozygous for the T-DNA insertions were identified by PCR using T-DNA left border- and gene-specific primers. T-DNA specific left border primers for SALK, GABI-Kat and WiscDsLox T-DNAs were 5′-GCGTGGACCGCTTGCTGCAACT-3′, 5′-ATATTGACC ATCATACTCATTGC-3′ and 5′-TGGCAGGATATATTG TGGTGTAAACA-3′, respectively. In combination with the respective left border primer we used the following gene-specific primers: 5′-GCTATGGCTTAAGCCTTAAG TTAAAAGG-3′ for SALK_006573, 5′-CGTAGCTGTC GTGGACTAGTGTCAATC-3′ for SALK_074426 and SALK_138712, 5′-AACCTCTGTTCGATACCAGCCA CAG-3′ for GABI-Kat 544F04 and 5′-AGCCATTGTAA CCTTATCGGAGAATGAG-3′ for WiscDsLox 457. The

stable *En*-1 insertion mutant 5ABA33-H1 (*spl9*-1) was obtained from the ZIGIA-population (Unte 2001). Plants homozygous for a four base pair insertion in the first exon of *SPL9* caused by the excision of the *En*-1 transposon were backcrossed with wild type twice to obtain plants exclusively containing the four base-pair insertion without any further transposon contamination. In order to identify plants containing the mutation we used the following primer combination: 5′-AGTAAGAGGAAACCACCATGG AGATGG-3′ (forward) and 5′-AACCTTCCACTTGG CACCTTGGTATA-3′ (reverse, recognises the insertion).

All plants were grown in plastic trays or pots filled with ready-to-use commercial, pre-fertilized soil mixture (Type ED73, Werkverband eV, Sinntal-Jossa, Germany). For stratification, seeds were kept on moist paper at 4°C in the dark for 4–5 days before transferring to soil (i.e. "sowing") in growth chambers at 22°C, 50% relative humidity. Germination and cultivation of the plants in long-day conditions (16 h light, 8 h dark) were either under approx. 70 μE/cm^2/s (LD1) or 175 μE/cm^2/s (LD2) light provided by fluorescent tubes (L58W/840 and L58W/25 Osram, Munich, Germany). Plants in short-day (SD) conditions (8 h light, 16 h dark) were cultivated under approx. 450 μE/cm^2/s light. To determine sensitivity to photoperiodic induction of flowering, stratified seeds were germinated in a modified SD with a 9 h light period. The developing plants were kept in these conditions for 21 days before they were transferred to similar growth chambers with continuous light provided by Osram HQIT 400 W lamps. Batches of plants were returned to the modified SD conditions after 1, 3 and 5 days.

Phenotypic analysis

Flowering time was measured as the time between sowing and anthesis (opening of first flower). Bolting time was recorded when the main inflorescence had reached a height of 0.5 cm. Inflorescence height was measured between the rosette and the first flower of the main inflorescence of plants with their first siliques fully ripened. In order to count the number of site shoots, all site shoots longer than 0.5 cm were scored. Abaxial trichomes were scored using a Leica MZFLIII stereomicroscope (Wetzlar, Germany). An estimation of the rosette leaf initiation rate (L/D day^{-1}) was obtained by dividing the number (L) of rosette leaves having reached at least 0.5 cm in length through the number of days (D) between sowing and determination. Note that this value reflects but does not equalize (average) plastochron as it should be corrected for the true start of initiation of the first leaf as well as the time needed for the first adult leaf to reach a size of 0.5 cm.

Histological analysis

Apical regions were isolated from plants grown in SD for 41 days by trimming with a razor blade. The tissue was fixed in 4% formaldehyde/0.1 M PO_4, pH 7.0 for 48 h and embedded in paraffin using a Leica ASP300 tissue processor (Wetzlar, Germany). Embedded apices were cut into 8 μm thin cross sections using a Jung Autocut 2055 and photographed using a Zeiss Axiophot microscope (Göttingen, Germany) equipped with a KY-F5U 28CCD camera (JVC, Yokohama, Japan). The first cross section in which the apex was visible plus two successive sections were used to determine the diameter of the apical region. From these three measured values the average was taken for comparison. In addition, cross sectional area and circularity factor ($=4\pi\ A\ P^{-2}$, A is area, P is perimeter) of the leaf primordia, outlined by hand on the photographs, were determined as well. The measurements were performed with help of the program ImageJ 1.35s (Wayne Rasband, National Institutes of Health, USA).

GA_3 treatments

Col-0, the *spl9 spl15* double mutant and the *35S::MIR156b* overexpressor were grown in LD1 conditions. Immediately after germination, half of the plants were treated by spraying 100 μM GA_3, 0.02% Tween 20 and this was repeated twice per week until they started flowering. The other half of the plants was similarly treated with 0.02% Tween 20.

Phylogenetic comparison

Multiple alignments of amino acid sequences were generated by the program ClustalW of the MacVector 7.2.2 software package (Accelrys Ltd., Cambridge, UK) using the BLOSUM 30 matrix with an open gap penalty of 10 and an extend gap penalty of 0.05. Only the SBP-domain was used for the phylogenetic reconstruction. The tree was constructed using the neighbour-joining algorithm of the MacVector 7.2.2 software package.

Quantitative real-time PCR analysis

To perform quantitative RT-PCR (using the iQ5 real-time PCR detection system, Bio-Rad, Munich, Germany) apical regions were collected (roots and as much of the leaves as possible were removed using tweezers) of plants cultivated 5, 9, 13, 27 and 32 days after sowing in LD1 conditions. Total RNA was extracted using the RNeasy plant mini kit

(Qiagen, Hilden, Germany), 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)12–18 primer (Gibco BRL, Karlsruhe, Germany). *SPL9*-specific primers, 5′-AGAACATTGGATACAACAGTGATGAGG-3′ (forward) and 5′-GTTTGAGTCG CCAATTCCCTTGTAGC-3′ (reverse) as well as *SPL15*-specific primers, 5′-TTGGGAGATCCTACTGCGTGG TCAACC-3′ (forward) and 5′-AGCCATTGTAAC CTTATCGGAGAATGAG-3′ (reverse), were designed to generate a PCR product of 171 and 300 bp, respectively. Based on the analysis of Czechowski et al. (2005) *PP2A* expression was used as reference for transcript normalization with the primer pair 5′-TAACGTGGC CAAAATGATGC-3′ (forward) and 5′-GTTCTCCA-CAACCGCTTGGT-3′ (reverse). The PCR efficiencies for *SPL9*, *SPL15* and *PP2A* primers were determined to be 96, 95.5 and 96%, respectively. Quantifications, in triplicate, were performed using the Brilliant SYBRGreen QPCR kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's protocol, in a final volume of 25 µl. PCR was carried out in 250 µl optical reaction vials (Stratagene) heated for 10 min at 95°C to hot-start the Taq polymerase, followed by 40 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C) and extension (30 s at 72°C).

Semi-quantitative RT-PCR analysis

Total RNA was extracted from seedlings of the Col-0 wild-type, mutant- and transgenic lines using the RNeasy plant mini kits (Qiagen). RT-PCR with equal amounts of RNA was performed using the one-step RT-PCR kit (Qiagen). *SPL9* knockout lines were identified using the following primer pair: 5′-GGTCGGGTCAGTCGGGTCAGATACC-3′ (forward) and 5′-ACTGGCCGCCTCATCACTCTTGTA TCC-3′ (reverse). *SPL9* mRNA is expected to yield a 415 bp fragment whereas genomic *SPL9* DNA is expected to yield a 1,138 bp fragment. *SPL15* knockout lines were identified using the following primer pair: 5′-AGAAG CAAGAACCGGGTCAATACC-3′ (forward) and 5′-AG CCATTGTAACCTTATCGGAGAATGAG-3′ (reverse). *SPL15* mRNA is expected to yield a 666 bp fragment whereas genomic *SPL15* DNA is expected to yield a 1,004 bp fragment. RT-PCR of the loading control (*RAN3*; At5g55190) was performed with the primer pair 5′-ACC AGCAAACCGTGGATTACCCTAGC-3′ (forward) and 5′-ATTCCACAAAGTGAAGATTAGCGTCC-3′ (reverse) to yield a fragment of 531 bp when derived from *RAN3* mRNA (genomic *RAN3* is expected to yield a 1,314 bp fragment).

Statistics

Graphical representations of numerical data were generated with the Microsoft Excel program (Microsoft Germany, Munich) and statistical tests were performed using the Student's *t*-Test within this program. *P*-values lower than 0.05 were considered to be statistically relevant and the data involved to represent significant differences.

Remaining techniques and methods

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

Results

Molecular characterization of the Arabidopsis SBP-box genes *SPL9* and *SPL15*

Mutant alleles for the Arabidopsis SBP-box genes *SPL9* (At2g42200) and *SPL15* (At3g57920) were obtained from screening publicly available electronic databases and seed stock centres for transposon or T-DNA tagged *SPL* genes. For *SPL9*, we identified three insertion alleles designated as *spl9*-1 to -3 and confirmed the nature and position of their mutations (see "Materials and methods"; Fig. 1a). The first allele, *spl9*-1, was identified in the En-transposon mutagenised ZIGIA population (Baumann et al. 1998; Unte 2001) and most likely resulted from the excision of an inserted *En*-1 transposon leaving behind a 4-bp insertion footprint in the first exon. The result of this is a frame shift in the coding sequence and the generation of a stop-codon 86 base pairs after the insertion site. Both *spl9*-2 and *spl9*-3 represent T-DNA insertion mutant alleles identified within, respectively, the SALK collection (Alonso et al. 2003) and the GABI-Kat collection (Li et al. 2007).

Also three independent T-DNA insertion lines for *SPL15* could be obtained and confirmed (see "Materials and methods"; Fig. 1a). Two alleles designated as *spl15*-1 and *spl15*-2 were identified within the SALK collection and one, *spl15*-3, within the WiscDsLox T-DNA collection.

According to data available from the AtGenExpress micro-array database (Schmid et al. 2005), both *SPL9* and *SPL15* transcript levels increase during development and are preferentially found in the shoot apical region and in young flowers. We confirmed this temporal expression pattern with the help of qRT-PCR (Fig. 1b). In LD1

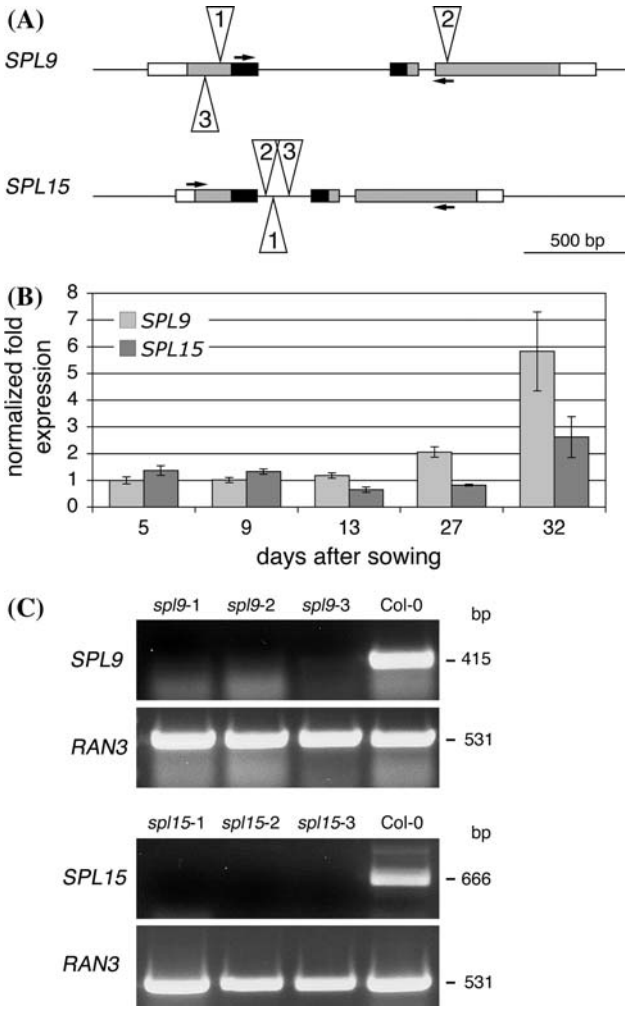

Fig. 1 Molecular characterization of *SPL9* and *SPL15*. (**a**) Schematic representation of the genomic loci of *SPL9* and *SPL15*. The positions of the mutations identified are indicated by open triangles, numbered according to the respective alleles. Boxes represent exons. The SBP-box sequences are depicted in black, the remaining coding sequences in grey and the untranslated 5′ and 3′ regions are left blank. (**b**) Changes in transcript levels of *SPL9* and *SPL15* in the shoot apical region during plant development in LD1 as determined with qRT-PCR and normalized against *PP2A*. For comparison, relative transcript levels were arbitrarily set to one for *SPL9* 5 days after sowing. Error bars indicate standard deviation. (**c**) Absence of *SPL9* and *SPL15* transcripts in seedlings of the respective mutants as validated by RT-PCR. Presence of the respective transcripts in Col-0 wild type seedlings is shown for comparison and the amplification of *RAN3* transcript as quality control and reference for quantification. Fragment lengths are indicated on the left in base pairs (bp)

growing conditions (see "Materials and methods"), *SPL9* and *SPL15* transcript levels remain comparable during the first 2–3 weeks. Thereafter, the expression level of *SPL9* starts to increase followed by that of *SPL15*. Around 32 days after sowing (DAS), at about the time Col-0 plants have undergone their reproductive phase transition, *SPL9* transcript levels have become approximately two and a half times higher in comparison to *SPL15* and six times in comparison to day 5. Arabidopsis lines carrying as a transgene a genomic fragment encompassing the locus for *SPL15* and with a GUS reporter gene inserted downstream of the ATG start codon, confirmed the predominantly apical expression of *SPL15* (Supplementary Fig. 1).

RT-PCR performed on mRNA isolated from whole seedling plants homozygous for any of the three *SPL9* or *SPL15* mutant alleles (see "Materials and methods") did not result in the detection of RNA derived of the respective genes (Fig. 1c). This strongly suggests that all mutant alleles isolated represent functional null-alleles. Accordingly, plants homozygous for any of the three *spl9* mutant alleles showed highly identical phenotypes, as did all three homozygous *spl15* mutants (see phenotypic analysis below; Supplementary Fig. 2). Allelic tests confirmed that the observed phenotypes are indeed due to mutation in either *SPL9* or *SPL15*, respectively (data not shown).

With over 75% of their amino acid residues identical, *SPL9* and *SPL15* show high similarity on the level of their proteins. Also a phylogenetic comparison based on the SBP-box of all 17 *SPL* genes in Arabidopsis revealed *SPL9* and *SPL15* as most closely related and most likely forming a pair of paralogous genes (Fig. 2). Based on this close relationship some degree of functional redundancy could be expected and, therefore, we created double mutant lines to uncover such redundancy. To ascertain that phenotypic changes in the mutant plants are solely due to the loss-of-function of *SPL9* and *SPL15* we generated two different homozygous double mutant lines with the allelic combinations *spl9*-1 *spl15*-1 and *spl9*-2 *spl15*-2, respectively.

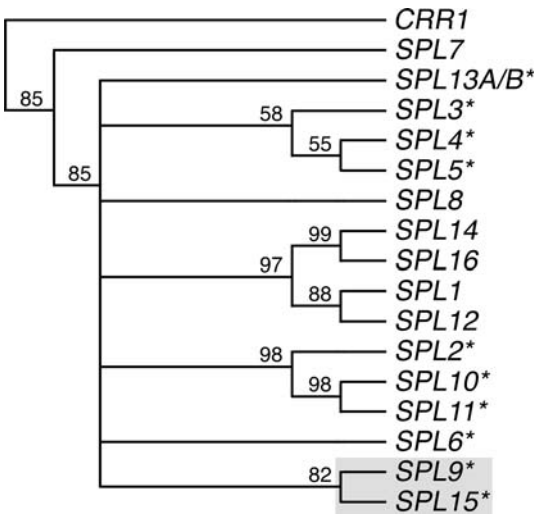

Fig. 2 Phylogenetic relationship of the Arabidopsis SBP-box genes as based on the conserved SBP-domain. The orthologous sequence of *Chlamydomonas* CRR1 has been used as outgroup. The likely paralogous pair *SPL9* and *SPL15* is boxed in grey. MiR156/157 targeted *SPL* genes are marked with an asterisk. Only bootstrap values over 50% are shown

Both lines exhibit the same phenotype as described in the next section. For further detailed analysis the *spl9*-1 *spl15*-1 line was chosen and in the following referred to as *spl9 spl15* for simplicity.

Phenotypic analysis of *spl9* and *spl15* mutants

For a phenotypic analysis, we compared *spl9* and *spl15* single mutants, *spl9 spl15* double mutants to Col-0 wild type as well as to an *35S::MIR156b* transgenic line (kindly provided by D. Weigel and R. Schwab).

An interesting aspect of the *MIR156b* over-expressing plants, as already noticed by Schwab and co-workers (2005) is an increased rate of rosette leaf initiation which, in combination with a modest delayed flowering, results in the obvious denser rosettes of fully developed plants (Fig. 3a). In addition, advanced *35S::MIR156b* plants became very bushy (Fig. 3c). We found these phenotypic aspects also displayed by the *spl9 spl15* double mutant, albeit less pronounced (Fig. 3a, b). To quantify the contribution of *SPL9* and *SPL15* to these phenomena, we compared the number of rosette and cauline leaves of the respective single and double mutants and of the *MIR156b* overexpressor to wild type (Table 1). Whereas in LD2 growing conditions, the *35S::MIR156b* line produced ca. eleven more rosette leaves in comparison to wild type, the single mutant lines produced, on average, only 1–2 rosette leaves more. Again, with ca. six extra rosette leaves, the *spl9 spl15* double mutant differed more from wild type than the single mutants and showed a stronger tendency towards the phenotype of the *MIR156b* overexpressor. The number of cauline leaves remained very comparable among all mutants and wild type, although some reduction may be observed particularly in the *spl9* mutants.

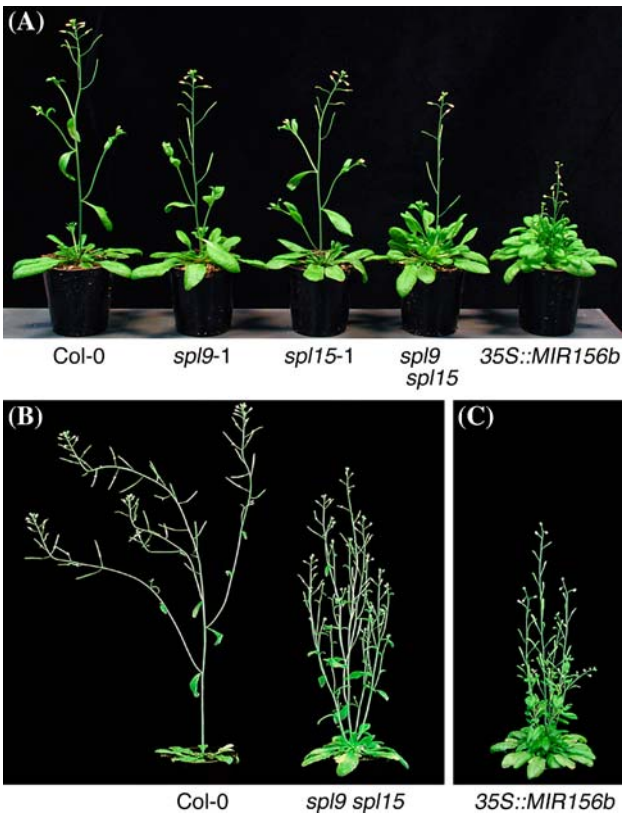

Fig. 3 Phenotypic analysis of *spl9* and *spl15* mutants. (**a**) Flowering *spl9*, *spl15* and *spl9 spl15* double mutant plants shown next to Col-0 wild type and the *MIR156b* overexpressor. Plants shown next to each other are of the same age and grown in parallel under LD2 conditions. (**b**, **c**) Col-0 wild type, *spl9 spl15* double mutant (**b**) and *MIR156b* overexpressor (**c**) at a more advanced stage of development in comparison to the plants shown in **a**

Also with respect to the development of side shoots, the *spl9 spl15* double mutant differed more from wild type than the single mutants. In fact, *spl9*-1 and *spl15*-1 single mutants were found not to differ significantly from Col-0 plants that had formed, on average, 0.9 ± 0.6 side shoots of at least 0.5 cm in length by the time that the first siliques ripened. With an average of 2.1 ± 1.1 side shoots, the *spl9 spl15* double mutant did significantly differ from wild type as did the *35S::MIR156b* transgenic line with, on average, 4.1 ± 0.8 side shoots. Taken together, the phenotypic data of the *spl9 spl15* double mutant clearly suggests a redundant function of *SPL9* and *SPL15* in shoot development and in the maintenance of apical dominance.

MiR156 is assumed to target, besides *SPL9* and *SPL15*, exclusively other *SPL* genes (Rhoades et al. 2002). These too were shown to be down regulated in *MIR156b* over-expressing plants (Schwab et al. 2005). As in comparison to the *spl9 spl15* double mutant the *MIR156b* over-expressor displays an even more severe aberrant phenotype, it can also be deduced that in addition to *SPL9* and *SPL15*, other miR156-controlled *SPL* genes act redundantly to control shoot development and apical dominance.

In addition to the number of leaves formed before the appearance of the first flowers, we also determined for the same plants the time they needed to bolt as well as to anthesis (Table 1). On average, the *spl9* and *spl15* single mutants behaved similar to wild type but, as expected based on the data of Schwab et al. (2005), the *35S::MIR156b* line bolted and flowered somewhat later. The *spl9 spl15* double mutants showed an intermediate behaviour. Whereas for the single mutants the few more leaves formed may be accounted for by the slight delay in the transition to flowering, this delay is unlikely to explain the increased rosette leaf number of the *spl9 spl15* double mutant. In line with the observation of Schwab and co-workers (2005) who reported a leaf-initiation rate per day in SD of 2.2 vs. 1.4 for the *MIR156b* overexpressor and the wild type, respectively, this is probably best explained by assuming a shortened plastochron during vegetative growth.

Table 1 Phenotypic evaluation of *spl9* and *spl15* mutant alleles in comparison to Col wt and a *35S::MIR156b* transgene under LD conditions

	Rosette leaves		Cauline leaves		Bolting (DAS)		Anthesis (DAS)		Juvenile leaves[a]		Infloresc. height[b] (cm)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Col-0 wt	13.1	1.1	3.9	0.5	16.3	1.2	20.9	1.5	5.5	1.2	12.1	1.3
spl9-1	*14.3*	1.1	3.4[c]	0.7	15.9	1.4	19.6[c]	1.7	*8.3*	0.8	*8.0*	1.0
spl9-2	*15.6*	1.2	3.5[c]	0.5	16.9	1.2	20.8	1.4	*9.2*	0.9	*8.2*	0.8
spl9-3	*15.7*	1.3	3.3[c]	0.6	17.5[c]	1.5	21.3	1.5	*9.6*	0.7	*8.6*	0.8
spl15-1	*15.6*	1.1	3.3[c]	0.4	16.6	0.8	20.9	1.0	*7.1*	0.7	*10.3*	1.2
spl15-2	*14.9*	1.0	3.5	0.5	17.1	1.9	20.9	1.9	*7.3*	0.8	11.4[c]	1.1
spl15-3	*16.1*	1.0	3.7	0.7	17.2[c]	0.8	21.8	1.1	*7.6*	0.7	11.1[c]	1.3
spl9-1 *spl15*-1	*19.5*[d]	1.4	3.4	0.9	*18.5*[d]	1.4	22.3[c]	1.8	*10.9*[d]	0.8	*6.9*[d]	1.4
spl9-2 *spl15*-2	*18.9*[d]	1.3	3.3[c]	0.6	*19.0*[d]	1.5	22.8[c]	1.8	*10.8*[d]	0.4	*6.8*[d]	0.8
35S::MIR156b	*24.4*[e]	2.4	3.2[c]	0.8	*19.3*	1.8	22.4[c]	2.1	*14.8*[e]	1.1	*2.3*[e]	0.7

16 plants per genotype were used for determination

DAS, days after sowing; SD, standard deviation

Values significantly different from Col-0 wt at 0.001 confidence level are shown in italics

[a] Number of rosette leaves formed before the first leaf with abaxial trichomes

[b] Measured from rosette to first flower

[c] Values significantly different from Col-0 wt at 0.05 but not at 0.001 confidence level

[d] Values significantly different from single mutants at 0.05 confidence level

[e] Values significantly different from double and single mutants at 0.05 confidence level

To uncover a possible cause or consequence for this increased rate of leaf initiation, we microscopically examined cross sections of the vegetative shoot apex to determine size and phyllotaxy of the *spl9 spl5* double mutant and the *MIR156b* overexpressor and compared these to wild type. To this purpose, plants were grown for 41 days in SD conditions, whereafter the number of rosette leaves having reached at least 0.5 cm in length were recorded and their apices dissected, fixed and embedded in paraffin (see "Materials and methods"). At this age, Col-0 plants were found to have formed on average 24.5 leaves of 0.5 cm or more, the double mutant 33.8 and the *MIR156b* overexpressor plants already 43.4 (Fig. 4a). As the plants were of the same age, these differences most likely reflect differences in plastochron. Alternatively, one may assume large temporal differences per genotype concerning initiation of the first leaf and/or development of the last leaf recorded to have reached 0.5 cm in length. However, we obtained no indications for such discrepancies and noted increasing differences in rosette density during the entire vegetative growth phase of wild type and mutants. From these data, a relative 1.8-fold (43.4/24.5) increase in leaf initiation rate of the *35S::MIR156b* transgenics over wild type can be deduced, a value that quite well matches the observation of Schwab et al. (2005). Leaf initiation rate of the double mutant seem to be increased by a factor of 1.4 (33.8/24.5) in comparison to wild type.

After sectioning the paraffin embedded material, a small but not significant difference in average SAM-diameter of the *spl9 spl15* double mutant and Col-0 wild type could be observed (Fig. 4b, e–f). However, with an average diameter of 104 μm, the *MIR156b* overexpressor showed also a slight but yet significant ($P < 0.05$) decrease in its SAM size compared to Col-0 (Fig. 4b, e, g). Furthermore, both the *spl9 spl15* double mutant and the *MIR156b* overexpressor exhibited the same phyllotaxy as wild type with rosette leaves initiated either clock- or anticlockwise with an angle of divergence of about 137.5° between successive leaves and forming a spiral lattice with a parastichy pair (3,5) (Fig. 4e–g). From these observations, it is concluded that the observed shorter plastochron is neither the result nor the cause of an altered phyllotaxy in the *spl9 spl15* double mutant or the *MIR156b* overexpressor. The shortened plastochron, however, seems to correlate with a reduced SAM size.

As obvious from cross sections shown in Fig. 4e, g, the young leaves of the *MIR156b* overexpressor appear more roundish in shape in comparison to wild type leaves at similar positions. In particular, the vacuolated cells surrounding their midveins seem larger and the developing laminas reduced, i.e. represented by less small cytoplasm rich cells along their lateral margins. In addition, the stipules of the *MIR156b* overexpressor seem to be more prominent. In these aspects of leaf development, the *spl9 spl15* double mutant seems to behave intermediate (Fig. 4e).

To quantify the difference in shape and size of the leaf primordia, we determined their circularity and cross

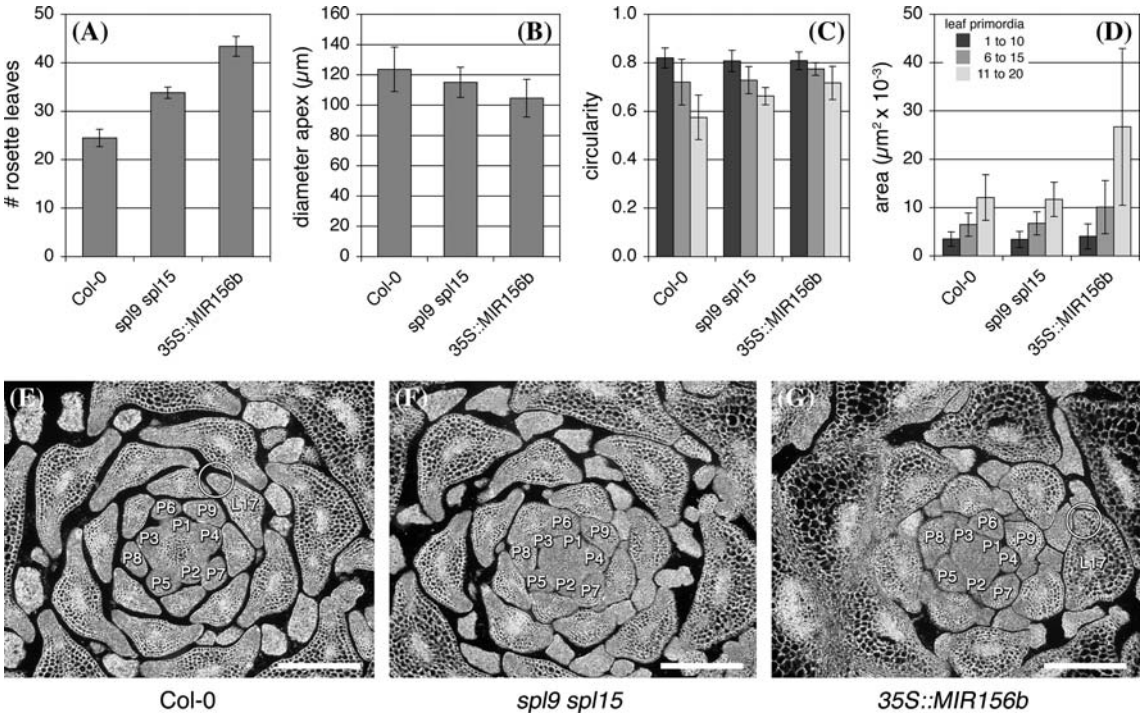

Fig. 4 Leaf formation of *spl9 spl15* double mutant in comparison to wild type and *MIR156b* overexpressor. (**a**) Determination of the average number of rosette leaves of at least 5 mm in length formed by the primary shoot and (**b**) of the average diameter of the primary shoot apex of *spl9 spl15* double mutants, Col-0 wild type and *MIR156b* overexpressor plants after having grown for 41 days in SD. (**c**, **d**) Average circularity (**c**) and cross sectional area (**d**) of leaf primordia as determined from cross section through primary shoot as shown in **e**–**g**. Values represent averages of 10 subsequent primordia as indicted in different shades of grey according to the legend shown in **d**. (**e**–**g**) Cross sections through primary shoot apices of a Col-0 wild type (**e**), a *spl9 spl15* double mutant (**f**) and a *MIR156b* overexpressor plant (**g**) after having grown for 41 days in SD. Error bars in **a**–**d** indicate standard deviation ($n = 6$). Successive leaf primordia in **e**–**g** are sequentially numbered starting with the youngest (P1). Marginal meristem on one side of leaf number 17 (L17; counted from the centre outwards) is encircled in **e** and **g**. Scale bar in **e**–**g** represents 200 μm

sectional area (see "Materials and methods") starting from the first leaf cross section found to be separated from the apical meristem. To reduce effects due to imperfect cross sectioning, i.e. not absolute perpendicular to the longitudinal axes, as well as in correlating sequentially numbered primordia of different sections, we averaged the values obtained over 10 successive primordia. As shown in Fig. 4c, circularity of the youngest 10 leaf primordia was highly similar between the different genotypes. Circularity of subsequent older primordia decreased in all, however, more rapidly in wild type such that, on average, leaf 10–20 did differ significantly between the genotypes. Interestingly, it cannot be excluded that this difference is a direct consequence of a shortened plastochron in the mutant lines. In particular as the leaf initiation rate of the *MIR156b* overexpressor line lies roughly one and a half times above that of wild type. Accordingly, and with respect to absolute age, leaf 11–20 of the *MIR156b* overexpressor may be more comparable to leaves 6–15 of wild type and to which indeed no significant difference in circularity was found. However, in cross sectional area these young leaves differed significantly. On average, $6.5 \pm 2.4 \times 10^3$ μm^2 for wild-type leaves 6–15 and $26.7 \pm 16.2 \times 10^3$ μm^2 for leaf 11–20 of the *MIR156b* overexpressor.

It is known that the shape and other characteristics of newly formed leaves progressively change in correlation with the vegetative phase transition (Telfer et al. 1997). Furthermore, likely due to a changed plastochron, the correlation between leaf number and flowering seems to differ for the mutants and wild type. Therefore, we further investigated the possibility that the observed differences correlate with relative altered timing of the vegetative phase transition.

Functional analysis of *SPL9* and *SPL15* during the vegetative phase transition

In order to determine the timing of the vegetative phase change we used the absence or presence of abaxial trichomes on rosette leaves as a morphological marker for leaves formed during the juvenile or adult growth phase, respectively (Telfer et al. 1997). On average, in our LD2 growing conditions, the first abaxial trichomes developed on rosette leaf number six of Col-0 wild-type plants

(Table 1). The *spl9 spl15* double mutants displayed its first abaxial trichomes on leaf number twelve and the *35S::MIR156b* overexpressor on leaf number 16. Although less than the *spl9 spl15* double mutant, the respective single mutants also developed significantly more juvenile leaves than wild type (Table 1).

We distinguished juvenile and adult growth phase based on a phase dimorphism, i.e. in abaxial trichomes. However, on a plant-physiological level the juvenile phase in Arabidopsis is characterized as being incompetent to respond to photoperiodic induction of flowering (Poethig 1990). To determine if this competence was indeed affected, small populations of 20–22 plants of wild type, the *spl9*, *spl15* single and double mutants and the *MIR156b* overexpressor were germinated and cultivated for 3 weeks in non-inductive SD conditions (see "Materials and methods"). The plants were then brought into continuous light and batchwise shifted back to SD after either 1, 3 or 5 days. Their flowering response was recorded within a 3-week period after this inductive treatment. Plants not flowering within this period also did not flower after 2 months like plants of a control group representing all genotypes that were kept continuously in SD. As shown in Table 2, the 5-day inductive treatment caused a flowering response in 100% of the plants of all genotypes. Three days also sufficed to induce all or almost all of the wild-type and single mutant plants, whereas the response of the double mutant and particular of the *MIR156b* overexpressor already declined. One day of continuous light, still enough to induce half or more of the wild-type and single mutant plants, did not induce flowering in any of the *MIR156b* overexpressor plants and only in one-tenth of the double mutants. These results thus demonstrate that also according to physiological criteria, *SPL9* and *SPL15* redundantly promote the juvenile-to-adult phase transition. In addition, other miR156-regulated *SPL* genes are expected to contribute as well based on the behaviour of the *MIR156b* overexpressor.

The role of gibberellin in the function of *SPL9* and *SPL15*

The plant hormone gibberellin (GA) is known to promote flowering in many plants and in Arabidopsis it is particularly required for flowering in SD (Wilson et al. 1992). Exogenous application of GA will induce abaxial trichomes on leaves where these are normally not present although they will not appear earlier than leaf three (Telfer et al. 1997). In order to test whether the *spl9 spl15* double mutant and the *MIR156b* overexpressor are defective in gibberellin sensitivity or biosynthesis, we exogenously applied GA_3 and compared the onset of abaxial trichome production to mock treated and wild-type plants grown in LD1 (Fig. 5). Like in wild type, the GA_3 treatment strongly reduced the number of rosette leaves without abaxial trichomes, i.e. juvenile leaves, with about a factor of three. This result shows that the *spl9 spl15* double mutant and the *MIR156b* overexpressor remained sensitive to GA_3. However, in both the *spl9 spl15* double mutant and the *MIR156b* overexpressor the amount of GA_3 applied could not reduce the number of juvenile leaves to that of obtained in GA_3 treated wild type.

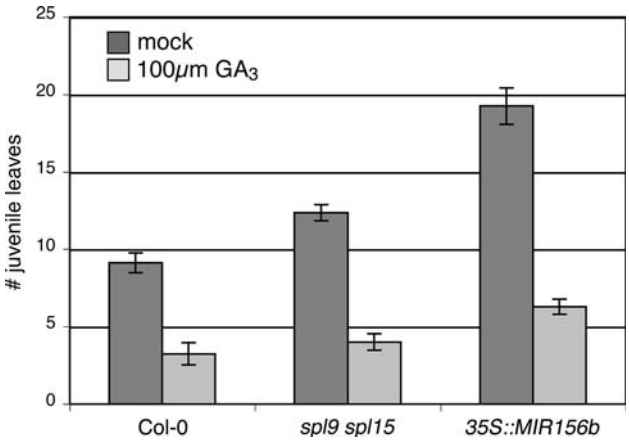

Fig. 5 Effect of GA on *spl9 spl15* double mutant and *MIR156b* overexpressor plants in comparison to wild type. The number of juvenile rosette leaves formed is shown for plants that were either regularly sprayed with GA_3 (100 μM GA_3) or mock treated. Error bars indicate standard deviation ($n = 8$)

Discussion

Transgenic plants constitutively over-expressing the plant specific miRNA156 have been described for Arabidopsis (Schwab et al. 2005; Wu and Poethig 2006) and rice (Xie

Table 2 Photoperiodic floral induction in wild type and *spl* mutants

	Percentage of plants[a] induced after treatment with continuous light for		
	1 D	3 D	5 D
Col-0 wt	60	100	100
spl9-1	59	100	100
spl15-1	50	95	100
spl9 spl15	9	86	100
35S::MIR156b	0	27	100

D, days

[a] Two populations of 10–11 plants per genotype were evaluated within a 3 week period following inductive treatment

et al. 2006). Recently, overexpression of a miR156 encoding locus has also been shown to be the cause of the natural maize mutant *Corngrass1* phenotype (Chuck et al. 2007). Interestingly, in all three different species, overexpression of this well conserved miRNA (Axtell and Bartel 2005; Arazi et al. 2005) causes a similar phenotype suggesting an evolutionary conserved role for the function of miR156 and its *SPL* target genes. Generally, in comparison to the respective wild type, miR156 over-expressing plants are smaller, flower later, tend to lose apical dominance and initiate more leaves with a shorter plastochron. MiR156 targets eleven SBP-box genes in Arabidopsis but the results presented here clearly show that already simultaneous silencing of the two likely paralogous target genes, *SPL9* and *SPL15*, well approximate the miR156 over-expressing phenotype regarding the traits mentioned above. *SPL9* and *SPL15* thus act as important and functionally redundant transcription factors regulating diverse processes in shoot maturation and most likely in combination with other miR156 regulated *SPL* genes. In agreement with this latter statement is our observation that in addition to *spl9*-1 and *spl15*-1, mutation of a third miR156 controlled gene, *SPL2* (At5g43270; T-DNA insertion line SALK_022235), results in triple mutant plants showing an even better approximation to the *MIR156b* overexpressor phenotype (Supplementary Fig. 2; despite the absence of detectable *SPL2* transcript, single homozygous *spl2*-1 mutant plants lack an obvious mutant phenotype, data not shown). In greenhouse LD conditions we found the triple mutant to have produced on average 17.3 ± 2.1 ($n = 16$) rosette leaves in comparison to 15.6 ± 2.6 for the *spl9 spl15* double mutant. Col-0 wild-type and the *MIR156b* overexpressor plants grown in parallel produced 12.9 ± 1.7 and 22.5 ± 3.5 rosette leaves before flowering, respectively.

SPL9 and *SPL15* positively regulate the juvenile-to-adult growth phase transition

It became clear with the detailed analysis of Arabidopsis *MIR156b* overexpressors by Wu and Poethig (2006) as well as with the description of the *Corngrass1* mutant in maize by Chuck et al. (2007), that one of the major phenotypic alterations in miR156 over-expressing plants is an extended juvenile growth phase. This suggests that one of the important functions of miR156 targeted SBP-box genes is to promote the vegetative phase change. In agreement with this observation, Wu and Poethig (2006) showed that overexpression of the miRNA156 regulated gene *SPL3* and its likely paralogs leads to a greatly shortened juvenile phase in Arabidopsis. Based on morphological markers (abaxial trichomes) and physiological parameters (response to photo-inductive stimulus) we found that the *spl9 spl15* double mutant exhibit a delayed vegetative phase transition and, therefore, conclude that both genes are very likely involved in the positive regulation of this developmental process in a redundant fashion. Most likely, because of this redundancy, photoperiodic induction of the single *spl9* and *spl15* mutants is not much affected. However, the effect on the appearance of abaxial trichomes as a marker for the juvenile-to-adult phase transition appears to be stronger in the *spl9* mutant in comparison to *spl15*. This may be due to the fact that in shoot apical development expression of *SPL9* starts to increase before that of *SPL15* (Fig. 1b).

As *SPL9* and *SPL15* promote the juvenile-to-adult growth phase transition and thus competence to respond to photoperiodic induction of flowering, it is interesting to note that both *SPL9* and *SPL15* themselves are strongly upregulated in the shoot apex upon such induction (Schmid et al. 2003). An additional role for these genes in establishing inflorescence or floral meristem identity may thus be suggested.

SPL9 and *SPL15* negatively regulate leaf initiation rate

Our data on the leaf initiation rate suggest that *SPL9* and *SPL15* act negatively on leaf initiation. Silencing of both genes leads to a shorter plastochron. Other miR156 regulated *SPL* genes may act similarly as the plastochron is even further shortened in the *MIR156b* overexpressor plants. A few mutants are known to cause a shortened plastochron and most of them simultaneously affect phyllotaxy. However, we found that shortening of the plastochron due to loss of *SPL* gene function is neither the cause nor the result of a changed spatial distribution of leaf primordia at the shoot apex. A shorter plastochron without an altered phyllotaxy has also been reported for two rice mutants, *plastochron1* and *-2* (*pla1, -2*; Itoh et al. 1998; Miyoshi et al. 2004; Kawakatsu et al. 2006). *PLA1* encodes a cytochrome P450 protein, whereas *PLA2* encodes a MEI2-like RNA binding protein. In both mutants the reduction in plastochron is accompanied by an increase in the size of the SAM and a higher rate of cell division. However, although the SAM of *pla2* is actually smaller than that of *pla1*, it has a shorter plastochron. Furthermore, higher cell division activity associated with constitutive overexpression of CyclinD shortened the plastochron in tobacco without altering SAM size (Cockcroft et al. 2000). These observations suggest, as already noticed by Kawakatsu et al. (2006), that not SAM size but rather cell division rate is decisive in plastochron duration. Also our results may lend support to this hypothesis as both the *spl9 spl15* double mutant and the *MIR156b* overexpressor

exhibit a clearly shorter plastochron than wild-type plants but their SAM sizes differ only marginally. Therefore, it will be of interest to determine if *SPL9*, *SPL15* and other miR156 regulated *SPL* genes control cell division rate in the SAM and, if so, in particular if their role is mediated through the phytohormone cytokinin. Not only is cytokinin a major positive regulator of cell proliferation and division in plants (Werner et al. 2001) it is also, in mutual dependence of auxin, a major determinant in the outgrowth of lateral shoots (Sachs and Thimann 1967; Chatfield et al. 2000). This latter aspect may explain the reduced apical dominance observed for the *spl9 spl15* double mutant and the *MIR156b* overexpressor. Finally, mutants disrupting cytokinin signalling are known to result in reduced leaf initiation rates in addition to a smaller SAM and other effects (Nishimura et al. 2004; Higuchi et al. 2004).

Do *SPL9* and *SPL15* negatively regulate leaf maturation rate?

Based on their observations of the *pla* mutants in correlation to the expression of the respective genes in leaf primordia but not in the SAM, Kawakatsu et al. (2006) proposed that the rate of leaf maturation plays a significant role in regulating the rate of leaf initiation. In addition, these authors postulated a model in which the inhibitory effect of pre-existing leaf primordia on the initiation of the next leaf is lost as they mature. Similarly, a shortened plastochron in the *spl9*, *spl15* mutants and the *MIR156b* overexpressor may also be due to precocious maturation of their leaves as suggested by our comparison of cross sections through successive leaf primordia of wild type and the *MIR156b* overexpressor. Even if the shape, i.e. circularity, of the primordia may not significantly differ after correction for an altered plastochron by comparing primordia based on age and not on serial sequence number, their cross sectional area seems to increase more rapidly in the *MIR156b* overexpressor. In particular, the cells surrounding the midvein in *MIR156b* overexpressor leaves appear to enlarge more rapidly.

SPL9 and *SPL15* do not modulate the role of GA in the vegetative phase change

Exogenous application of GA_3 has been found to accelerate abaxial trichome production in Arabidopsis suggesting that gibberellins function to regulate vegetative phase change (Telfer et al. 1997). These findings are also supported by mutant analysis. For example in *spindly* (*spy*) mutants, which undergo constitutive GA response, abaxial trichomes occur on leaves initiated significantly earlier than in wild type (Jakobsen and Olszewski 1993; Telfer et al. 1997). On the other hand, Telfer et al. (1997) found that mutants blocked in GA biosynthesis as well as GA insensitive mutants are significantly delayed in the appearance of abaxial trichomes. Loss-of-function mutants for the here examined *SPL9* and *SPL15* genes clearly delay the appearance of abaxial trichomes.

Our treatment of *spl9 spl15* double mutant and *35S::MIR156b* transgenic plants with high doses of GA_3 showed that, like in wild type, their number of juvenile leaves can be reduced but not to numbers equal to those found for similarly treated wild type. In fact the ratios of juvenile to adult leaves of wild type and mutant phenotypes remain highly comparable to untreated plants. From this we conclude that the role *SPL9*, *SPL15* and other miR156 controlled *SPL* genes play in the vegetative phase change, is unlikely to be GA mediated although a minor contribution to GA sensitivity can not be excluded.

Outlook

MiR156 targeted members of the SBP-box family of transcription factors in both mono- and dicots appear to play an important role as positive regulators of shoot maturation and of the vegetative to reproductive phase transition in particular. Both genetic factors, i.e. miR156 and SBP-box genes, have also been suggested to be major determinants in the transition from undifferentiated to differentiated embryogenic calli of rice (Luo et al. 2006). As the interaction between SBP-box genes and miR156 is of ancient origin in land plants (Arazi et al. 2005; Riese et al. 2007) it will be interesting to learn to what extent their molecular interplay is of importance to developmental phase transitions in plants in general.

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