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SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 2 controls floral organ development and plant fertility by activating *ASYMMETRIC LEAVES* 2 in *Arabidopsis thaliana*

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Abstract A network of genes is coordinately expressed to ensure proper development of floral organs and fruits, which are essential for generating new offspring in flowering plants. In *Arabidopsis thaliana*, microRNA156 (miR156) plays a role in regulating the development of flowers and siliques by targeting members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene family. Despite the important roles of the miR156/SPL network, our understanding of its downstream genes that are involved in floral organ and silique growth is still incomplete. Here, we report that the miR156/SPL2 regulatory pathway regulates pollen production, fertility rate, and the elongation of floral organs, including petals, sepals, and siliques in Arabidopsis. Transgenic plants exhibiting both overexpression of *miR156* and dominant-negative alleles of SPL2 had reduced *ASYMMETRIC LEAVES 2* (*AS2*) transcript levels in their siliques. Furthermore, their fertility phenotype was similar to that of the *AS2* loss-of-function mutant. We also demonstrate that the SPL2 protein binds to the 5′UTR of the *AS2* gene in vivo, indicating that *AS2* is directly regulated by SPL2. Our results suggest that the miR156/SPL2 pathway affects floral organs, silique development and plant fertility, as well as directly regulates *AS2* expression.

Keywords miR156 · *SPL2* · *AS2* · Floral organs · Siliques · Fertility

Abbreviations

SPL	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
GFP	Green fluorescent protein
ChIP	Chromatin immunoprecipitation
DAPI	4′,6-diamidino-2-phenylindole
NG RNA-Seq	Next generation RNA sequencing
AS2	ASYMMETRIC LEAVES 2

Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs of approximately 19–24 nucleotides in length that control gene expression at the posttranscriptional level either through transcript cleavage or translation inhibition (Bartel 2004; Cuperus et al. 2011; Nozawa et al. 2012; Sun 2012). Recently, miRNAs were shown to play a significant role in regulating various aspects of plant growth and development (Achard et al. 2004; Schwab et al. 2005). Among all miRNA families, miR156 is one of the most conserved families in plants. miR156 is involved in regulating multiple plant traits, including organ development, flowering time, seed and cell wall composition, and biotic and abiotic stress responses (Aung et al. 2014, 2015; Wang and Wang 2015; Wang et al. 2015).

In *Arabidopsis thaliana*, eight *miR156* isoforms have been experimentally identified so far, and all have near identical mature sequences (Xie et al. 2005). Overexpression

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of each of the six isoforms (*miR156a, miR156b, miR156c, miR156d, miR156e, miR156f*) in *Arabidopsis* results in similar phenotypes, indicating that they are functionally similar in this plant (Wu and Poethig 2006). miR156 targets *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes in *Arabidopsis*, which encode a family of plant-specific transcription factors (Jones-Rhoades et al. 2006). Members of the SPL protein family share a conserved Squamosa promoter binding protein (SBP) domain, which consists of about 80 amino acids (Yamasaki et al. 2004). The SBP domain binds to a DNA consensus (CNGTACM, N = any nucleotide, M = A or C) with a GTAC as core sequence in their target genes' upstream sequence to further regulate their expression (Birkenbihl et al. 2005; Cardon et al. 1999; Wei et al. 2012).

In *Arabidopsis*, 16 *SPL* genes have been identified, ten of which are targeted by miR156 (Preston and Hileman 2013). Several reports indicate that SPLs control diverse aspects of plant development through directly regulating downstream genes. For example, SPL9 regulates trichome distribution by directly activating two *MYB* family genes, *TRICHOMELESS1* (*TCL1*) and *TRIPTYCHON* (*TRY*), which are trichome development repressors (Yu et al. 2010). Also, SPL3 activates *LEAFY* (*LFY*), *FRUITFULL* (*FUL*), and *APETALA1* (*AP1*), which are floral meristem identity genes (Yamaguchi et al. 2009). In addition, the miR156/SPL regulatory network was reported to be required for proper anther development to maintain male fertility (Xing et al. 2010). The miR156 non-targeted *SPL* gene, *SPL8*, also functions redundantly with other miR156-targeted *SPLs* to control male fertility (Xing et al. 2010). Moreover, a *miR156* overexpression mutant, *sk156*, had dramatically smaller siliques and flowers (Wei et al. 2012). Overall, miR156 affects plant fertility and organ growth via *SPL* genes regulation, but research is still needed to uncover the downstream genes that are regulated by SPLs to affect plant organ development and fertility.

For flowering plants, the flower is where sexual reproduction takes place. Floral organs can be functionally grouped into two categories: vegetative and reproductive organs. The vegetative parts of the flower, including petals and sepals, provide a support structure to protect the sexual organs (Irish 2010). Petals and sepals have equally important roles during reproductive development as the reproductive organs. They support plant reproductive structures and build a protective surrounding to secure the normal development of sexual organs. Meanwhile, apart from petals and sepals, the development of reproductive organs can be adversely affected by a number of other factors, including drastic changes in environmental conditions, genetic mutations, and hormone deficiency, which may eventually result in sterility. For example, insufficient auxin has been shown to suppress anther filament elongation and to cause defective pollen development (Feng et al. 2006). Inviable pollen and insufficiency of pollen grains can cause embryo abortion which can also affect fertility rates (Bellusci et al. 2010; Niesenbaum 1999).

In *Arabidopsis*, reproductive development is a complex process regulated by many factors. For example, the MYB DOMAIN PROTEIN 26 (MYB26) has been identified as a key regulator of anther dehiscence, as the anthers of *myb26* fail to dehisce which results in inhibition of pollination and consequently male sterility (Steiner-Lange et al. 2003). A defective *NO EXINE FORMATION1* (*NEF1*) gene leads to the formation of an abnormal pollen wall and a large amount of aborted pollen in *Arabidopsis* (Ariizumi et al. 2004). The homolog of *Brassica campestris* POLLEN PROTEIN 1 (BCP1) is required for viable pollen grain development, and disruption of *BCP1* results in producing shriveled and aborted pollen grains (Xu et al. 1995). The *Arabidopsis* FIMBRIN5 (FIM5) plays an important role in regulating pollen tube growth; the formation of pollen tubes was inhibited in *FIM5* loss-of-function mutants (Wu et al. 2010). miR319 and its target *TCP FAMILY TRANSCRIPTION FACTOR4* (*TCP4*) are critical regulators of petal development, as evidenced by the ability of the *TCP4* mutant, which has a mutation in the miR319 binding site, to supresses the defective petal phenotype of the *miR319a* loss-of-function mutant (Nag et al. 2009).

Recent studies have indicated that *LATERAL ORGAN BOUNDARIES-DOMAIN* (*LBD*) genes encode a family of transcription factors that play key roles in regulating cell division and cell size and eventually affect organ growth (Xu et al. 2008). In addition to regulating organ development, *LBD* genes are also involved in controlling plant fertility (Nakazawa et al. 2003). For example, the interaction of LBD10 with LBD27 is essential in controlling pollen development as demonstrated by the loss-of-function *lbd10 lbd27* double mutant, which has dramatically lower pollen viability (Kim et al. 2015). To date, *LBD* family genes are also reported to be involved in organ formation, auxin response, nitrogen metabolism and several other aspects of plant development (Lee et al. 2009; Li et al. 2008; Rubin et al. 2009). *ASYMMETRIC LEAVES 2* (*AS2*) is a member of the *LBD* gene family, and is required for the formation of symmetric leaves and shoot development in *Arabidopsis* (Iwakawa et al. 2002; Rast and Simon 2012; Semiarti et al. 2001). AS2 is also involved in regulating the *KNOTTED1*-like homeobox genes (Xu et al. 2008), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1* (*KNAT1*) and *KNAT2*, which function in organ initiation and meristem growth (Hay and Tsiantis 2010). A MYB protein, AS1, is required for AS2 to form a complex, which can further bind to the *KNOX* genes' promoters to then regulate their transcription (Guo et al. 2008). Moreover, AS2 also controls petal and sepal development in *Arabidopsis* (Xu et al. 2008). These

pieces of evidence point to the essential role of AS2 in maintaining proper organ development in *Arabidopsis*.

Several reports in the literature have linked the floral development-related genes, *miR156* and *AS2*. For example, *POUND-FOOLISH* (*PNF*) and *PENNYWISE* (*PNY*), which are members of the *BEL1-LIKE HOMEODOMAIN* gene family, are expressed in floral meristem and are involved in floral organ development (Kanrar et al. 2008). *PNF* and *PNY* also participate in the regulation of the miR156/SPL network through activating *SPL3, SPL4* and *SPL5*, as well as repressing *miR156* (Lal et al. 2011). The involvement of both miR156 (Wei et al. 2012; Xing et al. 2010) and AS2 (Xu et al. 2008) in flower organ development raises the question as to whether the two act in concert or independently. In this study, global gene expression analysis was conducted to identify genes that are differentially expressed in siliques of *Arabidopsis miR156* overexpression plants. *AS2* was identified to be down-regulated in *miR156* overexpression plants. Further qRT-PCR experiments revealed that the transcription level of *AS2* was affected by miR156/SPL2 pathway. We then investigated the effects of altered expression of *miR156, SPL2*, and *AS2* on phenotypes and development of flower organs and siliques. We used Chromatin Immunoprecipitation-qPCR (ChIP-qPCR) to show that SPL2 can directly bind to the 5′UTR of *AS2*. This study establishes *AS2* as a direct target of SPL2, and thus builds a connection between the miR156/SPL network and *LBD* gene family.

Materials and methods

Plant materials and growth conditions

The EMS mutant *as2-101* (CS16274) (Xu et al. 2008) and *SPL10* overexpression line (*6mSPL10*) (Nodine and Bartel 2010) were obtained from the *Arabidopsis* Biological Resource Center. *as2101* is in the Landsberg *erecta* (L*er*) background. The *6mSPL10, 35S:miR156* (*miR156* overexpression line), *35S:amiR-SPL4/5* (artificial miRNA targets both *SPL4* and *SPL5*), *35S:SPL2SRDX* (SPL2 dominant-negative) and *35S:SPL10SRDX* (SPL10 dominant-negative) mutants are all in the Columbia (Col) background. Seeds of the *35S:miR156* and *35S:amiRSPL4/5* were kindly provided by Dr. Detlef Weigel (Wang et al. 2008, 2009), and seeds of *35S:SPL2SRDX* and *35S:SPL10SRDX* were kindly provided by Dr. Masaru Ohme-Takagi (Shikata et al. 2009). Wild type (WT) plants of Col and L*er* were used as controls. For stratification, seeds were kept at 4 °C for 2 days in the dark and then transferred to a growth room with long day conditions (16 h light, 8 h dark) and set at 22 °C, 70 % humidity, and a light intensity of 130–150 µmol/m^2/s.

Global gene expression analysis by next-generation RNA sequencing (NG RNA-Seq)

RNA was extracted from siliques of *35S:miR156* and WT Col that grew for 5 days after flowering. Four biological replicates were used for each genotype. NG RNA-Seq was performed by PlantBiosis (University of Lethbridge, Canada) under a fee for service contract. Illumina GAIIx was used to generate reads, and the quality was checked with FastQC software. The BOWTIER aligner was used to align sequences and remove contaminating sequences, including chloroplast and ribosomal RNA. The DESeq pipeline was used to map the filtered reads to the *Arabidopsis* genome (TAIR10, http://www.arabidopsis.org/). Statistical analysis was performed using the DESeq R/Bioconductor package.

Generation of transgenic *Arabidopsis* plants

To generate the *pSPL2:SPL2-GFP* construct, the *SPL2* genomic sequence without the stop codon, but including the 2 kb fragment upstream of the translation initiation site, was amplified by PCR from *Arabidopsis* WT Col genomic DNA using primers pSPL2:SPL2-GFP-5′ and pSPL2:SPL2-GFP-3′ (Table S1). The fragment was first cloned into the pENTR/TOPO-D directional cloning vector (Life Technologies, USA). After sequencing, the fragment was cloned into the binary expression vector pMDC107 (Curtis and Grossniklaus 2003) by recombination using LR Clonase II Enzyme Mix (Life Technologies, USA). The expression vector was then introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell 1986). Eventually the binary vector possessing *pSPL2:SPL2-GFP* was introduced into *Arabidopsis* WT Col plants using the floral-dip method (Zhang et al. 2006). The transformed plants were screened by using appropriate antibiotics and the presence of the transgene was further confirmed by PCR using primers SPL2-5′ and GFP-3′ (Supplemental Table S1).

Gene expression analysis

Gene expression was analyzed by determining transcript levels using quantitative real time-PCR (qRT-PCR). Total RNA was extracted from siliques of plants that grew for 5 days after flowering using the PowerPlant® RNA Isolation Kit (MO-BIO Laboratories, USA), and genomic DNA was removed using the TUBRO DNA-free Kit (Ambion, USA) according to the manufacturer's instructions. The RNA samples were then quantified by using Nano Vue (GE Healthcare, Canada). The first-strand of cDNA was synthesized with 1 µg of total RNA using qScript™ cDNA Super Mix (Quanta, USA). QRT-PCR was performed with PerfeCTa® SYBR® Green SuperMix (Quanta, USA) on a C1000 Thermal Cycler and CFX96 Real-Time System (Bio-Rad,

Canada). *PROTEIN PHOSPHATASE 2 A SUBUNIT A3* (*PP2AA3*) and *UBIQUITIN-CONJUGATING ENZYME 21* (*UBC21*) were used as internal controls to normalize data (Czechowski et al. 2005). Primers used to detect different genes are listed in Supplemental Table S2.

Phenotypic analysis

Length of siliques and seed sets were analyzed using the siliques that grew for 8 days post-flowering (the beginning of flowering for each flower was defined by visible white petals). The number of developing seeds and aborted embryos in the siliques were counted under a Nikon SMZ1500 stereomicroscope. Floral organs, such as petals and sepals, from fully opened flowers were imaged by using a Nikon DS-L3 camera controller. Cellular analyses were performed under the Leica DM IRE2 microscope. Cell area in sepals was measured for more than 200 cells using ImageJ software (National Institutes of Health, USA). The total cell number in sepals was measured as described in (Usami et al. 2009) using at least ten sepals from each genotype.

Testing for pollen viability

Pollen viability was assessed by using the Alexander staining method (Alexander 1969). Nondehiscent anthers were dissected and fixed with Alexander staining solution (10 mL 95% alcohol, 1 mL 1% malachite green in 95% alcohol, 54.5 mL distilled water, 25 mL glycerol, 5 mL 1% acid fuchsin, 0.5 mL 1% orange G, 4 mL glacial acetic acid) for at least 2 h. Viable pollen, as indicated by red staining, was counted under the Nikon Elipse-Ni microscope.

Detection of SPL2-GFP fusion proteins in *pSPL2:SPL2-GFP* transgenic *Arabidopsis*

Three-week-old leaves of *pSPL2:SPL2-GFP* transgenic *Arabidopsis* plants were first stained with the nuclear staining reagent, 4′,6-diamidino-2-phenylindole (DAPI), for 10 min at room temperature. The stained leaves were then examined on a DM IRE2 inverted microscope equipped with an HCX PL APO 1.20 63X water-immersion objective. Images were collected in a 512 × 512 format on a TCS SP2 confocal system (Leica Microsystems, Germany) using a scanning speed of 400 Hz. Three emission signals were collected between 390 nm and 450 nm for DAPI fluorescence; 490 nm and 530 nm for GFP fluorescence; 630 nm and 700 nm for auto-fluorescence of chloroplasts.

Protein-DNA binding analysis by ChIP-qPCR

ChIP-qPCR was performed to determine the capability of SPL2 protein to bind to the *AS2* promoter. The procedure was carried out as described in Gendrel et al. (2005). Briefly, 1 g of whole above-ground plant of three-week-old *Arabidopsis* WT and transgenic line expressing *pSPL2:SPL2-GFP* were fixed in 1% formaldehyde and ground in liquid nitrogen. The fine powder was resuspended in extraction buffer and nuclei lysis buffer (Gendrel et al. 2005). Then, the chromatin solution was sonicated at power three for 2 × 15 s using the Sonic Dismembrator (Fisher Scientific, USA). A portion of the sonicated DNA (20 µL) was reserved for the input control, and the rest was used for immunoprecipitation with the Ab290 anti-GFP antibody (Abcam, UK). QPCR was performed as described above and SPL2 occupancy on *AS2* was estimated by comparing the percentage of input (%input) in *pSPL2:SPL2-GFP* and WT plants (Yamaguchi et al. 2009). In the %input method, the Ct values for the ChIP sample are divided by Ct values for the input sample taking into account the dilution factor. The consensus sequence "GTAC" was identified as the SPL proteins' core binding motif (Birkenbihl et al. 2005; Klein et al. 1996). So we searched for the GTAC sequences in the 2 kb upstream fragment from the start codon of the *AS2* gene. Primers flanking the SPL2 binding core motif GTAC in the 5′UTR and promoter region of *AS2* were used to test for SPL2 occupancy. A fragment containing no SPL protein binding motif from the *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*EIF4A1*) gene promoter region was used as a negative control. Primers used for detection are listed in Supplemental Table S3.

Statistical analysis

The statistical significance of each individual sample versus control was determined by the Student's *t*-test. The Student's *t*-test was performed in Excel (Microsoft, USA). ANOVA was performed by using GraphPad Prism 6 software. The significant level was set at $P = 0.05$. The mean value for each experiment was derived from at least three independent biological replicates.

Results

AS2 is regulated by miR156/SPL2

To investigate the effect of *miR156* overexpression on global gene expression in *Arabidopsis* siliques, we performed high-throughput NG RNA-Seq. In our research, we were more interested in genes that are activated by SPLs, so only genes which have more than two-fold decreased expression level in *35S:miR156* were further investigated. We next searched for down-regulated genes having the putative SPL binding sites by using the consensus sequence

CNGTACM (N = any nucleotide, M=A or C) (Wei et al. 2012), and validated their expression profile by qRT-PCR. In total, 18 genes with at least one CNGTACM consensus sequence in 2 kb upstream of the translation start codon were found to have significantly decreased transcript levels in *35S:miR156* compared to WT Col (Table S4). Three of these genes, *ASYMMETRIC LEAVES 2* (*AS2*), *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*), and *FRUITFULL* (*FUL*) were chosen as candidate genes for further characterization based on their functional identity (Gu et al. 1998; Hu 2003; Lin et al. 2003). Of the *SPL* genes that have been shown to be targets of miR156, four were down-regulated in siliques of *35S:miR156* in NG RNA-Seq data. Significantly reduced transcript levels of *SPL2, SPL4*, and *SPL10* were further confirmed by qRT-PCR (Fig. 1a). To determine whether any of these candidates may be a direct target of SPL2, SPL4, or SPL10, the transcripts levels of *AS2, ARGOS* and *FUL* were examined in SPL2 and SPL10 dominant-negative mutants (*35S:SPL2SRDX* and *35S:SPL10SRDX*) (Shikata et al. 2009), one *SPL10* overexpression mutant (*6mSPL10*), and transgenic plants that express artificial miRNA targeted to *SPL4* and *SPL5* (*35S:amiR-SPL4/5*) (Wang et al. 2008), respectively. Our qRT-PCR results showed that while *AS2, ARGOS* and *FUL* were all affected in both *35S:SPL10SRDX* and *6mSPL10* (Fig. 1b), the transcript levels of these three genes did not follow the expected pattern in these two *SPL10* mutants (i.e. down-regulated in *35S:SPL10SRDX* and up-regulated in *6mSPL10*). There were no significant differences in gene expression between WT and *35S:amiR-SPL4/5;* however, *AS2* was significantly down-regulated in the *35S:SPL2SRDX* mutant (Fig. 1b). Together with the reduced *AS2* transcript levels in the *35S:miR156* mutant as demonstrated by NG RNA-Seq and qRT-PCR (Fig. 1b; Supplemental Table S4), our results suggested that *AS2* is regulated by the miR156/SPL2 pathway and that SPL2 acts upstream of *AS2*.

Furthermore, to examine the expression patterns of *AS2* and *SPL2* during floral organ development, we analyzed the transcript levels of *AS2* and *SPL2* in the buds, flowers and siliques of WT Col and found that transcript levels of both genes were highest in the flowers and lowest in siliques (Fig. 1c).

AS2 is required for silique development and floral organ elongation

Petals and sepals were noticeably smaller in *35S:miR156, 35S:SPL2SRDX* and *as2-101* mutants compared to their WT controls (Fig. 2a, b). The length of petals in WT Col was 2.075±0.144 mm, while those in *35S:miR156* and

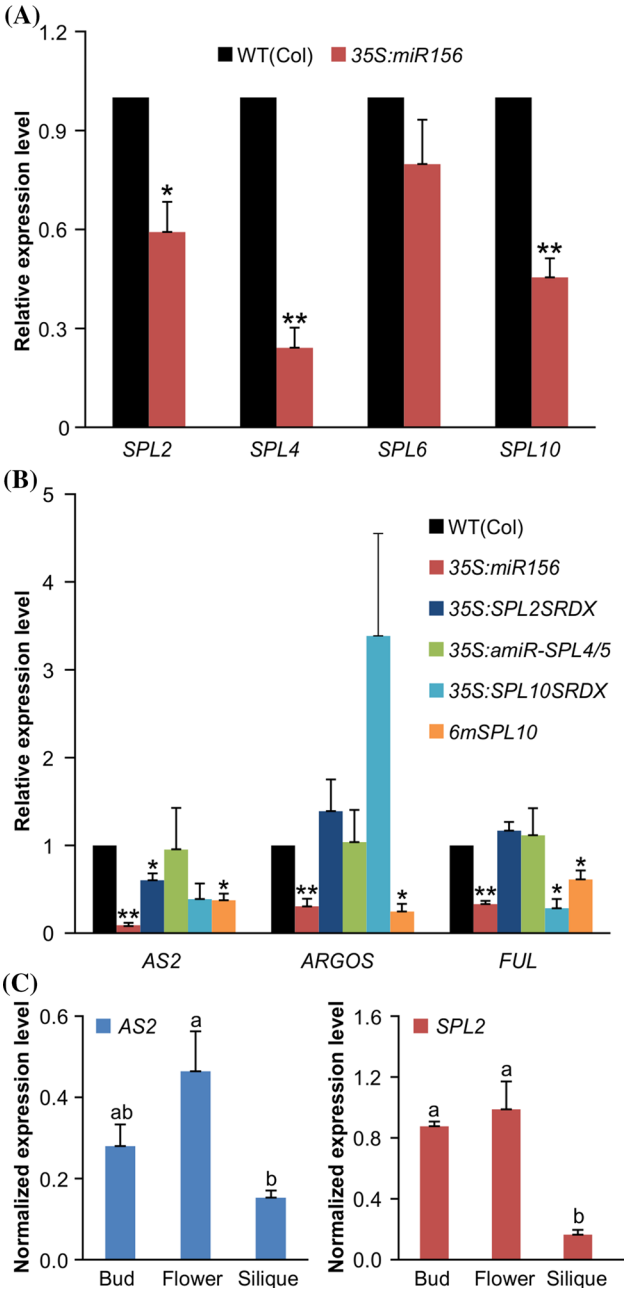

Fig. 1 Analysis of transcript levels of *SPL* and three silique development-related genes. **a***SPL* transcript levels in *35S:miR156* and WT (Col). Statistical significance was determined by Student's *t*-test. Transcript levels are given relative to WT (Col) (set to an arbitrary value of 1). **b***AS2, ARGOS*, and *FUL* in *35S:miR156, SPL* mutants and WT (Col). Statistical significance was determined by Student's *t*-test. **c** The transcript levels of *AS2* and *SPL2* in buds, flowers and siliques of WT (Col). Statistical significance was determined by ANOVA. All the values are the means of three biological replicates with *error bars* representing standard error (SE). Data were normalized using *PP2AA3* and *UBC21*. *Single asterisk* shows significant difference at $P<0.05$. *Double asterisks* show significant difference at $P<0.01$. *Different letters* indicate the significant difference at $P<0.05$

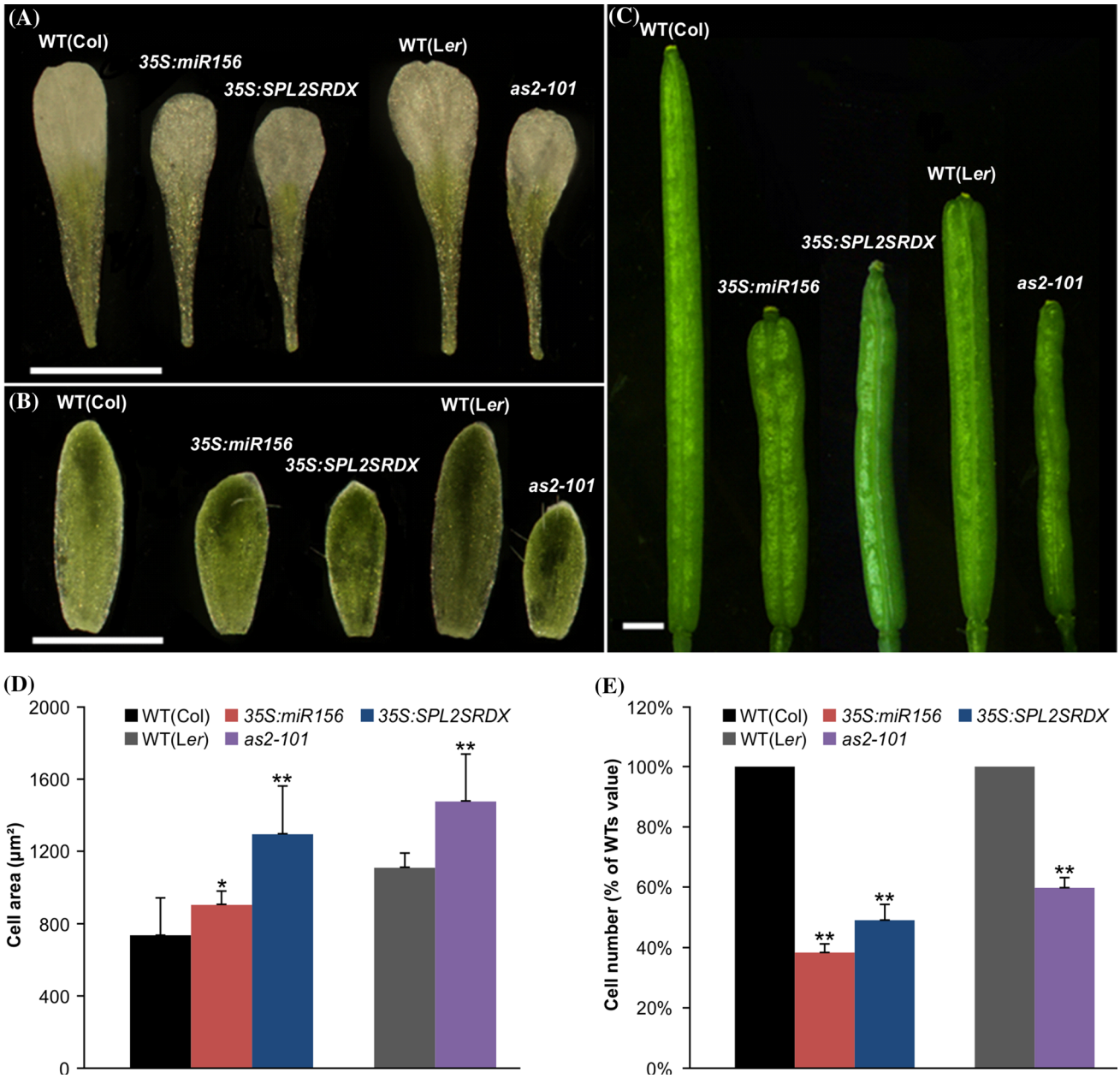

Fig. 2 Phenotypes of petals, sepals and siliques in *35S:miR156, 35S:SPL2SRDX*, and *as2-101* plants compared to WT (Col and L*er*) plants. **a** petals, **b** sepals, **c** siliques. *Scale bar* 1 mm. **d** Average cell area in the sepals of each genotype. **e** Cell number in the sepal of each genotype, and the values represent the percentage of WT controls. *Error bars* indicate standard deviation (SD). *Single asterisk* shows significant difference at $P < 0.05$. *Double asterisks* show significant difference at $P < 0.01$

35S:SPL2SRDX plants were notably reduced, having sizes about 82% of WT Col (Table 1). Similarly, sepals in *35S:miR156* and *35S:SPL2SRDX* plants were about 78% of the length of WT Col (Table 1). This reduction was even more pronounced in the *as2-101* mutant. The petals were 2.121 ± 0.138 mm in WT L*er*, while in *as2-101* the petal length was 1.697 ± 0.95 mm. The length of the *as2-101* sepals was only 66% of WT L*er* (Table 1). Moreover, shorter siliques were also observed in the *35S:miR156, 35S:SPL2SRDX*, and *as2-101* mutants (Fig. 2c; Table 1). In WT Col and WT L*er*, siliques were 14.76 ± 0.62 mm and 12.62 ± 0.87 mm in length, respectively. The siliques of *35S:miR156* and *35S:SPL2SRDX*, as well as of *as2-101*, were remarkably reduced by 20–30% of WT controls (Table 1). To further investigate factors contributing to the shorter floral organs in the mutants, we examined the cell size and total number of cells in the sepals. The cell area was significantly larger in all the three mutants (*35S:miR156, 35S:SPL2SRDX*, and *as2-101*) compared to WT controls (Fig. 2d), but fewer cells were produced in these mutants (Fig. 2e).

Table 1 Length of petals, sepals and siliques in different genotypes

Genotype	Petals		Sepals		Siliques	
	Length (mm)	% of WT	Length (mm)	% of WT	Length (mm)	% of WT
WT(Col)	2.075 ± 0.144		1.451 ± 0.084		14.75 ± 0.62	
35S:miR156	1.698 ± 0.076**	81.8 %	1.121 ± 0.084**	77.3 %	9.26 ± 0.44**	62.8 %
35S:SPL2SRDX	1.710 ± 0.121**	82.4 %	1.132 ± 0.062**	78.1 %	9.98 ± 0.75**	67.7 %
WT(L*er*)	2.121 ± 0.138		1.539 ± 0.076		12.62 ± 0.87	
as2-101	1.697 ± 0.095**	80.0 %	0.954 ± 0.109**	65.8 %	8.72 ± 0.72**	69.1 %

**Significant difference at $P < 0.01$

AS2 is required for fertility

As mentioned above, *Arabidopsis* mutants (*35S:miR156, 35S:SPL2SRDX* and *as2-101*) with reduced *AS2* expression had smaller siliques (Fig. 2c). To determine whether seed production was affected, we analyzed the siliques for their seed content. In WT Col and L*er*, about 47 seeds were produced per silique (Fig. 3a). In contrast, the number of seeds per silique in all three mutants was <30. Besides reduced seed set in the mutants, a partially sterile phenotype was also detected. In a fully fertile plant, a seed develops from an ovule and grows continually until it reaches the mature stage (Fig. 3b). In fully or partially sterile plants, some embryos abort development (Fig. 3c). In the WT plants (Col and L*er*), almost all of the seeds developed normally (Fig. 3d, g), while in the mutants, aborted embryos were observed (Fig. 3e, f, h). We calculated the sterility rate in each genotype and found that in WT Col and WT L*er* the sterility rates were only 2.51 and 3.05 % (Table 2), respectively. Compared with WT plants, *35S:miR156, 35S:SPL2SRDX*, and *as2-101* possess much higher sterility rates of 21.38, 29.90, and 37.28 %, respectively.

To further investigate the reasons for sterility of *35S:miR156, 35S:SPL2SRDX* and *as2-101* mutants, we analyzed pollen grains in all of these plants. We first tested for pollen viability, and found no inviable pollen grains in the three mutants or WT controls (Fig. 4a–e). However, the number of pollen grains in the mutants was significantly decreased compared to WT (Fig. 4f). Compared to Col and L*er* WT plants (Fig. 4a, d), the number of pollen grains produced per anther by each of the three mutants (Fig. 4b, c, e) was only about half of that produced by their respective WT controls (Fig. 4f).

Table 2 Sterility rate in Col, *35S:miR156, 35S:SPL2SRDX*, L*er* and *as2-101*

Genotype	Number of seeds	Number of aborted embryo	Total	Sterility rate (%)
WT(Col)	583	15	598	2.51
35S:miR156	364	99	463	21.38
35S:SPL2SRDX	367	142	509	29.90
WT(L*er*)	573	18	591	3.05
as2-101	244	145	389	37.28

SPL2 is a direct regulator of *AS2*

Given that *AS2* is reduced in *35S:SPL2SRDX* (Fig. 1b) and the fact that the 5′UTR and promoter region of *AS2* contains four putative SPL binding sites (Fig. 5b; Supplemental Fig. S1), we tested whether SPL2 is capable of binding to the regulatory region of *AS2* by using ChIP-qPCR on a transgenic line (*pSPL2:SPL2-GFP*) that expresses SPL2 as a translational fusion with GFP. First we produced transgenic plants harboring SPL2-GFP fusion proteins. The fusion protein was predominantly localized in the nucleus (Fig. 5a) indicating the fusion protein was intact. We then performed ChIP-qPCR to assess SPL2 occupancy in the three putative regulatory regions. Three regions (I, II and III) were identified as containing GTAC core sequences, and region II included two GTAC boxes (Fig. 5b; Supplemental Fig. S1). Region I is located in the promoter region of *AS2*, whereas regions II and III are in the 5′UTR. Fragments containing those consensus binding regions were amplified and the region in the *EIF4A1* gene promoter without a GTAC core sequence was amplified as a negative control. Strong binding capability of SPL2 to regions II and III was detected by ChIP-qPCR in the *pSPL2:SPL2-GFP* transgenic plants. Compared to WT, the occupied signals in these two regions were significantly higher than in the negative control (Fig. 5c). Two of the predicted SPL2 binding sites in region II are very close to each other (less than 100 bp apart), so we were unable to distinguish which is actually occupied by SPL2. Although, the signal in the *pSPL2:SPL2-GFP* plants at region I was stronger than in WT, in contrast to the other two regions, there was no significant difference from the negative control, so it was not considered as a SPL2 binding site. Hence, SPL2 can selectively occupy multiple binding sites in the *AS2* regulatory region to regulate *AS2* expression.

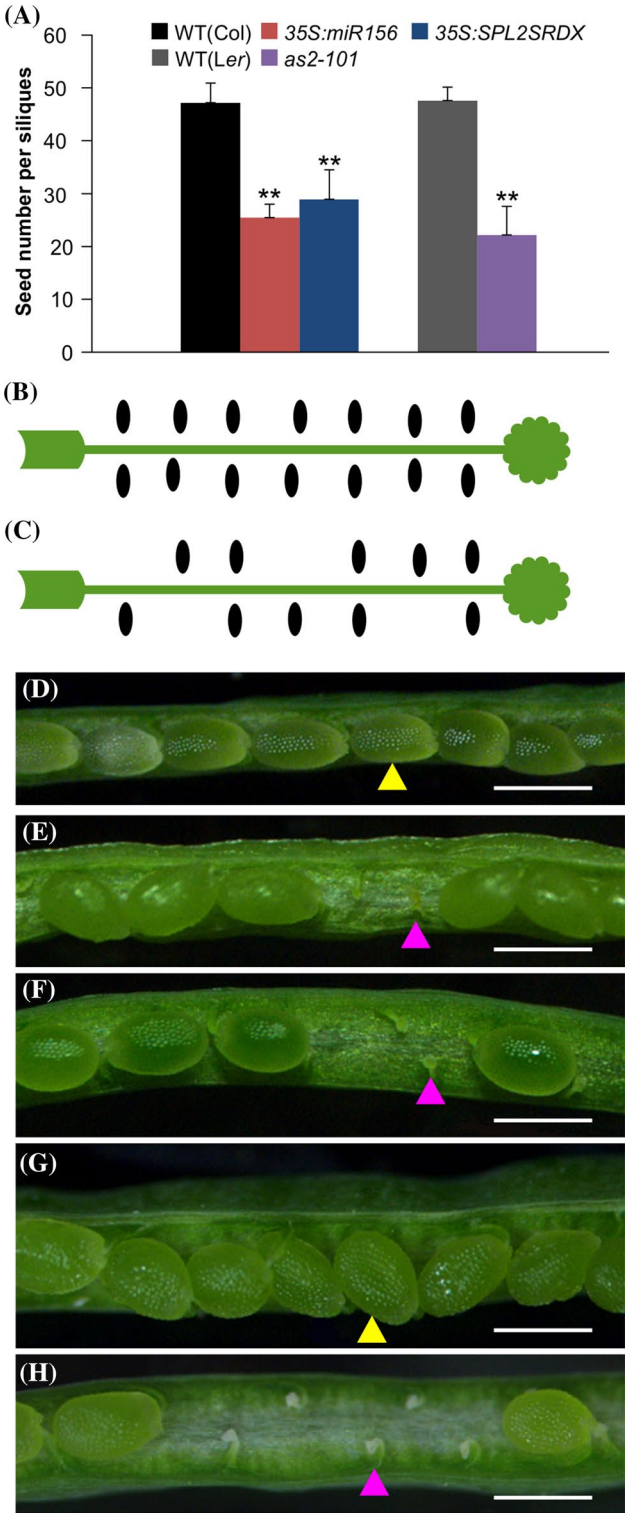

Fig. 3 Seed set analysis and evaluation of plant fertility. **a** Mean number of seeds set per silique in each genotype ($n \geq 15$). *Error bars* indicate standard deviation (SD). *Double asterisks* show significant difference at $P < 0.01$. Schematics of seed pattern of normal (**b**) and partially sterile (**c**) *Arabidopsis* plants. Seed patterns in the siliques of WT (Col) (**d**), *35S:miR156* (**e**), *35S:SPL2SRDX* (**f**), WT (L*er*) (**g**) and *as2-101* (**h**). *Purple arrows* show the aborted embryos, *yellow arrows* show the normal growing seeds. *Scale bar* 100 µm

The miR156/SPL2 pathway regulates plant organ growth- and fertility-related genes

To further investigate the influence of the miR156/SPL2 regulatory pathway on organ growth and plant fertility in *Arabidopsis*, we tested the expression levels of genes that control floral organ development or plant fertility, including *PNF, PNY, LFY, LBD10*, and *LBD27* (Kanrar et al. 2008; Kim et al. 2015; Weigel et al. 1992), in the three mutants and their respective WT controls (Fig. 6). These genes were chosen for their association with either the miR156/SPL network or AS2: *PNF* and *PNY* are involved in the regulation of *miR156* (Lal et al. 2011), *LFY* is a direct regulator of *AS2* (Yamaguchi et al. 2012), *AS2* regulates *KNAT1* (Guo et al. 2008), and *LBD10* and *LBD27* are from the same gene family as *AS2*. If the transcript level of these organ development- or fertility-related genes is reduced in *35S:miR156* and *35S:SPL2SRDX*, we can conclude that SPL2 acts upstream of these genes, and if their expression is altered in *as2-101*, then AS2 is involved in activating or repressing these genes. The expression of *KNAT1* was significantly increased in *35S:miR156, 35S:SPL2SRDX* and *as2-101*. A similar expression pattern was observed for *PNF*, indicating that *KNAT1* and *PNF* are downstream of AS2. *PNY* was repressed in *35S:miR156* and *35S:SPL2SRDX* compared to WT Col, but there were no significant differences between *as2-101* and WT L*er*, and thus *PNY* may be regulated through a pathway distinct from AS2. No significant difference was found in the expression of *LFY* in *35S:SPL2SRDX* and WT Col, but *LFY* transcript level was decreased in *as2-101*. The differential expression of *LBD10* in *35S:miR156* and WT Col indicates that its expression may be controlled by SPLs other than SPL2. *LBD27* expression was notably repressed in all mutants compared to WT in either background (Col or L*er*) indicating *LBD27* may act downstream of AS2.

Discussion

Proper development of sexual organs is critical for successful pollination and fertilization, leading to the formation of the embryo and endosperm (Goldberg et al. 1994; Horner and Palmer 1995; Lord and Russell 2002). Throughout these processes, the outer parts of the flower, such as petals and sepals, act as covers to protect reproductive organs, such as stamens and carpels. The development of embryo and endosperm can further determine the length of mature siliques (Chaudhury et al. 1997; Meinke and Sussex 1979; Ohad et al. 1999). On the other hand, siliques that produce fewer seeds are relatively shorter (Meinke and Sussex 1979); however, the growth of siliques can also be induced by

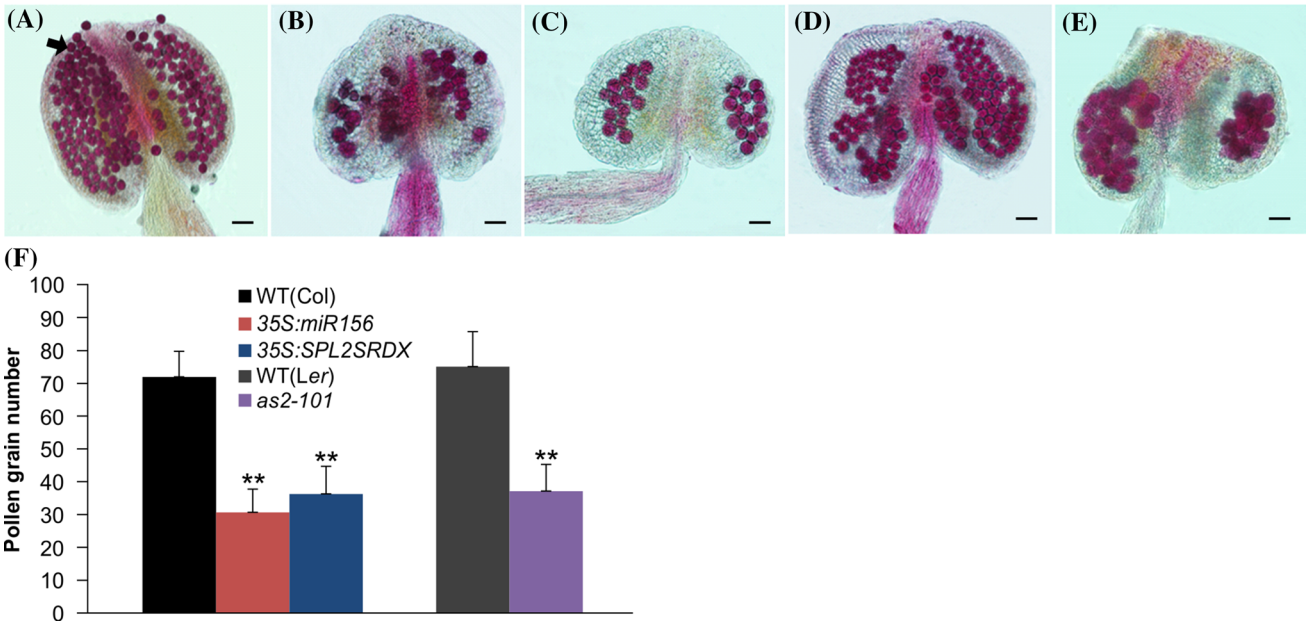

Fig. 4 Evaluation of pollen viability by Alexander staining. Alexander staining of pollen grains in WT (Col) (**a**), *35S:miR156* (**b**), *35S:SPL2SRDX* (**c**), WT (*Ler*) (**d**) and *as2-101* (**e**). *Scale bar* 50 µm. Viable pollen (indicated by an *arrow*) are stained in *red*. **f** Number of pollen grains per anther in each genotype. At least 13 anthers were used to count pollen number. *Double asterisks* show a significant difference at $P < 0.01$

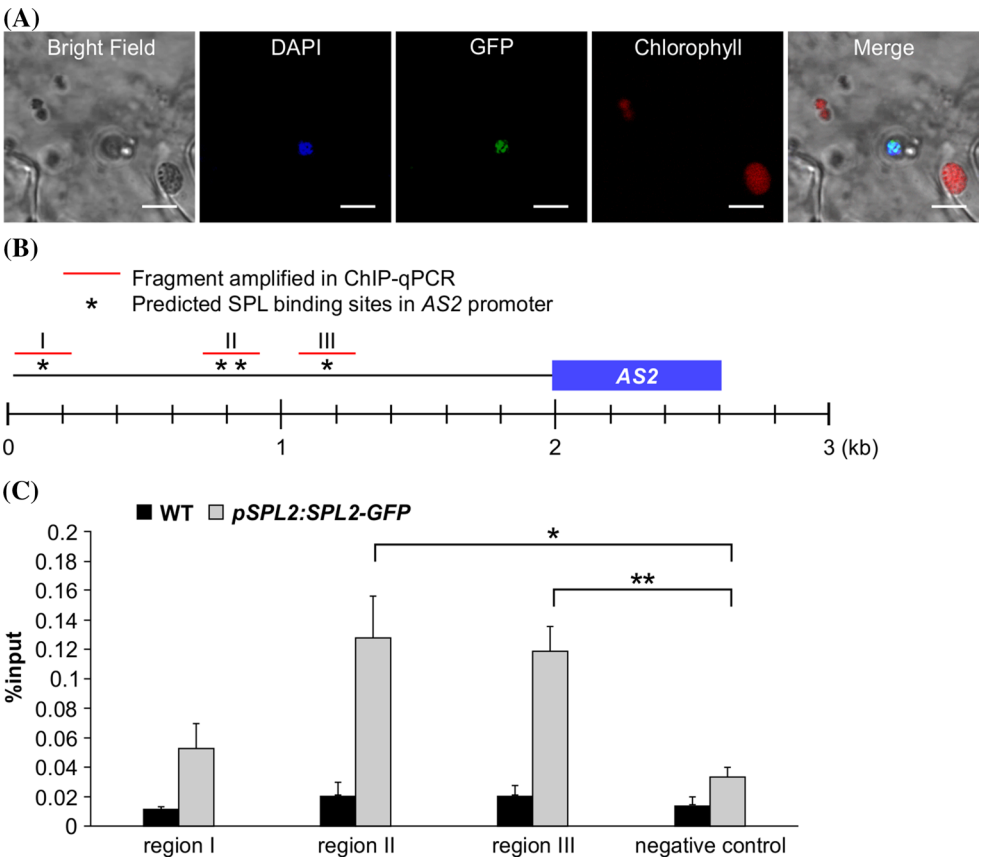

Fig. 5 Detection of SPL-GFP fusion protein and analysis of SPL2 binding to *AS2* promoter. **a** Cellular localization of SPL2-GFP fusion protein. The bright field and the fluorescence of DAPI (*blue*), GFP (*green*), and chlorophyll (*red*) are displayed. Merged image is shown on the *right*. *Scale bar* 5 µm. **b** Schematic of the *AS2* gene, *blue box* represents the coding sequence, *asterisks* indicate sites for putative SPL binding elements. *Red lines* and *Roman numerals* represent fragments amplified by qPCR. **c** qPCR analysis of SPL2 putative binding site abundance in *pSPL2:SPL2-GFP* and WT (Col). Percent input DNA was used to calculate immunoprecipitated DNA enrichment. Data are the mean of three biological replicates and the *error bars* represent SE. *Single asterisk* shows significant difference at $P < 0.05$. *Double asterisks* show significant difference at $P < 0.01$

fertilization-independent endosperm development (Chaudhury et al. 1997; Ohad et al. 1999). Recent reports in the literature point to the involvement of SPL transcription factors in maintaining organ growth and securing fertility (Aung et al. 2014; Silva et al. 2014; Wang et al. 2015; Xing et al. 2013). Several *SPL* genes, including *SPL2, SPL8, SPL9* and

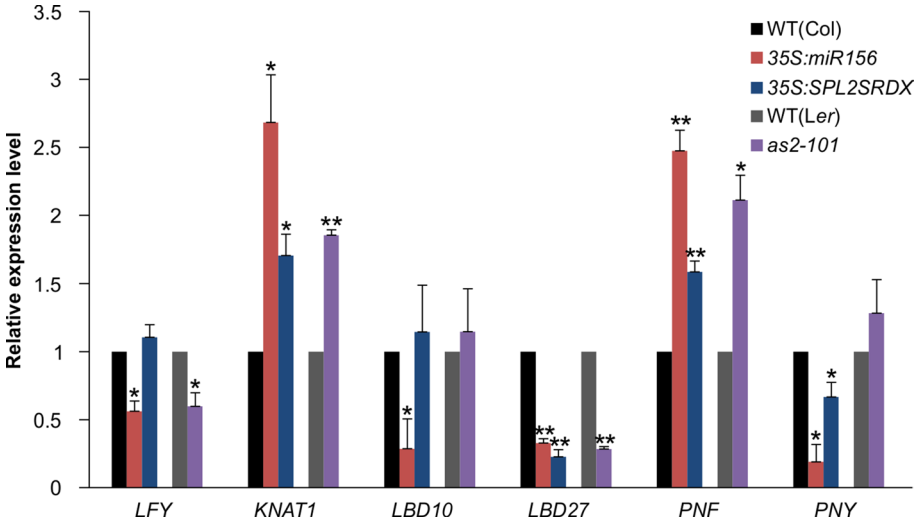

Fig. 6 Expression analysis of genes related to growth of floral organs and plant fertility in siliques. Transcript levels in *35S:miR156* and *35S:SPL2SRDX* were calculated relative to WT (Col) (set to an arbitrary value of 1), and expression levels in *as2-101* were relative to WT (L*er*) (set to an arbitrary value of 1). The expression data are the mean of the three biological replicates. *Error bars* represent SE. *Single asterisk* shows significant difference at $P < 0.05$. *Double asterisks* show significant difference at $P < 0.01$

SPL15 function redundantly to maintain male fertility in *Arabidopsis* (Xing et al. 2010). Knocking-out of miR156-targeted and non-targeted *SPL* genes reduces pollen production and seed set (Xing et al. 2010). Together, these findings point to an essential role for the miR156/SPL network in the determination of organ growth and plant fertility. Here, we characterized the role of SPL2 in maintaining plant fertility and floral organ development. Our results establish a functional link between the miR156/SPL regulatory network and a member of the *LBD* gene family, *AS2*.

Repression of *AS2* inhibits floral organ growth and causes sterility

The floral organs and siliques of *35S:miR156, 35S:SPL2SRDX* and *as2-101* plants were smaller than those of WT plants. The extent to which an organ grows relies on both cell proliferation and cell expansion, and the coordination of these two processes determines the overall organ size (Beemster et al. 2003; Mizukami 2001). Overexpression of *miR156* leads to increased cell size and decreased cell number by silencing *SPL* genes, whereas overexpression of *SPL3, SPL4, SPL5* and *SPL15* causes a reduction in cell size and an increase in cell number, and phenotypes that are opposite to those effectuated by *miR156* overexpression (Usami et al. 2009). This phenotype that induced by ectopic expression of *miR156* was also detected in my research. The cell sizes in *35S:miR156* and *35S:SPL2SRDX* plants were significantly increased, while the numbers of cell were decreased (Fig. 2d, e). This demonstrates the involvement of miR156 and SPLs in regulating plant organ size. Also, larger size and smaller amount of cells were observed in *as2-101* (Fig. 2d, e). Overall, the reduced cell number is the factor that caused inhibiting the growth of floral organs and siliques.

In *Arabidopsis, AS2* is a direct repressor of *KNAT1* (Guo et al. 2008), which is a negative regulator of cell division (Truernit and Haseloff 2008). An increase in the transcripts level of *KNAT1* in *35S:miR156* and *35S:SPL2SRDX* was accompanied by a reduction in *AS2* expression in these plants, as well as in *as2-101* (Fig. 6). This is consistent with previous results (Guo et al. 2008; Lin et al. 2003), which showed direct repression of *KNAT1* by AS2. Our results establish a function for *miR156* in controlling organ growth through affecting cell division, and identify a genetic mechanism for regulating organ growth. In addition, the expression of two transcription factor coding genes, *LFY* and *PNF*, was affected in *as2-101* (Fig. 6). LFY is a master regulator of floral development (Huala and Sussex 1992; Schultz and Haughn 1991; Weigel et al. 1992) and PNF is also involved in regulating organ development-related genes, such as *SHOOT MERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*) (Ung et al. 2011). The involvement of these two transcription factors further suggests the possibility of controlling floral organ and silique development by other genetic pathways that are independent of the miR156/SPL regulatory network.

We also noticed a significantly higher sterility rate in *35S:miR156, 35S:SPL2SRDX* and *as2-101* compared to WT plants (Table 2). To further investigate the factors that caused sterility, we stained the anthers using the Alexander staining method. While no inviable pollen was found in any of the plant lines, a much smaller number of grains were produced in *35S:miR156, 35S:SPL2SRDX* and *as2-101*. An abundant number of pollen grains is critical to secure the chance for sufficient pollen to adhere to the stigma. Lower number of pollen grains often results in reduced fertilization rates, and subsequently partial sterility (Ter-Avanesian 1978). The abnormal pollen grain production combined with the under-developed siliques that are caused by *miR156* overexpression, and by *SPL2* repression, suggests that miR156/SPL regulatory network plays a role in maintaining plant fertility.

SPL2 is a direct activator of *AS2*

Several transcription factors have been shown to directly regulate *AS2* expression. For example, BLADE-ON-PETIOLE1 (BOP1) is a direct activator of *AS2*, and BOP2 is also required for *AS2* activation during leaf development (Jun et al. 2010). Furthermore, another transcription factor, KANADI1 (KAN1), can control leaf polarity by binding to the promoter of *AS2* to repress *AS2* transcription (Wu et al. 2008). In this report, we discovered genetic evidence that SPL2 also activates *AS2* expression. Three regions upstream of the translational initiation site of *AS2* contain putative SPL binding sites and were tested for their SPL2 binding capability. ChIP-qPCR assay indicated that two regions (regions II and III) closer to the translational start codon have strong SPL2 occupation signal, while the distant region (region I) did not show significant binding of SPL2 (Fig. 5b, c). Previous investigations showed that the GTAC core sequence is necessary for SPL binding (Cardon et al. 1999), but this sequence by itself is not sufficient for binding (Yamaguchi et al. 2009; Yu et al. 2010), thus DNA sequences flanking GTAC and its position within the regulatory region may be determining factors in SPL binding.

We also tested the mRNA expression pattern of *AS2* and *SPL2* in different floral tissue (Fig. 1c). The mRNA level of *SPL2* was significant higher in buds than siliques; while no significant different of transcript level of *AS2* in buds and siliques were found. It is likely that some other pathways are also involved in regulating *AS2* during the early floral organs development stages. The transcript levels of both *AS2* and *SPL2* were significantly decreased in siliques compare to flowers, which further prove the activator role of SPL2 to *AS2* in the flowers and siliques.

Proposed model for miR156/SPL regulatory network

Based on the expression patterns of *miR156, SPL2* and *AS2*, as well as those of organ development- and fertility-related genes (Figs. 1, 6), we propose a regulatory model that centers on the miR156/SPL*2* pathway (Fig. 7). *LFY* is necessary for flower formation (Weigel et al. 1992; Weigel and Nilsson 1995) and directly induces the transcription of *AS2* through binding to specific regulatory regions of *AS2* (Yamaguchi et al. 2012). We also found that *LFY* is feedback activated by *AS2* (Fig. 6). SPL3 binds to the first exon and intron of *LFY* to regulate its expression (Yamaguchi et al. 2009), but in our study, the expression of *LFY* was not affected in *35S:SPL2SRDX* indicating that SPL2 is not functionally redundant with SPL3 in regulating *LFY*. The expression of three other genes, *KNAT1, PNF* and *PNY*, was altered by SPL2. Previous research showed LBD10 and LBD27 to be crucial for maintaining plant fertility, and our data revealed that the *LBD27* is regulated through the

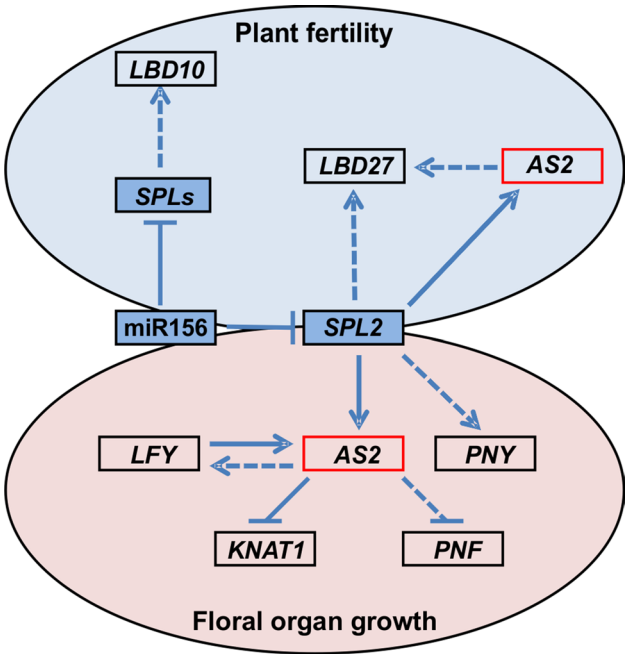

Fig. 7 A proposed model for the regulatory pathway of floral organs and fertility involving miR156/SPL network and *AS2* gene. *Solid arrows* indicate the direct activation and *dashed arrows* show direct or indirect activation; *blunted lines* show direct repression and *dash blunted lines* show direct or indirect repression. *Pink ellipse* indicates genes involved in floral organ growth; *blue ellipse* shows genes involved in plant fertility

miR156/SPL2 pathway, and by AS2 (Fig. 6). Moreover, the genomic sequence of *LBD27* contains both SPL and LBD putative binding sequences (Husbands et al. 2007), which suggests that both SPL2 and AS2 are upstream regulators of *LBD27*, but further investigation will be needed to prove this hypothesis. Thus, miR156/SPL2 pathway appears to act upstream of *KNAT1, PNF* and *LBD27*. Reduced *LBD10* transcript level in *35S:miR156* indicates *LBD10* might be regulated by a pathway independent of miR156/SPL2.

Conclusion

Our results indicate that the miR156/SPL*2* regulatory pathway is necessary for the regulation of plant floral organ growth and fertility by directly regulating the expression of *AS2*. Besides *AS2*, several other genes with diverse functions also act in the pathway. These regulatory interactions within different transcription factor families broaden the regulatory network of miR156/SPL as manifested by the disparate sets of traits that are affected by this network in plants, ranging from plant yield, to stress tolerance, to flowering time, to mention a few (Aung et al. 2015; Wang and Wang 2015). However, the *SPL2* loss-of-function mutant showed a similar visible phenotype to that of WT (Schwarz et al. 2008), which may indicate functional redundancy between

some SPLs. In our qRT-PCR and NG RNA-Seq data, *SPL4* and *SPL10* also showed decreased transcript levels in the *miR156* overexpression line. So it is likely that other SPLs also act in concert with SPL2 in controlling the development of siliques. Besides, the expression patterns of *SPL2* and *SPL10* are similar in different tissues, and the protein sequences share three conserved domains in the N-teminus (Riese et al. 2007). Moreover, the redundant function of SPL2 and SPL10 during leaf growth has been reported (Shikata et al. 2009). Collectively, these data suggest that SPL10 might be also functionally redundant with SPL2 in regulating silique development.

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Author contributions AH conceived of the project and secured funding. ZW, YW and LA conducted experiments, analyzed data and drafted the manuscript. AH and SEK supervised the research.

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