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Functional Analysis of the miR156 Regulatory Network in Arabidopsis Siliques

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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Siliques**

(Thesis format: Monograph)

by

Zhishuo, Wang

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Siliques are photosynthetically active seed capsules and their development is strongly influenced by embryo development. MicroRNA156 (miR156)-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) network is involved in regulating plant growth and development, but the downstream genes of this network are still not fully elucidated. Here, I show that the miR156/SPL2 pathway controls the development of floral organs, regulates pollen production, and thus affects male fertility in *Arabidopsis thaliana*. I present evidence that SPL2 binds to the 5'UTR of the *ASYMMETRIC LEAVES 2 (AS2)* gene *in vivo*, indicating that *AS2* acts downstream of SPL2. When compared to wild-type plants, *AS2* loss-of-function mutants share certain similarities with SPL2 dominant-negative mutants and *miR156* overexpression plants, including defective floral organ growth and partially sterile phenotypes. Furthermore, the transcript levels of *AS2* were decreased in both *miR156* overexpression plants and SPL2 dominant-negative mutant. My results suggest that the miR156/SPL2 pathway and *AS2* affect floral organs and silique development as well as plant fertility, and *AS2* is directly activated by SPL2.

Keywords: miR156, SPL2, AS2, plant fertility, floral organ, siliques development

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List of Abbreviations

Note: International System of Units (SI) are not listed

<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
bp	Base pair
cDNA	Complimentary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
Col	Columbia
CRES-T	Chimeric repressor gene-silencing technology
CTAB	Hexadecyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
GFP	Green fluorescent protein
LB	Luria broth
<i>Ler</i>	Landsberg <i>erecta</i>
miR156	MicroRNA156
miRNA	MicroRNA
mRNA	Messenger RNA

MS	Murashige and skoog medium
NG RNA-Seq	Next generation RNA sequencing
PCR	Polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
TAIR	The Arabidopsis information resource
Tris	Tris-(hydroxymethyl)-methylamine

CHAPTER I. INTRODUCTION

During their life cycle, plants go through different phases, with reproduction being one of the most important ones. Flowering plants produce new individuals by either sexual or asexual reproduction. Sexual reproduction creates new offspring via producing seeds, and abnormal development of sexual organs usually results in full or partial sterility. Petals and sepals surround the reproductive parts of the flower to protect sexual organs. Female gametophyte forms in an ovule, and ovule locates inside ovary. Compared to female gametophytes, male gametophytes are less protected from the environment, and this results in the higher possibility of male sterility (Schnable and Wise, 1998). There are many causes to male sterility, including severe environmental conditions and genetic factors (Budar and Pelletier, 2001).

MicroRNA156 (miR156) is known as a crucial regulator of plant growth and development (Wu, 2013). The *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* family of genes, which code for plant-specific transcription factors, are reported to be the target of miR156 (Jones-Rhoades et al., 2006). miR156 is required for multiple aspects of growth and development in plants, such as organ development, flowering time, seed and cell wall composition, and biotic and abiotic stress responses (Aung et al., 2015a, 2015b; Wang and Wang, 2015).

Given all the reports in the literature about a role for miR156/SPL in plant fertility and organ development, research is needed to identify miR156-regulated molecular factors that affect these processes. In this dissertation, the model organism

Arabidopsis thaliana was used to investigate the role of miR156 and its target *SPL2* in controlling male fertility and floral organ development.

1.1 Development of Floral Organs and Siliques

Floral organs can be functionally divided into two types: vegetative and reproductive. The vegetative part of the flower, including petals and sepals, provides a support structure to protect sexual organs. In *Arabidopsis*, several genes are reported to be involved in the growth of sepals and petals. For example, disruption of *ROXY1* causes petal failing initiation and also reduces petal size during later floral development (Xing et al., 2005).

Successful pollination and fertilization lead to the formation of the embryo and endosperm (Goldberg et al., 1994; Horner and Palmer, 1995; Lord and Russell, 2002). The development of embryo and endosperm further determine the length of mature siliques (Meinke and Sussex, 1979). Siliques that produce fewer seeds are shorter (Meinke and Sussex, 1979). However, the growth of siliques can also be induced by fertilization-independent endosperm development (Chaudhury et al., 1997; Ohad et al., 1999). So far, many genes have been reported to affect silique morphology. The length of silique in *ARABIDOPSIS THALIANA GTP BINDING PROTEIN BETA 1 (AGBI)* loss-of-function mutant is decreased, while the width is increased (Lease et al., 2001). Reduced expression of *ARABIDOPSIS THALIANA SHAGGY-LIKE PROTEIN KINASES 32 (ASK32)* causes reduced seed production, which further inhibits the growth of siliques (Dong et al., 2015).

12 Pollination and Double Fertilization in Flowering Plants

Pollinations can be classified into two categories: cross-pollination and self-pollination. Cross-pollination occurs in many types of plants, such as *Medicago sativa* (alfalfa), in which pollen grains are delivered from one plant to fertilize the flower of another plant. Arabidopsis is a typical self-pollinating plant. In the middle stage of flower development, the stigma on the mature gynoecium becomes receptive and stamen filaments elongate and when the elongation process has past the stigma, self-pollination occurs. Fertilization happens when the pollen tube extends down to the ovule (Berger, 2008). Proper development of reproductive organs ensures efficient fertilization, and leads to the formation of seeds (Goldberg et al. 1994). For example, insufficient auxin has been shown to suppress anther filament elongation, and thus pollen grains fail to adhere onto the stigma (Feng et al., 2006; Saito et al., 2015). In addition, both inviable pollen and insufficient number of pollen grains can cause embryo abortion (Niesenbaum, 1999; Bellusci et al., 2010).

13 Male Sterility in Flowering Plants

There are several possible causes for male sterility. For example, anthers produce inviable or insufficient quantity of pollen grains can result in sterility (Niesenbaum, 1999; Bellusci et al., 2010). This indicates that the development of pollen grains is crucial for ensuring fertility. In Arabidopsis, male organ development is a complicated process, and many genes have been reported to be involved in controlling male fertility (Ryan et al., 2015). MYB26 is one of the transcription factors that control anther dehiscence. The loss-of-function mutant, *myb26*, produces

non-dehiscent anthers and therefore increases the difficulty of pollen grains to adhere onto the stigma and results in male sterility (Steiner-Lange et al., 2003). A T-DNA mutant line of *NO EXINE FORMATION 1 (NEF1)* is defective in forming pollen walls, and hence pollen grains cannot develop during the flowering period in the mutant (Ariizumi et al., 2004). The *HOMOLOG OF BRASSICA CAMPESTRIS POLLEN PROTEIN 1 (BCP1)* is involved in controlling the development of viable pollen grain, and reducing the expression of *BCP1* results in producing inviable pollen and pollen grains are shriveled (Xu et al., 1995). The Arabidopsis *FIMBRIN5 (FIM5)* plays an important role in pollen tube growth regulation, and a loss-of-function mutant inhibits the formation of pollen tubes, which affects fertilization (Wu et al., 2010b).

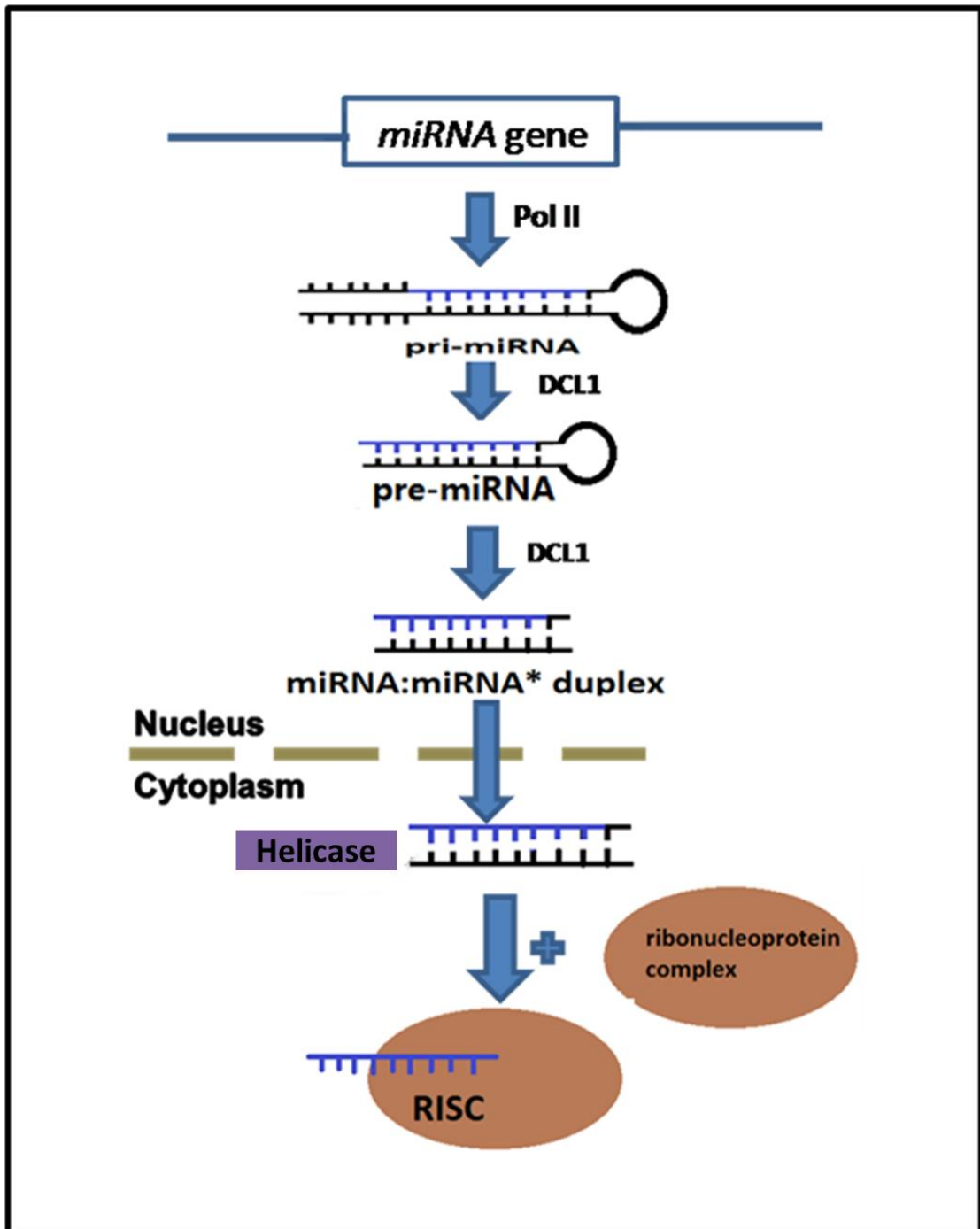
14 Plant miRNAs

MicroRNAs (miRNAs) are a class of non-coding RNAs with mature sequences of approximately 20 ~ 24 nucleotides (Wu, 2013). miRNAs play important roles in plants, animals and some viruses (Du and Zamore, 2005). In animals, miRNAs can affect cell differentiation, programmed cell death and can also be involved in human disease (Ambros, 2004). Plant miRNAs were discovered 10 years after animal miRNAs (Llave et al., 2002; Reinhart et al., 2002), and since then they have been shown to be important regulators of plant development, as many transcription factor coding genes are identified to be targeted by miRNAs in plants. For example, microRNA319 (miR319) controls leaf morphogenesis by targeting TCP-domain family genes (Palatnik et al., 2003); miR160 represses *AUXIN RESPONSE FACTOR*

(*ARF*) family genes, and reducing expression of both *ARF10* and *ARF16* causes producing root cap in irregular shape (Wang et al., 2005); miR172 controls flowering time and floral organ growth through regulating its target *APETALA 2 (AP2)* (Chen, 2004).

Most plant genomes encode at least 100 different types of miRNA (Millar and Waterhouse, 2005). The plant primary miRNA (pri-miRNA) is first transcribed by RNA polymerase using a *miRNA* gene as a template (Figure 1). The pri-miRNA is processed to precursor miRNA (pre-miRNA) by the RNase-III-like protein DICER-LIKE 1 (DCL1). After the pre-miRNA is produced, DCL1 catalyzes the formation of the miRNA:miRNA* duplex. Next, the duplex is exported from the nucleus to the cytoplasm and unwound into a single strand by HELICASE-LIKE enzyme. The “miRNA” strand of miRNA:miRNA* duplex is then incorporated into the RNA-induced silencing complex (RISC), while the miRNA* strand is degraded. Once RISC is formed, miRNAs can directly downregulate the target gene by either transcriptional or post-transcriptional mechanisms. For the post-transcriptional mechanism, plant miRNAs usually have perfect or near perfect complementary binding within the target messenger RNA (mRNA) and RISC can either directly cleave the mRNA of specific genes after miRNA binding to the target site or inhibit translation of the target transcript (Chen, 2005). miRNAs can also downregulate gene expression at the transcriptional level by DNA methylation (Wu et al., 2010a). Interestingly, for this particular pathway, miR1873 can also direct methylation at its own locus; the methylated sites are always located in the center of its mature

Figure 1. The biogenesis of miRNA in plants. miRNA is transcribed from miRNA gene and mature miRNA is subsequently produced in the cytoplasm (see details in section 1.4) (Bartel, 2004). Purple box shows the HELICASE enzyme; brown box shows the ribonucleoprotein complex.



sequence region.

Plant miRNAs are repressors of many genes, but the regulation of the *miRNA* genes themselves is not yet fully understood. Plant *miRNAs* are reported to be regulated at multiple levels. Firstly, the enzymes involved in miRNA biogenesis, such as HYPONASTIC LEAVES1 (HYL1) and DCL1, are crucial regulators of *miRNA* (Kurihara and Watanabe, 2004; Li et al., 2012). Also, miRNAs can methylate themselves to regulate their own expression (Wu et al., 2010a). In addition, some transcription factor families, such as MYB, WD40-repeat and SPL, also act as regulators of miRNA (Wu et al., 2009; Zhang et al., 2013, 2014).

15 miR156 in Plants

miR156 is a highly conserved miRNA family in plants. To date, miR156 has been confirmed to regulate plant growth and development in many species. For example, miR156 is involved in regulating nodulation and root elongation in *Lotus japonicas* (Wang et al., 2015); overexpression of miR156 in *Medicago sativa* results in enhancing shoot branching (Aung et al., 2015b); in *Solanum lycopersicum*, increased miR156 transcript level results in reducing fruit number (Ferreira e Silva et al., 2014); overexpressing *miR156* in *Zea mays* shows longer juvenile phases and delayed flowering time (Chuck et al., 2007).

In Arabidopsis, eight *miR156* isoforms (*miR156a* to *miR156h*) have been experimentally identified so far, and they all have near identical mature sequences (Xie et al., 2005). Overexpression 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). In *Arabidopsis*, miR156 plays diverse functions. For example, in the vegetative phase, miR156 can regulate *miR172*, and thus affect the transition from the juvenile to the adult phase via regulating miR172 targets *TARGET OF EARLY ACTIVATION TAGGED 1 (TOE1)* and *TOE2* (Wu et al., 2009). In this pathway, miR156-regulated genes, *SPL9* and *SPL10*, form a connecting link to activate the expression of *miR172*. For the flowering pathway, miR156 can control the expression of *FRUITFULL (FUL)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, which are MADS box genes. These MADS box genes in turn activate *LEAFY (LFY)* and *APETALA1 (API)* to affect flowering time (Wang et al., 2009). In the reproductive phase, miR156 is required for proper anther development to maintain male fertility (Xing et al., 2010). The miR156 non-targeted *SPL* gene, *SPL8*, also functions in parallel to control male fertility (Xing et al., 2010). Like other miRNAs, miR156 can regulate its target genes at the post-transcriptional level, but the regulation of miR156 itself is still unclear. Much evidence suggests that *SPLs* modulate the *miR156* expression through a feedback regulatory loop. Increasing the transcript levels of miR156-resistant forms of *SPL9* and *SPL10* were shown to activate expression of *miR156* (Wu et al., 2009). Some other transcription factors can also regulate *miR156* expression. The *BELL1-like* homeobox genes *PENNYWISE (PNY)* and *POUND-FOOLISH (PNF)*, which are essential genes for floral meristem development, were reported to negatively regulate *miR156* in shoots and therefore affect transcript level of *SPLs* (Lal et al., 2011); AGAMOUS-LIKE 15 (AGL15) can bind to the

miR156a and *miR156c* promoter regions and directly activate their expression (Serivichyaswat et al., 2015). In this regulation pathway, AGL15 formed a complex with AGL18 and both of these two proteins were essential for the regulation of *miR156* (Serivichyaswat et al., 2015).

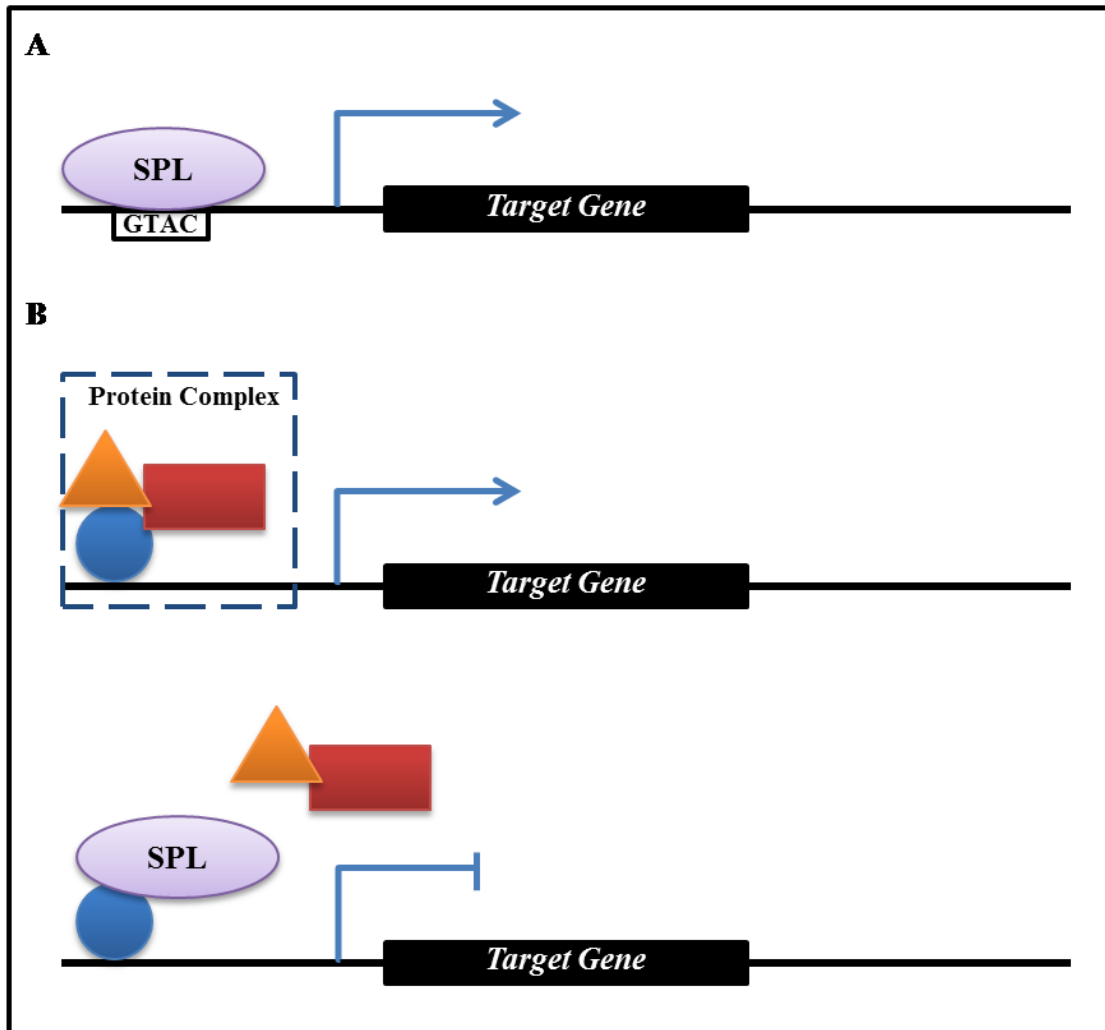
1.6 SPL Transcription Factor Family

SPL genes code for a family of plant-specific transcription factors. These transcription factors have various primary structures, but all of them share a conserved SQUAMOSA PROMOTER BINDING PROTEIN (SBP) domain (Preston and Hileman, 2013). This SBP domain consists of about 80 amino acids, and it contains two zinc binding motifs, which are responsible for DNA binding (Yamasaki et al., 2004). Based on the results of random primer selection experiments and electrophoretic mobility shift assays (EMSA), the SBP domain was determined to recognize a specific consensus sequence, which contains GTAC as a core element, in the promoter regions of target genes to regulate their expression (Cardon et al., 1999; Birkenbihl et al., 2005).

The *SPL* gene family was first discovered in *Antirrhinum majus* (Klein et al., 1996), where *SPL* transcription factors can recognize a specific element in the promoter region of the MADS-box gene *SQUAMOSA* and regulate its expression. *SPL* genes have been characterized in many other plant species, including maize (Moreno et al., 1997), silver birch (Länneppää et al., 2004) and Arabidopsis (Cardon et al., 1999). In Arabidopsis, 16 *SPL* genes have been identified, 10 of which are targeted by miR156 (Preston and Hileman, 2013). The miR156-targeted *SPLs* are

crucial regulators of many aspects of plant growth and physiology. For example, *SPL15* is involved in regulating carotenoid levels, and decreased *SPL15* expression in *Arabidopsis* causes an increase in the accumulation of carotenoids (Wei et al., 2012). *SPL2*, *SPL10* and *SPL11* function redundantly in controlling fruit development in the reproductive phase (Shikata et al., 2009). *SPL9* directly activates two MYB family genes, *TRICHOMELESS1 (TCL1)* and *TRIPTYCHON (TRY)*, which are trichome development repressors, to regulate trichome distribution (Yu et al., 2010). Also *SPL3* is a direct activator of the transcription factor coding genes *LFY*, *FUL* and *API*, which are vital regulators of flowering time (Yamaguchi et al., 2009). The miR156 non-targeted *SPLs* are also involved in plant development. For example, *SPL8* is essential for plant reproductive organ development (Xing et al., 2010, 2013). *SPL8* and miR156-targeted *SPL* genes, such as *SPL2*, are also involved in regulating anthers and gynoecium development; reducing transcript level of *SPL8* together with increasing transcript level of *miR156* result in producing smaller reproductive organs (Xing et al., 2010, 2013). *SPLs* are bifunctional transcription factors, as they can directly regulate target gene expression through binding to the promoter region (Figure 2A), and also indirectly regulate gene expression by interacting with other transcription factor complexes (Figure 2B) (Gou et al., 2011). For example, the anthocyanin biosynthetic gene *DIHYDROFLAVONOL 4-REDUCTASE (DFR)* is activated by the MYB-bHLH-WD40 complex, and the presence of *SPL9* can interfere with the formation of this complex and cease its activity, which results in reduced

Figure 2. Different regulation mechanism of SPLs. (A) SPLs directly bind to target promoter region to active gene expression. (B) SPL down-regulates target gene by interfering with the formation of an activator protein complex. Blue circle, red square, and orange triangle represent the proteins which form a complex to activate target genes.



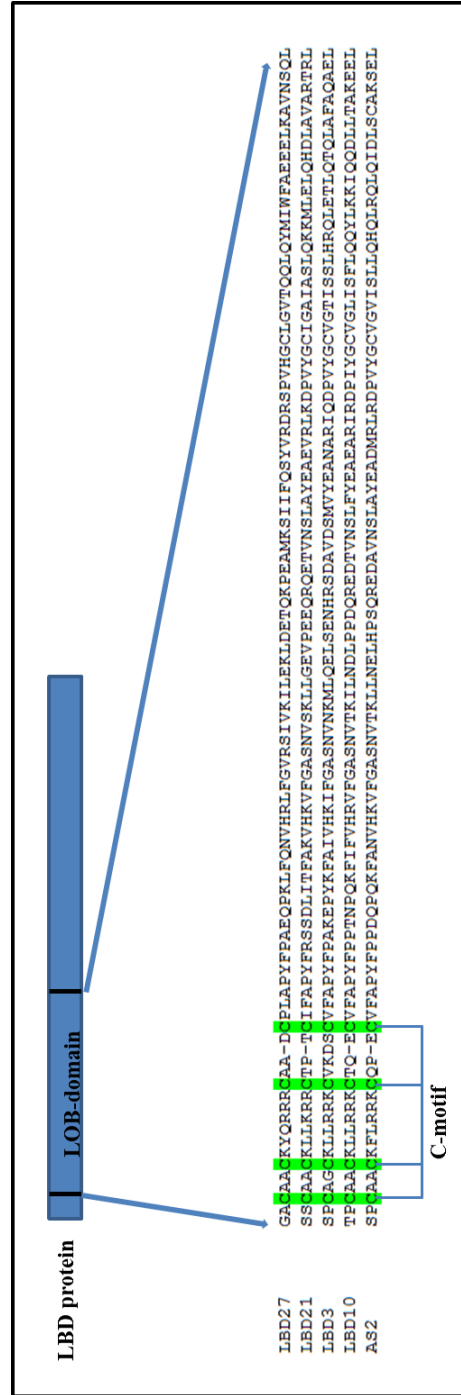
anthocyanin biosynthesis (Gou et al., 2011).

1.7 *AS2* and *LBD* Gene Family

ASYMMETRIC LEAVES 2 (AS2) belongs to the *AS2/LATERAL ORGAN BOUNDARIES-DOMAIN (LBD)* gene family. The *LBD* genes code for a class of plant-specific DNA-binding transcription factors and their expression is enriched at the boundaries of lateral organs (Shuai et al., 2002). To date, the *LBD* family genes have been reported to be involved in organ formation, auxin response, nitrogen metabolism and several other plant development aspects (Li et al., 2008; Lee et al., 2009; Rubin et al., 2009). In *Arabidopsis*, there are 42 members belonging to the *LBD* family, and all these members contain a conserved *AS2/LOB* domain which is about 100 amino acids in length (Figure 3). There are four cysteine residues that separately locate in the N-terminal conserved region of the *LOB* domain which are named C-motif, and this motif is essential for *LBD* function (Matsumura et al., 2009; Luo et al., 2012). *LBD* proteins function by interacting with some other proteins, such as members of the *bHLH* and the *MYB* family (Xu, 2003; Husbands et al., 2007).

Recent studies indicate that the *LBD* genes also influence plant fertilization (Kim et al., 2015). The *LBD10* is highly expressed in pollen and gradually increases during the early pollen growth stages (Kim et al., 2015). The *LBD10* loss-of-function mutant showed a significantly higher ratio of aborted pollen than the wild-type (WT) plants, which in turn affected fertilization and resulted in producing less seeds in the mutant (Kim et al., 2015). The sterile phenotype of the *LBD10* and *LBD27*

Figure 3. The sequence of LOB-domain. The amino acid sequences of LOB-domain in different LBD family proteins were aligned. The C-motif, which is highlighted by green in the N-terminal, is essential for LBD function.

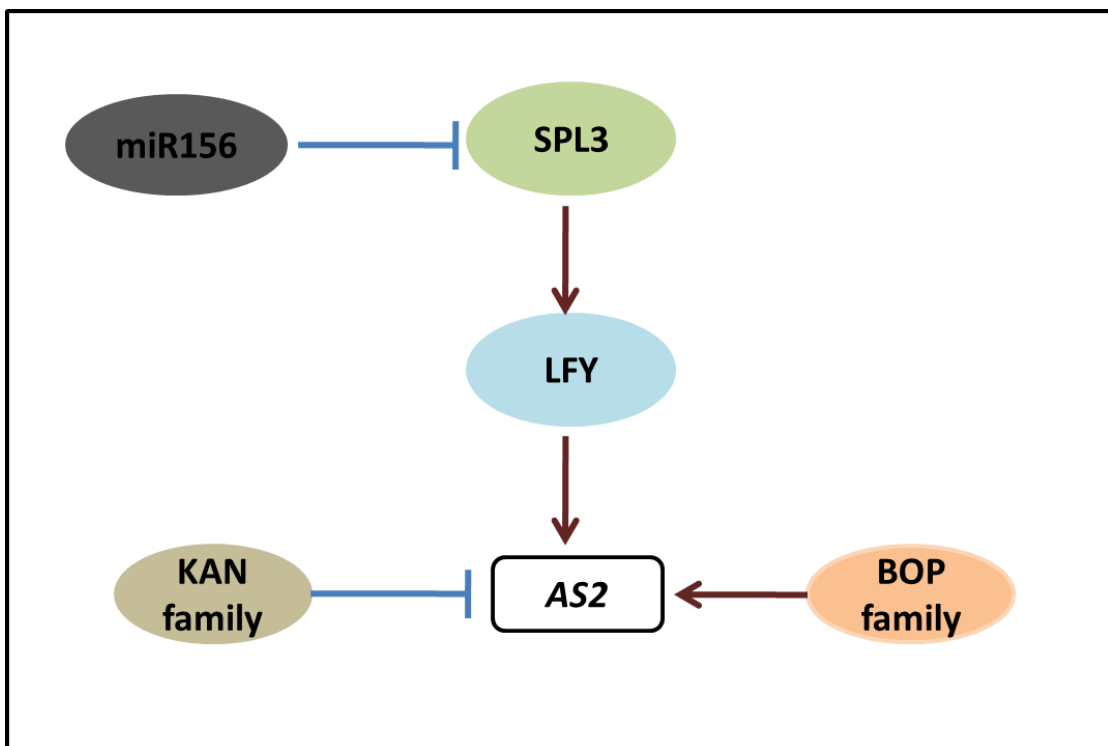


loss-of-function double mutant *lbd10lbd27* was more severe than the single mutants (Kim et al., 2015). Moreover, the LBD10 formed heterodimers with the LBD27, which indicates that both LBD10 and LBD27 are crucial for pollen development (Kim et al., 2015). AS2 is involved in regulating *KNOX* genes, which function in organ initiation and meristem growth (Hay and Tsiantis, 2010). To mediate regulation of the *KNOX* genes, a MYB domain protein AS1 is also required. AS2 forms a complex with AS1 and this complex can bind to a specific element in the promoters of the target genes to regulate their expression (Guo et al., 2008b). Intriguingly, this complex can regulate *LBD* genes as well (Byrne et al., 2002). As a transcription factor, AS2 increases or decreases the transcript level of downstream genes through direct or indirect regulation (Semiarti et al., 2001; Lin et al., 2003; Guo et al., 2008b). Conversely, *AS2* expression is also regulated by other transcription factors. For example, BLADE-ON-PETIOLE1 (BOP1) directly activates *AS2*; KANADI1 (KAN1) binds to the *AS2* promoter to silence expression (Wu et al., 2008; Jun et al., 2010). In addition, the *LFY*, which is a direct target of the SPL3 (Yamaguchi et al., 2009), also acts as a direct activator of *AS2* (Figure 4) (Yamaguchi et al., 2012).

1.8 Proposed Research and Hypothesis

Proper development of plant organs is essential for normal plant growth, development and reproduction. Sexual organs, such as anthers, are particularly important for flowering plants, and normal development of these organs ensures continuous sexual reproduction. miR156 is involved in controlling multiple aspects of plant organ growth, including shoot branching, leaf elongation, and root formation

Figure 4. The regulation of *AS2*. LFY and members of the BOP transcription factor family act as direct activators of *AS2* (Jun et al., 2010; Yamaguchi et al., 2012). Members of the KAN transcription factor family act as direct repressors of *AS2* (Wu et al., 2008). Solid arrows indicate the direct activation; blunted lines show direct down-regulation.



(Wei et al., 2012; Aung et al., 2015a, 2015b). In addition, ectopic expression of *miR156* causes both male and female plant sterility by affecting the development pattern of anthers and gynoecium in *Arabidopsis* (Xing et al., 2010, 2013). However, to my knowledge no miR156/SPL network-regulated gene has been reported to participate in controlling floral organ development and fertility in *Arabidopsis*.

Taken together, these pieces of evidence led me to investigate whether *AS2* is directly regulated by the miR156/SPL2 pathway. I hypothesized that SPL2 is a direct upstream activator of *AS2*, and that the differential expression level of *AS2* in *miR156* and *SPL2* mutants will cause several defective phenotypes in floral organs and siliques.

The objectives of this study are:

- To characterize the phenotypes of the *miR156* overexpression line, the *SPL2* dominant-negative mutant and the *AS2* EMS mutant,
- To examine the regulation of *AS2* by miR156/SPL2 pathway,
- To test *in vivo* interaction of SPL2 with *AS2* promoter.

CHAPTER II. MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

The *Arabidopsis* ecotype Columbia (Col) and Landsberg *erecta* (*Ler*) WT plants were used as controls. Seeds of *35S:miR156* (*miR156* overexpression) and *35S:amiR-SPL4/5* (artificial miRNA targets both *SPL4* and *SPL5*) were obtained from Dr. Detlef Weigel (Wang et al., 2008, 2009); *SPL2* and *SPL10* dominant-negative mutants (*35S:SPL2SRDX* and *35S:SPL10SRDX*) were kindly provided by Dr. Masaru Ohme-Takagi (Shikata et al., 2009). Seeds of the *AS2* EMS mutant (*as2-101*) and *SPL10* overexpression line (*6mSPL10*) were obtained from the Arabidopsis Biological Resource Center (ABRC). The *35S:miR156*, *35S:amiR-SPL4/5*, *35S:SPL2SRDX*, *35S:SPL10SRDX* and *6mSPL10* are all in the Col background, while *as2-101* is in *Ler* background.

For plants grown in soil, the PRO-MIX® BX MYCORRHIZAE growing medium was used. Seeds were sown onto the surface of soil in plastic pots. The tray containing pots was covered with a plastic lid to maintain humidity. The growing condition was described by ABRC (<http://abrc.osu.edu/seed-handling>). Briefly, the tray was kept at 4°C for 2-4 days to stratify the seeds. After stratification, seeds were transferred to growth room. Plants were grown at 22°C with 70% humidity. The photoperiods were 16 h light and 8 h dark with a light intensity of 130-150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$. The plastic lid was removed after seeds germinated. After two weeks, plants were fertilized with fertilizer 20-20-20 (1 g/L).

2.2 Arabidopsis RNA Extraction and cDNA Synthesis

Total RNA was extracted with the PowerPlant® RNA Isolation Kit (Mo-Bio Laboratories, USA). About 30 mg of plant tissue was used for RNA extraction. The RNA was eluted with 50 μ L of RNase-Free water. The purity of RNA was tested by Nano Vue Plus Spectrophotometer (GE healthcare, Canada), and only the RNA with $A_{260/280}$ and $A_{260/230}$ ratio around 2.0 was used for cDNA synthesis. Contaminating DNA was removed using a TURBO DNA-free Kit (Ambion, USA). Briefly, 5 μ L of TURBO DNase Buffer and 1 μ L of TURBO DNase were added to the RNA, and the mixture was incubated at 37°C for 30 min. Six μ L of DNase inactivation reagent was added to the mixed solution and incubated at room temperature for 10 min. In the final step, the mixture was centrifuged at 12,000 g for 2 min and the supernatant containing RNA was transferred to a fresh tube. The concentration of RNA was determined by Nano Vue Plus Spectrophotometer.

The cDNA used for qRT-PCR was synthesized with a qScript™ cDNA SuperMix Kit (Quanta, USA), and 1 μ g of total RNA was used for each reaction. The reaction was performed at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. For cloning purposes, the cDNA was synthesized with SuperScript® III First-Strand Synthesis System (Life Technologies, USA) by using oligo dT primers. For each reaction, 2 μ g of RNA, oligo dT primer, and dNTPs were added and incubated at 65°C for 5 min to remove RNA secondary structure. Then the mixture was placed on ice for 5 min and 10 μ L of cDNA Synthesis Mix (2 μ L RT buffer, 4 μ L 25 mM MgCl₂, 2 μ L 0.1 M Dithiothreitol (DTT), 1 μ L RNaseOUT and 1 μ L SuperScriptIII RT) was added. The

reaction was then incubated at 50°C for 50 min, 85°C for 5 min. Subsequently, 1 µL of RNase H was added and incubated at 37°C for 20 min. The cDNA was stored at -20°C after reverse transcription.

2.3 Arabidopsis Genomic DNA Extraction

The Arabidopsis genomic DNA was extracted by cetyltrimethyl ammonium bromide (CTAB) method (Allen et al., 2006). Plant leaf tissue was ground in liquid nitrogen using a cold mortar and pestle. The powder was scraped into a fresh Eppendorf tube and 0.5 ml of DNA extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM Ethylenediaminetetraacetic acid (EDTA), 100 mM pH=8.0 Tris-Cl) was added. The solution was vortexed for 15 s to mix completely. Next, 0.5 ml chloroform was added to the mixture and mixed well by gently inverting the tube for 4-6 times. The mixed solution was centrifuged at 10,000 g for 5 min, and the aqueous phase supernatant containing DNA was transferred into a fresh tube. Then, 400 µL of isopropanol was added into the supernatant and mixed briefly. The mixed solution was centrifuged at 13,000 g for 15 min. Finally, the supernatant was discarded and the pellet in the bottom was washed with 500 µL of 70% ethanol. The pellet was air-dried and 50 µL of double distilled H₂O was added to elute DNA. The DNA solution was stored at -20°C for further experiments.

2.4 Quantitative Real-Time PCR

The cDNA, which was synthesized as previously described in section 2.2, was diluted 4 times with double distilled H₂O. Quantitative real-time PCR (qRT-PCR) was performed on a C1000 Thermal Cycler and CFX96 Real-Time System (Bio-Rad,

Canada) with PerfeC_Ta® SYBR® Green SuperMix (Quanta, USA). *PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3)* and *UBIQUITIN-CONJUGATING ENZYME 21 (UBC21)* were used as internal controls to normalize data (Czechowski et al., 2005). Gene-specific primers used for qRT-PCR are given in Appendix 1A. Each qRT-PCR reaction contained 5 µL of 2X PerfeC_Ta® SYBR® Green SuperMix, 3 µL of 1 µM primers and 2 µL of diluted cDNA. The program was performed as one cycle at 95°C for 30 s and followed by 35 cycles at 95°C for 5 s and 58°C for 30 s. Transcript levels were then analyzed by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). A total of six replicates were used for each tested gene (three biological replicates and two technical replicates for each), the mean value and standard error for tested genes were calculated based on three biological replicates.

2.5 Preparation of Chemically Competent *E. coli* Cells

Chemically competent *Escherichia coli* (*E.coli*) cells were prepared by using the method as described by Chung and Miller (1988). A single colony from streaked *E.coli* TOP10 cells (Invitrogen, USA) was cultured in 3 mL of LB broth medium (Appendix 2) at 37°C overnight. The culture was then used to inoculate into 250 mL LB broth medium until OD₆₀₀ reached 0.3-0.5. The bacterial culture was centrifuged at 4,000 g for 10 min at 4°C, the supernatant was discarded. The pellet was resuspended in chilled 100 mL 0.1 M CaCl₂ and left on ice for 30 min, and then centrifuged at 4,000 g for 10 min at 4°C. The washing procedure by CaCl₂ was repeated twice, and finally the pellet was resuspended in 2 mL of chilled 0.1 M CaCl₂ and 2 mL of 40% glycerol. The stock was then divided into 50 µL aliquots in 1.5 mL

Eppendorf tubes and stored at -80°C for future use.

2.6 Preparation of Electro Competent *A. tumefaciens* Cells

The starting culture of *Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV3101 was incubated in LB broth medium at 28°C for 2 days. Five mL of the culture was then used to inoculate 250 mL LB broth medium and incubated with shaking at 28°C until OD₆₀₀ reached around 0.5. The bacterial suspension was centrifuged at 4,000 *g* for 10 min at 4°C, and the supernatant was discarded. The pellet was resuspended in 50 mL pre-chilled H₂O and left on ice for 30 min. The bacterial cell solution was then centrifuged at 4,000 *g* for 10 min at 4°C. The pellet was washed with H₂O twice, and finally the pellet was resuspended in 2 mL pre-chilled 40% glycerol. The competent cells were aliquoted into 1.5 mL Eppendorf tubes with 30 µL for each and stored at -80°C for future use.

2.7 Heat Shock Transformation of *E. coli*

For chemical transformation of competent *E.coli* cells, 50 ng of plasmid DNA was added to 30 µL competent cells and mixed gently by pipetting. The mixture was kept on ice for 20-30 min. Then the competent cells were heat-shocked at 42°C for 1 min and transferred on ice immediately to incubate for another 2 min. The 200 µL LB broth medium was then added to the mixture and placed at 37°C for 45-60 min with shaking. Finally, 100 µL of bacterial culture was spread on an LB plate containing appropriate antibiotics and incubated at 37°C overnight.

2.8 Transformation of *A. tumefaciens* by Electroporation

For electroporation of *A.tumefaciens*, 50 ng of plasmid DNA was mixed with 30 µL

competent cells. The mixture was added into an electroporation cuvette (Bio-Rad, Canada) and kept on ice for 15 min. Transformation was performed by using the MicroPulser™ Electroporator (Bio-Rad, Canada) with the voltage of 2.2 kV. The cuvette was transferred back on ice immediately after transformation. Two hundred μL of LB broth medium was added to the bacterial cell suspension, and the mixture was incubated at 28°C for 1 h with shaking. Fifty μL of bacterial suspension solution was spread on LB plate containing appropriate antibiotics and incubated at 28°C for 2 days.

2.9 Plasmid DNA Extraction

Plasmid DNA extraction was performed as described with some modification (Birnboim and Doly, 1979). A single positive *E.coli* colony was cultured overnight in 3 mL LB broth medium with appropriate antibiotic at 37°C in a shaking incubator. One mL of the bacterial culture was transferred into a 1.5 mL Eppendorf tube and centrifuged at 13,000 g for 1 min. The supernatant was discarded, and the pellet was resuspended in 200 μL of pre-chilled Solution I (0.9% glucose, 25 mM Tris, 10 mM EDTA, 100 mg/L RNase) and vortexed thoroughly. Next, 200 μL of Solution II (0.2 M NaOH, 1% sodium dodecyl sulfate (SDS)) was added to the mixture and mixed by inversion 6 times. The pre-chilled 200 μL Solution III (3 M CH_3COOK , 11.5% glacial acetic acid) was then added to the mixture and mixed by inverting tube 6 times. The mixed solution was then kept on ice for 5-10 min and followed by centrifuging for 5 min at 13,000 g. The supernatant was transferred into a fresh tube. To precipitate DNA, 1 ml of 95% ethanol was added into the supernatant, and the mixture was

incubated at -20°C for at least 30 min. Finally, the solution was centrifuged at 13,000 g for 10 min, and the pellet containing DNA was washed with 500 μL of 70% ethanol. The pellet was air dried completely, and subsequently eluted by 50 μL double distilled H_2O . The extracted DNA was stored at -20°C until use.

2.10 Generation of *pSPL2:SPL2-GFP* Construct

To make the *pSPL2:SPL2-GFP* construct, the *SPL2* genomic sequence including the 2 kb fragment upstream of the ATG translation start codon, but without the stop codon was amplified from Arabidopsis WT Col genomic DNA. PCR was performed with a Phusion High-Fidelity DNA Polymerase Kit (New England Biolabs, Canada) using primers *pSPL2:SPL2-GFP-5'* and *pSPL2:SPL2-GFP-3'* (Appendix 1B). The thermocycling conditions of PCR was: 98°C for 30 s, followed by 35 cycles at 98°C for 30 s, 55°C for 30 s, 72°C for 4 min, then the final extension was at 72°C for 10 min. Next, the size of the PCR product was tested by agarose gel electrophoresis, and the product with the expected size was purified using a QIAquick Gel Extraction Kit (Qiagen, Canada). The construct was generated by Gateway cloning, whereby the purified PCR product was first cloned into pENTR directional TOPO cloning vector (Life technologies, Canada). The reaction was performed by adding 1 μL purified PCR product, 0.5 μL salt solution and 0.5 μL TOPO vector, then the reaction was kept at room temperature overnight. One μL of the reaction was used to transform the vector into *E. coli* TOP10 competent cells, which were then cultured on LB agar plates (Appendix 2) with kanamycin (50 $\mu\text{g}/\text{mL}$) as a selectable agent. DNA was extracted from positive colonies that grew on the plate, and subjected to PCR. Only the colonies

that had the expected PCR product size were used for the next steps. The selected colony was used to inoculate in 5 mL LB medium (recipe is given on Appendix 2) containing 50 µg/ml kanamycin overnight. One mL of bacterial culture was used for plasmid DNA extraction (section 2.9), and the plasmid DNA was then sent for sequencing. The clone that had an identical sequence to that of the *SPL2* listed by The Arabidopsis Information Resource (TAIR) was transformed into the destination vector pMDC107 by using the Gateway cloning method (Curtis and Grossniklaus, 2003; Karimi et al., 2007). The plasmid DNA was recombined into pMDC107 vector with LR Clonase II Enzyme Mix (Life Technologies, Canada). The reaction was prepared by mixing 1 µL of 100 ng/µL pENTR vector that carried the *SPL2* genomic sequence, 0.5 µL pMDC107 vector at a concentration of 150 ng/µL and 0.5 µL LR Clonase II Enzyme. The reaction was kept at 25°C overnight. The reaction was then terminated by adding 0.5 µL of proteinase K solution and incubated at 37°C for 10 min. The construct was transformed into *E.coli* TOP10 competent cells, and positive colonies were confirmed by PCR using primers SPL2-5' and GFP-3' (Appendix 1C). DNA was extracted from positive colonies (section 2.9) and subjected for sequencing. The destination vector was introduced into *A. tumefaciens* strain GV3101 (Koncz and Schell, 1986).

2.11 Arabidopsis Transformation

The Arabidopsis transformations were performed by using the floral dip method as described (Zhang et al., 2006b). Briefly, a single colony of *A. tumefaciens* strain that contained a vector with the transgene was used to inoculate into 5 mL LB broth

medium containing appropriate antibiotics. The culture was incubated at 28°C for 2 days with shaking at 200 rpm. The culture was then transferred into a flask that contained 300 mL LB broth medium with appropriate antibiotics and incubated at 28°C overnight. Next day, the *A. tumefaciens* cells were collected by centrifugation at 4,000 g for 10 min, and the supernatant was discarded. The *A. tumefaciens* cells were resuspended in 300 mL 5% sucrose and 60 µL Silwet L-77 was added to the solution. The Arabidopsis plants that grew for 1-2 weeks after bolting were used for transformation. To increase transformation efficiency, the siliques of Arabidopsis plants were removed before floral dip. Arabidopsis plants were inverted and the aerial parts were soaked into the previously prepared *A. tumefaciens* cell suspension solution for 45-60 s. The dipped plants were then covered with a plastic bag and left at room temperature in the dark for 16-24 h. The next day, the plastic cover was removed and treated plants were transferred back to the growth chamber to grow another 3-4 weeks. Seeds that were harvested from the transformed plants were used for screening on half-strength Murashige and Skoog ($\frac{1}{2}$ MS) medium plates (Appendix 2) with appropriate antibiotics.

2.12 Screening of Transformed Arabidopsis

Seeds collected from transformed Arabidopsis were first surface sterilized by soaking in sterilizing solution (70% ethanol and 0.5% Triton X-100) for 10 minutes with rotation. Next, seeds were rinsed with 1 mL of 95% ethanol and poured onto a sterilized filter paper. The seeds were air-dried completely in the hood for 20-30 min and then sown on $\frac{1}{2}$ MS medium plates with appropriate antibiotics. The plates were

then placed at 4°C for 2 days in the dark for stratification and subsequently transferred to the growth chamber. The growth condition was described in section 2.1. After growing the seeds for two weeks, the healthy transgenic seedlings were transferred to soil, and plants were transferred to the growth room. DNA was extracted from transgenic seedlings and PCR was performed to confirm the presence of the transgene by using primers SPL2-5' and GFP-3' (Appendix 1C). Only the plants that contained the transgene were used for future experiments.

2.13 Analysis of SPL2-GFP Fusion Expression

To confirm the expression of SPL2-GFP fusion protein, confocal microscopy was performed. The *pSPL2:SPL2-GFP* transgenic plants were grown on ½MS with 50 µg/mL hygromycin. Three-week-old seedlings were used, and the leaves were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. The emission signal was collected between 370 nm and 430 nm for DAPI fluorescence, between 490 nm and 530 nm for GFP fluorescence, and between 630 nm and 700 nm for chlorophyll auto-fluorescence.

2.14 ChIP-qPCR Assay

The chromatin immunoprecipitation (ChIP)-qPCR assay was used to test the occupancy of SPL2 on the regulatory region of the *AS2* gene. ChIP was performed according to a previously published protocol (Gendrel et al., 2005) with some modifications. Briefly, 1 g of 3-week-old seedlings from WT and *pSPL2:SPL2-GFP* transgenic plant was collected and washed three times with double distilled H₂O, and dried with blotting paper. The tissues were then soaked into 1% formaldehyde for

20 min under vacuum to cross-link bound proteins to DNA. Glycine was added to the mixture to a final concentration of 0.125 M, and the mixed solution was incubated under vacuum for an additional 5 min to terminate cross-linking. The tissues were washed with double distilled H₂O three times and ground into powder by using a mortar and a pestle with liquid nitrogen. The powder was homogenized in 20 mL Extraction Buffer 1 (Appendix 3). The solution was filtered twice through two layers of Miracloth (Millipore, Canada). The filtered mixture was then centrifuged at 4,000 g at 4°C for 20 min. The supernatant was discarded and the pellet was resuspended in 1 mL Extraction Buffer 2 (Appendix 3) then centrifuged at 12,000 g at 4°C for 10 min. The supernatant was removed and the pellet was resuspended in 300 µL Extraction Buffer 3 (Appendix 3). The mixed solution was then layered over the 300 µL Extraction Buffer 3 and centrifuged at 16,000 g for 1 h at 4°C. The pellet containing chromatin was resuspended in 300 µL Nuclei Lysis Buffer (Appendix 3) by gentle pipetting. The chromatin solution was sonicated twice at power 3 on a Sonic Dismembrator (Fisher Scientific, USA), 15s for each time. After sonication, the chromatin solution was centrifuged at 12,000 g for 5 min at 4°C. Twenty µL of chromatin solution was moved into a fresh tube and stored at 4°C. This was used as input control, and the rest of the solution was used for immunoprecipitation. The volume of chromatin solution was brought to 1.5 mL by adding ChIP dilution buffer. Thirty µL protein A-agarose beads (Millipore, Canada) and 5 µL Ab290 GFP antibody (Millipore, Canada) were added to the chromatin solution and the mixture was kept at 4°C overnight with gentle rotation. The next day, the chromatin solution was

centrifuged at 3,500 g for 1 min at 4°C, and the supernatant was removed. The beads were washed with low salt wash buffer, high salt wash buffer, LiCl wash buffer and Tris-EDTA buffer for 10 min of each at 4°C with rotation. After each wash, the tube was centrifuged at 3,800 g for 30 s at 4°C and the supernatant was discarded. Next, the immuno-precipitated complex was eluted by 250 µL Elution Buffer (Appendix 3), and incubated at 65°C for 15 min with gentle agitation. The mixture was centrifuged at 3,800 g at 23°C for 2 min and the supernatant was transferred to a fresh tube. The cross-link for all samples, including input DNA, was reversed by adding 5 µL of 5 M NaCl and incubating at 65°C for 5 h. To each mixed solution, 10 µL of 0.5 M EDTA, 20 µL of 1 M Tris-HCl (pH=6.5) and 2 µL of 10 mg/ml proteinase K (Sigma-Aldrich, Canada) were added and the mixture was incubated at 45°C for 1 h. The DNA was precipitated with 550 µL of phenol/chloroform (1:1, vol/vol) and centrifuged at 16,000 g for 5 min. The aqueous phase was transferred to a fresh tube, and then precipitated with 95% ethanol, 0.3 M sodium acetate (pH=5.2) and 2 ng glycogen at -20°C overnight. DNA samples were centrifuged at 16,000 g for 20 min and the pellet was washed with 70% ethanol. The pellet was subsequently eluted with 100 µL double distilled H₂O. To test the occupancy of the SPL2 to the *AS2* regulatory region, qPCR was performed with gene-specific primers that flank the SPL binding motifs GTAC in the *AS2* regulatory region. For the qPCR reaction, 5 µL of 2X PerfeC_Ta® SYBR® Green SuperMix, 3 µL of 1 µM primers and 2 µL of extracted DNA were used. The qPCR data were analyzed with percent input method by using the formula as described (<http://www.lifetechnologies.com>). A fragment containing no SPL

protein binding motif from the *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*EIF4A1*) promoter region (Appendix 6) was used as a negative control (Yamaguchi et al., 2009). The primer sequences are listed in Appendix 1D.

2.15 Phenotypic Analysis

Siliques that grew for eight days post flowering, and petals and sepals that grew for one day post flowering were used to measure the length. Length was measured by using a Nikon DS-L3 camera controller under Nikon SMZ1500 stereomicroscope (Nikon Corporation, Japan). For seed set analysis, siliques that grew for at least 6 days were collected and dissected by using tweezers and fine needles. The number of developing seeds and aborted embryos in each silique were counted under a Nikon SMZ1500 stereomicroscope. The NIS-Elements software was used to take images of siliques.

2.16 Alexander Staining for Pollen Viability

Pollen viability was assessed by the Alexander staining method as described (Alexander, 1969). Briefly, flower buds were collected and dissected to release anthers. Anthers were fixed with Alexander staining solution (10 mL 95% alcohol, 1 mL of 1% malachite green in 95% alcohol solution, 54.5 mL distilled water, 25 mL of 100% glycerol, 5 mL 1% acid fuchsin, 0.5 mL 1% orange G, 4 mL glacial acetic acid) for at least 2 h. Pollen number was determined with a Nikon Elipse-Ni microscope (Nikon Corporation, Japan). Images were taken using NIS-Element software.

2.17 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).

The mean value for each experiment was derived from at least three independent biological replicates. The DNA sequence analysis was performed by the DNASTAR multiple program package (DNASTAR Inc., USA).

CHAPTER III. RESULTS

3.1 Changes in Global Gene Expression in Siliques of *35S:miR156* Line

To investigate the transcriptome differences between WT and *miR156* overexpression (*35S:miR156*) plants, Ying Wang performed high-throughput Illumina Next-generation RNA sequencing by using RNA extracted from siliques. The results indicated that 498 (Appendix 4) genes were differentially expressed by at least two-fold in *35S:miR156* relative to WT(Col) siliques. Among these genes, 305 were down-regulated and 193 genes were up-regulated. Since SPLs are known to function as activators of gene expression via direct binding to the promoter region of target gene, I focused only on the down-regulated genes as they are more likely to be directly regulated by SPLs.

3.2 SPL Binding Site Analysis and Expression Data Validation

As these down-regulated genes are either directly or indirectly regulated by SPLs, only promoters containing the core 'GTAC' sequence can be bound by SPLs. Among these down-regulated genes, 210 genes contain at least one GTAC element in their 2kb upstream of the translation start codon (Appendix 4). To further narrow the data, I manually searched for the consensus CNGTACM (N=any nucleotide, M=A or C) with GTAC as the core sequence (Wei et al., 2012). Only 60 of the 210 genes matched this criterion (Appendix 4).

3.2.1 Selection of candidate genes

To validate NG RNA-Seq data, I performed qRT-PCR to determine the transcript

level of these 60 genes that contain at least one of the CNGTACM consensus sequences. RNA samples extracted from siliques of *35S:miR156* and WT(Col) plants were used in this qRT-PCR analysis. The analysis indicated that the transcript level of 18 genes was significantly decreased in *35S:miR156* plants compared to WT(Col) (Figure 5), while the transcript level of the other 44 genes was inconsistent when comparing the three biological replicates. Then I checked the putative functions of these 18 genes using the TAIR database (Table 1). Based on this *in silico* analysis, 10 genes either have known functions or encode transcription factors, with 5 genes (*AS2*, *WD40 REPEAT-LIKE SUPERFAMILY GENE (WD40-LIKE)*, *USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 30 (UMAMIT30)*, *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS)* and *FRUITFULL (FUL)*) being involved in plant organ development and secondary metabolism, and 5 genes (*CYTOCHROME P450, FAMILY 96, SUBFAMILY A, POLYPEPTIDE 15 (CYP96A15)*, *GLAUCE (GLC)*, *BRANCHED-CHAIN AMINOTRANSFERASE4 (BCAT4)*, *GALACTOKINASE 2 (GALK2)*, *GATED OUTWARDLY-RECTIFYING K⁺ CHANNEL (GORK)*) participating in ion homeostasis, enzymatic reactions and other activities (Hu et al., 2003; Leshem et al., 2012; Schuster et al., 2006; Tran et al., 2013; Wen and Jetter, 2009; Yamaguchi et al., 2009; Zhao et al., 2013). In my project, I focused on genes that function in controlling plant development, and I selected organ development and secondary metabolism related genes: *AS2*, *WD40-LIKE*, *UMAMIT30*, *ARGOS* and *FUL* as my candidate genes.

Figure 5. qRT-PCR validation of differential expression of genes in siliques of *35S:miR156* and WT(Col). The expression of genes that contain the CNGTACM consensus was significantly decreased in *35S:miR156* compared to WT(Col). Data presented as the mean \pm standard error. Statistical significance was determined by Student's *t* test. Single asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$. Three biological replicates were used.

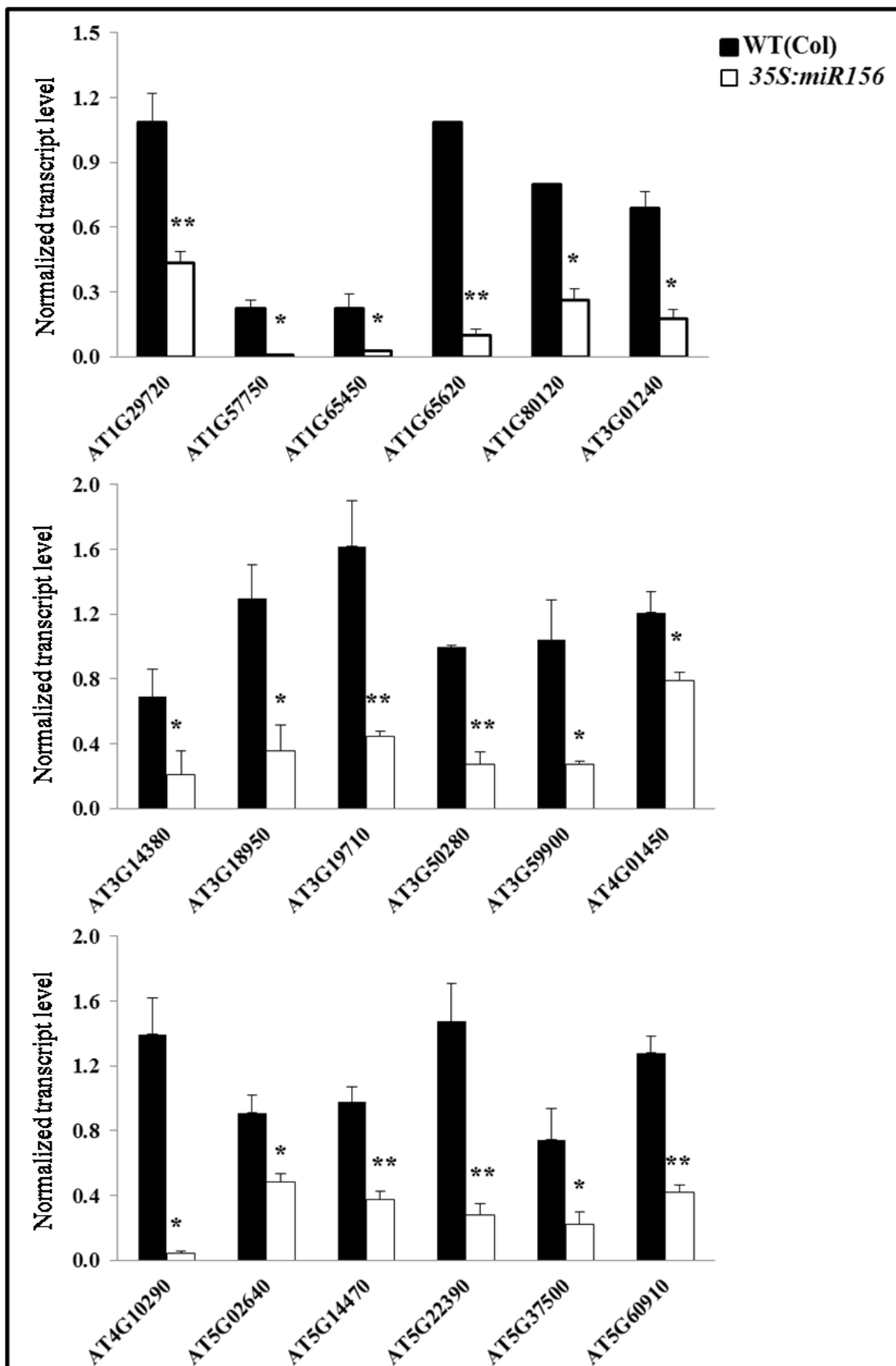


Table 1. The genes that were differentially expressed in 35S:miR156 compared to WT(Col), and putative function of their coding proteins.

Locus	Gene names	Numbers of putative SPL binding elements	Putative function ^a
AT1G29720		4	Encodes a leucine-rich repeat transmembrane protein kinase. Involved in protein amino acid phosphorylation.
AT1G57750	<i>CYP96A15</i>	8	Encodes a CYP96A15 protein, involved in cuticular wax biosynthesis.
AT1G65450	<i>GLC</i>	6	Involved in double fertilization
AT1G65620	<i>AS2</i>	8	Required for formation of a symmetric flat leaf lamina and floral organ development
AT1G80120		20	Unknown
AT3G01240		4	Unknown
AT3G14380		14	Unknown
AT3G18950		10	Encodes for a WD40 repeat-like superfamily protein
AT3G19710	<i>BCAT4</i>	6	Belongs to the branched-chain amino acid aminotransferase gene family. Involved in the biosynthesis of methionine-derived glucosinolates.
AT3G50280		4	Unknown
AT3G59900	<i>ARGOS</i>	10	Inducible by auxin, involved in lateral organ size control

AT4G10290		4	Unknown
AT4G01450	<i>UMAMIT30</i>	8	Encodes for a nodulin MtN21-like transporter family protein
AT5G02640		8	Unknown
AT5G14470	<i>GALK2</i>	8	Involved in carbohydrate metabolic process and phosphorylation
AT5G22390		6	Unknown
AT5G37500	<i>GORK</i>	10	Encodes a guard cell outward potassium channel, involved in ion transport
AT5G60910	<i>FUL</i>	10	Belongs to MADS box gene family, involved in fruit development, floral organ development and anthocyanin biosynthesis process

^aThe putative functions were based on TAIR.

3.2.2 Differential transcript level of *SPLs* in *35S:miR156* plants

In the NG RNA-Seq data, four *SPLs* also showed a reduced transcript level in the siliques of *35S:miR156* plants compared to WT(Col). I used qRT-PCR to confirm these data, and found that the expression of *SPL2*, *SPL4* and *SPL10* was indeed decreased in *35S:miR156*, but the expression of *SPL6* was not (Figure 6). These three *SPLs* were further examined, as they may be responsible for the down-regulation of the 5 candidate genes chosen in Section 3.2.1.

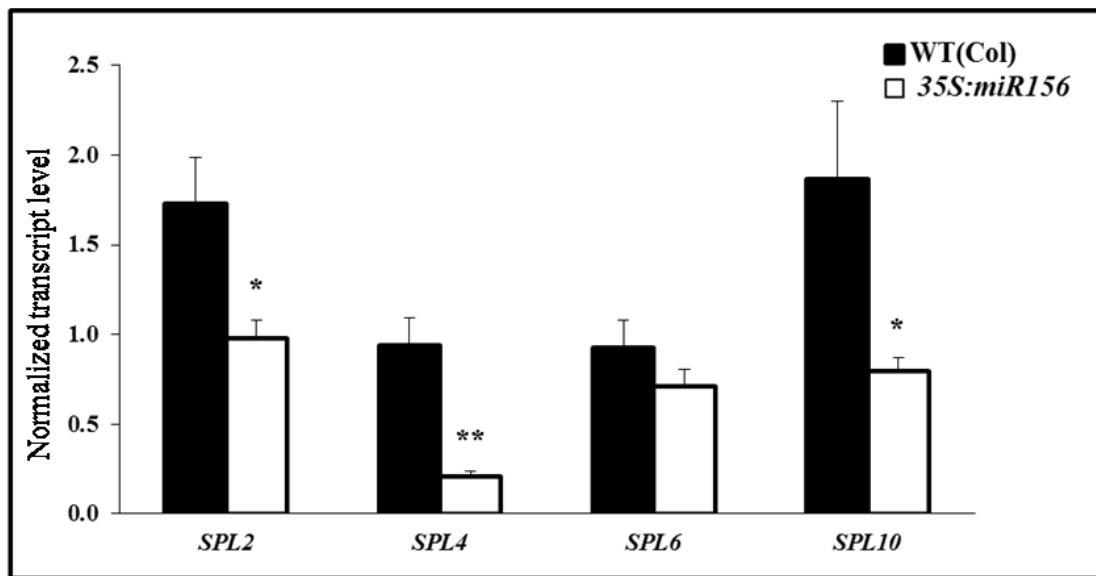
3.3 Regulation of Candidate Genes by *SPLs*

The five candidate genes (*AS2*, *WD40-LIKE*, *UMAMIT30*, *ARGOS*, and *FUL*) were identified after qRT-PCR expression analysis and *in silico* functional characterization (Section 3.2.1), and therefore were investigated to determine if their expression is regulated by the three *SPLs* (*SPL2*, *SPL4*, and *SPL10*) that were downregulated in *35S:miR156* plants. To investigate which *SPL* protein may directly regulate these candidate genes, I determined the transcript levels of the 5 candidate genes in *SPL* mutants.

3.3.1 *SPL4* and *SPL10* are not direct regulators of candidate genes

To analyze the transcript levels of candidate genes in *SPLs* mutants, I obtained transgenic Arabidopsis plants transformed with an artificial miRNA that targets both *SPL4* and *SPL5* (*35S:amiR-SPL4/5*), in which the transcript levels of *SPL4* and *SPL5* are decreased (Wang et al., 2008). Also, I obtained an *SPL10* overexpression transgenic plant (*6mSPL10*) (Nodine and Bartel, 2010), and a dominant-negative mutant *35S:SPL10SRDX* that was created by chimeric repressor gene-silencing

Figure 6. qRT-PCR validation of *SPLs* expression in the siliques of *35S:miR156* and WT(Col). The expressions of *SPL2*, *SPL4* and *SPL10* were significantly decreased in *35S:miR156* compared to WT(Col). Data presented as the mean \pm standard error. Statistical significance was determined by Student's *t* test. Single asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$. Three biological replicates were used.



technology (CRES-T) (Shikata et al., 2009). In the *35S:SPL10SRDX* mutant, SPL10 was fused to a repression domain (SRDX). This fusion protein can still bind to target genes' regulatory region, but will function as a repressor instead of an activator. The chimeric repressor allows it to overcome the redundant function with other SPL family proteins. Then, I used qRT-PCR to test the expression levels of these five candidate genes in the siliques of *SPL* mutants compared to WT (Figure 7, 8). In *amiR-SPL4/5* siliques, none of the five genes' transcript level was significantly decreased compared to WT(Col) in the three biological replicates (Figure 7A). Similarly, in *6mSPL10* and *35S:SPL10SRDX* siliques, the expression levels of the candidate genes did not correspond to the expression level of *SPL10* in each mutant (i.e. if SPL10 is a direct activator, the expression of the target gene will increase in *6mSPL10* and decrease in *35S:SPL10SRDX* compared to WT(Col)) (Figure 7B). In summary, SPL4 and SPL10 were not activators of these five genes. Alternatively, they might be direct repressors for *ARGOS*, but this needs to be further investigated.

3.3.2 AS2 acts downstream of SPL2

The T-DNA insertion line of *SPL2* showed no visible phenotypes, probably due to the functional redundancy of SPLs in Arabidopsis (Wang et al., 2008; Yamaguchi et al., 2009). To analyze the transcript level of the candidate genes in the *SPL2* mutant, *35S:SPL2SRDX* transgenic plants were used (Shikata et al., 2009). The expression of the candidate genes in *35S:SPL2SRDX* compared to WT(Col) was tested using

Figure 7. Candidate genes expression in *SPL4* and *SPL10* mutants. The transcript levels of the candidate genes in *35S:amiR-SPL4/5* (A), and *6mSPL10*, *35S:SPL10SRDX* (B). Data presented as the mean \pm standard error. Statistical significances were determined by Student's *t* test. Single asterisks showed significant differences at $P < 0.05$, double asterisks showed significant differences at $P < 0.01$. Three biological replicates were used.

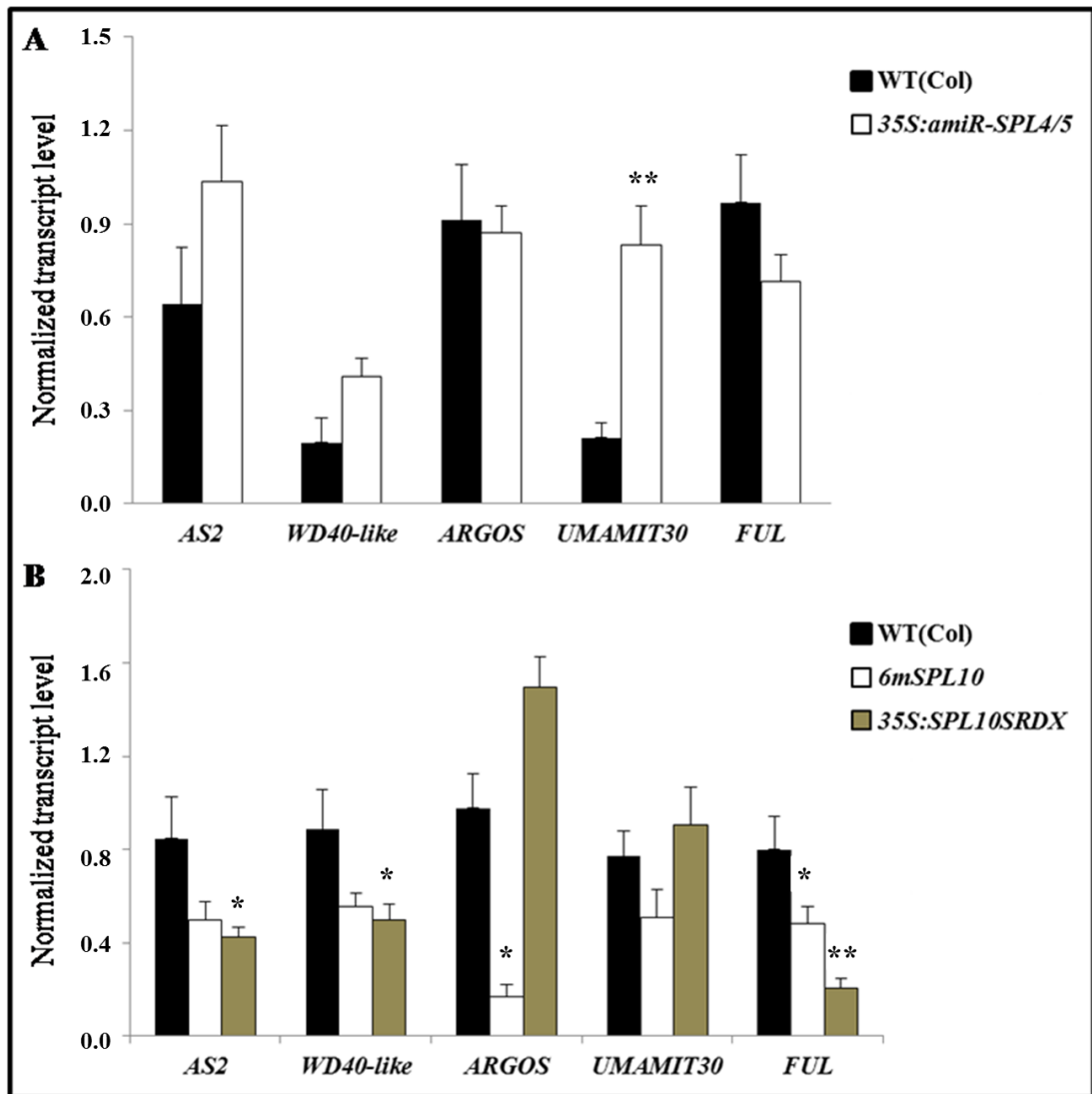
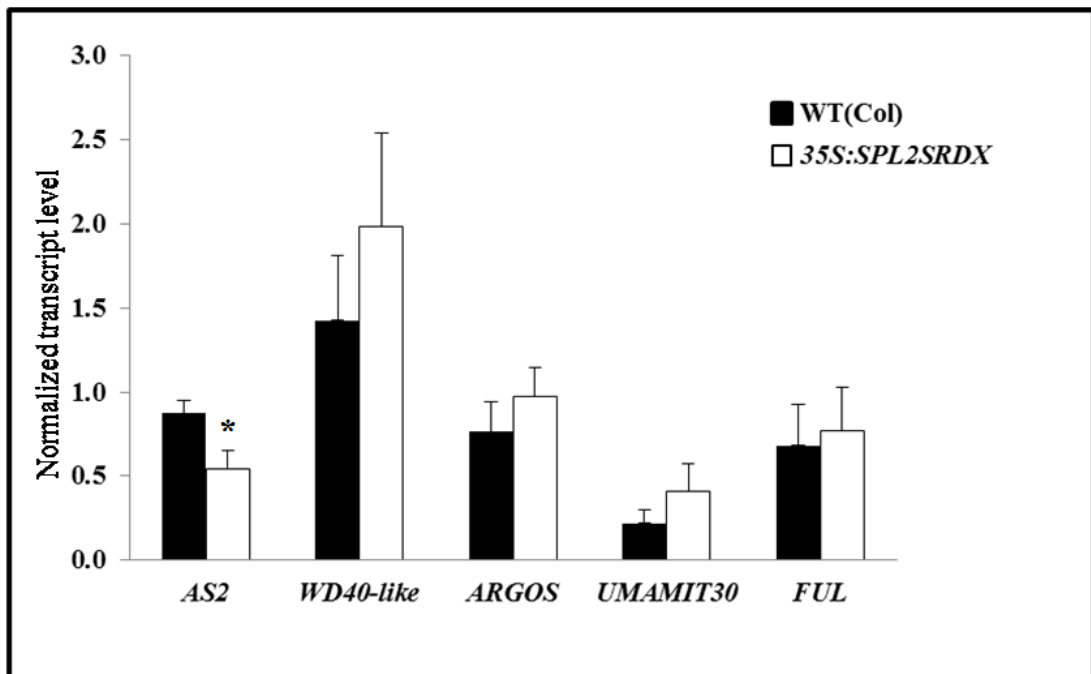


Figure 8. The transcript level of candidate genes in *35S:SPL2SRDX* and WT(Col).

The transcript level of *AS2* was significantly decreased in *35S:SPL2SRDX* compared to WT(Col). Data presented as the mean \pm standard error. Statistical significances were determined by Student's *t* test. Single asterisks showed significant differences at $P < 0.05$. Three biological replicates were used.

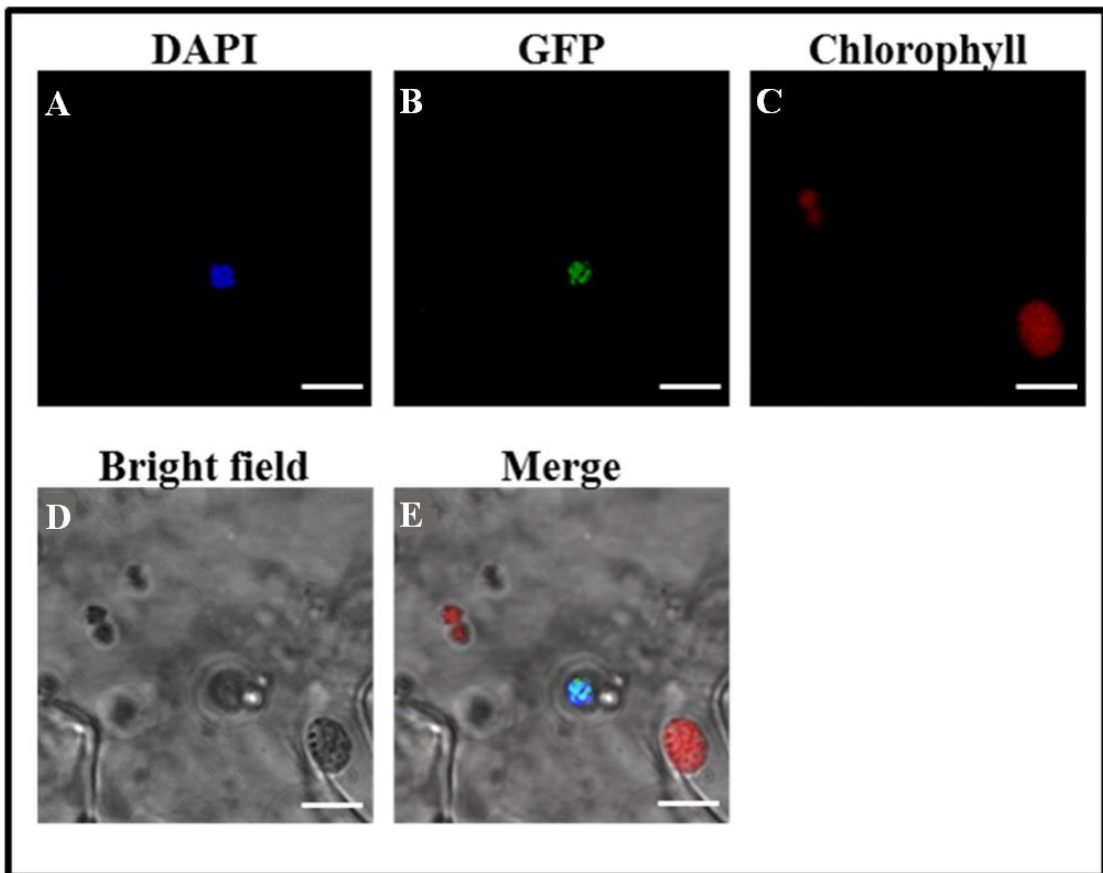


qRT-PCR, and the data revealed that only the transcript level of *AS2* was significantly decreased in *35S:SPL2SRDX* transgenic plants, while the expression of other candidate genes did not show significant differences compared to WT(Col) (Figure 8). Together with the decreased *AS2* transcript level in the *35S:miR156*, my data provide evidence that *AS2* is regulated by the miR156/SPL2 pathway and that SPL2 is an activator of *AS2*. However, these data do not show whether *AS2* is directly or indirectly regulated by SPL2.

3.4 SPL2 Directly Binds to *AS2* 5'UTR

The fact that SPL2 is a DNA-binding transcription factor (Yamasaki et al., 2004) and given my finding that *AS2* is activated by SPL2 (Figure 8), I hypothesized that SPL2 can bind directly to the *AS2* promoter to regulate *AS2* expression. To investigate this hypothesis, I used ChIP-qPCR to examine whether SPL2 binds to *AS2* promoter. The transgenic Arabidopsis line (*pSPL2:SPL2-GFP*) that expresses SPL2 as a translational fusion with GFP was used for the ChIP-qPCR assay. The production of the SPL2-GFP protein was detected using a confocal microscope. The cells were stained with DAPI, which is a stain to identify nuclei, and it was determined that the fusion protein was predominantly localized in the nucleus (Figure 9). Chromatin extracted from 3-week-old seedlings was immuno-precipitated with an anti-GFP antibody, and qPCR was used to analyze the abundance of the SPL binding region. Since an element sequence GTAC was identified as the SPLs' core binding motif (Birkenbihl et al., 2005), I searched for GTAC sequences in the 2 kb upstream fragment from the translation start codon of the *AS2*. Three regions (I, II and III) were identified to

Figure 9. Detection of SPL-GFP fusion protein. Cellular localization of SPL2-GFP fusion protein. The fluorescence of DAPI (A), GFP (B), chlorophyll (C) and the bright field (D) are shown. A merged image is shown (E). Scale bar = 5 μm .



contain GTAC boxes, and region II covered two GTAC boxes (Figure 10A; Appendix 5). Fragments that flanked these putative binding regions were amplified and a region in the *EIF4A1* promoter without a GTAC box was amplified as a negative control (Appendix 6).

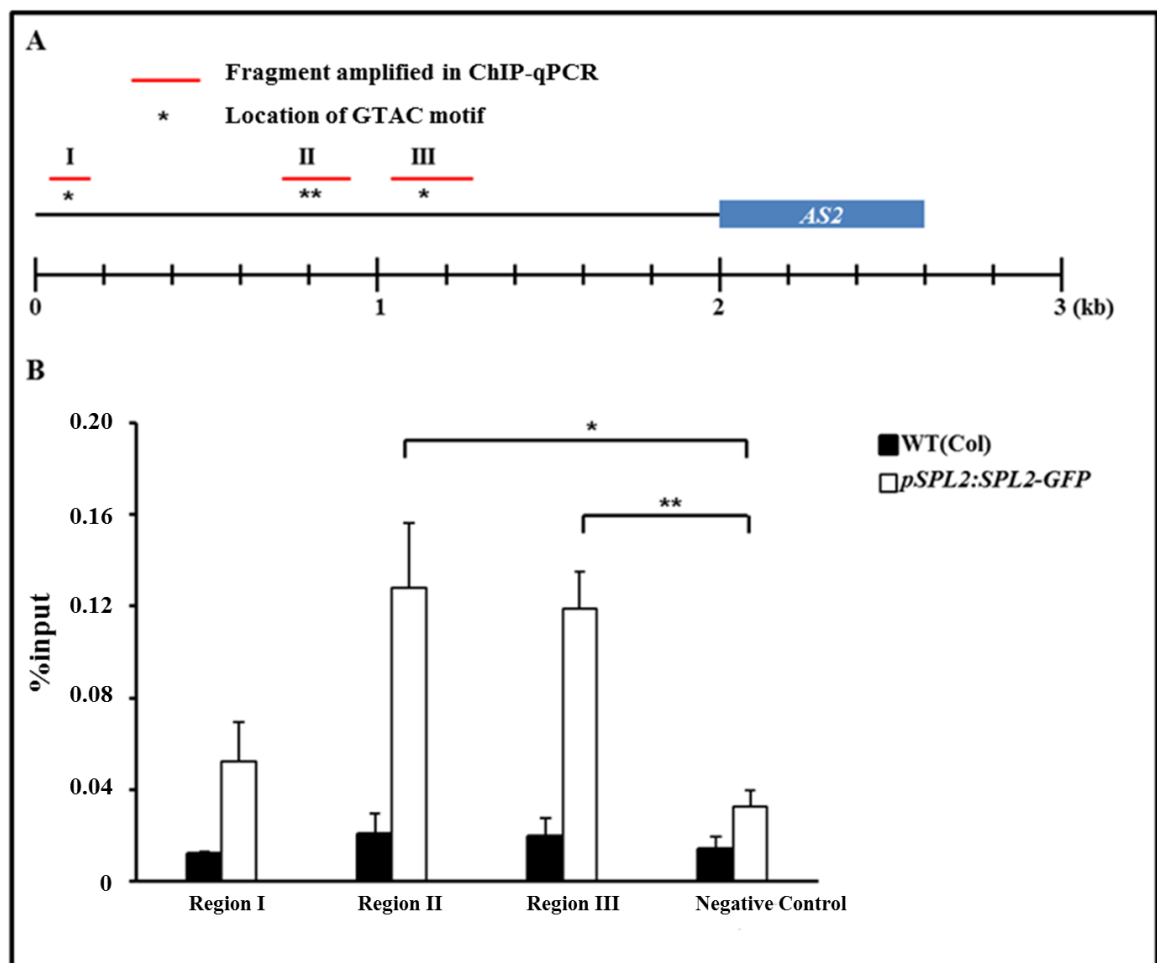
The strong binding of SPL2 at regions II and III was detected by ChIP-qPCR in the *pSPL2:SPL2-GFP* transgenic plants. In *pSPL2:SPL2-GFP* transgenic plants, the occupied signals in these two regions were significantly higher than that in the negative control (Fig. 10B). Two of the predicted SPL2 binding sites in region II were very close (less than 100 bp apart). Since the average size of the sonicated chromatin fragment is about 500 bp, I was unable to distinguish which one was actually occupied by the SPL2 in this region. By contrast, the signal in the *pSPL2:SPL2-GFP* plants at region I was stronger than in WT(Col), but there was no significant difference from the negative control, so it was not considered to be a putative binding site. Hence, SPL2 can selectively occupy multiple binding sites in the *AS2* regulatory region to activate *AS2* expression.

3.5 Functional Analysis of *AS2*

Based on ChIP-qPCR results, SPL2 appears to be a direct activator of *AS2*. Next, to know what role(s) *AS2* may play in plant development, I compared the phenotypes of the *AS2* loss-of-function mutant *as2-101* with *35S:miR156* and *35S:SPL2SRDX* transgenic plants. The *as2-101* is an EMS mutant which is in the *Ler* background. Thus, the Col and *Ler* wild-type were both used as controls.

Figure 10. Determination of SPL2 binding to *AS2* regulatory region *in vivo*. (A)

Schematic of *AS2*. Blue box represents exon; asterisks indicate GTAC motif. Red lines and Roman numerals represent fragments amplified by qPCR; (B) qPCR analysis of SPL2 putative binding site abundance in *pSPL2:SPL2-GFP* and WT (Col). Percent input DNA was used to calculate immune-precipitated DNA enrichment. Data were the mean of three biological replicates and the error bars represent SE. The statistical significance of SPL2 occupancy in each region compared to negative control in *pSPL2:SPL2-GFP* was determined by Student's t test. Single asterisk shows significant difference at $P < 0.05$, double asterisks show significant difference at $P < 0.01$. Three biological replicates were used.



3.5.1 *AS2* is involved in controlling growth of floral organs and siliques

Compared with WT(Col) plants, the petals and sepals in *35S:miR156* and *35S:SPL2SRDX* plants were smaller, and this defective phenotype was also observed in *as2-101* when compared to WT(*Ler*) plants. Following measurement of the petals within each genotype, it was found that the length of petals in WT(Col) were 2.075 ± 0.144 mm, while the petal lengths of the *35S:miR156* and *35S:SPL2SRDX* plants were notably reduced, as the sizes were about 82% of WT(Col) (Figure 11A, Table 2). Similarly, the length of sepals in *35S:miR156* and *35S:SPL2SRDX* plants were decreased, with only about 78% of WT(Col) (Figure 11B, Table 2). More severely defective phenotypes were observed in the *as2-101* mutant. The petals were 2.121 ± 0.138 mm in WT(*Ler*), while in *as2-101* the petal length was 1.697 ± 0.095 mm. The length of *as2-101* sepals was only 65% of WT(*Ler*). Moreover, shorter siliques were also observed in *35S:miR156*, *35S:SPL2SRDX*, and *as2-101* mutants (Figure 11C, Table 2). In WT(Col) and WT(*Ler*), 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 markedly reduced (Table 2).

To summarize, *AS2* regulates growth of floral organs and siliques, and reduced transcript level of *AS2* in *35S:miR156* and *35S:SPL2SRDX* plants might cause production of smaller floral organs and siliques.

3.5.2 Decreased of *AS2* transcript level causes partial sterility

The three mutants, *35S:miR156*, *35S:SPL2SRDX* and *as2-101*, with reduced *AS2* expression had smaller siliques (Fig. 11C), suggesting they also may have decreased

Figure 11. Phenotypes of petals, sepals and siliques in *35S:miR156*, *35S:SPL2SRDX*, and *as2-10* plants compared to WT (Col and *Ler*) plants. (A) petals; (B) sepals; (C) siliques. Scale bar = 1 mm.



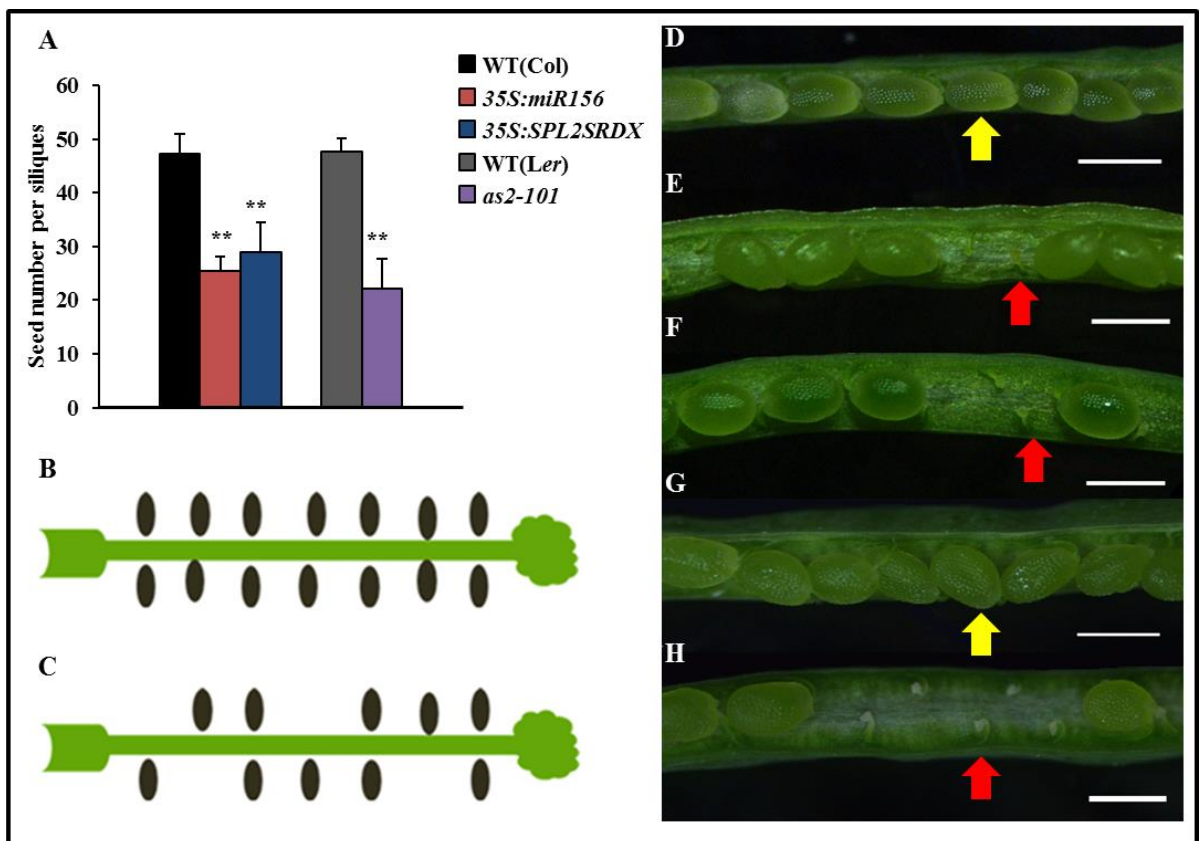
Table 2. Length of petal, sepal and siliques in different genotypes.

Genotype	Petal		Sepal		Silique	
	Length (mm) ^a	% of WT	Length (mm) ^a	% of WT	Length (mm) ^a	% of WT
WT(Col)	2.075±0.144		1.451±0.084		14.75±0.62	
<i>35S:miR156</i>	1.698±0.076**	81.8%	1.121±0.084**	77.3%	9.26±0.44**	62.8%
<i>35S:SPL2SRDX</i>	1.710±0.121**	82.4%	1.132±0.062**	78.1%	9.98±0.75**	67.7%
WT(Ler)	2.121±0.138		1.539±0.076		12.62±0.87	
<i>as2-101</i>	1.697±0.095**	80.0%	0.954±0.109**	65.8%	8.72±0.72**	69.1%

^aDouble asterisk shows significant difference at $P < 0.01$. At least 15 petals, sepals and siliques were measured.

Student's *t* test was used to compare the statistical significances of *35S:miR156* and *35S:SPL2SRDX* to WT(Col), and *as2-101* to WT(Ler).

Figure 12. Seed set analysis and evaluation of plant fertility. (A) mean number of seeds set per silique in each genotype. Error bars indicate standard deviation (SD). Student's *t* test was used to compare the statistical significance of *35S:miR156* and *35S:SPL2SRDX* to WT(Col), and *as2-101* to WT(Ler). Double asterisks show significant difference at $P < 0.01$. At least 15 siliques were used for each genotype. 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 (Ler) (G) and *as2-101* (H). Red arrows show the aborted embryos, yellow arrows show the normal growing seeds. Scale bar = 100 μ m.



seed production. To that end, I analyzed the siliques for their seed production. I dissected siliques and counted seeds of each genotype. In WT(Col) and WT(Ler), about 47 seeds were produced per silique (Figure 12A). In contrast, the number of seeds in the mutants (*35S:miR156*, *35S:SPL2SRDX* and *as2-101*) were all less than 30. Besides the reduction of seed sets in the mutants, a partial sterility phenotype was also detected. Generally, seeds develop from the ovule and grow continually until they reach the mature stage (Figure 12B). In fully or partially sterile plants, some embryos abort (Figure 12C). In WT plants (Col and Ler), almost all the seeds developed normally (Figure 12D, G), while in the mutants, aborted embryos were observed (Figure 12E, F, H). I calculated the sterility rate for each genotype and found that in WT(Col) and WT(Ler) the sterility rates were 2.51% and 3.05%, respectively (Table 3). In *35S:miR156*, the sterility rate was much higher (21.38%). The sterile phenotype was much more severe in *35S:SPL2SRDX* and *as2-101* with rates of 29.90% and 37.28%, respectively.

3.5.3 AS2 regulates pollen production

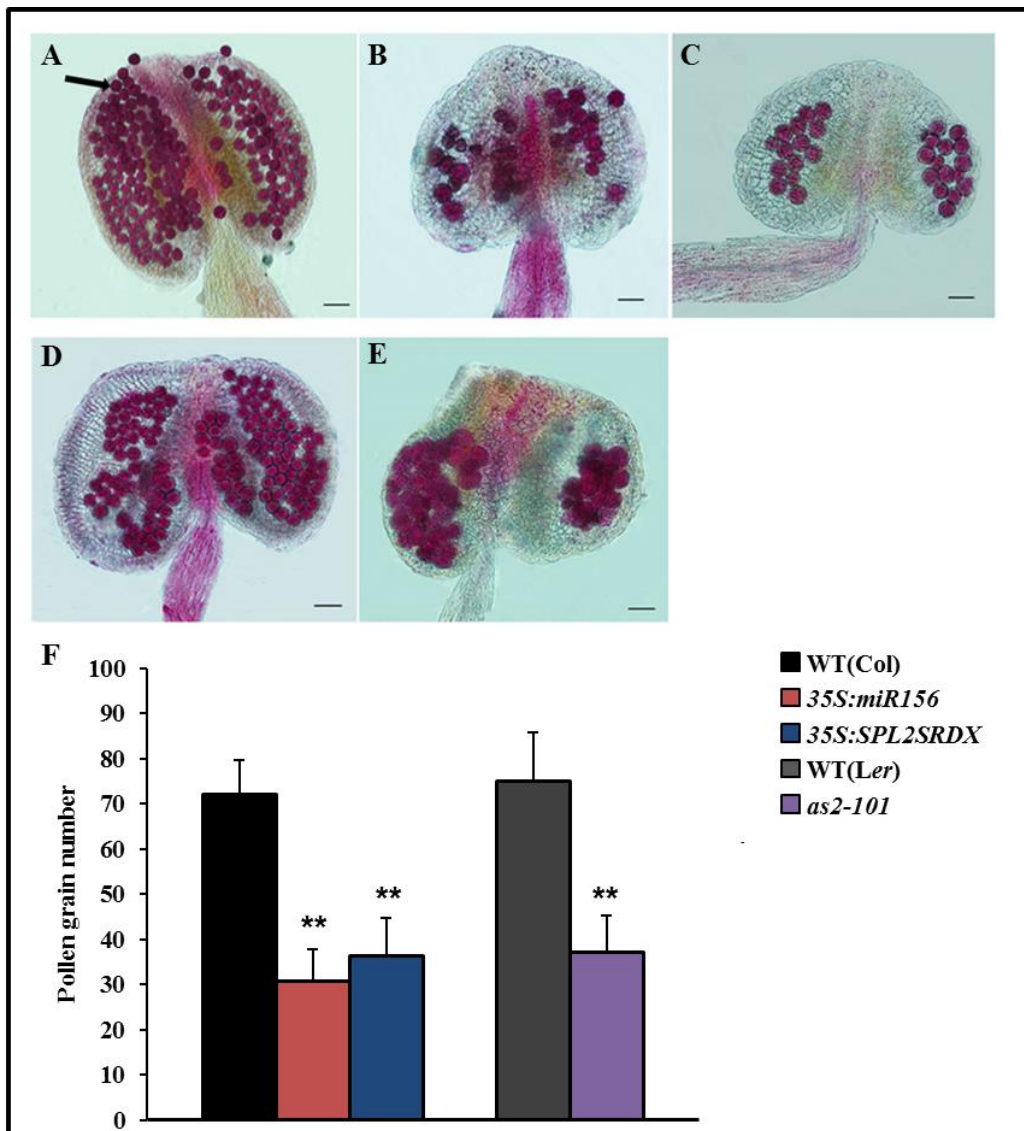
To further investigate the factors causing the sterile phenotypes that were observed in the mutants, I examined the pollen grains in all the genotypes. I used the Alexander staining method to examine the viability of pollen grains in the anthers (Alexander, 1969). In the Alexander staining method, the viable pollen grains are stained red, and inviable pollen grains are stained blue-green. In my result, no inviable pollen grains were found in any of the genotypes (WT controls, *35S:miR156*, *35S:SPL2SRDX* and *as2-101*). Interestingly, the number of pollen grains in the

Table 3. Sterility rates of WT(Col), 35S:miR156, 35S:SPL2SRDX, WT(Ler) 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 (Ler)	573	18	591	3.05%
as2-101	244	145	389	37.28%**

^aDouble asterisk shows significant difference at $P < 0.01$. At least 13 siliques were examined. Student's t test was used to compare the statistical significances of 35S:miR156 and 35S:SPL2SRDX to WT(Col), and as2-101 to WT(Ler).

Figure 13. 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 μ m. Viable pollen grains (indicated by an arrow) are stained in red. (F) The number of pollen grains in each genotype. At least 13 anthers were used to count pollen number. Student's *t* test was used to compare the statistical significance of *35S:miR156* and *35S:SPL2SRDX* to WT(Col), and *as2-101* to WT(*Ler*). Double asterisks show significant difference at $P < 0.01$.

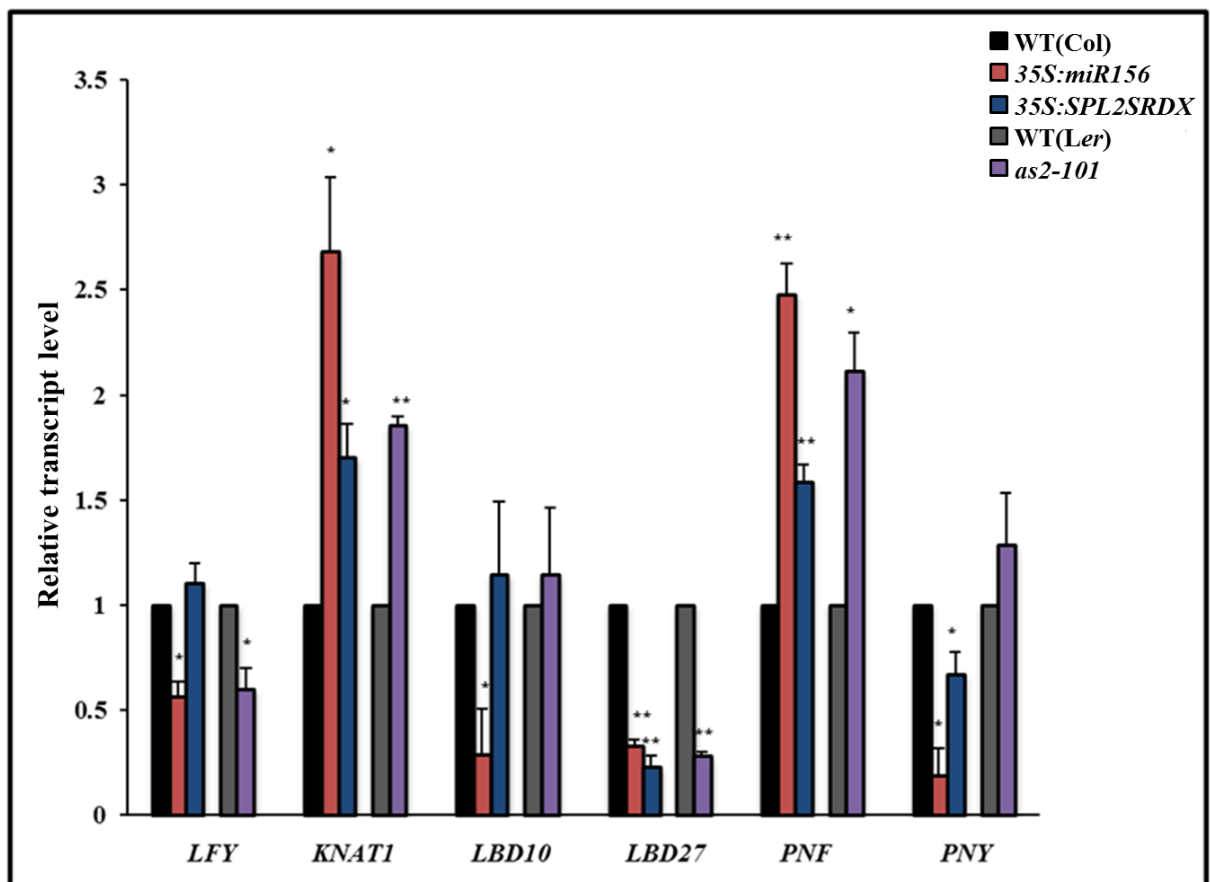


mutants was dramatically decreased. When compared with WT(Col) and WT(Ler) plants, the mutants produced less pollen in the anthers (Figure 13A, B, C, D and E). I counted the number of pollen grains, and found that in WT(Col), 72 ± 7.8 pollen grains were produced, and a similar number was produced in WT(Ler) (75.1 ± 10.8) (Figure 13F). The number of pollen grains that were produced by the mutants were only about half of those produced by WT plants in each background.

3.6 Regulation of Plant Development Related Genes by miR156/SPL2 Pathway

To further investigate the influence of the miR156/SPL2 regulatory pathway on organ growth and plant fertility in Arabidopsis, I identified several genes that either control floral organ development or plant fertility, *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1 (KNAT1)*, *LFY*, *PNF*, *PNY*, *LBD10* and *LBD27* (Guo et al., 2008b; Kim et al., 2015; Weigel et al., 1992; Yu et al., 2009). I then tested the transcript levels of these genes in siliques of *35S:miR156*, *35S:SPL2SRDX*, *as2-101*, WT(Col) and WT(Ler) (Fig. 14). The transcript level of *KNAT1* was significantly decreased with reduced transcript level of *AS2*, which is consistent with previous results (Guo et al. 2008b). A similar expression pattern was observed for *PNF*, suggesting that *PNF* is downstream of *AS2*. *PNY* had decreased transcript level in *35S:miR156* and *35S:SPL2SRDX* compared to WT(Col), but no significant changes in *PNY* were found between *as2-101* and WT(Ler). Thus, the regulation of *PNY* might occur through a pathway independent of *AS2*. No significant differences in *LFY* expression were detected between *35S:SPL2SRDX* and WT(Col). The differential expression of *LBD10* in *35S:miR156* and WT(Col) indicates that its

Figure 14. 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 level in *as2-101* was relative to WT (*Ler*) (set to an arbitrary value of 1). The expression data were the mean of three biological replicates. Error bars represent SE. Student's *t* test was used to compare the statistical significance of *35S:miR156* and *35S:SPL2SRDX* to WT(Col), and *as2-101* to WT(*Ler*). Single asterisk shows significant difference at $P < 0.05$, double asterisks show significant difference at $P < 0.01$.



expression may be controlled by SPLs other than SPL2. *LBD27* transcript level was notably decreased in all mutants compared to the WT controls (Col or *Ler*). Therefore, both miR156/SPL2 pathway and AS2 can regulate the transcript level of *LBD27*.

CHAPTER IV. DISCUSSIONS

4.1 Research Overview

In plants, miRNAs function by building genetic regulatory networks via targeting several transcription factors, such as SPL and MYB transcription factor families (Jones-Rhoades et al., 2006). Among plant miRNAs, miR156 is one of the most conserved miRNA families (Islam et al., 2015). Functional characterization in different species has revealed that miR156 plays versatile essential roles in different phases of plant development (Jiao et al., 2010; Aung et al., 2015b; Wang et al., 2015). Recent research indicates miR156 regulates multiple aspects of growth and secondary metabolism in Arabidopsis, such as organ elongation, flowering time, carotenoid accumulation and flavonoid synthesis (Wang et al., 2009; Gou et al., 2011; Wei et al., 2012). Moreover, miR156 together with its target, *SPLs*, are reported to regulate fertility and the miR156 non-targeted gene *SPL8* is also involved in this aspect (Xing et al., 2010, 2013). Although the miR156/SPL network is required for maintaining normal fertility and proper organ development, the downstream genes of this regulatory network that are involved in these processes are still not fully elucidated.

In this study, I investigated the function of miR156 and SPL2 in reproductive development of Arabidopsis. Both the *miR156* overexpression line and SPL2 dominant-negative mutant showed a sterile phenotype and inhibited elongation of floral organs (section 3.5). Furthermore, the NG RNA-Seq and qRT-PCR results indicate that *AS2*, which belongs to the *LBD* gene family, was regulated by the

miR156/SPL2 pathway (section 3.32). Interestingly, the defective phenotypes which were detected in *35S:miR156* and *35S:SPL2SRDX* were also observed in the *AS2* loss-of-function mutant. These pieces of evidence led me to hypothesize that SPL2 is a direct regulator of *AS2*, and the abnormal development of the organs in *35S:miR156* and *35S:SPL2SRDX* might be caused by the reduced transcript level of *AS2*.

4.2 miR156 and SPL2 Pathway Involved in the Development of Siliques and Floral Organs

Based on previous phylogenetic analyses, the miR156-targeted *SPL* genes in Arabidopsis can be divided into four groups: *SPL2/SPL10/SPL11*, *SPL3/SPL4/SPL5*, *SPL6/SPL13* and *SPL9/SPL15* (Guo et al., 2008a). The diverse functions of SPLs in plant development have been previously reported and several SPLs are known to be involved in organ growth (Wang et al., 2008; Wu et al., 2009). In the NG RNA-Seq and qRT-PCR data, three *SPL* genes (*SPL2*, *SPL4* and *SPL10*) showed reduced expression in the siliques of *miR156* overexpression plants. I also determined that *SPL2*, *SPL4* and *SPL10* have a high transcript level in the early seed development stage according to the Arabidopsis eFB Browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). This reveals that miR156 targets these three *SPLs* to control the development of siliques in Arabidopsis. Moreover, the expression patterns of *SPL2* and *SPL10* are similar in different tissues, and their protein sequences share certain similarities (Riese et al., 2007; Shikata et al., 2009). In addition, the knock-out mutant of *SPL2* showed a similar visible phenotype to that of WT (Schwarz et al.,

2008), and the redundant function of *SPL2* and *SPL10* during leaf growth has been reported (Shikata et al., 2009). These evidently suggest that *SPL10* might be functionally redundant with *SPL2* during silique development.

4.3 Functional Connection Between miR156/SPL and AS2

In *Arabidopsis*, *AS2* was first characterized as a gene that is required for the formation of symmetric leaves (Semiarti et al., 2001; Iwakawa et al., 2002; Rast and Simon, 2012). The loss-of-function mutant of *AS2* produces curled leaves, as a consequence of an increasing ratio of cell numbers in adaxial side and abaxial side of the mutant leaves (Iwakawa et al., 2007). Previous research indicates *AS2* loss-of-function mutants are inhibited in growth of petals and sepals (Xu et al., 2008). This defective phenotype was also observed in my research (Figure 11; Table 4). A reduction in producing pollen grains was also found in *as2-101* (Figure 13). This indicates that the function of *AS2* is not limited to regulating leaf growth, and that this transcription factor is required for the proper development of siliques and floral organs as well. In *Arabidopsis*, miR156 reaches its highest level at the early juvenile phase and gradually decreases as plants grow (May et al., 2013). Contrary to miR156, the highest transcript level of *SPL2* occurs in the floral part (Shikata et al., 2009). Moreover, analysis using the *Arabidopsis* eFB Browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) showed the expression of *AS2* in the flowers to be higher than in other parts of the plant, and a relatively high level of *AS2* expression was also detected in anthers (Keta et al., 2012). The similar expression patterns of *SPL2* and *AS2* gives further proof that *SPL2* is an upstream activator of *AS2*.

Table 4. SBP-domain binding sequences in target genes of SPLs.

Genes	SPL binding sequence	Binding protein
<i>AS2-1</i>	CTCTAGTACCAAAT	SPL2
<i>AS2-2</i>	AATTAGTACTAGTC	SPL2
<i>AS2-3</i>	ACACGGTACAAATT	SPL2
<i>API-1</i>	CCTGCGTACAAATC	SPL3 ¹
<i>API-2</i>	GGTCCGTACAATGT	SPL3 ¹
<i>FUL-1</i>	AGTCCGTACGGTAC	SPL3 ¹
<i>FUL-2</i>	GTACGGTACGGAAA	SPL3 ¹
<i>LFY</i>	GAACGGTACGGTAT	SPL3 ¹
<i>MiR398c</i>	TCGCAGTACACAAT	SPL7 ²
<i>TCL1-1</i>	ATGGTGTACCTATA	SPL9 ³
<i>TCL1-2</i>	ATCATGTACCGTTC	SPL9 ³
<i>TCL1-3</i>	TCATCGTACCACAT	SPL9 ³
<i>TRY-1</i>	GTAGGGTACTTTAT	SPL9 ³
<i>TRY-2</i>	GAAGCGTACTTTAT	SPL9 ³
<i>TRY-3</i>	CATACGTACTATAT	SPL9 ³
<i>DFR-2</i>	GTTACGTACCACAC	SPL9 ⁴
<i>DFR-3</i>	GATTTGTACCGAAC	SPL9 ⁴

¹(Yamaguchi et al., 2009); ²(Yamasaki et al., 2009); ³(Yu et al., 2010) ; ⁴(Gou et al., 2011)

Recent research indicates that the miR156/SPL network is involved in the auxin signal response; the expressions of *miR156*, *SPL9* and *SPL10* increase with higher levels of indole-3-acetic acid (IAA) (Yu et al., 2015). In the NG RNA-Seq data, several genes were found to participate in auxin signaling, such as the *AUXIN RESISTANT 5* (*AXR5*, AT4G14560) (Appendix 4), the expression of which can be up-regulated by auxin (Abel et al., 1994). Interestingly, it was also reported that *AS2* is involved in auxin transport; as auxin in the leaves of *AS2* loss-of-function mutant was asymmetrically distributed, and this was suggested to cause the abnormal pattern of cell division in this mutant (Zgurski et al., 2005). In addition, the interaction between *AS2* and auxin-related genes reveals the involvement of *AS2* in the auxin response pathway. For example, *AS2* is a direct transcriptional repressor of *AUXIN RESPONSE TRANSCRIPTION FACTOR 3* (*ARF3*) (Iwasaki et al., 2013), and *ARF3* can be up-regulated by exogenous auxin (Cheng et al., 2013). Furthermore, auxin is essential for flower formation and growth (Nemhauser et al., 1998; Cheng and Zhao, 2007). These pieces of evidence suggest that *AS2* might be one of the miR156/SPL network downstream genes that are required for auxin response and transport.

4.4 SPL2 is a Direct Activator of AS2

AS2 is involved in regulating plant development, but how *AS2* itself is regulated is still not completely understood. So far, only several transcription factors are known to directly regulate *AS2*, such as *BOP1* and *KAN1* (Jun et al., 2010; Wu et al., 2008). In my study, I identified *SPL2* as a direct regulator of *AS2*. The transcript level of *AS2* was significantly decreased in the *SPL2* dominant-negative mutant and *miR156*

overexpression line compared to WT (Col). This indicates that SPL2 is an activator of *AS2*. Next, ChIP-qPCR assay was performed to test the *in vivo* interaction between SPL2 and *AS2*, and three regions located upstream of the translation initiation site and containing putative SPL binding elements were tested. The data indicate two regions, which are closer to the coding region, had strong binding to SPL2. The distant region (region I) did not show significant binding (Fig. 10). These results provide direct proof that SPL2 can selectively bind to the regulatory elements of *AS2*. This seems to confirm results of previous research (Cardon et al., 1999) that the GTAC core sequence is necessary for the binding of SBP-domain, but it by itself is not sufficient for binding.

Although I showed that SPL2 can directly bind to the regulatory region of *AS2*, the mechanism by which SPL2 activates *AS2* is still unknown. It will be interesting to further identify the proteins that interact with SPL2 to form a complex and activate *AS2*.

4.5 Diversity of SPL Binding Sites in Arabidopsis

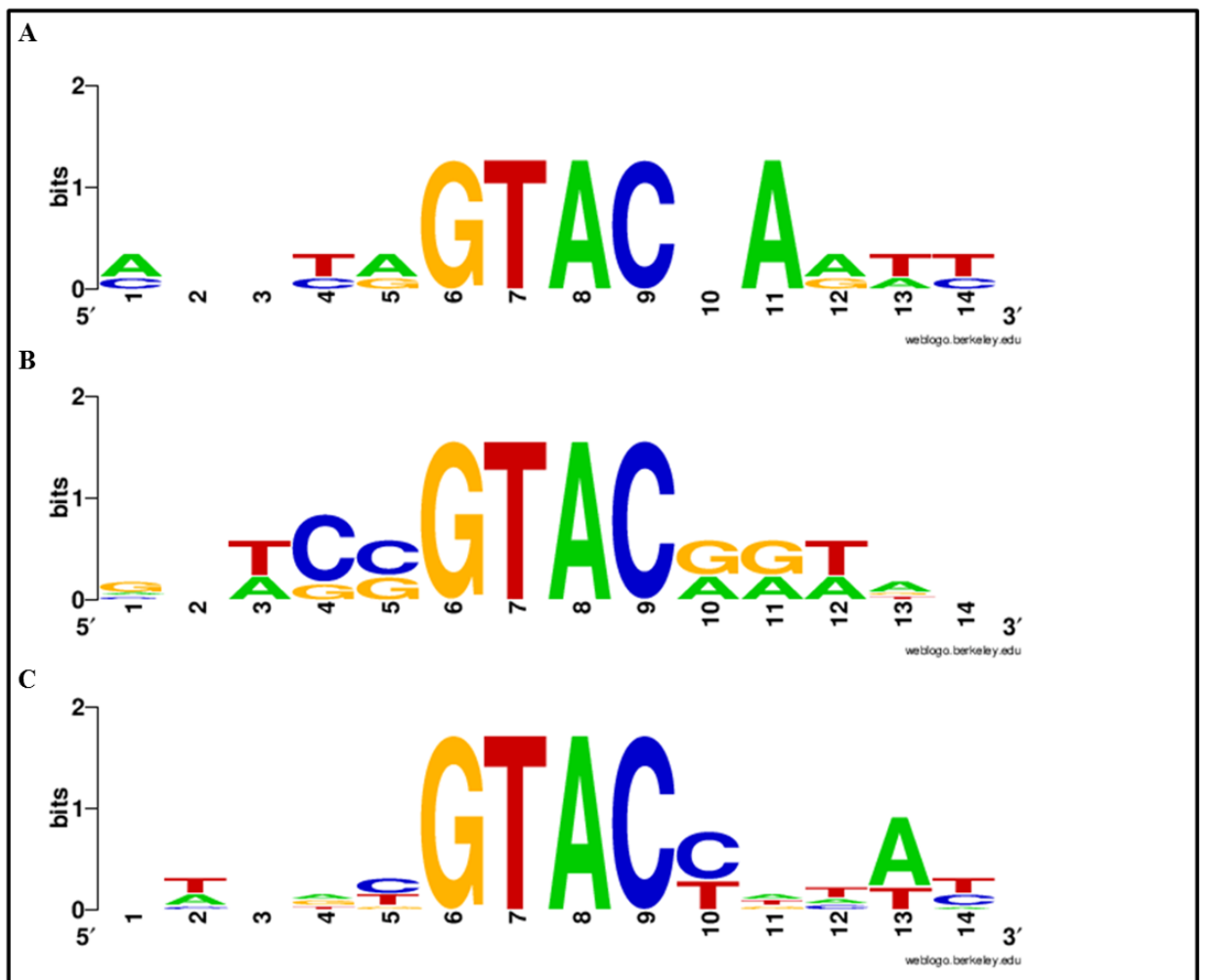
In Arabidopsis, several genes have been identified as direct targets of SPLs. For example, SPL3 can bind to specific regions that contain GTAC in *API*, *FUL* and *LFY* (Yamaguchi et al., 2009), and SPL9 can bind to the promoter regions of *TCL1*, *TRY* and *DFR* (Yu et al., 2010; Gou et al., 2011). Since previous reports demonstrate that the consensus “CNGTACM” is more likely to be bound by SPLs, I analyzed whether the recently identified target genes of SPLs also contain this consensus. I compared SBP-domain binding sequences from *AS2* and other SPL-targeted genes using

Weblogo (<http://weblogo.berkeley.edu/logo.cgi>) (Figure 15; Table 4). Among the three SPL2 binding sites in *AS2* regulatory region, only one site contains the consensus "CNGTACM" sequence (Table 4). The nucleotide "A" was found at the same position in all these three SPL2 binding sites on *AS2* (Figure 15). The second nucleotide upstream of "GTAC" from SPL3 binding sites is more likely to be "C" (Figure 15; Table 4). SPL9 occupies diverse binding sites in the target gene promoter regions (Figure 15; Table 4). Moreover, the binding sites of SPL7 and SPL14 tend to be closer to the consensus "CNGTACM" (Liang et al., 2008; Yamasaki et al., 2009). Therefore, the sequence near the "GTAC" element in the SBP binding element may vary from gene to gene in Arabidopsis. More downstream genes regulated by SPL would need to be identified and characterized to come up with a more representative consensus sequence for SPL binding.

The SBP proteins were first identified in *Antirrhinum majus*, and these proteins can bind to the promoter region of *SQUAMOSA* (Klein et al., 1996). SBP protein family was later shown to activate expression by occupying specific regions in the promoters of target genes in both *Chlamydomonas reinhardtii* and Arabidopsis (Kropat et al., 2005; Yu et al., 2010). It has also been reported that SPL3 can activate the expression of *LFY* and *FUL*, by binding to GTAC-containing consensus sequences in the exon of *LFY* and the intron of *FUL* (Yamaguchi et al., 2009). In my study, the sites that were occupied by SPL2 locate to the 5'UTR of *AS2*. Therefore, the SPL binding elements are not limited to the promoter region of the target genes, but rather they can also be found in the UTR or exons.

Figure 15. SBP-domain binding sequences present in the target genes of SPLs.

Consensus SPL binding sequences in target genes of SPL2 (A), SPL3 (B) and SPL9 (C) were aligned by Weblogo. The height of the letter shows the frequency of each nucleotide at each position.



4.6 Repression of *AS2* Inhibits Growth of Floral Organs and Causes Partial

Sterility

To functionally characterize the role of miR156, SPL2 and AS2 in the siliques and floral organs of Arabidopsis, I analyzed the phenotypes of these organs in *35S:miR156*, *35S:SPL2SRDX*, and *as2-101*. Since these three mutants are from different accessions, both the WT(Col) and WT(*Ler*) were used as controls. In comparison to WT(Col), smaller petals and sepals were observed in *35S:miR156* and *35S:SPL2SRDX*. Similar defective phenotypes were also found in *as2-101*. Moreover, the growth of siliques in all mutants was inhibited. The extent to which the organ growth relies on both cell proliferation and cell expansion, and the coordination of these two processes determines the overall organ size (Mizukami, 2001; Beemster et al., 2003). *miR156* overexpression-mediated silencing of *SPLs* was shown to increase cell size and decrease cell number to control leaf size; whereas overexpression of *SPL3*, *SPL4*, *SPL5*, and *SPL15* caused phenotypes opposite to these effectuated by *miR156* overexpression (Usami et al., 2009). This demonstrates the involvement of miR156 and SPLs in regulating organ size in plants. In Arabidopsis, AS2 is a direct repressor of *KNATI* (Guo et al., 2008b), which is a negative regulator of cell division (Truernit and Haseloff, 2008). To mediate regulation of *KNATI*, the MYB domain protein AS1 is also required. AS2 forms a complex with AS1 and this complex can bind to the target genes' specific motif in the promoter region (Guo et al., 2008b). In my research, the increased transcript level of *KNATI* in *35S:miR156* and *35S:SPL2SRDX* was accompanied by a

reduction of *AS2* expression in these plants (Fig. 14). This further demonstrates the function of miR156 in controlling organ growth through affecting cell divisions. SPL2 direct activation of *AS2* and further repression of *KNAT1* might be a genetic mechanism for regulating organ growth in Arabidopsis.

Additionally, I found a notably higher sterility rate occurring in *35S:miR156*, *35S:SPL2SRDX* and *as2-101* compared to WT controls (Table 3). To further investigate the factors that cause the sterility, I stained the anthers with the Alexander staining method. No inviable pollen was found in any genotypes, and therefore I concluded that the sterile phenotype was not caused by the lack of viability of the pollen grains. Intriguingly, I found far fewer pollen grains were produced in *35S:miR156*, *35S:SPL2SRDX* and *as2-101*. I suggest the low number of pollen grains might be an important factor that results in high sterility rate in the mutants, since the smaller the number of pollen grains that the anther produces, the lower the chance that pollen will have to adhere to the stigma. An insufficient amount of viable pollen results in reducing the possibility of fertilization, and subsequently causes sterile phenotypes (Ter-Avanesian, 1978).

In my research, the function of *AS2* in controlling plant fertility and organ growth was identified. The decreased *AS2* transcript level was detected in *35S:miR156* and *35S:SPL2SRDX*, and similar phenotypes, such as shorter organs and high sterility rate, were observed in these two mutants. However, I cannot rule out the possibility that other factors, in addition to repression of *AS2*, may have contributed to the defective phenotypes in *35S:miR156* and *35S:SPL2SRDX* plants.

4.7 Relationship Between miR156/SPL2 Pathway and Organ Growth- and Fertility-Related Genes

In this study, I tested several genes that are involved in plant development. Firstly, *PNF* and *PNY*, which are members of the *BELI-LIKE* homeodomain gene family, are expressed in floral meristem and involved in floral organ development (Kanrar et al., 2008). *PNF* and *PNY* also participate in the regulation of the miR156/SPL network through increasing transcript level of *SPL3*, *SPL4* and *SPL5*, as well as decreasing the transcript level of *miR156* (Lal et al., 2011). *KNAT1*, which is a member of the class I *KNOTTED1-LIKE* homeobox gene family, has been reported to be a direct target of AS2 and is also involved in floral organ development (Guo et al., 2008b; Kanrar et al., 2008; Zhao et al., 2015). Based on the expression analysis of these genes, *KNAT1*, *PNF* and *PNY* were affected by miR156/SPL2 pathway, and AS2 is involved in the down-regulation of *KNAT1* and *PNF*. Furthermore, *LFY* directly activates expression of *AS2* through occupying specific regulatory regions of *AS2* (Yamaguchi et al., 2012), and *SPL3* can directly activate *LFY* by binding to the first exon and intron of *LFY* (Yamaguchi et al., 2009). However, the expression level of *LFY* was not affected in *35S:SPL2SRDX*, so *SPL2* is not functionally redundant with *SPL3* in regulating *LFY*.

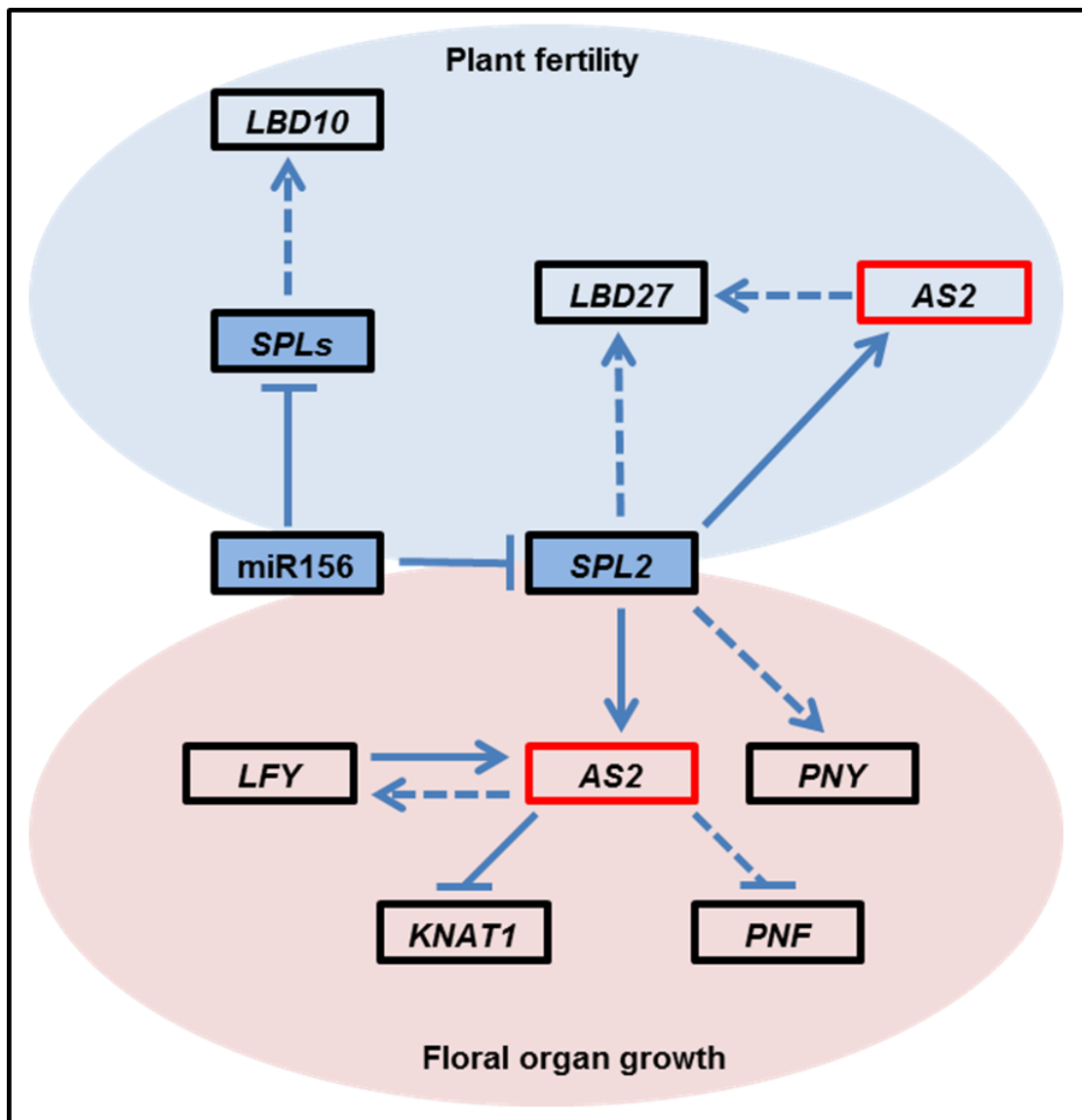
Previous research showed *LBD10* and *LBD27* were crucial for maintaining plant fertility in *Arabidopsis* (Kim et al., 2015). My analysis revealed that *LBD27* was regulated by miR156/SPL2 pathway, as well as AS2 (Figure 14). Moreover, the genomic sequence of *LBD27* contains both SBP-domain and LBD core putative

binding sequences (Husbands et al., 2007), which suggests that both SPL2 and AS2 are upstream regulators of *LBD27*.

4.8 Proposed Model for miR156/SPL2 Regulatory Pathway

My results indicate that the miR156/SPL2 regulatory pathway is necessary for the regulation of plant floral organ growth and fertility, and one of the SPL2 target genes, *AS2*, was also found to control these processes. Besides *AS2*, several other genes with diverse functions, such as *PNF* and *LBD27*, were also found to be regulated by this pathway. I also found that *LFY* was feedback activated by *AS2*. 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., 2015a; Wang and Wang, 2015). Finally, I propose a regulatory model that centers on the miR156/SPL2 pathway (Figure 16). Thus, the miR156/SPL2 pathway appears to act upstream of *KNAT1*, *PNF* and *LBD27*. Reduced *LBD10* transcript levels in *35S:miR156* indicates *LBD10* might be regulated by a pathway independent of miR156/SPL2.

Figure 16. A proposed model for the regulatory pathway that controls organ growth and fertility involving miR156/SPL network and AS2. The transcript levels of the organ growth- and fertility-related genes were tested in WT(Col), *35S:miR156*, *35S:SPL2SRDX*, WT(*Ler*) and *as2-101*. 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.



CHAPTER V. CONCLUSIONS AND PROSPECTIVES FOR FUTURE RESEARCH

The miR156 regulatory network is vital for maintaining proper development of plants (Aung et al., 2015a; Wang and Wang, 2015). In my study, I showed that the miR156/SPL2 pathway was involved in controlling floral organ development and plant fertility, and found that *AS2* acts as a direct target of SPL2. This finding helps to understand the new pathway that participates in plant reproductive development.

So far, several reports indicate that the miR156/SPL regulatory network is essential for plant development in the reproductive phase (Xing et al., 2010; Li et al., 2013; Ferreira e Silva et al., 2014), but no downstream genes have been identified to govern this process. Moreover, *AS2* is reported to directly reduce the transcript level of a cell division-related gene *KNAT1* (Guo et al., 2008b; Truernit and Haseloff, 2008), which might be the cause of smaller floral organs in *as2-101*. The evidence that SPL2 activates the expression of *AS2* provides a connection between the SPL and *LBD* gene families. In addition, the ChIP-qPCR assay results indicate the direct binding of SPL2 to the *AS2* upstream regulatory region, which further demonstrates *in vivo* interaction between SPL2 and *AS2*. Finally, the decreased transcript level of *AS2* in the *miR156* overexpression line and the SPL2 dominant-negative mutant is one of the factors that leads to the sterility phenotype and defective development of floral organs. For future research, it will be also interesting to perform a genetic complementation experiment to test whether the decreased transcript level of *AS2* is the dominant cause of these phenotypes.

Versatile functions for miR156 have been reported in many plant species over the last 10 years (Llave et al., 2002; Reinhart et al., 2002; Aung et al., 2015b; Wang et al., 2015). The miR156/SPL network is a master regulator of plant development, and it is thus necessary to fully understand all the genes that are involved in it. To this end, my research provides a new insight into the function of miR156, its target *SPL2*, and another downstream target gene of *SPL2*, *AS2*.

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Appendices

Appendix 1. List of primers used in expression analysis

Appendix 1A. Primers used in gene expression analysis

Gene	Accession number	Sequence	
		Forward	Reverse
unknown	AT1G75620	TGACACGTGTGCGA GAATCA	CAAGCAGCTGTCCC TGAAGA
unknown	AT5G22580	TGCTTAGACAAGGA TTCACTCA	ACGGGGAAATCGA GGAGAAC
<i>ATDSEL</i>	AT4G18550	AGGTATGGCCCAAC AAGACG	GTTTCCAAACATCA ACACTTGACA
unknown	AT1G80120	GTTGTCCTCATGGA CGCTCA	TTCCTTGCCCCGAA ACTACC
unknown	AT1G31250	ATCGAGGCTAGGGA AGGCAA	GGAGGTATCGAAAG AGAAGCGT
unknown	AT1G47395	CTCATCACACTTCTT CACTCGG	CACGGAAGCACGG TCAAAAC
<i>AST91</i>	AT1G23090	ACGAGTGGTCATGG CAAACA	AGCTTCGGCTTCTT CATGCT
<i>CASPL2A2</i>	AT3G14380	ACTGAGAAATCGGC AGAGGC	ACGCCATTAGCACT CACCAA
<i>ATSDII</i>	AT5G48850	GAAGAACCCGGAG ATGGCAA	CTTGGAACAACGAG GACGGA
unknown	AT3G21460	GAGCCCGGCAATTG TAGAGA	CCCTCCAACAAAA CCGCAG
<i>GAMMA-TIP1</i>	AT2G36830	CAGATGAAGCCACC CGTCC	GAGACAGCGACGA AGAGTCC
unknown	AT3G27025	ATGTCCAAGACATC CGCACA	CAGGGTCTGTACCA AGCGTC
<i>SAUR31</i>	AT4G00880	ACAAGCGACTTCAC CATCCA	TGCCATGGTTTCTG TCTCTCC
unknown	AT3G62960	CCTGAGCCCTCAGA CCAAAA	TTCTCGATCTCACG GCAGTC

<i>CEP2</i>	AT3G48340	AACGCGGCTCAA ACAGTTC	GCTACGGTAGAGAA TGCCCA
<i>HIPP21</i>	AT5G17450	AAATGGACTGTGAT GGTTGTGAAA	GCCAGGAGCAAAA GGGTAATAGAC
unknown	AT3G18950	GGCGTCTACGGTTA TTGGCT	AGCCGGCCATAAAA ACTTGC
<i>FUL</i>	AT5G60910	TCAGGGTTGTCGTT TCTCTCTC	TCAGCATCGCAGAG AACAGAG
unknown	AT5G16350	AGCGTTGGAGAGA GAGAATAAA	AGTCTATGTCCGGC GATTGG
unknown	AT2G16630	TGAAACGTTTGCTG CGGATG	GCTGTGGGTGGTTT AGGACA
<i>ARGOS</i>	AT3G59900	CGGCGCAGAAAAG GATGATG	AGGTAACGGCGGC AAAATA
<i>ATDIP2</i>	AT5G03210	GTGGTGGATGGAGG CTGTTC	CGGTCTCTCCGCCT TCTAAA
<i>GORK</i>	AT5G37500	TCAAAGGCTGCTCC ACAGAG	ATCCACGACGTTTC CTTGCT
<i>NAC074</i>	AT4G28530	TGCACGAGTTCCGT CTTGAG	ACTTCAAGCCTTTG CGTCTG
<i>MES18</i>	AT5G58310	GGGCATGGAGGATG GTGTT	GGGAAGTTGGGAG AGGAAGG
<i>CYP96A15</i>	AT1G57750	AATTCTGGTCCGAG AACTTGCT	GGAATTGGTTCGAC CTTGTGAC
unknown	AT5G02640	GTCGTCGTGGTGAG AAGAGG	TCAACGGTAGCAGC ACAAGA
unknown	AT1G20070	TACAGCTCCGGTGA AAGGTG	CAAGACAGACCCA AAGCCCA
<i>GIS3</i>	AT1G68360	CGGAAGAAGTGGC GGTGGTGT	ATTTGCGGCTGCGT TTCGTGTA
<i>AS2</i>	AT1G65620	GCCCATCGAGAGCC TCAAAA	ACAGATGGGATCAA TGGTGTCT
<i>ATGALK2</i>	AT5G14470	AAGTTCACTGGAAG CGGAGG	TCAGAACTACTGAG GCGGGT
<i>TED6</i>	AT1G43790	ACCGTGAGAGATCT GAAGCG	CACGAACGGGAAA CGACTGA
unknown	AT1G52810	TCCAATGGGACTCG GTGAGA	AGAAGCATCGAAA ACCGCCT
<i>MYB16</i>	AT5G15310	ACAACAGCTTGAAT CTCCGACA	AGAGACCCGTGAA CCCTTCT

<i>DELTA-TIP1</i>	AT3G16240	AGTTGCCTTTGGTT CCTTTGATGA	CTAGTCCC GG TGTA TCAAGAGCAG
unknown	AT5G62720	GTCTCCTATTGGCTA AGAAAACAGA	GTTCCGGCGAAGAA GAGGTA
<i>UMAMIT30</i>	AT4G01450	CGTTATAGCGGGAG TGGTTG	AGATCCAATAACGC TTCCA
<i>GLC</i>	AT1G65450	CGTACCAAGAATCT CCACCGT	CGCTGGTTTCTTCA GATGGG
unknown	AT5G22390	GCCGAGAACAGCA GTCTTCA	CTTCGTGGTTCCCA CTCCTC
<i>MYB86</i>	AT5G26660	AGCTTATAGAACCT CCAAGAACCA	GGACAGA ACTCCA ACAGCCA
unknown	AT4G18425	TGGAGATCAAAGTT GACGAAGG	CGAGAACGGTTCCT GTTGGT
<i>RNS3</i>	AT1G26820	ACGTACAAAGTTTC GCCCAAGATT	GCATCCACCGGTTT TG TAGTT
<i>PXG4</i>	AT1G70670	TGCAGAGACATGTC GCTTTTT	GCTGCTGAGACCTA TGTTTATGAA
<i>BCAT4</i>	AT3G19710	AGTGGGAAGAGTT GGCATTCA	CCCTGGCCATACTG AAGAACA
unknown	AT1G54095	TTTGTGGTACGGCG GTGATT	ACCTCGCCGGCAAA TAAAGT
<i>ATFROI</i>	AT1G01590	CGTCACCCGAGAAG AGAAAAC	GCCCGAGGATTGGT GAGATG
unknown	AT1G58170	TGTAACCGGCCAAG ATTCGT	TGTGCCCTTCCCAC CATTTT
unknown	AT1G70000	AGTCTCTGTGTTGT TTTGGTCA	ACGGCAAATTACTG GAAAAGTC
unknown	AT2G37880	ACTCCGACGAATCC AACCAC	GTAGTCGACGGCGG AAGTAG
unknown	AT3G62040	CCCTTTGAGCATCG GAATCA	ACCATAACCTTGA GACCAGC
<i>AGP6</i>	AT5G14380	AATCTCCATCACCC ACTGCC	AGATGATTTAGGTG CGGCGG
unknown	AT4G10290	GCTCCTGAGGTTCC CACTG	TAACCCCGAGTTCA GCAAGC
<i>ATMC9</i>	AT5G04200	GGCAGTCCTTGACC ACTTGA	CCTGACACCCGCTC ATCAAT
unknown	AT1G67870	TGTGGAGTCATCAG GAGGCT	GTCCGGCTGCTCTC AAAGAA

unknown	AT3G01240	GGGAAACCTGAGCT AGAAAAACC	CAACGTTTTACCT CAGATTTCAA
<i>ATGPX7</i>	AT4G31870	ACCCTTCAATGGTT TCGCTG	GCAGCCCTTGCATA GACAC
unknown	AT3G50280	TTGTGTCAAGCTCT CCTCGG	TCACAAGTACGTCTG GGCAAT
<i>ATACAI</i>	AT3G52720	AAAGGGAACCACA CAGCACA	GACATTGACCTCAC CTTGCC
<i>ATWRKY23</i>	AT2G47260	CCATGCACACGAAC CAACAC	TTGGTCAACGCGTA AGTCAGA
unknown	AT1G29720	AGGAGAGCTGTCTG GATGGAA	AACTCGCGGTTTCC TTGACT
<i>LFY</i>	AT5G61850	TGAAGAAGCAACA GCAGCAG	GGATGCTCCCTCTG TCTCTC
<i>KNAT1</i>	AT4G08150	GATCGGGAACCTCAA GAACCA	TTGTAATGCAACTC CCACCA
<i>LBD10</i>	AT2G23660	CTTAAGTGGGGATG GCTTCA	GCGTTGATCAGGTG GAAGAT
<i>LBD27</i>	AT3G47870	GGCAAGGAGATTCT GACCAA	TTCCACTCGCTGAT GATTTG
<i>PNY</i>	AT5G02030	AGTCCTCTCACCGC ACAGAT	CAGCGTTGTCTGTTG TTGTCT
<i>PNF</i>	AT2G27990	CTCAGCAGCAGCAT CAGAAC	AGGGCCTTGTGGGT CTCTAT
<i>SPL2</i>	AT5G43270	TTGGGACTTCCAGT GACTCTGGCTTC	CATCAAACCTCAGAG AGACAGTGGAACC
<i>SPL4</i>	AT1G53160	GAGAAGGATCAGGT CGGAGAGGAAT	CTGTGGTCGCTTGA ATGATGAGTTT
<i>SPL6</i>	AT1G69170	CCACCGTACAAGTA GACTCGTGAG	GAGATTTTGGTTGG GTTGGGTGA
<i>SPL10</i>	AT1G27370	GTGGGAGAATGCTC AGGAGGC	GAGTGTGTTTGATC CCTTGTGAATCC
<i>UBC21</i>	AT5G25760	CTGAGCCGGACAGT CCTCTTAACTG	CGGCGAGGCGTGTA TACATTTGTG
<i>PP2AA3</i>	AT1G13320	TAACGTGGCCAAAA TGATGC	GTTCTCCACAACCG CTTGGT

Appendix 1B. Primers used in cloning

Primer	Gene	Sequence (5' to 3')
pSPL2:SPL2-GFP-5'	<i>SPL2</i>	CACCATGGCATATGCGAAAAAGTCTA
pSPL2:SPL2-GFP-3'	<i>SPL2</i>	GTTATAAACTGGTTCAAGCTGAAGTA

Appendix 1C. Primers used in screening of transformed plants

Primer	Gene	Sequence (5' to 3')
SPL2-5'	<i>SPL2</i>	TTCCGATACCGAGCACAATAG
GFP-3'	<i>GFP</i>	GGTAATGGTTGTCTGGTAAAAGG

Appendix 1D. Primers used in ChIP-qPCR

Gene	Sequence (5' to 3')	
	Forward	Reverse
<i>AS2-I</i>	CTCTCCGTTTCCTTAGCCTC	AGAAAAGAGGAAGGCATAGA
<i>AS2-II</i>	CTCCTTAGAACTCGCAACT	TGAGGCTCTCGATGGGCAAA
<i>AS2-III</i>	CTCCTTCTTCTCTCTTTC	GATATAGAGGAACTTGTAG
<i>EIF4A1</i>	TGTTTTGCTTCGTTTCAAGGA	GCATTTTCCCGATTACAAC

Appendix 2. Recipes of growth media

Medium	Chemicals	Concentration (g/L)
Lysogeny broth (LB) medium	Tryptone	10
	Yeast extract	5
	NaCl	10
Half-strength Murashige and Skoog ($\frac{1}{2}$ MS) medium (pH=5.7)	Sucrose	10
	MS salt ¹	2.165

¹(PhytoTechnology Laboratories, USA)

Appendix 3. Recipes of buffers used in ChIP assay

Buffers	Chemicals	Final concentration
Extraction buffer 1	Sucrose	0.4 M
	Tris-HCl (pH=8)	10 mM
	MgCl ₂	10 mM
	β-ME	5 mM
	PMSF	0.1 mM
	Protease inhibitor ¹	2 tablets/100mL
Extraction buffer 2	Sucrose	0.25 M
	Tris-HCl (pH=8)	10 mM
	MgCl ₂	10 mM
	Triton X-100	1%
	β-ME	5 mM
	PMSF	0.1 mM
	Protease inhibitor ¹	1 tablet/10mL
Extraction buffer 3	Sucrose	1.7 M
	Tris-HCl (pH=8)	10 mM
	MgCl ₂	2 mM
	Triton X-100	0.15%
	β-ME	5 mM
	PMSF	0.1 mM
	Protease inhibitor ¹	1 tablet/10mL
Nuclei lysis buffer	Tris-HCl (pH=8)	50 mM
	EDTA	10 mM
	SDS	1%
	Protease inhibitor ¹	1 tablet/10mL
ChIP dilution buffer	Triton X-100	1.1%
	EDTA	1.2 mM
	Tris-HCl (pH=8)	16.7 mM

	NaCl	167 mM
Elution buffer	SDS	1%
	NaHCO ₃	0.1M
Low salt wash buffer	SDS	0.1%
	Triton X-100	1%
	EDTA	2 mM
	Tris-HCl (pH=8)	20 mM
	NaCl	150 mM
High salt wash buffer	SDS	0.1%
	Triton X-100	1%
	EDTA	2 mM
	Tris-HCl (pH=8)	20 mM
	NaCl	500 mM
LiCl wash buffer	LiCl	0.25 M
	IGEPAL-CA630	1%
	Deoxycholic acid	1%
	EDTA	1 mM
	Tris-HCl (pH=8)	10 mM
TE buffer	EDTA	1 mM
	Tris-HCl (pH=8)	10 mM

¹(Sigma-Aldrich, Canada)

Appendix 4. List of differentially expressed genes tested by NG RNA-Seq in the siliques of *35S:miR156* compared to WT^{a, b}

More than 2-fold up-regulated genes in <i>35S:miR156</i> compared to WT	AT1G52040	AT2G39330	AT5G26000
	AT5G44120	AT1G52400	AT4G30270
	AT1G62070	AT1G74020	AT1G52000
	AT4G23600	AT4G30975	AT3G15450
	AT5G24780	AT3G62820	AT4G28520
	AT5G50950	AT2G05520	AT1G20620
	AT3G16470	AT1G19670	AT3G57520
	AT4G35770	AT2G45180	AT3G57260
	AT1G62000	AT1G52410	AT4G12870
	AT4G27150	AT4G27160	AT1G55020
	AT2G01450	AT1G62225	AT2G36710
	AT5G45770	AT2G44500	AT5G35935
	AT3G52500	AT2G38540	AT1G24070
	AT4G00120	AT2G05540	AT3G63210
	AT4G30280	AT2G16660	AT2G43530
	AT5G57800	AT1G62080	AT1G72180
	AT3G57510	AT1G03880	AT4G17220
	AT4G27170	AT4G36850	AT1G62500
	AT1G02720	AT4G15110	AT3G28220
	AT5G18840	AT2G47750	AT2G34810
	AT3G52840	AT3G04910	AT3G62570
	AT4G27860	AT4G16590	AT1G52030
	AT4G27140	AT1G23760	AT4G17470
	AT1G43590	AT5G49520	AT5G05020
	AT1G72140	AT1G59870	AT3G04720
	AT3G22640	AT1G52700	AT5G06870
	AT4G19430	AT3G44860	AT1G75040
	AT3G23410	AT3G24480	AT1G62220
	AT1G54020	AT1G58270	AT5G47140
	AT1G44350	AT1G73480	AT1G66760
	AT4G13340	AT4G26690	AT2G19800
	AT2G15970	AT2G33060	AT3G03480
	AT3G55130	AT3G45060	AT5G66920
	AT1G15040	AT5G11920	AT1G56650
	AT1G22500	AT5G05340	AT5G50335
	AT1G73325	AT1G03010	AT5G54740
	AT2G43570	AT3G16360	AT2G36120
	AT1G21890	AT1G74430	AT1G72120
	AT5G39440	AT2G47780	AT4G16870
	AT3G13900	AT1G22380	AT2G25640

AT1G72520	AT2G07680	AT1G01070
AT2G36090	AT5G24160	AT4G14130
AT4G02520	AT4G19530	AT1G76960
AT1G52440	AT1G09080	AT5G66150
AT3G48290	AT2G47000	AT4G39950
AT1G28130	AT2G38890	AT4G03820
AT2G28490	AT2G38470	AT2G04032
AT1G14390	AT5G06839	AT3G02470
AT5G62280	AT2G01860	AT4G23300
AT1G05810	AT1G12150	AT4G24275
AT4G25835	AT2G38790	AT4G26740
AT4G02780	AT1G49200	AT2G27380
AT2G41480	AT5G59660	AT4G25390
AT3G22410	AT1G35140	AT5G46040
AT4G23560	AT5G26810	AT5G39520
AT5G24920	AT4G22753	AT4G28040
AT3G44990	AT5G18360	AT1G35230
AT1G66180	AT2G16980	AT4G26050
AT3G21520	AT1G63210	AT3G60890
AT3G27730	AT2G30310	AT5G46730
AT5G45890	AT3G44260	AT1G32560
AT1G80970	AT3G04200	AT5G06865
AT5G60830	AT1G27710	AT4G21640
AT2G45900	AT3G09930	AT4G18990
AT2G35670		

More than 2-fold down-regulated
genes in *35S:miR156* compared to WT

AT5G37600*	AT3G01500	AT1G64370
AT3G59845	AT1G67865*	AT2G37170*
AT5G38420	<u>AT1G67870*</u>	AT4G33790*
AT1G37130*	AT5G10770*	<u>AT3G19710*</u>
AT5G56540*	AT4G26530*	AT2G47880*
AT3G62410*	AT2G18328*	<u>AT2G36830*</u>
AT4G24780*	<u>AT1G20070*</u>	<u>AT3G52720*</u>
AT4G04020*	AT1G64360*	AT4G00780*
AT1G64770*	AT5G14740*	<u>AT5G04200*</u>
AT1G04250*	AT3G01345	AT2G40100*
AT4G30450*	AT5G37300*	<u>AT1G70670*</u>
AT2G30766	<u>AT5G48850*</u>	AT4G30460*
AT1G13650*	AT3G24460*	AT1G76680*
AT3G56290*	AT3G21550*	<u>AT5G22580*</u>
AT2G27420*	<u>AT1G57750*</u>	AT2G32160*
<u>AT3G16240*</u>	AT1G67860	AT5G23750*
AT2G15020*	AT1G66725	AT1G07440*
<u>AT3G62960*</u>	AT5G63850*	<u>AT3G48340*</u>

AT2G03200 AT4G18550* AT4G00880*
 AT3G26280* AT1G75620* AT1G19050*
 AT1G56600* AT5G53200* AT5G16570*
AT4G18425* AT5G16600* AT5G17450*
 AT3G61210* AT1G11190 AT1G14280*
 AT1G76520* AT5G21430 AT3G50560*
AT1G26820* AT4G39710* AT1G47400*
 AT5G62920 AT1G14150 AT5G58310*
 AT5G42250* AT4G18640* AT4G10290*
 AT4G23680* AT5G13320* AT1G20850*
 AT3G24420* AT3G22840* AT2G29310*
 AT1G33170* AT1G70680* AT4G08150*
 AT2G29490 AT1G72930 AT5G56860*
 AT3G26960* AT3G21460* AT5G60490*
 AT2G30770* AT3G62040* AT3G49580*
 AT5G65380* AT2G29320 AT5G02020
 AT1G05680* AT1G54790 AT1G47395*
 AT5G47350* AT1G67195 AT1G61093*
 AT2G37750* AT4G00050* AT5G55132*
 AT2G10940* AT1G02790* AT4G31870*
 AT2G14247* AT5G52780 AT1G30260*
 AT5G52570 AT4G01450* AT1G56430*
 AT3G62950* AT3G49570* AT1G33110
 AT2G17280* AT5G22390* AT5G39260
 AT3G30122 AT4G00870 AT2G21465*
AT1G65450* AT3G22240 AT5G51190*
 AT3G01270 AT4G28530* AT3G05640*
AT1G23090* AT5G26660* AT4G18280*
 AT4G29110* AT3G50280* AT1G70000*
 AT1G02820* AT5G45820* AT3G06145*
 AT1G12670 AT5G46950 AT4G16000*
 AT1G10682 AT5G25840* AT4G32100
 AT2G14095* AT5G60910* AT2G05910*
 AT2G26695* AT1G14455 AT1G53160*
 AT1G73640 AT5G16350* AT4G35350*
 AT1G73010* AT1G43790* AT5G66400*
 AT2G44460* AT1G79420* AT2G16630*
 AT4G34930 AT5G59670* AT2G30810*
 AT1G02630* AT5G42210* AT4G16008
AT1G58170* AT1G45191 AT5G02640*
AT1G80120* AT2G40080 AT1G31250*
 AT1G56415* AT3G01240* AT3G29670*
 AT5G45280 AT5G37500* AT3G14170
 AT5G24155* AT4G14560* AT2G30540*

AT3G18950* AT5G62720* AT4G08160*
 AT2G03710* AT4G11211* AT5G40020*
AT1G65620* AT3G12700* AT1G17030
 AT1G58370* AT3G57020 AT3G61750*
AT5G15310* AT3G22231 AT3G20460*
 AT4G19370* AT4G15735 AT1G29720*
 AT2G36590* AT3G24510* AT5G56840*
 AT2G43920* AT2G04925* AT5G01610*
 AT4G33450* AT1G78950* AT3G62930
 AT1G63055 AT3G27025* AT2G14935*
AT1G68360* AT5G35480 AT1G70800*
 AT5G24105* AT5G58580* AT3G17050
 AT1G68825* AT3G16920* AT3G14380*
 AT5G04890 AT2G02498* AT5G60660
 AT4G36410* AT3G59900* AT3G50770*
 AT4G13800* AT1G54200 AT5G67340
AT1G52810* AT1G61930* AT5G46960
AT5G14470* AT2G47260* AT1G68200
 AT1G13260 AT2G41690* AT4G26255
 AT3G07820* AT3G18773 AT2G41610
AT1G54095* AT1G64380 AT3G50020*
 AT3G28750 AT2G36724* AT5G45880
 AT2G20562* AT3G57690* AT5G04180
 AT2G44300* AT3G25620 AT1G48745*
 AT3G49540 AT3G22540* AT1G02220
 AT1G61667 AT3G22120 AT5G56320
AT2G37880* AT3G21870 AT3G05650
 AT1G61566* AT5G16340 AT2G47030
 AT3G23165 AT2G19500* AT1G14420
 AT1G10770 AT5G03210* AT5G54610
 AT4G33985 AT3G51171* AT1G01590*
 AT5G39693 AT4G03945 AT1G07473
 AT5G45070 AT5G51310 AT1G80520
 AT1G07450* AT3G01230* AT5G54095*
 AT3G19270* AT1G61563* AT5G11410
 AT4G26260 AT2G44260 AT3G48950
 AT1G01250 AT4G33905 AT1G24520
 AT2G31945* AT1G24580 AT4G03290
 AT4G39917 AT4G18596 AT2G04034
AT5G14380* AT3G54800

^aGenes have at least one GTAC in the 2kb upstream of the translation start codon are indicated by asterisk

^bGenes have at least one CNGTACM consensus in the 2kb upstream of the translation start codon are underlined

Appendix 5. Genomic sequence of AS2 gene and 2 kb upstream from the translation start codon. Red letters (upper case) represent *AS2* coding sequence.

Black letters (lower case) represent non-coding sequence. Green letters represent GTAC core sequence. The fragments that are amplified by qPCR are underlined.

ctctctctatctctataatcgcacacacataaaagtgttcttctctccggttctcttagcctctgcatctggcgattttctct
ttctatctccctacaccttagggagtactga tcctaaactcatcatctttctatgcttctctctttctcttctacttatttctttt
ctatctttttctcttcttactttgctgtaaaaatagcgagtcataaaataagacactgcatgatcctaataaataatattatattg
taattagagaaaagagagagaaagaaagaaagtggcagtagatcttta tggat tttgg tataagaa acatg aaaaa atgggt
ttgaccgaagaaactttgaggcggtaacttcatta tcaac ttttc gactgt tattat atatt ttagcttt gtcaa accgt atcata
taataattatgcgaattactagtagagagta taata atagt ataaa caaaa tgctt ggtta ctgt atgta aatcc taga caaaa
gaataagaataa aaagag caaaat cttcat cccaag tccat caggagag tcagag tcag acc catct ctcat ttccc atctc
cttttcacatttatttctttttttctttctaga tactt tttta tttg aaatt taata ctaa taact ataca tttat tg ttat
tattg ttg ttag gtct atgc taggg tcca agggt catcc caata tcct tctc ctct tagaa actcg caact tctc acatg
aatcaat ttccc tctag tacc aaatc ctatt actat tga agg tata ctcta tag ttat ttat tttag taagtata tata taaaa
taataa catta attag tact agtct tatca tattt attag ctcat tttct tataa aatac taata ataat aggaa ttta agcaca
agatgagctttgccc atcgag gcct caaaa cccaa aagtc tctc ttctt ca gagat tcca atc ttt g cttt tag ta attaa
tacatct tctc cttct cttc tctct ttctc ttccc tctct catac ctacc tata agca acaca aga ac ccat tgat ccatc
gtttgt tcct atatt tcgt taatc cat ccat acg catt cca act ac cg gtaca aat ttc cat ctct ttta ttgt ttta
aatgatt ttaaa tctct cagg ttgtc tctag actct taggt ctagt tacca aaatt tgact agca atcata tagc taca ag ttcc
ctatat cata tag ctac acata atctc taggt ctgaa atgat ctttt atctc caag catat atta tatat ttgga tgca ac ctt
atg ttg act ttcg aaa agca cctt cat gtt act ca tttt tctc tatt g cttt ca ac ag ttg aca aga aggt tgga cag tat
atata ag tata catata cact tatgt gtgtg tgtag tgat gtgca tatat ag tc ac caaa at ctt ta act actaa ttg caa
ag ttct cttat tttac agct ttt gt atg ca acc caaa at ag cag ctta agaa gcag ag gag gag at ctt cttt att cc ctc
gag ca ac aga ag ccatt at tca aga aag gc ttctt taatt tact cg cagg taaa ag cttt ctta ttcta tag ag att tga aca act
tttttt tttt taatt gaca aca ataa aga ttgag caact tctct acatt attc ttct ttat tttct cact taatt cat att
tgatt caaaaa cagata atg taata tctc aaaat cacaa ttgat ttca ata at ctttt ttg ttcc ttata ctt ct tac ctc
caaaa ac taaa tcaaa aacc cttat ttctc tcaat tttca atgg cg gctt tgtgt ag gga gag g gaa aaa gagaa aaaat tgaaa
actccat ttca agtca ttaaa **ATGGCATCTCTTCAACAACTCAACATGCGCCGCTTGCAAATTCCTCGGCGAAAATGTCAAC**
CGGAATGTGTATTCGCGCCCTATTTCCACCGGACCAGCCACAAAAATTCGCAAACGTTCACAAAGTGTGGAGCAAGTAACGTG
ACAAAGCTCCTCAACGAGCTTCACTTCCACAACTGAAGAAGCAGTGAACCTTTGGCCTATGAAGCCGACATGCGCCTCCGTGA
CCCTGCTACGGCTGCGTCGGCGTCACTCTCTCCTCCAACATCAGCTTCTGTCAGCTTCCAGATAGATCTCAGCTGTGCTAAATCTG
AGCTCTCTAAGTACCAAAGCCTCGGTATCCTCGCCGCACTCATCAGAGTCTTGGCATCAACTTCTGCGCGGAGCAGCAGATGGA
ACAGCCACCGCCGTGAGAGACACTATCACCACCACAGTTTTTCTAGAGAACAATGTTGGTGGCTTGGATGTTCCGGCCGG
TAACAACACGACGGTGGGATCTTTGCCATTGGACAGATCACTCAGTTTCAGCAGCAGAGAGCCGCGCTGGAGATGATGTCGCC
GTACTGTTGATCGTCTTGA

Appendix 6. The non-coding region of EIF4A1 gene. Black letters (lower case)

represent EIF4A1 noncoding sequence. The fragments that are amplified by qPCR are underlined.

aattctaccataaaccgatataaaagtatttaaacccgactaattaagcttcacaaatctcggtttgtaactttattata
 ttgggcccattattattctctgagcttttgogtctcaccaaaagacagagatcatcaggtgogtttagtgattaogt
 aggcattctaatgaaocggcagggatttagtcaaaacttattaatgggcccatactttggcccacogttttccctcgattc
 ctgtcacacaaaaaacctctagctcttctctctacctacacaagctaaatacatatTTTTTgcttatacctaagcatca
 tgattatgTTTTgcccctctccagctTTTTcttcaatggcaacagattcctaagaaagtctcttgaggctaaaaatcaaa
 gcattTTTTgttgaagatagatagcaacttgctgctctctcatactagctagttaccttctctcttactcaatgtggt
ttgcttcogtttcaaggattcttcatatcacttggtggaacaattacatgattaaacattcaacatagagagatagatg
tgtaataagtaaagacattttcagataaaaogtctctatcagtcactttattcttctaatacctcgttghtaatog
 ggaaaatgctttgtaacgtcaaaaagcaataaaagtgcaggagaaagaaagctttggaaaagaaaaataaataaat
 gaagaatattttctttgctagtcacaaaaataatgaagaatttatgctcctaattcccactagatatttgtttatt
 tattttgcaaaaatcaagttaagacaatgagctaagtgttggaacacctgtccgagccaaaagagtaaaaagaaa
 gggaaataaaggggtaaaaccggaaaatccgaaaaagaaaaggagaagatttccaaaggagaaaaccctaaagacggag
 tataaaacaaggtaacoggtttctctcagcctctttoggatattccaccagctctctcgcaatctctcgtctctctc
 tttgctctctctctcaacogcggttcagatccgagtttgggagattcaagctccctgaaaaagccctttaactctc
 gcttaccaactttgctccaggttcgcacctatcoggattgtctatctattatctcgatgtaacttttaogattcatg
 attgtttctttaacttttgattgttctgattgtttttttgtggtttccgatagatttggtttcaactttgttgagt
 tgatttgaagttttgattttctgtaacttcaagatcgtttttttgctgtggaagtgtcttctgttttgtctctt
 tattagattctgagctgctgatgatgacgattataaatttaggatctacacatctgtagatttgttgatgggtttg
 tagattttgttaocgctagggttagtctctcaatagaatgaaatgaaatcgtcttcaagctcagatgtctctgtotta
 aactcacttggtttttgtctgattgaaatgttttttttgtaatttgagagaaataggttgagtggtgtctatgtttaa
 tagataaccatattgtgcctttatacattttctccgcacatcatctgagggcagatattaatgcttgttttctctctt
 tatggacttogagtttggtttcttgtcacatttctcctttgcaatcatattattaacgatataaaaataggtggt
 gtctctcttcttttgatatgaaatctgattgtttttgtctttttatgttagc

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(2015). "Ectopic expression of miR156 represses nodulation and causes morphological and developmental changes in *Lotus japonicus*." *Mol Genet Genomics* 290: 471-484.

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Poster presentation:

Wang, Z., Wang Y., Kohalmi SE., Amyot L, Hannoufa A. (2015). "The miR156/SPL network determines floral organ development and plant fertility by regulating AS2 gene." *The Canadian Society of Plant Biologists*, Edmonton, Alberta, Canada.