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Arabidopsis mutant *sk156* reveals complex regulation of *SPL15* in a *miR156*-controlled gene network

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Abstract

Background: The *Arabidopsis* microRNA156 (*miR156*) regulates 11 members of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL)* family by base pairing to complementary target mRNAs. Each *SPL* gene further regulates a set of other genes; thus, *miR156* controls numerous genes through a complex gene regulation network. Increased axillary branching occurs in transgenic *Arabidopsis* overexpressing *miR156b*, similar to that observed in loss-of-function *max3* and *max4* mutants with lesions in carotenoid cleavage dioxygenases. *Arabidopsis miR156b* was found to enhance carotenoid levels and reproductive shoot branching when expressed in *Brassica napus*, suggesting a link between *miR156b* expression and carotenoid metabolism. However, details of the *miR156* regulatory network of *SPL* genes related to carotenoid metabolism are not known.

Results: In this study, an *Arabidopsis* T-DNA enhancer mutant, *sk156*, was identified due to its altered branching and trichome morphology and increased seed carotenoid levels compared to wild type (WT) ecovar Columbia. Enhanced *miR156b* expression due to the 35S enhancers present on the T-DNA insert was responsible for these phenotypes. Constitutive and leaf primodium-specific expression of a *miR156*-insensitive (mutated) *SPL15 (SPL15m)* largely restored WT seed carotenoid levels and plant morphology when expressed in *sk156*. The *Arabidopsis* native *miR156*-sensitive *SPL15 (SPL15n)* and *SPL15m* driven by a native *SPL15* promoter did not restore the WT phenotype in *sk156*. Our findings suggest that *SPL15* function is somewhat redundant with other *SPL* family members, which collectively affect plant phenotypes. Moreover, substantially decreased *miR156b* transcript levels in *sk156* expressing *SPL15m*, together with the presence of multiple repeats of *SPL*-binding GTAC core sequence close to the *miR156b* transcription start site, suggested feedback regulation of *miR156b* expression by *SPL15*. This was supported by the demonstration of specific *in vitro* interaction between DNA-binding SBP domain of *SPL15* and the proximal promoter sequence of *miR156b*.

Conclusions: Enhanced *miR156b* expression in *sk156* leads to the mutant phenotype including carotenoid levels in the seed through suppression of *SPL15* and other *SPL* target genes. Moreover, *SPL15* has a regulatory role not only for downstream components, but also for its own upstream regulator *miR156b*.

Background

The *Arabidopsis* *miR156* family has eight members and is highly conserved in the plant kingdom. It has been identified in 45 plant species [1]. *MiR156* is known to repress *SPL (SQUAMOSA PROMOTER BINDING*

PROTEIN-LIKE) genes [2-4], which are plant-specific transcription factors containing the SBP (*SQUAMOSA* promoter binding protein) box [5]. In *Arabidopsis*, 11 *SPL* genes are targeted by *miR156* [2,4,6,7], and *in silico* full genome analysis showed that no other genes in *Arabidopsis* have the segment complementary to *miR156* [2]. Some details of the relationship between the *miR156* regulatory network downstream of *SPL* genes and flowering enhancement and flavonoid metabolism have been revealed

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[8-10]. But this is clearly an area of research that still needs strong attention.

Diverse and redundant roles of some individual *SPL* genes in plant morphology and development have been reported. The *SPL* genes targeted by miR156 can be grouped into four major clades: *SPL3/SPL4/SPL5*, *SPL2/SPL10/SPL11*, *SPL9/SPL15*, and *SPL6/SPL13* [7]. *SPL3*, *SPL4* and *SPL5* exhibit partially redundant effects on plant juvenile-to-adult transition [4,11,12]. *SPL9* and *SPL3* directly activate *MADS* box genes that promote flowering [8]. In addition, *SPL9* and *SPL15* interchangeably control shoot maturation and leaf initiation [13]. *SPL10* and *SPL9* expression in leaf primordia modulated by miR156 affects initiation of new leaves at the shoot apical meristem [14]. *SPL2*, *SPL10* and *SPL11* were each able to control leaf lamina shape in association with shoot maturation in the reproductive phase [15]. Moreover, *SPL* genes (represented by *SPL9*) regulate trichome development via direct interaction with miR172 [9] and the MYB transcription factor genes *TRICHOMELESS1* (*TCL1*) and *TRIPTYCHON* (*TRY*) [16].

Increased axillary branching occurs in transgenic *Arabidopsis* lines expressing *miR156b* under the control of a cauliflower mosaic virus 35S promoter (CaMV35S) [6], similar to that observed in loss-of-function *max3* and *max4* mutants with lesions in carotenoid cleavage dioxygenases, *CCD7* and *CCD8*, respectively [17-19]. These *max* mutations cause defective biosynthesis of strigolactones; a group of carotenoid-derived hormones that inhibit shoot branching [20,21]. The morphological similarities between these transgenic and mutant lines suggest a link between *miR156b* expression and carotenoid metabolism. This was partially confirmed when *Arabidopsis miR156b* was found to enhance carotenoid levels and reproductive shoot branching when expressed in *Brassica napus* [22].

In this study, we report on a new *Arabidopsis* activation tagged mutant, *sk156*, with strongly enhanced expression of *miR156b*. MiR156b-induced *SPL15* suppression was partially responsible for the increased seed carotenoid abundance and altered plant morphology observed in *sk156*. In addition, we highlight a new *SPL15* feedback loop which controls expression of *miR156b*, likely via a physical interaction between the *SPL15* SBP domain and the promoter of *miR156b*.

Results

Phenotypes of the *sk156* mutant

The *sk156* mutant was selected from an *Arabidopsis* activation-tagged mutant population that was developed using a T-DNA construct containing four CaMV35S enhancers [23]. Compared to the parental wild type (WT) Col-4, *sk156* exhibited the following morphological changes: increased numbers of rosette leaves

which were slightly pale, smaller and rounder, increased reproductive branching, ectopic expression of trichomes on flower sepals and shoot tips, delayed bolting, severely stunted cauline stems, and decreased flower and silique sizes (Figure 1).

For comparison, we examined the phenotypes of *max3-9* [17] and *max4-1* [19] mutants, which had been confirmed previously to contain defective *CCD7* and *CCD8* genes, respectively (Additional file 1). In our hands, these *max* mutants showed increased secondary and tertiary branches (Figure 1G), which was consistent with an earlier report that *max* mutants showed increased inflorescence number compared to WT plants [24]. However the cauline stems of these two *max* mutants were not significantly stunted as in *sk156*. The branching pattern of *sk156* was also somewhat distinct compared with *max* mutants; *sk156* had more quaternary and quinary branches and the *max* mutants mainly had increased secondary and tertiary branches.

The abundance of five major carotenoid compounds was examined in the mature seeds and leaves of *sk156* and WT plants. Levels of lutein, β -carotene, violaxanthin, and zeaxanthin were 2.3-, 11.7, 5.5-, and 1.8- fold higher in *sk156* seeds than in WT seeds (Figure 2A and B). Slight decreases (but not statistically significant) in the levels of major carotenoids in leaves were found in *sk156* compared to WT (Figure 2C and D). Cryptoxanthin was undetectable in either tissue of the mutant, but present at 0.62 $\mu\text{g g}^{-1}$ FW (fresh weight) in WT seeds and 4-fold higher in WT leaves.

Molecular analysis of *sk156*

Segregation analysis of heterozygous *sk156* mutant offspring revealed that the ratio of resistant-to-sensitive seedlings (271:109, respectively) was well within the expected 3:1 ratio (χ^2 , $p = 0.05$), indicating that there was a single T-DNA insertion site. This was confirmed by Southern blot analysis (Figure 3A). Sequencing of the T-DNA-flanking regions indicated that the T-DNA was located in AT4G30980 (Figure 3B), which encodes a basic helix-loop-helix (bHLH) family protein bHLH069 [25,26]. CaMV 35S enhancers in the T-DNA were within close proximity to five other gene loci: AT4G30975 (unknown RNA gene), AT4G30972 (*miR156b*), AT4G30970 (unknown protein) and AT4g30960 (SOS2-like protein kinase PKS4).

The *sk156* phenotypes were dominant when crossed with WT, suggesting that the altered phenotypes in *sk156* were tightly linked to enhanced expression of gene (s) adjacent to the T-DNA, rather than gene disruption. Thus, transcript levels of genes close to the T-DNA insertion site were compared in the mutant and WT plants using quantitative RT-PCR. Transcript levels of AT4G30972 (*miR156b*) and the unknown RNA gene

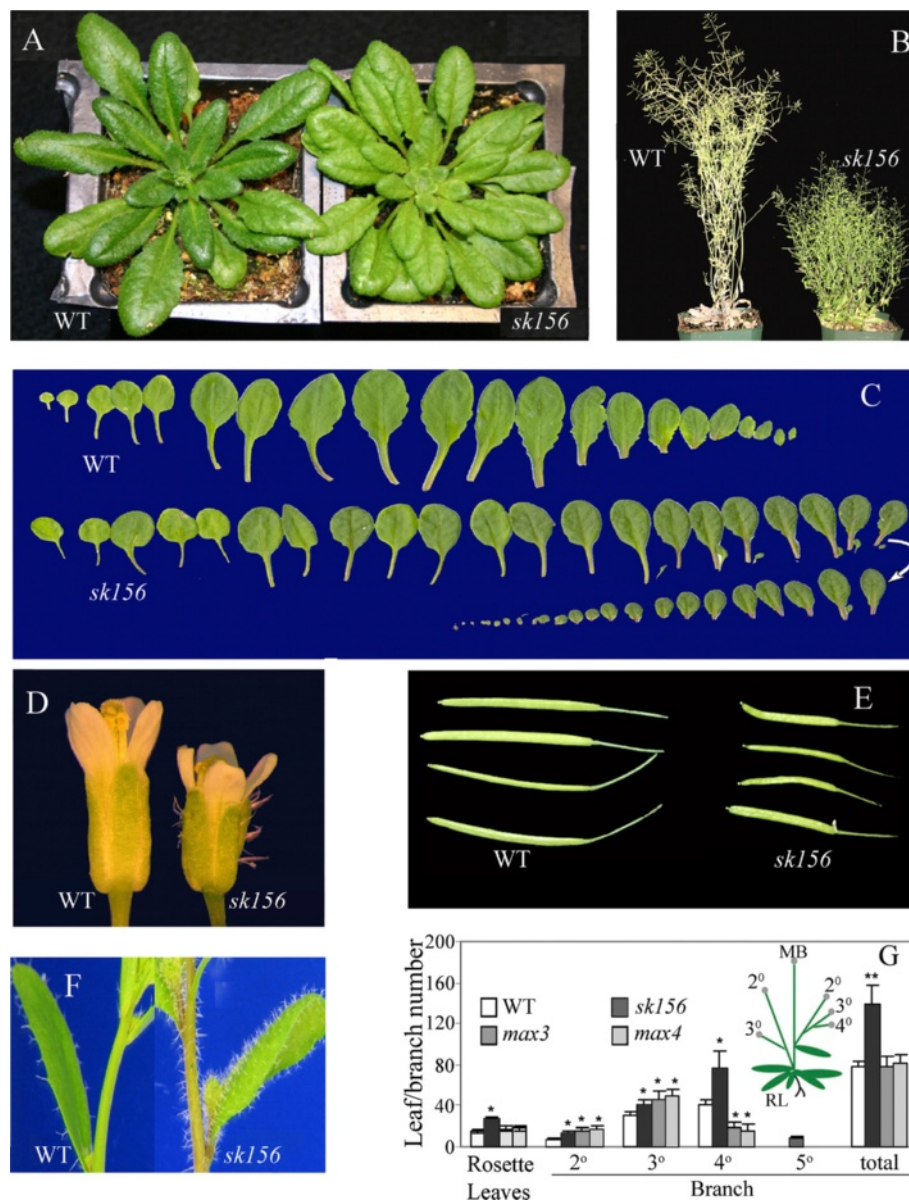
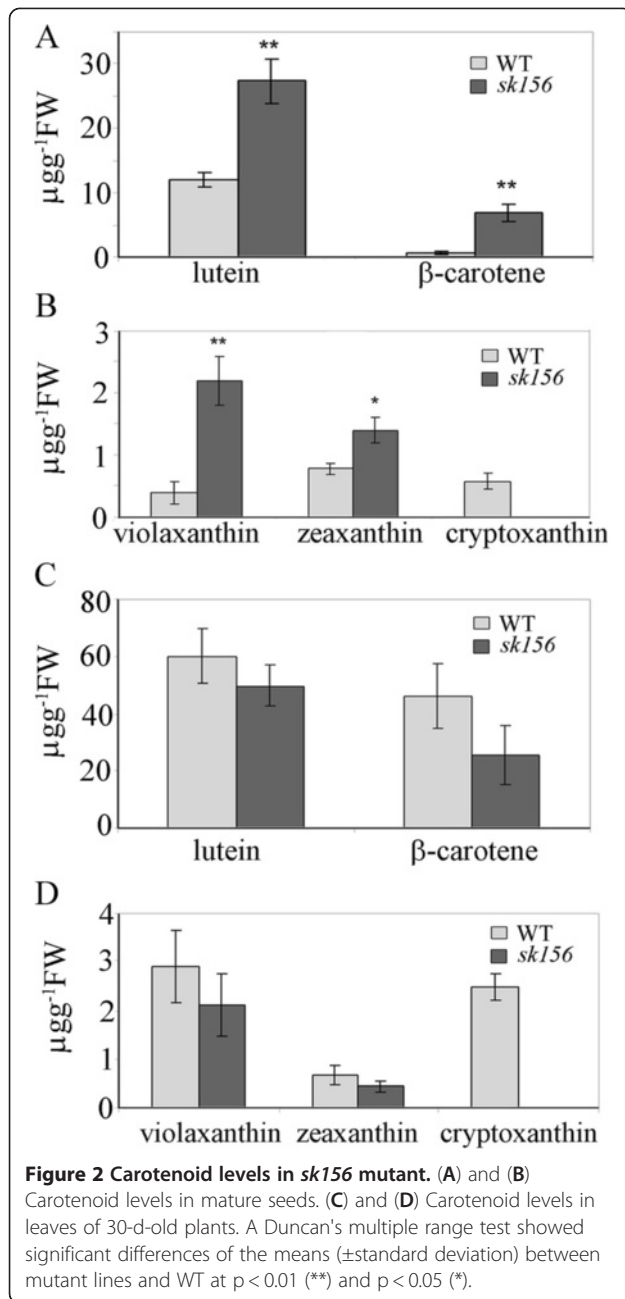


Figure 1 Morphological phenotype of the *Arabidopsis sk156* mutant. (A) 30-d-old rosette plants. (B) 8-week-old mature plants. (C) 30-d-old plant leaves. WT on top, *sk156* on bottom. (D) Flowers, showing trichome-bearing *sk156* sepals (right). (E) Siliques at 14 days post-anthesis. (F) Trichome enhancement on cauline stems and leaves of *sk156*. (G) Enhanced rosette leaf and branch numbers (insert defines branch order). Data were the mean of thirty 8-week-old plants. MB, main stem; RL, rosette leaves. A Duncan's multiple range test showed significant differences of the means (\pm standard deviation) between mutant lines and WT at $p < 0.01$ (**) and $p < 0.05$ (*).

AT4G30975 were increased 91.4-fold (\pm 25.8) and 95.8-fold (\pm 18.6), respectively, in *sk156* (Figure 3C), while the T-DNA-disrupted AT4g30980 (*bHLH069*) was repressed 11.1-fold (Figure 3D). No significant changes in transcript levels were detected for the other adjacent genes (data not shown). This indicated that the four CaVM 35S enhancers inserted into *bHLH069* caused the activation of *miR156b* and the unknown RNA gene.

To determine which of the three T-DNA affected genes was responsible for the altered phenotypes in

sk156, including enhanced seed carotenoid levels, four types of plants were obtained and examined (Table 1). These included a *bHLH069* knockout mutant S468 (SALK_032468) (Figure 4A), transgenic *sk156* overexpressing a *35S:bHLH069* cDNA (Figure 4B) transgenic WT carrying *35S:AT4G30975* cassette (A975, the unknown RNA gene) (Figure 4C and D), and transgenic *Arabidopsis* WT carrying a *35S:miR156b* cassette (T156b) (Figure 4E and F) [12]. Transcript analysis showed that expression of *bHLH069* was defective in



both S468 and *sk156* but enhanced in *sk156* over-expressing *bHLH096* (Figure 4A). Morphology was indistinguishable between *sk156* and *sk156* over-expressing *bHLH096* (Figure 4B) and between the SALK mutant and WT (data not shown). Additionally, over-expression of the unknown RNA cDNA in WT (line A975) (Figure 4C) did not produce the *sk156* mutant phenotype (Figure 4D). However, the 35S promoter-driven *miR156b* overexpression resulted in morphological characteristics in line T156b similar to those of *sk156* (Figure 4F).

Carotenoid analysis further confirmed the role of enhanced *miR156b* expression in generating the *sk156*

phenotypes (Figure 4G and H). No significant differences in seed carotenoid abundance were found between the defective *bHLH069* mutant S468 and WT ($p \leq 0.05$). Complementation of *sk156* with *bHLH069* did not restore *sk156* seed carotenoid levels back to WT levels, nor did over-expression of the unknown RNA in WT result in enhanced seed carotenoid levels. Only in line T156b overexpressing *miR156b* were seed carotenoid levels raised to levels found in *sk156* seeds (Figure 4G and H). These data clearly indicate a role for *miR156b* in the phenotypes of *sk156*.

Due to similar enhanced transcript levels of *miR156b* and AT4G30975 in the *sk156* mutant, yet unchanged morphology and seed carotenoid accumulation in the AT4G30975 over-expression lines (line A795, Table 1), we examined the relationship between *miR156b* transcripts and AT4G30975 cDNA. These two transcripts are separated by a 17 bp intergenic fragment (TAIR, version 9). Sequencing of cDNA obtained from the leaves of 4-week-old WT plants revealed a *miR156b* transcript which included both the 17 bp fragment and the full segment of the AT4G30975 transcript (sequencing data not shown). These data show that enhanced expression of *miR156b* is the reason for the phenotypic changes in the *sk156* mutant line, regardless of AT4G30975 co-transcription.

Suppression of *SPL15* is involved in the *sk156* phenotype

Arabidopsis Columbia has 11 *SPL* genes (two loci for *SPL13*) that are regulated by *miR156* [7,9]. To determine which *SPL* genes were most likely involved in altered carotenoid profiles of *sk156* seeds, mutant lines with confirmed T-DNA insertion knockouts for all *miR156*-target *SPL* genes (except *SPL5* where mutants were unavailable) were obtained (Additional file 2). The mutant lines were compared for morphology and carotenoid differences with their corresponding ecotype Columbia and Wassilevskija controls. While levels of carotenoids were modestly increased in three *spl15* mutant lines (Figure 5A insert), they were less affected in the remaining *spl* mutants (Figure 5A). Even in the *spl15* mutant lines, the enhanced total carotenoid levels ($21.5 \pm 1.7 \mu\text{g/g.FW}$) (Figure 5 insert) were not as high as the sum of each individual carotenoid compound observed in *sk156* ($37.8 \pm 5.5 \mu\text{g/g.FW}$) (Figure 6C and D).

The three different *SPL15* "knockout" mutants, CS852117, CS856815 and SALK_138712 (Figure 5A insert) all exhibited reduced *SPL15* transcript levels similar to levels found in the *sk156* mutant and the *miR156b* over-expression line T156b (Figure 5B; Table 1). However, other *spl* mutants had normal cauline stem lengths (data not shown), while *spl15* mutants had slightly stunted stems (Additional file 3) as reported previously [13]. Assays using rapid

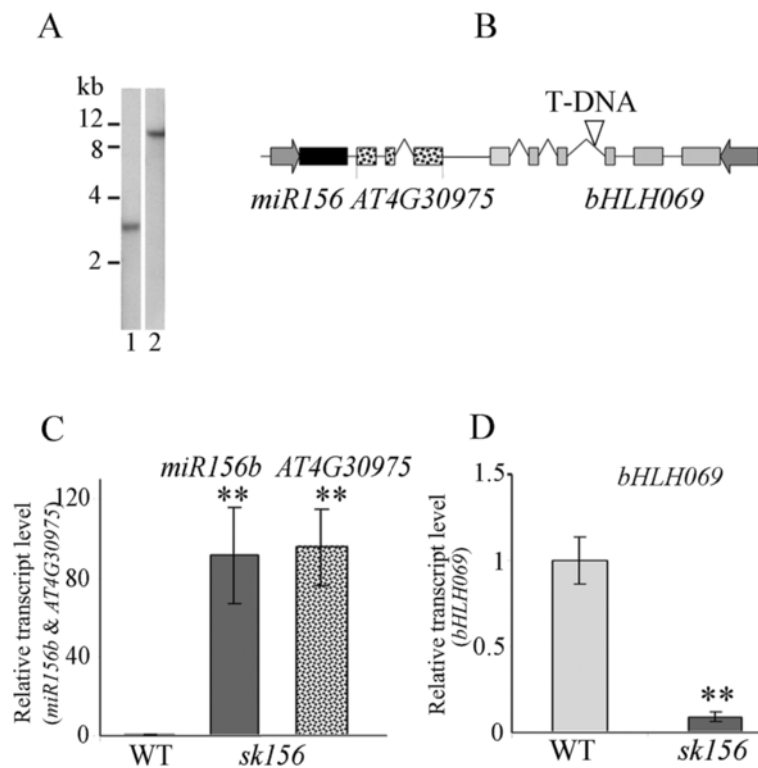


Figure 3 T-DNA insertion site and affected genes in *sk156* mutant. (A) Southern blot analysis of *sk156* showing a single T-DNA insertion. Lane 1, digested with *Bam*HI. Lane 2, digested with *Hind*III. (B) Schematic of T-DNA inserted in the third intron of *bHLH069* shown by '∇' (empty triangle). Boxes, exons; Lines, introns and intergenic regions. Thick grey arrows show promoter regions and their transcription direction. (C) Transcript levels for *miR156b* and unknown RNA gene (AT4G30975) in *sk156* relative to WT (set at 1) using qPCR. (D) Transcript levels for *bHLH069* in *sk156* relative to WT (set at 1) using qPCR. A Duncan's multiple range test in (C) and (D) showed significant differences of the means (\pm standard deviation) between *sk156* and WT Col-4 at $p < 0.01$ (**) and $p < 0.05$ (*).

amplification of 5' complementary DNA ends (5'-RACE) indicated that three cleavage sites exist within the region of the native *SPL15* transcript that is complementary to the miR156 mature sequence, with one site being used more frequently than the other two (Figure 6A). Collectively, these results suggest that *SPL15* is a direct target of miR156 and that it plays a role in the morphology and seed carotenoid phenotypes found in *sk156* mutant line.

Expression of miR156-insensitive *SPL15m* affects the phenotype of *sk156*

Since *spl15* mutants had several phenotypes similar to those of *sk156*, two different *SPL15*-complemented *sk156* lines were developed to determine whether the WT phenotype could be restored in a miR156b-enhanced *sk156* background (Table 1). The first transgenic *sk156* carried a 35S:*SPL15n* (native *SPL15*) cassette to avoid any endogenous transcriptional regulation which might be present if using a native *SPL15* promoter (Figure 6A). The second carried 35S: (mutated *SPL15*), which contains 11 mutated sites within the *SPL15* segment

complementary to mature miR156 so that *SPL15m* becomes insensitive to miR156 regulation (Figure 6A). PCR analysis using gene-specific primers confirmed both pSKI015 T-DNA and either *SPL15n* or *SPL15m* within these transgenic lines (Additional file 4). Sequencing of these PCR products confirmed that all *SPL15m*-expressing lines had a *SPL15*-mutated segment (Figure 6A) as expected. *SPL15* transcript levels (sum of *SPL15m* and endogenous *SPL15*) within the 35S:*SPL15m*⁺ lines were increased by 86.2-fold to 617.4-fold compared to WT (Figure 6B). No significant differences were found in the amount of lutein, violoxanthin, zeaxanthin and β -carotene in seeds between these 35S:*SPL15m*-transformed plants and wild type control plants (Figure 6C and D). The transgenic plants showed a largely restored WT morphology in leaf and branch numbers, bolting time, cauline stem length, flower and silique size, and trichome number in reproductive shoots and tissues (Figure 6E). Only leaf morphology was different between transgenic and non-transgenic lines (Figure 6F). This contrasted with miR156-controlled

Table 1 Summary of transgenic and mutant *Arabidopsis* lines characterized in this study

Plant Lines	Construct, key element and genetic background	Gene expression vs. WT control	Key phenotypes
<i>sk156</i>	pSKI015, 35S enhancer, Col-4 background	91.4- and 95.8-fold higher expression for <i>miR156b</i> , At4g30975	Increased seed carotenoids and branching
S468	pROK2, SALK_032468, Col-0 background	11.1 fold less expression for AT4G30980	Similar to WT
<i>35S:bHLH069</i>	Modified pBI121, 35S:At4g30980 cDNA, <i>sk156</i> background	708.4 fold higher expression for bHLH069	Similar to <i>sk156</i>
T156b	Modified pBI121, 35S: <i>miR156b</i> , Col-4 background	2134.6 fold higher expression for <i>miR156b</i>	Similar to <i>sk156</i>
A975	Modified pBI121, 35S:At4g30975 cDNA, Col-4 background	332.5 fold higher expression for At4g30975	Similar to WT
CS2117	pDs-Lox, CS852117, Col background	4.3 fold less expression for <i>SPL15</i>	Slightly stunted cauline stem and increased seed carotenoids.
Col background	4.3 fold less expression for <i>SPL15</i>	Slightly stunted cauline stem and increased seed carotenoids.	
CS6815	pDs-Lox, CS856815, Col background	5.2 fold less expression for <i>SPL15</i>	Similar to CS2117.
S8712	pROK2, SALK_138712, Columbia background	4.7 fold less expression for <i>SPL15</i>	Similar to CS2117.
<i>35S:SPL15n</i>	Modified pBI121, 35S: <i>SPL15n</i> (miR156 sensitive), <i>sk156</i> background	4.7 fold less expression for <i>SPL15</i> .	Similar to <i>sk156</i>
<i>35S: SPL15m</i>	Modified pBI121, 35S: <i>SPL15m</i> (miR156 insensitive), <i>sk156</i> background	22.8-613.6 fold higher expression of <i>SPL15</i>	Similar to WT, changed leaf shape.
<i>ASI:SPL15n</i>	Modified pBI121, <i>ASI:SPL15n</i> (miR156 sensitive), <i>sk156</i> background	3.2 fold less expression for <i>SPL15</i>	Similar to <i>sk156</i> , slightly changed leaf color
<i>ASI:SPL15m</i>	Modified pBI121, <i>ASI:SPL15m</i> (miR156 insensitive), <i>sk156</i> background	4.6 fold higher expression for <i>SPL15</i>	Similar to WT, changed leaf shape
<i>SPL15:SPL15m</i>	Modified pBI121, <i>SPL15:SPL15m</i> (miR156 insensitive), <i>sk156</i> background	1.6 fold higher expression for <i>SPL15</i>	Similar to <i>sk156</i> , changed leaf shape

35S:SPL15n⁺ lines (Table 1), which displayed *sk156* phenotypes.

Since constitutive expression of *SPL15m* predominantly restored *sk156* phenotypes, native *SPL15n* and mutated *SPL15m* under the control of the young leaf primordia promoter from the *ASYMMETRIC LEAVES 1* (*ASI*) gene [27] were used to generate two additional complemented sets of *sk156* lines. This was to find out whether *SPL15* functions at the leaf primordia within the shoot apex as occurs with certain *SPL* genes controlling plastochron length [14]. PCR analyses using promoter-specific and gene-specific primers (Additional file 5, see materials and methods) confirmed the presence of the transgene in these transgenic plants (Additional file 6). *SPL15m* transcript levels were increased in *ASI* promoter-driven *SPL15m* transgenic *sk156* lines, but not in *ASI* promoter-*SPL15n* lines, compared to WT and *sk156* mutants (Figure 7A). As with *SPL15m* expression directed by the 35S promoter, expression of *SPL15m* under the control of the *ASI* promoter in the *sk156* background restored WT morphology at different growth stages, whereas *ASI:SPL15n* did not (Figure 7B, C, and D). Moreover, the levels of seed carotenoids in

these *ASI:SPL15m* plants were not significantly different from those in WT ($p > 0.05$), while seed carotenoid abundance was significantly higher in plants carrying the *ASI:SPL15n* cassette ($p > 0.05$) (Figure 7E and F). These changes in the phenotype of transgenic *sk156* plants expressing the miR156-insensitive *ASI:SPL15m* suggested that *SPL15m* could function independently of miR156b and that expression of *SPL15* is effective at the leaf primordia within the shoot apex.

Since the native *SPL15n* appeared to be ineffective at restoring the WT phenotype in the enhanced *miR156b* environment of *sk156*, another set of complemented *sk156* lines was generated to express the miR156-insensitive *SPL15m* under the control of the native *SPL15* promoter (Table 1). Morphological traits and carotenoid levels remained unchanged for these transgenic *SPL15:SPL15m* plants compared to uncomplemented *sk156*, except that the leaf shape was similarly elongated as were *35S:SPL15m* plants compared to WT plants (Figure 6F). These data suggested that overwhelming miR156b levels within the *sk156* mutant negatively regulated the expression of the *SPL15n* under its native promoter and prevented restoration of WT seed carotenoid abundance

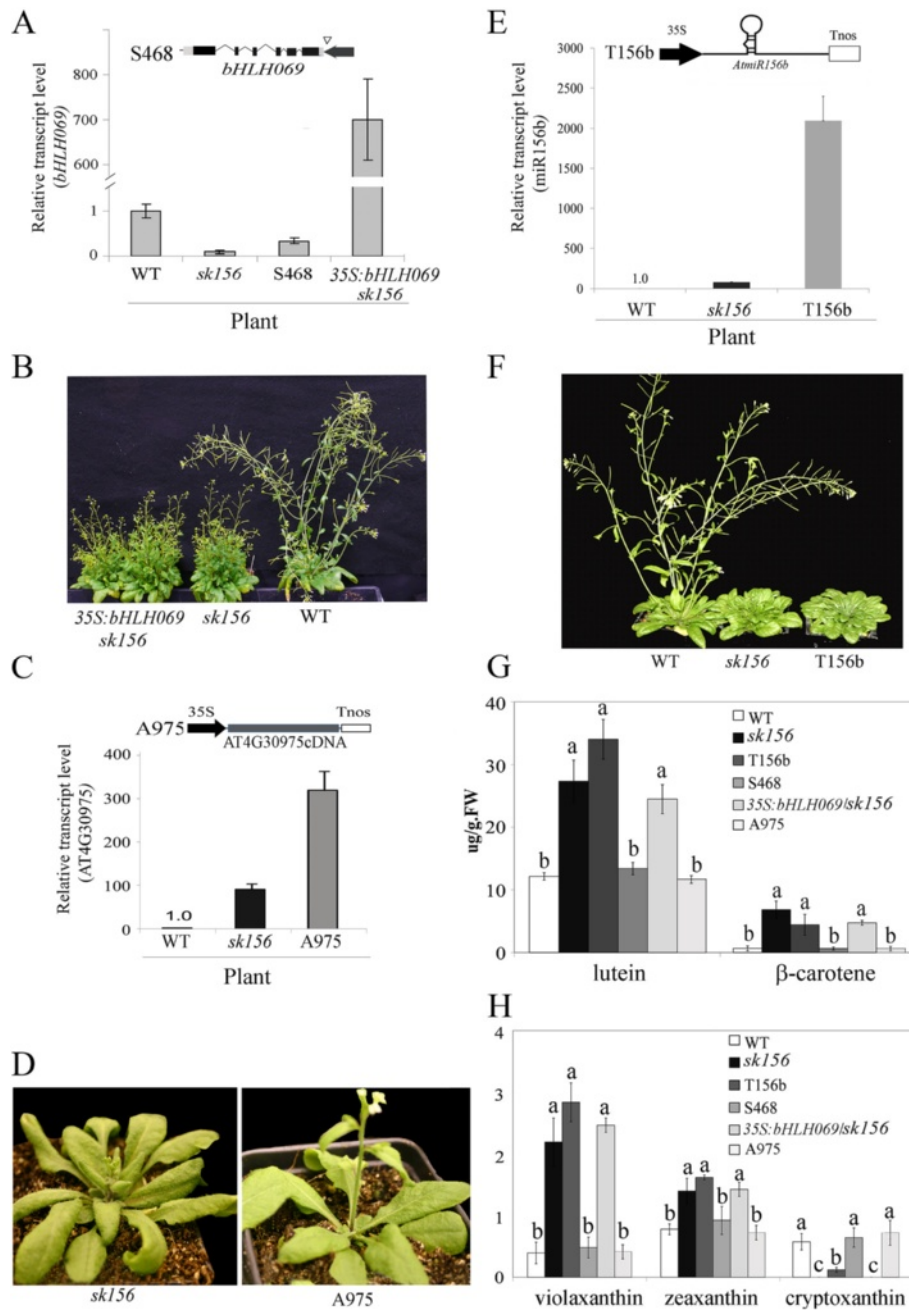
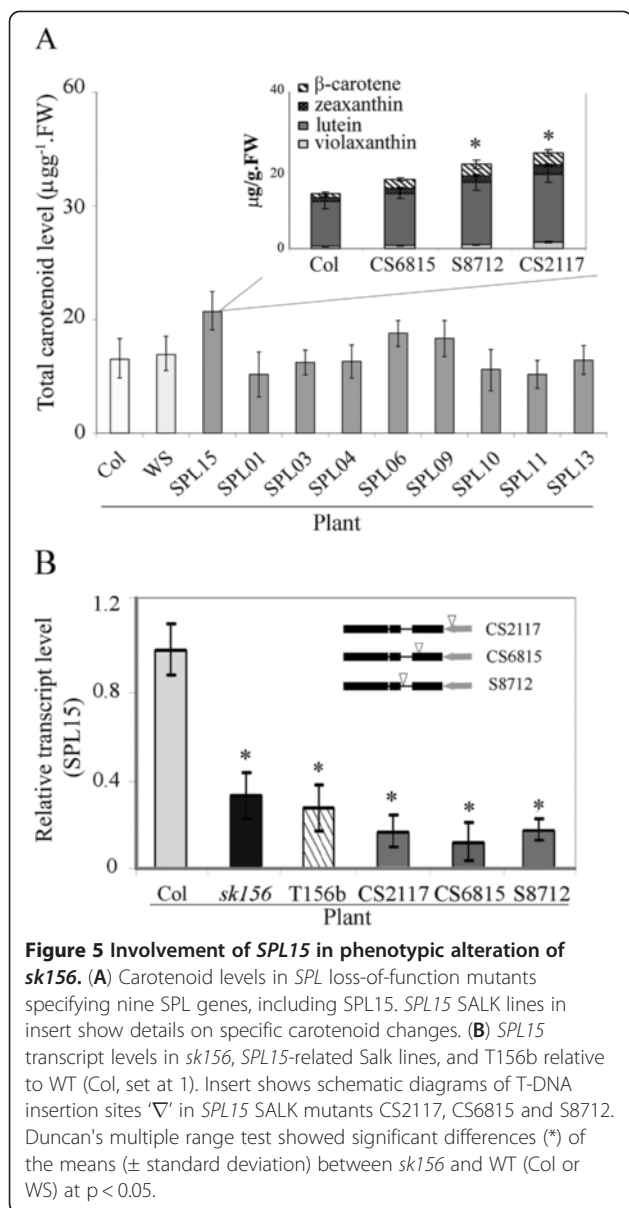


Figure 4 Involvement of *miR156b*, *bHLH069*, and the unknown RNA *AT4G30975* in the phenotypes of the *sk156* mutant. (A) Transcript levels of *bHLH069* in WT (set at 1), *sk156*, mutant line S468 (SALK_032468), and in *sk156* transformants complemented with a *35S:bHLH069* construct relative to WT (set at 1). Insert, schematic of T-DNA insertion site (triangle) in *bHLH069* (*AT4G30980*) in S468 shows above. Exons (black boxes), untranslated regions (light grey boxes), promoter direction (dark arrow). (B) Morphology of 45-d-old transgenic *sk156* complemented with *35S:bHLH069*. *Sk156*, and WT plants are also shown. (C) Transcript levels of unknown RNA gene *AT4G30975* in WT (set at 1), *sk156*, and *35S:AT4G30975cDNA*-complemented *sk156* (A975) plants. Schematic of *AT4G30975* expression construct shows above. (D) Morphology of 35-d-old transgenic plant A975 compared to *sk156*. (E) Transcript levels of *miR156b* in WT, *sk156*, and *35S:miR156b*-complemented *sk156* (T156b) plants. Schematic of *35S:miR156b* expression construct shows above. (F) Morphology of 40-d-old transgenic plants from *sk156* and *35S:miR156b*-over-expressing T156b lines compared to WT. (G) and (H) Seed carotenoid levels in *sk156*, T156b, A975, S468, *35S:bHLH069/sk156*, and WT plants. A Duncan's multiple range test showed significant differences of the means (\pm standard deviation) compared to WT at $p < 0.05$. Means containing the same letter for the same compounds are not significantly different.



and plant morphology. Moreover, the inability of *SPL15*:*SPL15m* plants to restore most of the WT phenotype as did *35S:SPL15m*⁺ lines suggested that the enhanced seed carotenoid level and morphological traits observed in the *sk156* mutant were the collective result of miR156 suppression of at least some other *SPL* genes rather than *SPL15* alone and that *SPL15* functions redundantly with some other *SPL* gene products.

Transcription of *miR156b* is affected by over-expression of *SPL15m*

Wu and Poethig [12] reported earlier that expression of miR156-insensitive *SPL3* under the control of the CaMV35S promoter causes reduced levels of *miR156* transcripts in *Arabidopsis*. To investigate the ability of

SPL15m to affect expression of its regulatory *miR156* genes, we examined *miR156b* transcript levels in the *sk156* mutant and in complemented *sk156* carrying the *35S:SPL15m* cassette (Table 1). Primers for premature *miR156b* rather than mature *miR156* sequence were used so that measurement of transcripts originating from other *miR156* genes would be excluded. In 30-day-old plants, *miR156b* premature transcript levels were reduced from 82.2-fold above WT levels in *sk156* leaves down to a mean of 7 ± 5.5 fold above WT in the leaves of these complemented lines, with a minimum of 1.4-fold in line 7 and a maximum of 19.6-fold in line 9 (Figure 6G). Thus, *miR156b* transcription was depressed substantially such that ~50% of the complemented lines had WT levels. A reduction of *miR156b* transcript level concurrent with an increase in *SPL15m* transcripts suggests that *SPL15* may have two regulatory functions; one controlling feedback regulation of its cognate regulator *miR156* and one controlling downstream genes, such as *miR172*, *APETALA2-LIKE* (*AP2-like*) transcription factors *TOE1*, and *TOE2*, in the miR156 controlled gene network [9].

Physical interaction between the *SPL15* DNA binding domain and GCAT motifs in the *miR156b* promoter

To investigate whether *SPL15* interacts directly with the *miR156b* promoter to affect transcription, we identified putative *SPL15* binding sites in the *miR156b* promoter based on the consensus sequence for SBP binding domain (Figure 8A). To define the consensus sequence, we compared *cis* elements previously identified as SBP box interacting sequences in *Chlamydomonas reinhardtii* [28,29], *Antirrhinum majus* [30] and *A. thaliana* [5,11,31,32] using WebLogo [33]. The consensus SBP domain binding sequence was determined to be 5'-NNGTACR-3', where frequently N=C and R=A (Figure 8A). This consensus sequence was used to search for putative *SPL* binding sites in the promoters of *miR156* genes. Putative *SPL* binding sites with a GTAC core sequence were repeatedly present in the *miR156b* promoter [-1 to -1500], according to the gene transcript start site revealed by Schwab [34]. Of these, a region containing three repeats of the core sequence appeared between -200 bp to -220 bp (Figure 8B) suggesting that *miR156b* expression could be directly controlled by *SPL15*. This was tested using a His-tagged *SPL15* recombinant SBP DNA-binding domain (Figure 8C and D) in an electrophoretic mobility shift assay with a labeled DNA fragment (46-mer) containing a motif from -200 bp to -220 bp from the *miR156b* promoter. The recombinant SBP domain of *SPL15n* specifically bound to this region of the *miR156b* promoter to yield a less mobile promoter fragment (Figure 8E), and competition with 100-fold unlabeled *miR156b* promoter DNA was required to displace the SBP domain. This result suggested that a direct physical

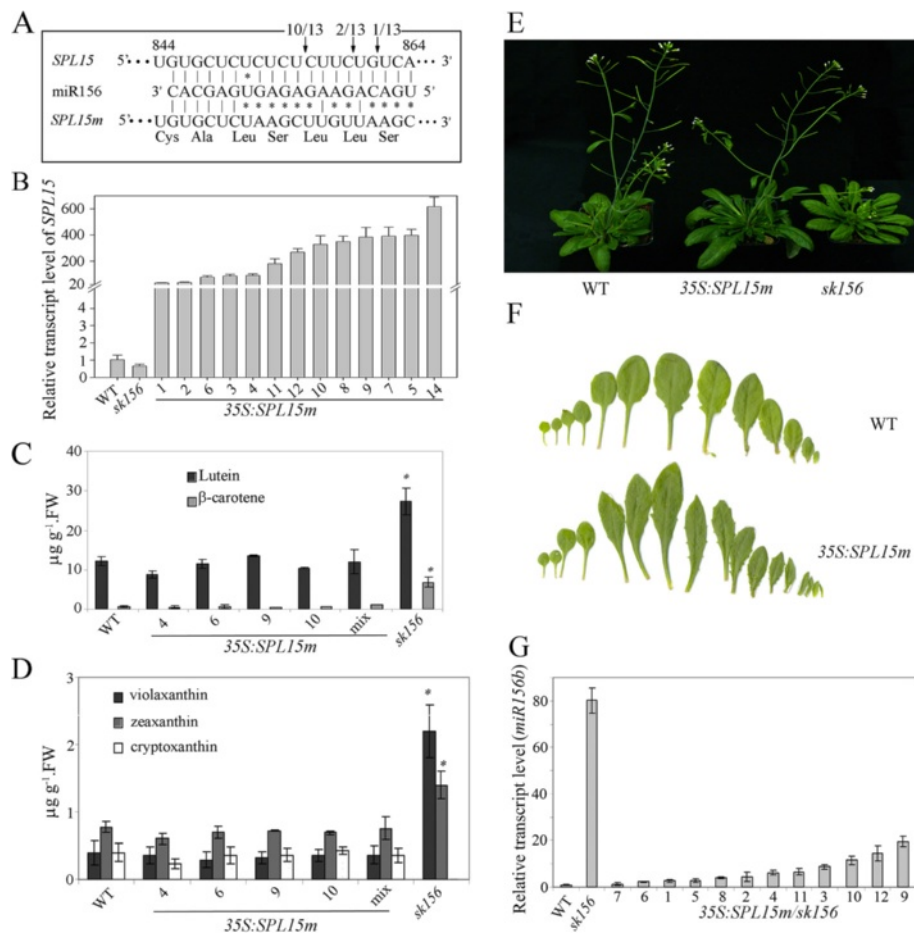


Figure 6 Expression of mutated *SPL15m* in *sk156* results restores a WT phenotype and down regulates *miR156b* transcription. (A) Complementarity of *miR156b* with *SPL15* sequences. Arrows show three cleavage sites (one used 10 out of 13 times) in *SPL15* mRNA due to interaction with *miR156b*. *SPL15m* shows 11 nucleotides mutated in DNA sequence but unchanged in amino acid sequence. (B) *SPL15* transcript levels in independent transgenic *sk156* lines carrying 35S:*SPL15m* compared to WT (set at 1) and *sk156*. Error bars are standard deviations of the means. (C) and (D) Restoration of WT-like carotenoid levels in dry seeds of transgenic *sk156* expressing *SPL15m*. Cryptoxanthin was undetectable in the mutant. (E) Restoration of WT-like plant growth in 35-d-old plants of *sk156* expressing *SPL15m*. (F) Distinctive elongated leaf morphology of *SPL15m*-transformed plants compared to WT plants. (G) *miR156b* transcript levels in different transgenic *sk156* lines expressing *SPL15m* compared to WT (set at 1) and *sk156*. A Duncan's multiple range test was conducted to separate significantly different means. Panels B, G, ± standard deviation; Panels C, D, ± standard error; p < 0.01 (***) and p < 0.05 (*).

interaction can occur between the *SPL15* DNA binding domain and the *miR156b* promoter through the GTAC motif.

Discussion

Enhanced expression of *miR156b* in *sk156*

In this study, we highlight a new *Arabidopsis* mutant, *sk156*, with enhanced expression of *miR156b* due to the insertion of a T-DNA activation tag. The elevated *miR156b* transcript levels in *sk156* are responsible for the full spectrum of morphological phenotypes observed for this mutant. Increased seed carotenoid levels and enhanced branching are consistent with phenotypes observed in *B. napus* expressing *AtmiR156b* under the control of the CaMV35S promoter [22]. These results confirm the role for *miR156b* in seed carotenoid accumulation, and are in

agreement with morphological changes reported in earlier studies [3,6,12,14,35].

Differences in transcriptional regulation for the native *miR156b* promoter, the CaMV35S promoter, and the enhanced *miR156b* native promoter, in addition to different T-DNA insertion sites, can lead to substantial differences in transcript levels between individual transgenic lines and, consequently, to variation in phenotype. For example, *miR156b* transcription driven by the CaMV 35S promoter was more than 2000-fold higher than native *miR156b* transcription in WT plants, whereas *miR156b* transcription in the *sk156* mutant was ~80-fold higher than in WT plants. Such differences in *miR156b* expression levels may result in distinct phenotypes between the *sk156* mutant and 35S:*miR156b* lines. For example, numerous small rosettes and

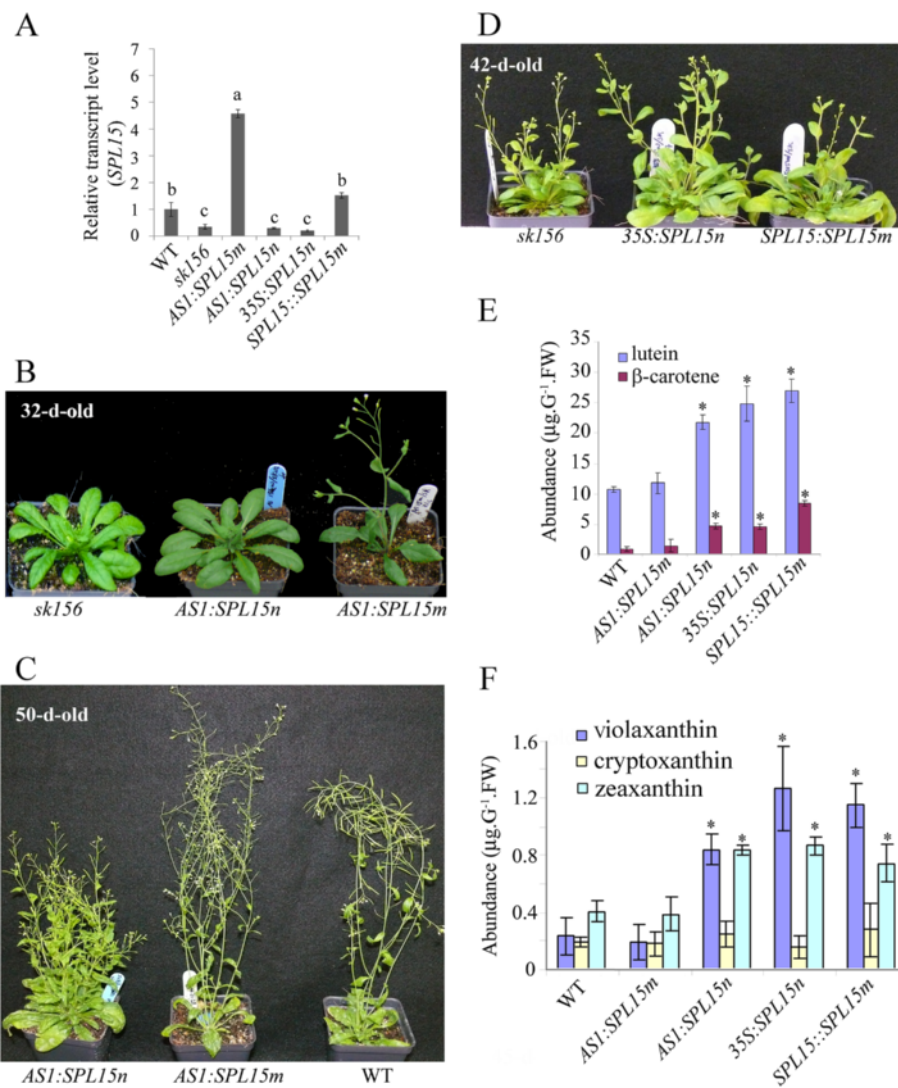


Figure 7 Leaf primodium-dependent miR156-insensitive *SPL15m* restores WT phenotypes when expressed in *sk156*. The *sk156* background was transformed with native miR156-sensitive *SPL15n* or miR156-insensitive *SPL15m* expression cassettes each controlled by either an AS1 or 35S promoter. **(A)** *SPL15* transcript levels by qPCR (\pm standard deviation) in transgenic *sk156* plants complemented with *AS1:SPL15m*, *AS1:SPL15n*, *35S:SPL15n* or *SPL15:SPL15m* compared to WT (set at 1) and *sk156* ($p < 0.05$). **(B)** and **(C)** Morphology at bolting (32 d) and maturity (50 d) for plant expressing *SPL15n* or *SPL15m* under the control of AS1 promoter compared to *sk156*. **(D)** Morphology of 42 d flowering lines for *sk156* plant carrying *35S:SPL15n* or *SPL15:SPL15m* compared to *sk156*. **(E)** and **(F)** Carotenoid levels (\pm standard error) in seeds of *sk156* complemented with *AS1:SPL15m*, *AS1:SPL15n*, *35S:SPL15n* or *SPL15:SPL15m* compared to those of WT and *sk156*. Panels **A**, **E**, **F**: A Duncan's multiple range test was conducted to separate significantly different means for more than 10 independent transgenic lines (each measured with triplicated seed batches) relative to WT plants at $p < 0.05$ (*).

tiny leaves were produced in *sk156* (Figure 1C), and comparatively much smaller and many more rosette leaves were noted in the *35S:miR156b* lines in this and previous studies [6].

Strigolactone-independent seed carotenoid increase in *sk156*

Our data indicated that morphological traits and seed carotenoid profiles are affected in *sk156*. A link between altered carotenoid profiles and branching was previously

observed in *max* mutants [17,19] and in an *Arabidopsis* histone methyltransferase (SDG8)-defective mutant [36]. This was largely due to the effect on the biosynthesis of carotenoid-derived strigolactone branching inhibitors [20,21]. In the current study, we also demonstrated that the elevated levels of seed carotenoids in *Arabidopsis* mutant *sk156* were due to the enhancer-driven expression of *miR156b*. However, morphological phenotypes of the T₁ progeny of *sk156* crossed with WT (in which *CCD7* and *CCD8* are transcribed normally) and of

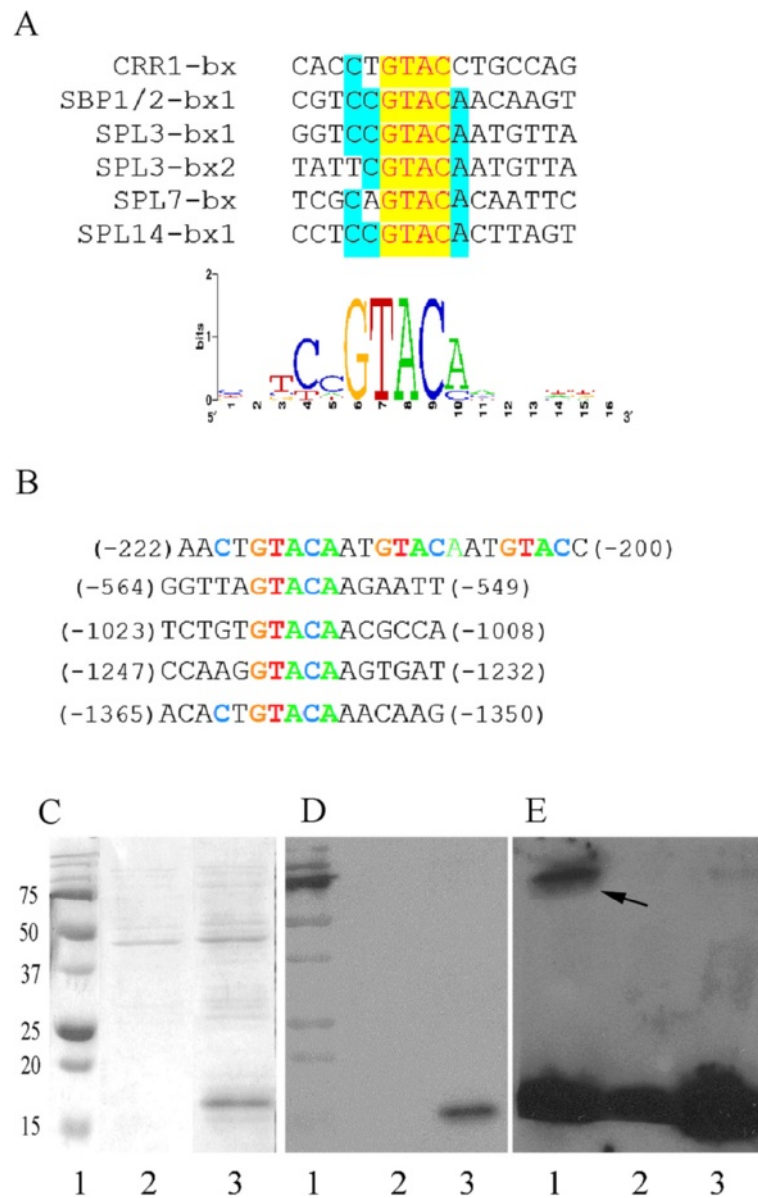


Figure 8 Interaction of the *miR156* SBP-binding motif (GTAC core sequence) with the SBP protein domain of *SPL15*. (A) Consensus DNA sequence present in the promoter region of six previously reported genes interacting with SBP DNA binding domains. CCR1-bx represents the GTAC motif binding to SBP protein CCR1 from *Chlamydomonas reinhardtii* [28,29]; SBP1/2-bx1 represents the GTAC motif binding to SBP1 and SBP2 in *Antirrhinum majus* [30]; SPL3-bx1 and -bx2 [5,11], SPL7-bx [32]; and SPL14-bx [31] represents the GTAC motif binding to corresponding SPL proteins from *Arabidopsis thaliana*. Yellow boxes, 100% conserved; green boxes, 67-83% conserved. The degree of conservation is indicated in the schematic by the height of the letters (measured as bits). (B) GTAC core repeats present in the *miR156b* promoter sequence (from bp -1 to -1700). (C) SDS-PAGE analysis of His-tagged recombinant SBP peptide expressed in *E. coli*. Lane 1, protein marker; lane 2, protein extract of non-induced *E. coli* cells carrying the SBP domain in pET28a; lane 3, protein extract of induced *E. coli* carrying the SBP domain. (D) Western blot of resolved proteins in C using anti-His antibody. (E) Electrophoretic mobility shift assay illustrating His-tagged SBP peptide bound to a labeled *miR156b* promoter fragment containing tandem repeats of the GTAC core. Lane 1, labeled DNA plus SBP peptide. lane 2, labeled DNA only; lane 3, labeled DNA plus SBP protein and 100-fold excess unlabeled DNA. Black arrow shows the shifted band in lane 1.

transgenic WT overexpressing *CCD7* or *CCD8* (data not shown) remained almost the same as in *sk156*. Exogenous application of the artificial strigolactone GR24 (2'-epi-5-deoxystrigol) to *sk156* seedlings for six weeks by supplementing the chemical to the *in vitro* plant

growing in MS media did not affect *sk156* phenotypes (data not shown). These data suggest that ectopic expression of the *miR156b*-induced branching phenotype in *sk156* might not be directly related to the strigolactone pathway. *In silico* analysis revealed that no known

carotenoid biosynthesis or catabolism genes in *Arabidopsis* possess sequences complementary to the mature sequence of *miR156b* [2]. As well, transcript levels of β -ring carotenoid hydroxylase, ϵ -ring carotene hydroxylase, lycopene β -cyclase, lycopene ϵ -cyclase, phytoene desaturase, phytoene synthase or ζ -carotene desaturase were not significantly different in leaves and siliques of *sk156* and WT (data not shown). Thus, *miR156b* likely affects the seed carotenoid pathway indirectly through the regulation of SPL networks as demonstrated in the *miR156*-regulated accumulation of anthocyanin via several *SPL* genes [10]. However, we cannot exclude the possibility of changes to seed carotenoid enzyme activities in the absence of transcription changes, such as occurred with phytoene synthase in etiolated *Arabidopsis* [37]. Also, the impact on seed carotenoid accumulation may not necessarily be due to biosynthesis, but rather to improved carotenoid sequestration and storage as demonstrated in the *Or* mutant of *Brassica oleracea* [38]. These possibilities point to the need for additional investigation to unravel the biological basis for increased carotenoid accumulation in *sk156* seeds.

In *sk156*, a substantial increase in seed carotenoid levels was detected in all except cryptoxanthin. This minor carotenoid, however, was reduced in *sk156* seeds. Cryptoxanthin may be more efficiently converted to zeaxanthin in *sk156* than in WT *Arabidopsis*. This possibility could arise potentially due to differences in transcription of the related genes between WT and *sk156* and should be tested in the future. For example, in *Zea mays*, a β -carotene hydroxylase variant, ZmBCH1, converts β -carotene to β -cryptoxanthin and zeaxanthin, whereas ZmBCH2 can only convert β -carotene to β -cryptoxanthin [39]. Also, in *Arabidopsis* two variants of β -carotene hydroxylase were found to function differently [40].

A slight decrease in carotenoid abundance was observed in leaves of the *sk156* line even though carotenoid levels were increased in *sk156* seeds. Transgenic *B. napus* in which the gene encoding lycopene ϵ -cyclase was constitutively down-regulated also showed increased carotenoids in seeds, but not in leaves [41]. Different mechanisms control carotenoid metabolism in green and non-green tissues, including seeds [42-44]. In developing chloroplasts, the synthesis of carotenoids, such as lutein, β -carotene, zeaxanthin and violaxanthin, is regulated in concert with the light-regulated assembly of light-receiving antennae and photosystem centers into which the carotenoids are integrated [45,46]. In contrast, carotenoid compounds in non-green tissue plastids vary widely in quantity and composition and their synthesis is not necessarily regulated similarly to chloroplasts. In seeds, carotenoids are stored in elaioplasts (lipid storing plastids) or amyloplastids (starch storing plastids) [42,47].

Evaluating the impact of *miR156b* on the transcription of genes specifying differentiation into these carotenoid-rich plastid types could lead us to understand why *miR156b* regulates carotenoid accumulation differently in seeds and leaves of *sk156*.

SPL15 regulation of the morphology and carotenoid alterations in *sk156*

Our 5' RACE assays showed that *SPL15* mRNA was cleaved at three cleavage sites in the segment located near the middle of the coding sequence and is complementary to mature *miR156*, suggesting a role for *miR156*-directed cleavage in *SPL15* transcript processing. However, distinct morphologies were often difficult to find between many of the *Arabidopsis* mutants defective in individual *SPL* genes both in our study and in others [8]. In the present study, *spl15* mutants exhibited slightly stunted shoot growth and a modest increase in carotenoid levels, which were some (but not the full spectrum) of the phenotypes of *sk156*, while the other *spl* mutants examined in this study did not exhibit any discernible phenotypes. Consistently, phenotypes specified by the *sk156* mutant were largely restored to WT phenotypes in *sk156* complemented with *SPL15m* expressed under the control of the CaVM35S promoter or the *ASI* promoter. These pieces of evidence clearly suggest that *miR156* suppresses *SPL15* in the *sk156* mutant and causes much of its phenotypic iteration.

Other *SPL* genes must contribute somewhat to the *sk156* mutant phenotype development, since *spl15* mutants did not show the entire spectrum of *sk156* phenotypes and *sk156* was not fully restored to a WT phenotype by *SPL15m* expressed from a native *SPL15* promoter. These data not only indicate that *SPL15* is functionally redundant with other *miR156*-targeted *SPL* genes, but that this redundancy probably is limited by the endogenous regulation of temporal and spatial gene expression. Our data derived from *ASI* promoter-driven *SPL15m* experiments show that the leaf primordium within the shoot apex is a crucial site for *SPL15m* expression to restore *sk156* phenotypes. This is consistent with previous findings that the shoot apex is a predominant expression site for some *SPL* genes [2,5,13] and that *ASI* promoter-driven *miR156* promotes leaf initiation by suppressing *SPL* genes at the shoot apical meristem [14]. *ASI* is expressed only in emerging lateral organ primordia, and in seeds its expression is detectable only in limited subepidermal cells corresponding to cotyledon initials at the heart stage [48]. In our hands, *ASI:GUS* + plants did not show visible GUS staining in seeds but did show staining in shoot tips, suggesting that *SPL15m* transcript level might not be significantly elevated in *ASI:SPL15m*⁺ seeds compared to *sk156*. The morphological changes in transgenic *sk156* expressing

35S/ASI:SPL15m point to functional redundancy of *SPL15* with at least *SPL9* and *SPL10* on plastochron length [8], *SPL9* on shoot development [13], and *SPL3*, *SPL4*, and *SPL5* on flowering stage transition [4,12].

Expression of miR156b-insensitive *SPL15m* modifies leaf shape in the *sk156* lines carrying *35S:SPL15m* or *SPL15p:SPL15m* constructs. A role for *SPL15* in leaf morphological traits was also revealed by Usami et al [49]. The enhancement of *miR156b* expression in *sk156* possibly prevents the plants from maintaining sufficient *SPL* transcript levels required for normal control of leaf architecture, including those from *SPL15*. These findings are consistent with reduced expression of 10 *SPL* genes in the inflorescences of *Arabidopsis* lines over-expressing *miR156b* [6]. Expression of *SPL15* gene in leaf primordia was also important for rescuing WT-like seed carotenoid and morphology traits in *sk156*. This was supported partially by the finding that leaf primordia are a site where *SPL* factors control the rate of leaf initiation [8].

SPL15 feedback regulation of miR156b

Feedback loops, in which a miRNA-targeted transcription factor either increases or decreases the expression of its cognate miRNA, have been reported in animals [50,51]. However, the mechanism underlying this feedback regulation is unclear, particularly in plants. In our hands, complementation of *sk156* with *SPL15m* leads to increased levels of *SPL15* transcripts and decreased levels of *miR156b* transcripts. The reduction in *miR156b* expression due to increased *SPL15* was consistent with the inverse correlation of *SPL3* and mature *miR156* transcripts in WT *Arabidopsis* at the early vegetative stages (19- and 26-d-old) [12]. These data suggest that negative feedback regulation by *SPL15* may exist for *miR156b*. A recent study on the regulation of *miR156a* and *miR172b* in early vegetative stages proposed feedback regulation between the miRNAs and their targets but did not further investigate the underlying mechanism [9].

All *SPL* family members contain a conserved SBP DNA binding domain [5,30] with variations outside the domain. Moreover, DNA sequences in promoters of *SPL*-regulated genes contain a GTAC core [5,28,29,31,32,52], which is a key element for transcription regulation [53]. Our gel shift assay demonstrated a direct physical interaction between the *SPL15* SBP domain and the promoter region of *miR156b* containing three repeats of the GTAC core sequence. This suggests a mechanism for the underlying feedback regulation of *miR156b* by its target *SPL15*. Support for this finding comes from reports by Birkenbihl et al [52] and Yamasaki et al [32] which indicated that such an interaction also exists between the SBP domain for *SPL1*, -3, -7 and -8 and the GTAC element.

Conclusions

In the present study we showcase a new mutant, *sk156*, in which a T-DNA insert containing four CaMV35S enhancers hyper-induced *miR156b*. We documented phenotypes for several *sk156* lines complemented with different expression cassettes for *miR156b*-sensitive and *miR156b*-insensitive *SPL15*, and showed for the first time a direct interaction between *SPL15* and *miR156b*. These data and the inverse pattern of *miR156b* and *SPL15* expression in mutant *sk156* lead us to conclude that negative feedback regulation of *miR156b* by *SPL15* exists. A second finding is that *miR156b* regulates seed carotenoid abundance in *Arabidopsis* differently than in leaves although the underlying molecular basis remains a subject for further investigation.

Methods

Plants and growth conditions

The *sk156* mutant was isolated from a T-DNA activation-tagged mutant population of *Arabidopsis* Col-4 [23]. The population was generated at the AAFC Saskatoon Research Centre using the pSKI015 binary vector containing a T-DNA with a *Bar* gene and 4 tandem CaMV enhancers [54]. SALK and FLAG T-DNA insertion lines with knockouts in *SPL* genes were obtained from the Arabidopsis Biological Resource Center and the FST (flanking insertion sites) project (www.arabidopsis.org; Additional file 2). *max* mutants were kindly provided by Dr. M-S Peng (University of Guelph) and transgenic *Arabidopsis* lines over-expressing *miR156b* under the control of the CaMV35S promoter were provided by Dr. R. S. Poethig (University of Pennsylvania). To recover homozygous mutant lines, segregating offspring grown were grown on half-strength Murashige-Skoog (MS) basal medium (Sigma, M5519-50L) with 0.8% agar and a selective agent (7.5 mg/L phosphinothricin or 50 mg/L kanamycin) and were screened by PCR using primers specific for each insertion site (Additional file 5). Homozygous plants were transplanted into growth chamber pots for further characterization.

For phenotypic analysis, plants were grown in 32-well flats containing Co-Co Mix soil mixture consisting of compacted coconut fibre/peat moss/vermiculite (1/3/3, v/v/v) and 15-9-12 "Osmocote PLUS" controlled release fertilizer (Scotts Company LLC). Plant age was recorded from the time seeds were imbibed. All plants were kept in a controlled environment growth chamber under a 16/8 h light/dark photoperiod with a light intensity of 230 $\mu\text{E}/\text{min}/\text{m}^2$ and temperature of 20°C/17°C. Leaf and branch number means were calculated from 30 individual plants of mutant or WT lines. A Duncan's multiple range test was conducted to show significant differences between the means at $p < 0.01$ and $p < 0.05$.

Carotenoid extraction and quantification

For carotenoid analysis, leaves of 28-day-old plants were excised and samples immediately ground in liquid nitrogen. Mature seeds harvested from different plants were kept at -80°C for further analysis. Tissues were maintained at -80°C before extraction to minimize carotenoid degradation. Approximately 100 mg of leaf tissue powder and 150 mg of homogenized seeds were used for carotenoid extraction. Carotenoid extraction and HPLC quantitative analysis were carried out as previously described for seeds [41]. HPLC chromatograph peaks were identified by comparing their retention times and absorption spectra to authentic standards, and quantified using standard curves corresponding to carotenoid compounds. Pure chemical standards for β -carotene and lutein were purchased from Sigma. β -cryptoxanthin, zeaxanthin and violaxanthin were purchased from CaroteNature (Lupsingen, Switzerland). Each sample was pooled from the leaves or seeds of 6-10 different plants. Six replicates (two biological replicates with three technical replicates) were used for carotenoid analysis. A Duncan's multiple range test was used to separate statistically different means at $p < 0.01$ and $p < 0.05$.

Molecular characterization of the *sk156* mutant

Plant genomic DNA was extracted to determine T-DNA copy number in the *sk156* mutant. Southern analysis was conducted using 15 μg of genomic DNA digested with *Bam*HI and *Hind*III. The 1.4 kb fragment was excised from a pSKI015 plasmid using *Hind*III and *Eco*RI, labeled with ^{32}P , and used as a hybridization probe. To identify the T-DNA insertion site of *sk156*, genomic DNA was digested with each of four blunt-end restriction enzymes provided in the GenomeWalk kit (Clontech), ligated with adaptors, and flanking regions amplified by nested PCR with the T-DNA specific primers pSKI015-GW-LB1 and pSKI015-GW-LB2 according to the manufacturer's instructions (Additional file 5). Cloned GenomeWalk PCR products were sequenced using the pSKI015-GW-LB2 primer (Additional file 5) and the T-DNA insertion site was determined by BLAST analysis to the TAIR sequence database (Version 9) (<http://www.arabidopsis.org/Blast/index.jsp>). Genetic segregation of plants with T-DNA inserts was completed in triplicate by recording T-DNA insertion and the number of surviving seedlings that germinated out of ~ 100 seeds growing in half-strength MS media containing 7.5 mg/L phosphinotricin or 50 mg/L kanamycin, followed by χ^2 analysis.

Constructs and plant transformation

Total RNA for cDNA synthesis was extracted from 3-week-old *Arabidopsis thaliana* ecotype Columbia plants

using the QIAGEN RNeasy kit (Qiagen) according to the manufacturer's instructions. DNase I was used for on-column DNA digestion to minimize genomic DNA contamination. First-strand cDNA was synthesized by reverse transcription of 300 ng of total RNA in a final reaction volume of 20 μL using random primers and 200 units of SuperScriptTM II Reverse Transcriptase (Invitrogen). Full length cDNA of *miR156b*, *SPL15*, AT4G30975 and *bHLH069* was amplified using Platinum[®] Taq High Fidelity DNA Polymerase (Invitrogen) and gene specific primer pairs (*miR156b_XbaI_F* / *miR156b_SacI_R*; *pXbaI_SPL15F* / *pSacI_SPL15R*; *p30975BamHI_F* / *p30975SacI_R*; and *pbHLH_F* / *pbHLH_R*, respectively), with addition of restriction enzyme sites at the 5' and 3' ends of the genes (Additional file 5). *SPL15m* was generated by introducing 11 mutations into the predicted *miR156* binding site using the PCR primers pSPL15m851F and pSPL15m865R containing mutated sequences (Additional file 5). The *AS1* promoter was isolated from *A. thaliana* Columbia genomic DNA using pAS1_HindIII_F and pAS1_XbaI_R (Additional file 5) according to Wang et al [8], while a 3 kb DNA fragment upstream of the *SPL15* transcription start site including both the 'proximal promoter' and 'distal promoter' regions [55] was amplified by PCR using the primers pSPL15Pr3_HindIII_F and pSPL15Pr3_XbaI_R (Additional file 5) and used as the native *SPL15* promoter. Amplified PCR fragments were cloned into pCR2.1-TOPO vector (Invitrogen) and verified by DNA sequencing. The fragments were retrieved with corresponding restriction enzymes and inserted into the binary vector pBI121 digested with the same pair of restriction enzymes (replacing the original *gusA* gene or *CaMV35S* promoter in the vector). The resulting binary expression vectors contained an *nptII* gene and transgenes under the control of either the *CaMV35S* promoter, the *AS1* promoter, or the native *SPL15* promoter and were introduced into *Agrobacterium tumefaciens* GV3101pMP90 and used to transform *Arabidopsis* using the standard floral dip method. Putative transgenic lines that survived antibiotic selection were transplanted into the greenhouse for further analysis.

5'RACE

5'RACE was carried out using the FirstChoice RLM RACE Kit (Ambion) following the manufacturer's instructions specially for the detection of miRNA degradation products [56]. Briefly, total RNA was isolated from 3-week-old seedlings as described above. Five micrograms of total RNA was ligated to the RNA adapter and a random-primed reverse transcription reaction was performed to synthesize cDNA. A second round of PCR was carried out using a nested adapter primer and primers specific for *SPL15* (Additional file

5). The RACE products were cloned into the pGEM T Easy vector (Promega) for sequencing.

Quantitative Real Time PCR analysis

Total RNA extraction, on-column genomic DNA digestion, and first-strand cDNA synthesis were conducted as described above. Real time quantitative RT-PCR (qPCR) analyses were performed with gene specific primers listed in supplemental Table S1. To quantify miR156b, primers based on the stem sequence of the pre-miR156b hairpin structure were designed to measure premature miR156b according to Schmittgen et al. [57] (Additional file 5). qPCR mixtures contained 10 μ l of diluted cDNA, 12.5 μ l of 2X SYBR Green qPCR Master Mix (Cat No. 11735-040, Invitrogen) and 200 nM of each gene-specific primer in a final volume of 25 μ l. qPCR reactions were conducted using the StepOne Plus system and software (Applied Biosystems) using a relative standard curve method and default reaction parameters. The relative index of the standard curve was over 98%. Control PCR reactions with no templates were also performed for each primer pair. The specificity of amplicons was verified by melting curve analysis (60 to 95°C) after 40 cycles and by agarose gel electrophoresis. All samples were assayed in triplicate from two independent RNA preparations. Mean expression values of all replicates were calculated and normalized to the expression of *PEROXIN 4* (*PEX4*, set at 1), a suitable endogenous reference gene because of its stable and low level expression in *Arabidopsis* [58]. All PCR reactions displayed efficiencies between 87 and 115%. Normalized means were analyzed for significant differences by a Duncan's test ($p < 0.05$).

Expression and extraction of SBP-domains

For recombinant protein expression in *Escherichia coli*, a coding sequence fragment encoding the SPL15 SBP domain (80 amino acid residues) plus 5 amino acid residues both upstream and downstream [48] was amplified by PCR using the primers p15SBP-BamHI-F and p15SBP-SalI-R with addition of *Bam*HI at the 5' end and *Sal*I and a TGA stop codon at the 3' end (Additional file 5). The PCR product was digested with *Bam*HI and *Sal*I and cloned into the pET28a vector (Novagen) between the *Bam*HI and *Sal*I sites. The resulting chimeric recombinant protein fused with a His tag (16.3kD) was expressed in *E. coli* strain Rosetta 2(DE3) pLacI (Clontech) by induction with 0.8 mM isopropyl- β -D-thiogalactopyranoside. Cells were harvested by centrifugation and resuspended in BugBuster[®] Master Mix (Novagen) to lyse the cells. Recombinant His-tagged SBP proteins were detected in the inclusion bodies using anti-His antibody (G-18) (Santa Cruz Biotechnology) in Western blot assays. Proteins in inclusion bodies were solubilized

with 1.5% sarkosyl (N-laurylsarcosine) according to Frangioni and Neel [59], and recombinant SBP protein recovered and re-folded using a protein refolding kit (TB234, Novagen) according to the manufacturer's instructions. Protein concentration was measured in lysates by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and proteins separated in 13% SDS-polyacrylamide gels. The protein was stored at 4°C until use in gel shift assays.

Electrophoretic mobility shift assays

The ability of SPL15 SBP to bind to the *miR156b* promoter DNA was examined using electrophoretic mobility shift assays. A 46 residue DNA fragment, which included the three repeated GTAC core sequences close to the transcriptional start site of *miR156b* (shown in Figure 8B), was synthesized using primers R156b-bx1-U and R156b-bx1-L (Additional file 5), hybridized and labeled with digoxigenin using a DIG Gel Shift kit (second generation) (Roche). The labeled DNA fragment (~20 fmol) was incubated for 30 min at 25°C with or without the SPL15 SBP protein (~500 ng) in 20 μ l of reaction buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 5 mM DTT, 2.5% glycerol, and 0.05% NP-40. Then, a 100-fold excess of the unlabeled promoter DNA fragment was added to the reactions. After incubation, the mixtures were separated by polyacrylamide electrophoresis (7.5% gel) at 4°C for 1.5 h (0.8 V/cm²) in 0.5 X TBE (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA at pH 8.0). DNA was blotted onto a nylon membrane and mobility changes for the labeled *miR156b* promoter fragment detected with digoxigenin-specific antibodies.

Additional files

Additional file 1: Schematic diagram of disrupted carotenoid cleavage dioxygenase genes *CCD7* and *CCD8* in the *max3-9* and *max4-1* mutants used in this study. Boxes represent exons and lines represent introns. Triangles show the T-DNA insertion sites. The *max* mutants were previously reported by (Booker et al. 2004).

Additional file 2: SALK and FLAG T-DNA insertion lines for miR156-targeted *SPL* genes.

Additional file 3: Reduced lengths of cauline stem basal internodes in three *spl15* mutants compared with WT *Arabidopsis*. Length of the cauline stem basal inter-node was measured from the rosette core up to the first visible basal node for WT, three *spl* mutants and *sk156* plants grown for 6 weeks.

Additional file 4: Confirmation by PCR of transgene presence in *sk156* lines transformed with a 35S:*SPL15m* gene. The miR156 insensitive *SPL15m* contained 11 mutated nucleotides as described in Materials and Methods. Primer sequences are listed in Additional file 5. (A) Activation-tag from pSKI015 T-DNA detected in transgenic *sk156* plants carrying 35S:*SPL15m* cassette (lanes 0-11) and in the *sk156* background alone (lanes 12 and 13). Primers SK2222-F (430bp upstream) and SK2222-R (1830bp downstream) flanking the T-DNA insertion site and primer pSKI015-GW-LB2 (439bp to the T-DNA left border) were used to detect the insert. In WT lane, no T-DNA insert was detected and only a

fragment close to 1.9kb was present due to genomic DNA amplified with the primers flanking the T-DNA insertion site. In homozygous *sk156* plants which did not carry *35S::SPL15m*, a single T-DNA fragment (869bp) was generated. 1kb, 1-kb Plus DNA ladder (Invitrogen); WT, Col-4; pSKI015, plasmid containing the activation tag present in *sk156*. (B) Transgene *SPL15m* detected in the transgenic *sk156* lines carrying *35S::SPL15m* (lanes 0-14), but not in *sk156* alone using primers 35SF3 and SPL15R.

Additional file 5: Primers used in this study.

Additional file 6: PCR confirmation of transgene presence in four different miR156-sensitive or miR156-insensitive transgenic Arabidopsis populations used in this manuscript. (A) Lanes 2-8, PCR product (818bp) for 7 transgenic plants carrying a *35S::AT4G30975* cassette in a WT background using primers 35S-F3 and p795-3R. P, binary plasmid pBI121 containing *35S::AT4G30975* as a positive control. WT, Col-4. (B), (C) and (D) Transgene PCR product (636bp) carrying *AS1::SPL15m*, *AS1::SPL15n* and *SPL::SPL15m* cassettes in a *sk156* background, respectively, using primers SPL15-871F and NosTer-R6. P, plasmid containing *35S::SPL15* as a positive control. Black arrows points to the DNA marker. Primer sequences are listed in Additional file 5. 1kb, 1-kb Plus DNA ladder (Invitrogen).

Authors' contributions

SW designed the experiments and conducted the majority of the experimentation, analyzed data, and drafted the manuscript; IAP Parkin constructed and provided the *Arabidopsis* activation-tagged population; BY was involved in construction of SPL15 binary vectors; MJG performed computational sequence analysis of the miR156b promoter binding motif; MYG, and AH provided critical feedback on experimental concepts. MYG, GGK, DDH and AH revised the manuscript. All authors read and approved the final manuscript.

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