12-3-2015 12:00 AM

Functional Analysis of the miR156 Regulatory Network in Arabidopsis Siliques

Zhishuo Wang, *The University of Western Ontario*

Supervisor: Abdelali Hannoufa, *The University of Western Ontario*
Joint Supervisor: Susanne Kohalmi, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

Functional Analysis of the miR156 Regulatory Network in Arabidopsis Siliques

Zhishuo Wang

Supervisor
Abdelali Hannoufa
The University of Western Ontario

Graduate Program in Biology

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

Functional Analysis of the miR156 Regulatory Network in Arabidopsis 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

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

Acknowledgements

I would like to thank my supervisor, Dr. Abdelali Hannoufa, for giving me the opportunity to conduct this research. I appreciate his patience, advice and encouragement from the beginning of experiments to the thesis writing.

I would like to acknowledge the suggestions and support provided by my co-supervisor, Dr. Susanne Kohalmi during my research. Also, I would like to thank my advisory committee members, Dr. Denis Maxwell and Dr. Mark Gijzen, for their kind guidance and help.

Special thanks to my lab member and friend, Ying Wang, and our technician, Lisa Amyot, for their suggestions and technical support. I would like to extend my thanks to my colleagues: Banyar Aung, Muhammad Arshad, Omari Khaled, Ruimin Gao and Sneha Challa.

I appreciate the help from the staff of the University of Western Ontario and Agriculture and Agri-Food Canada.

My final acknowledgement is to my parents, who always give me continuous support during my time in Canada. Without their encouragement, I would never have gotten to where I am today.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

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

A. tumefaciens	*Agrobacterium tumefaciens*
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
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
GFP	Green fluorescent protein
LB	Luria broth
L*er*	Landsberg *erecta*
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-(hydroxylmethyl)-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* (*AGB1*) 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).

1.2 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).

1.3 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).

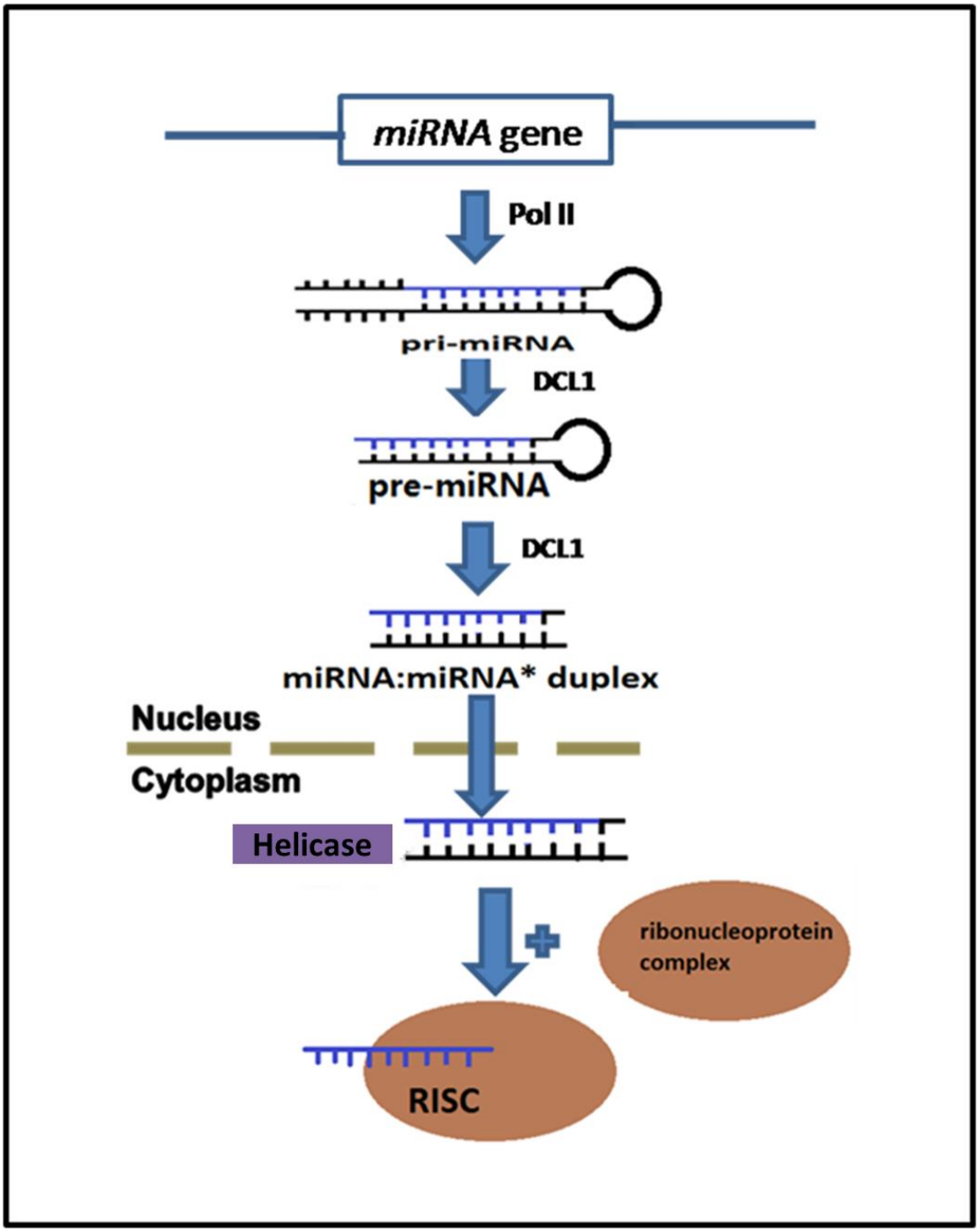
1.4 Plant miRNAs

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.


miRNA gene
Pol II
pri-miRNA
DCL1
pre-miRNA
DCL1
miRNA:miRNA* duplex
Nucleus
Cytoplasm
Helicase
ribonucleoprotein complex
RISC

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).

1.5 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* (*AP1*) 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).

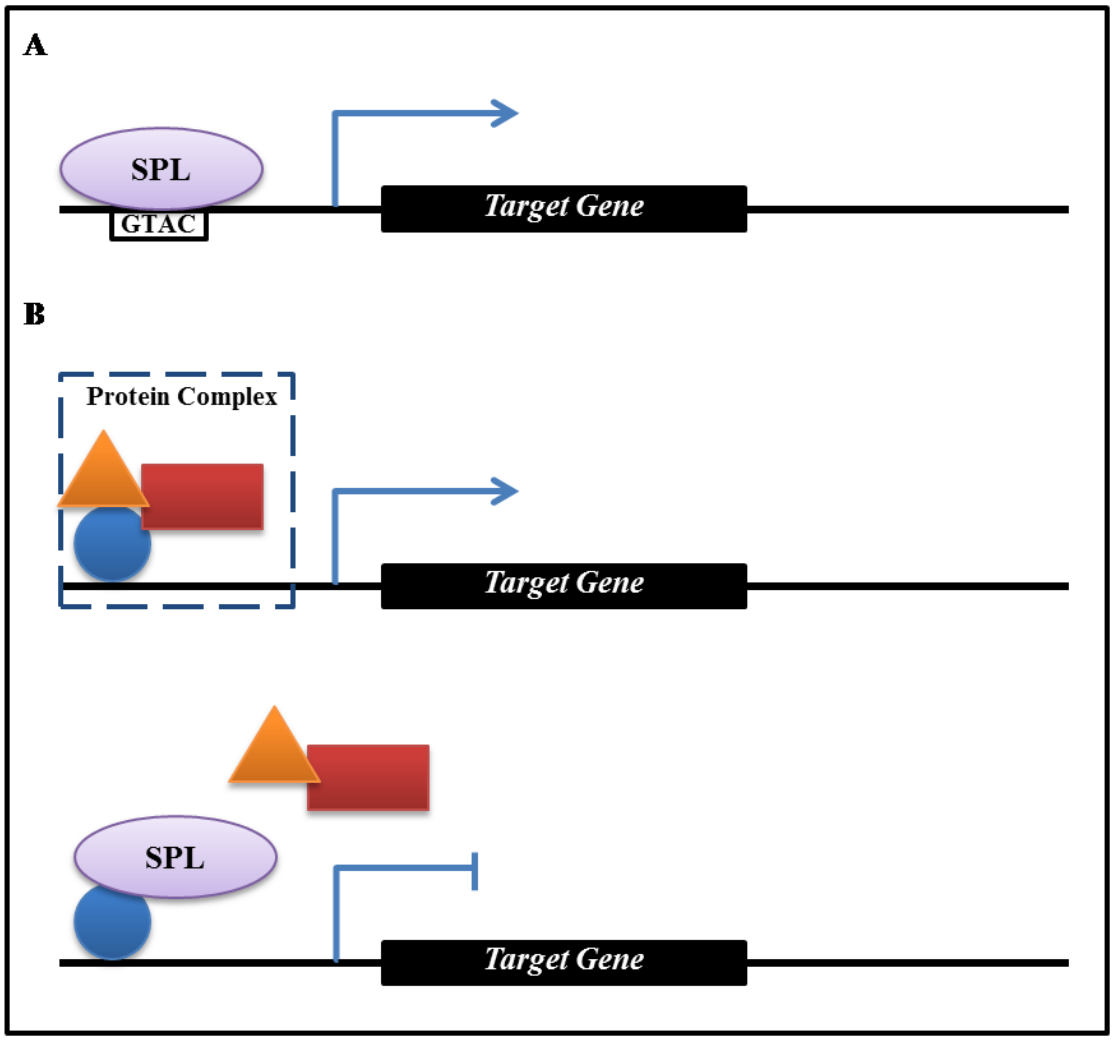
1.6 SPL Transcription Factor Family

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 (Lännenpää 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 *AP1*, 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.


A
SPL
GTAC
Target Gene
B
Protein Complex
Target Gene
SPL
Target Gene

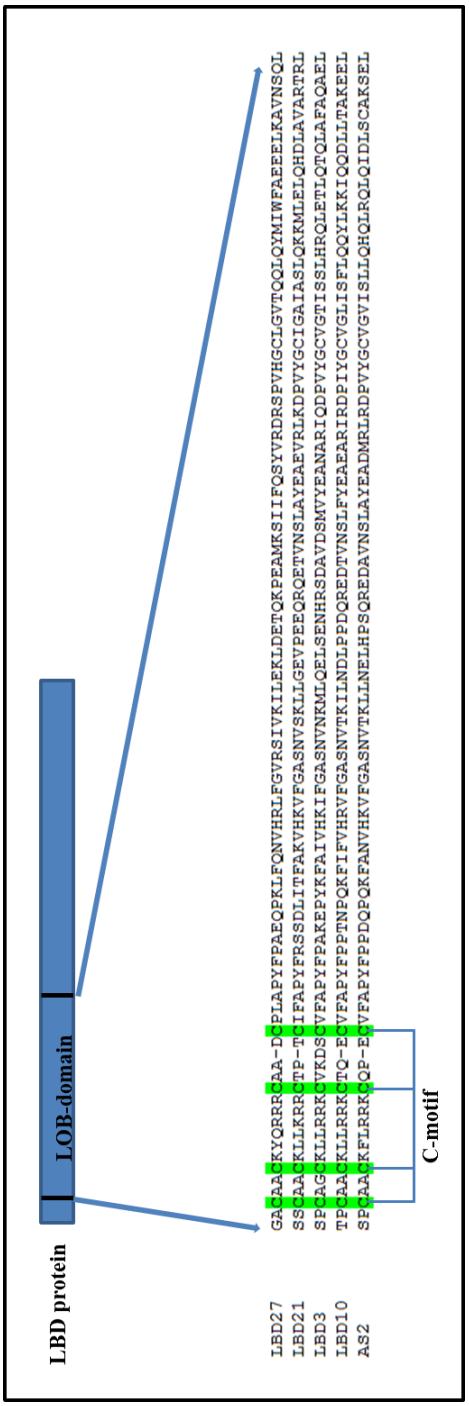
anthocyanin biosynthesis (Gou et al., 2011).

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.

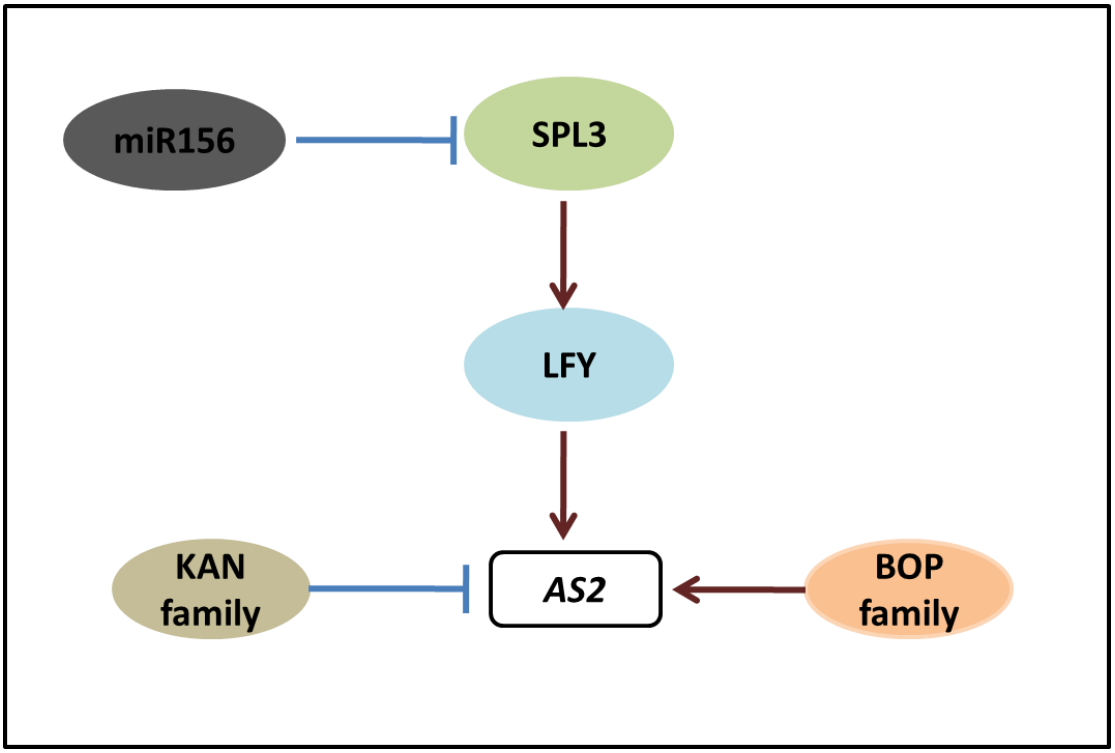

LBD protein
LOB-domain
C-motif
LBD27
LBD21
LBD3
LBD10
AS2

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.


miR156
SPL3
LFY
KAN family
AS2
BOP family

(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* (L*er*) 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 L*er* 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 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. 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 µL 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 TUBRO DNA-free Kit (Ambion, USA). Briefly, 5 µL of TURBO DNase Buffer and 1 µL of TURBO DNase were added to the RNA, and the mixture was incubated at 37°C for 30 min. Six µL 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^{TM} cDNA SuperMix Kit (Quanta, USA), and 1 µg 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 µg 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 µL of cDNA Synthesis Mix (2 µL RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M Dithiothreitol (DTT), 1 µL RNaseOUT and 1 µL 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 extract 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 H_2O 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 H_2O. Quantitative real-time PCR (qRT-PCR) was performed on a C1000 Thermal Cyclerand CFX96 Real-Time System (Bio-Rad,

Canada) with PerfeCTa® 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 PerfeCTa® 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 OD_{600} 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 $CaCl_2$ and left on ice for 30 min, and then centrifuged at 4,000 *g* for 10 min at 4°C. The washing procedure by $CaCl_2$ was repeated twice, and finally the pellet was resuspended in 2 mL of chilled 0.1 M $CaCl_2$ 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 OD_{600} 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 H_2O 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 H_2O 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 µg/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. tumefcaiens* cells were collected by centrifugation at 4,000 *g* for 10 min, and the supernatant was discarded. The *A. tumefcaiens* 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. tumefcaiens* 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 (½MS) 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 ½MS 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 H_2O, 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 H_2O 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 (Appendix3) 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 H_2O. 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$_T$a® 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 AMINOT—RANSFERASE4 (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 ± 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.

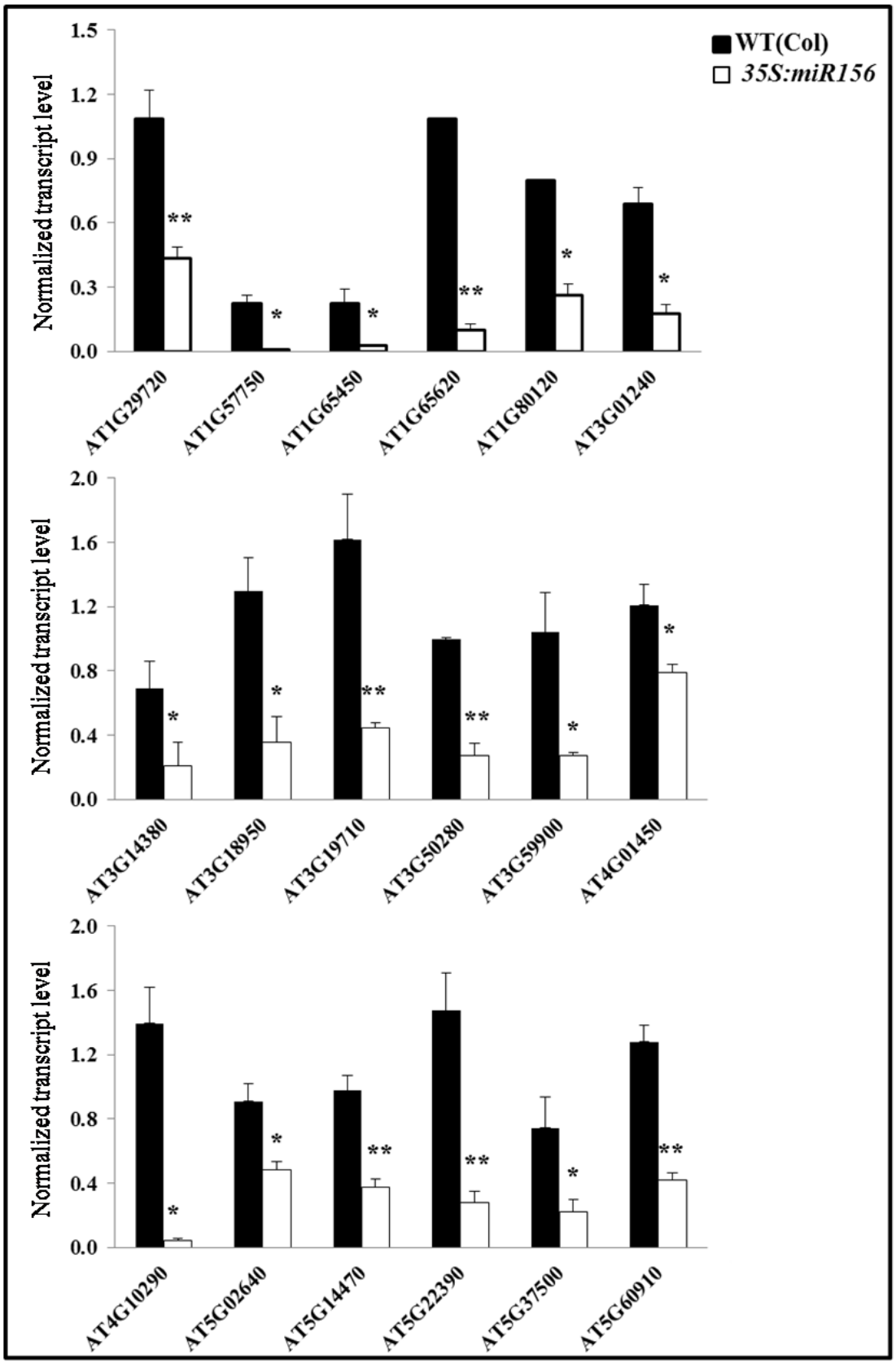

WT(Col)
35S:miR156
Normalized transcript level
1.5
1.2
0.9
0.6
0.3
0.0
AT1G29720
AT1G57750
AT1G65450
AT1G65620
AT1G80120
AT3G01240
2.0
1.6
1.2
0.8
0.4
0.0
AT3G14380
AT3G18950
AT3G19710
AT3G50280
AT3G59900
AT4G01450
AT4G10290
AT5G02640
AT5G14470
AT5G22390
AT5G37500
AT5G60910

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	*CYP96A15*	8	Encodes a CYP96A15 protein, involved in cuticular wax biosynthesis.
AT1G65450	*GLC*	6	Involved in double fertilization
AT1G65620	*AS2*	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	*BCAT4*	6	Belongs to the branched-chain amino acid aminotransferase gene family. Involved in the biosynthesis of methionine-derived glucosinolates.
AT3G50280		4	Unknown
AT3G59900	*ARGOS*	10	Inducible by auxin, involved in lateral organ size control

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

[a]The 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**.

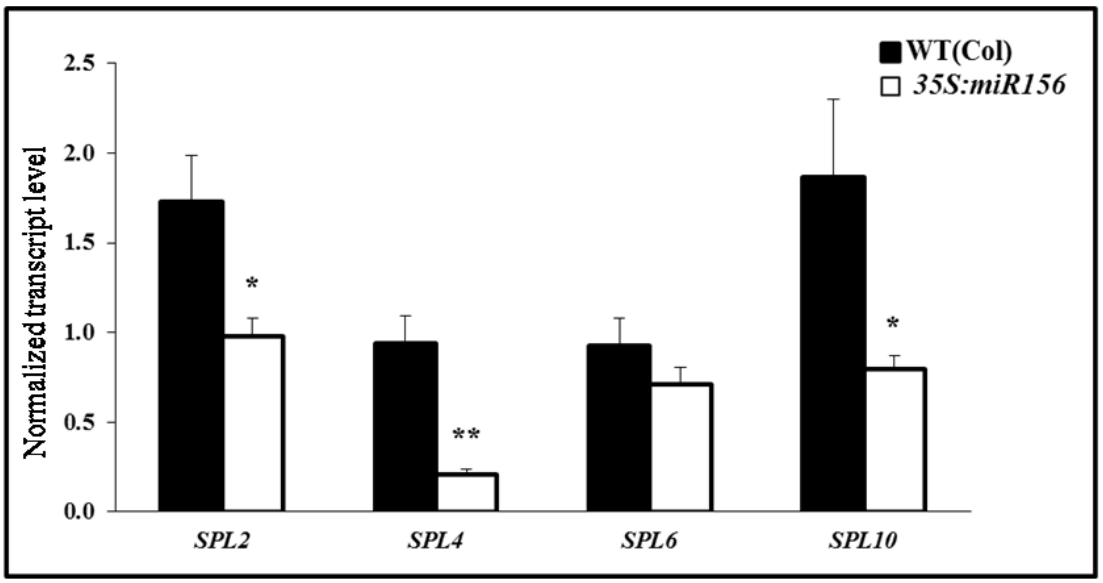
3.3 Regulation of Candidate Genes by SPLs

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 ± 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.


WT(Col)
35S:miR156
Normalized transcript level
2.5
2.0
1.5
1.0
0.5
0.0
*
**
*
SPL2
SPL4
SPL6
SPL10

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 ± 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.

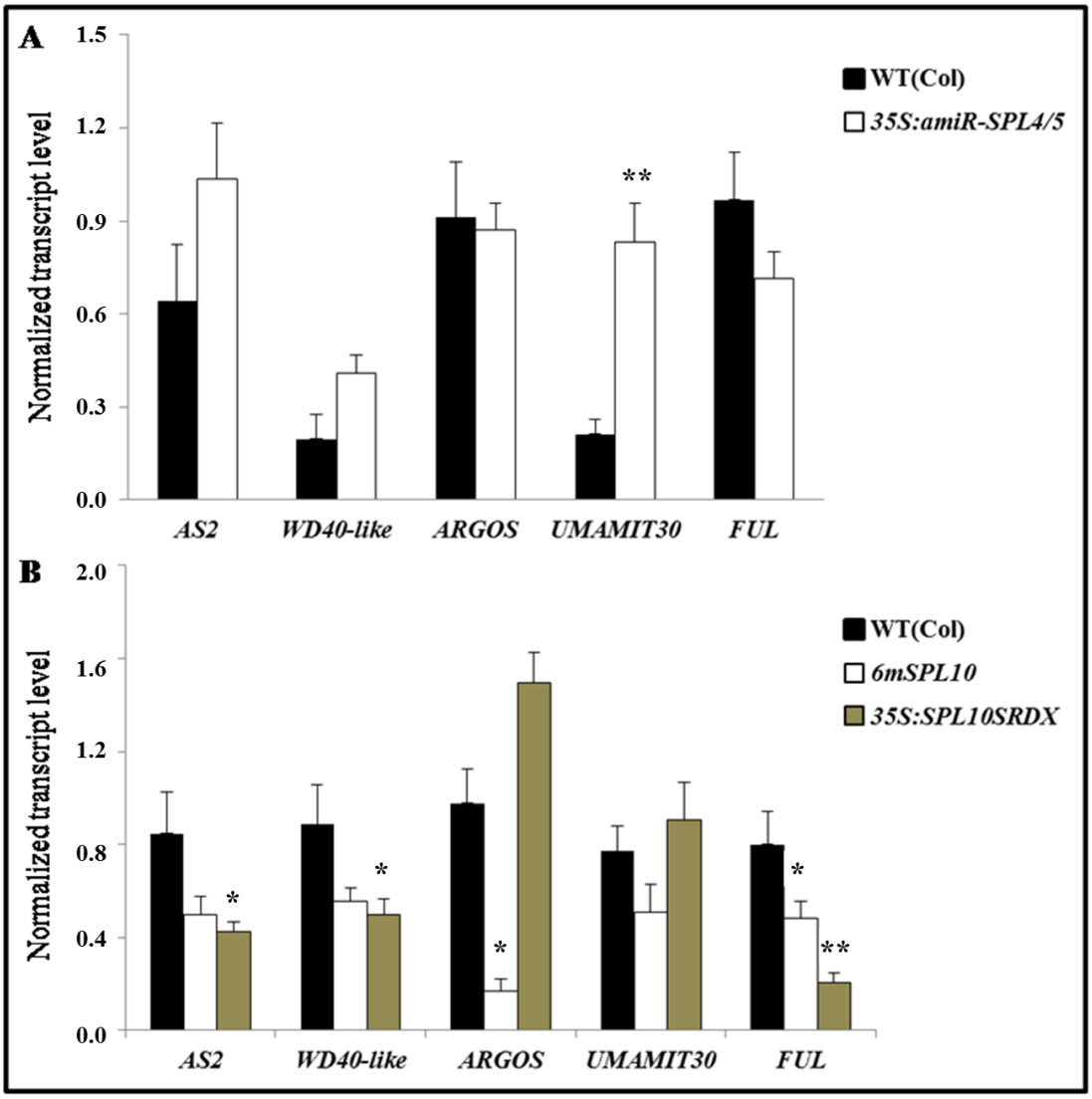
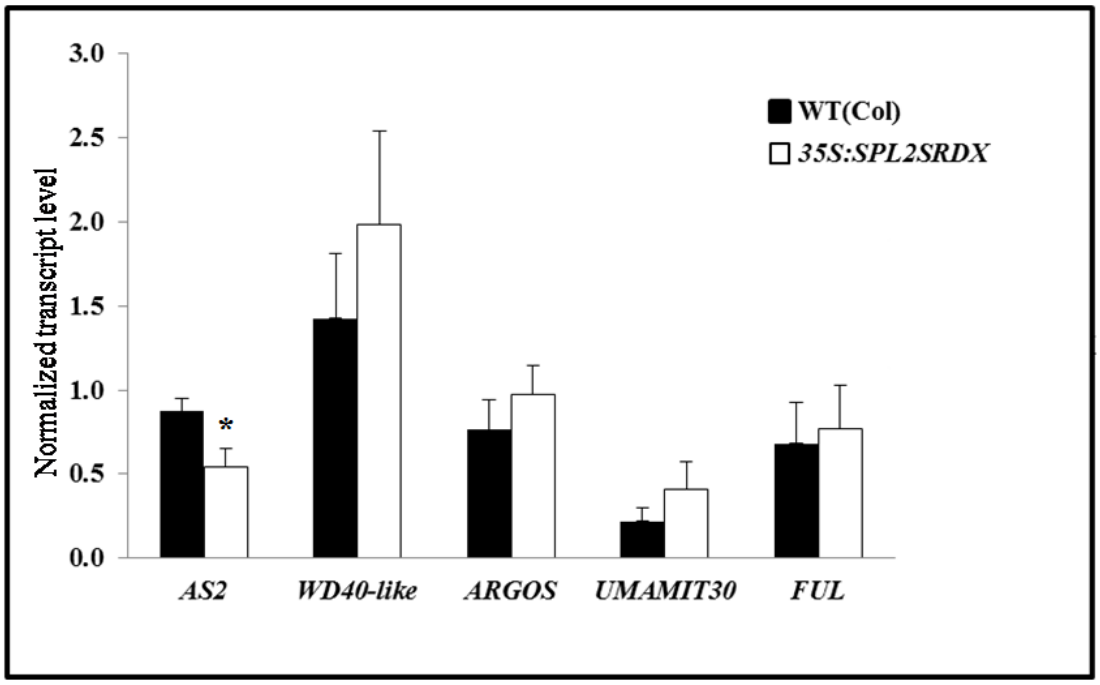

A
Normalized transcript level
1.5
1.2
0.9
0.6
0.3
0.0
WT(Col)
35S:amiR-SPL4/5
**
AS2
WD40-like
ARGOS
UMAMIT30
FUL
B
Normalized transcript level
2.0
1.6
1.2
0.8
0.4
0.0
WT(Col)
6mSPL10
35S:SPL10SRDX
*
*
*
**
AS2
WD40-like
ARGOS
UMAMIT30
FUL

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 ± 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.

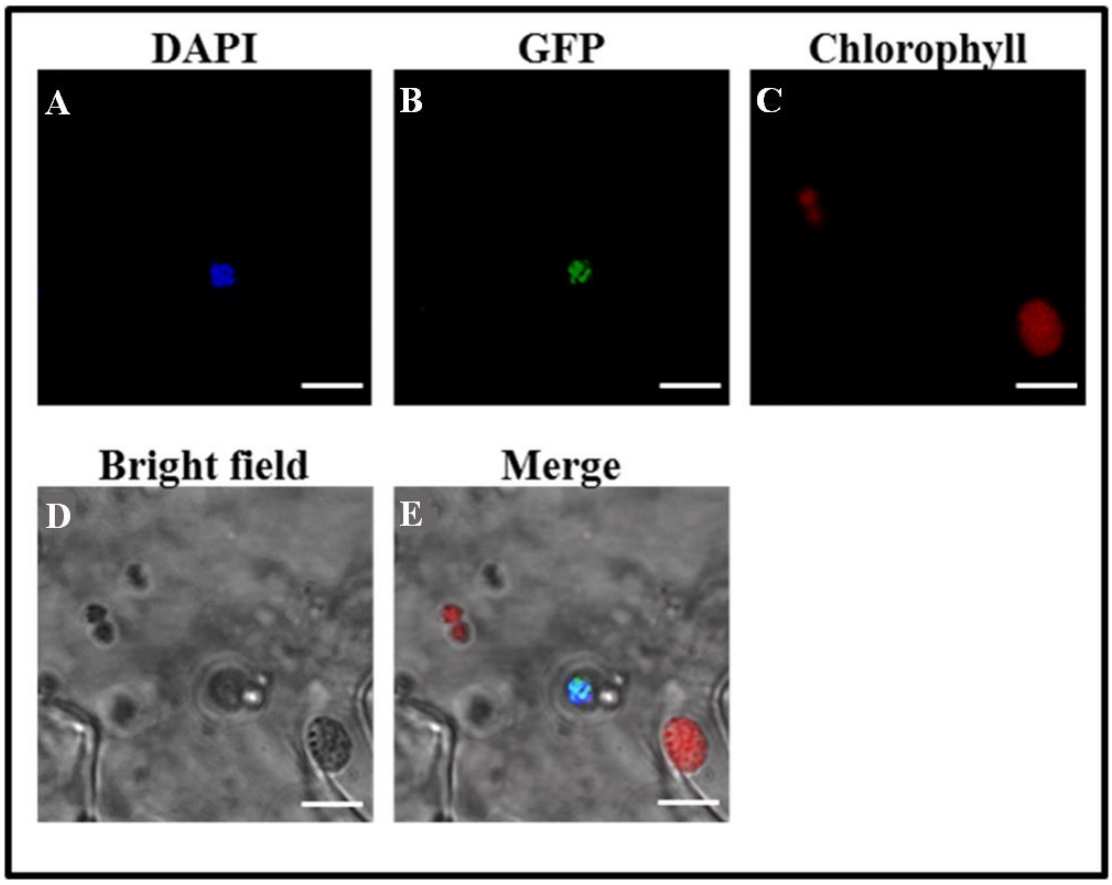

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Normalized transcript level
WT(Col)
35S:SPL2SRDX
*
AS2
WD40-like
ARGOS
UMAMIT30
FUL

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.


DAPI
GFP
Chlorophyll
A
B
C
Bright field
Merge
D
E

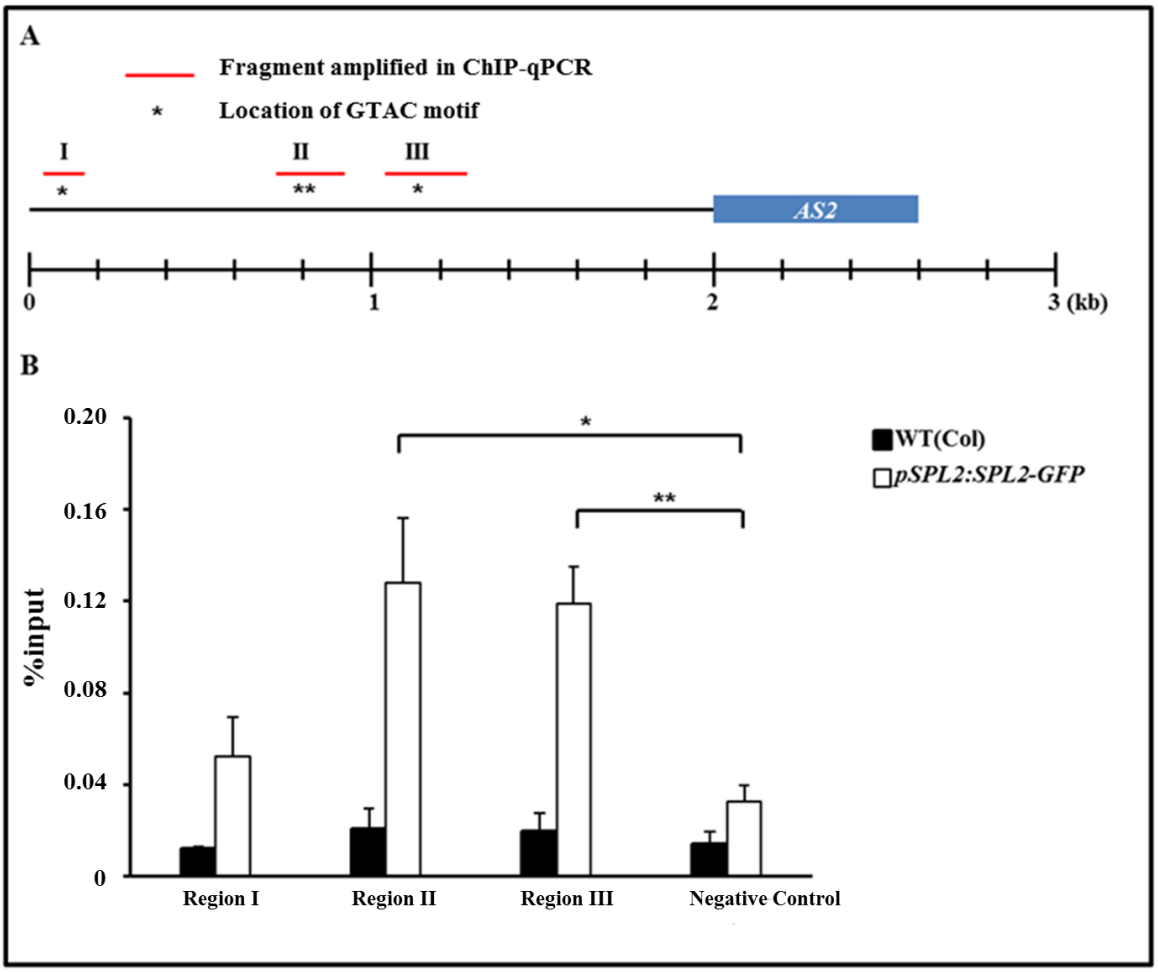
contain GTAC boxes, and region II covered two GTAC boxes (Figure 10A; Appendix 5). Fragments that flanked these putative binding regions were amplified and a region in the *EIF4A1* promoter without a GTAC box was amplified as a negative control (Appendix 6).

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 L*er* background. Thus, the Col and L*er* 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.


A
Fragment amplified in ChIP-qPCR
Location of GTAC motif
I
II
III
*
**
*
AS2
0
1
2
3 (kb)
B
0.20
0.16
0.12
0.08
0.04
0
%input
*
**
WT(Col)
pSPL2:SPL2-GFP
Region I
Region II
Region III
Negative Control

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(L*er*) 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(L*er*), while in *as2-101* the petal length was 1.697±0.095 mm. The length of *as2-101* sepals was only 65% of WT(L*er*). Moreover, shorter siliques were also observed in *35S:miR156*, *35S:SPL2SRDX*, and *as2-101* mutants (Figure 11C, Table 2). In WT(Col) and WT(L*er*), siliques were 14.76±0.62 mm and 12.62±0.87 mm in length, respectively. The siliques of *35S:miR156* and *35S:SPL2SRDX,* as well as of *as2-101,* were 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 L*er*) plants. (A) petals; (B) sepals; (C) siliques. Scale bar = 1 mm.

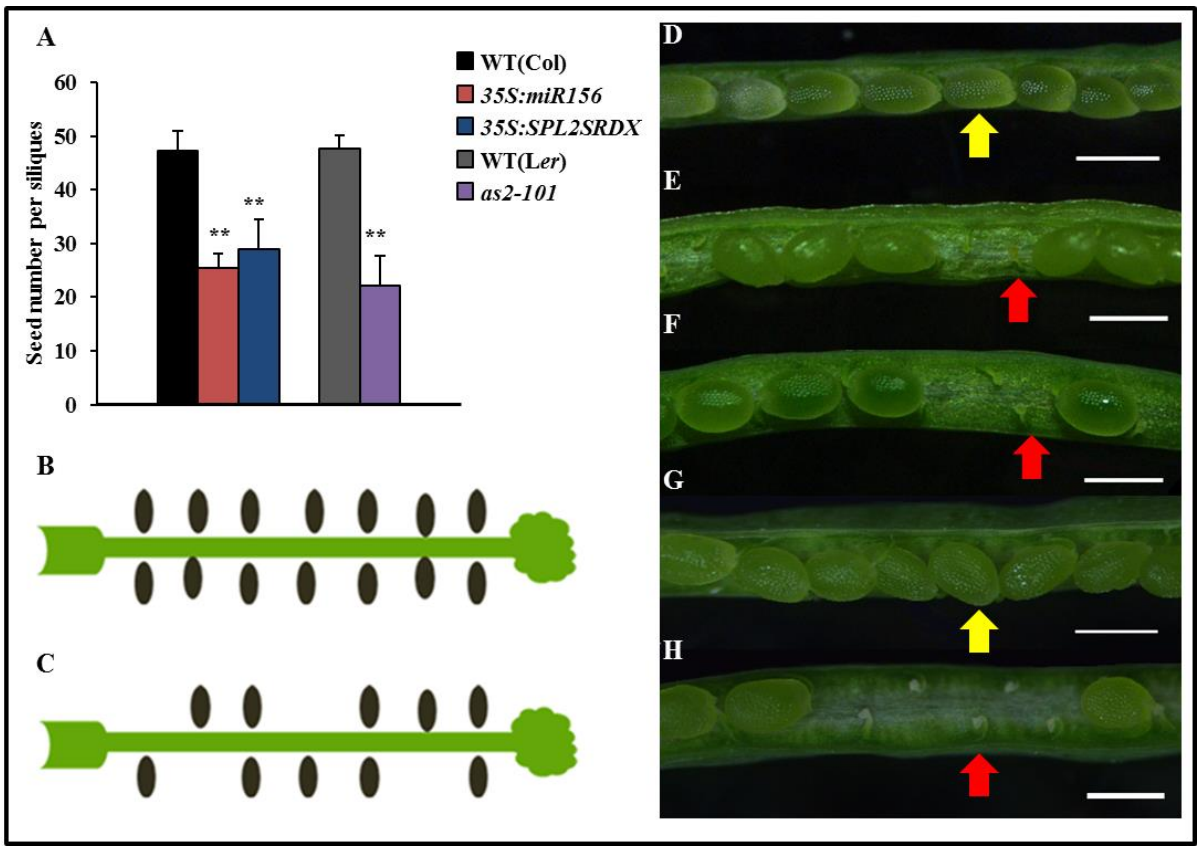

A
WT(Col)
35S:miR156
35S:SPL2SRDX
WT(Ler)
as2-101
B
WT(Col)
35S:miR156
35S:SPL2SRDX
WT(Ler)
as2-101
C
WT(Col)
35S:miR156
35S:SPL2SRDX
WT(Ler)
as2-101

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	
35S:miR156	1.698±0.076**	81.8%	1.121±0.084**	77.3%	9.26±0.44**	62.8%
35S:SPL2SRDX	1.710±0.121**	82.4%	1.132±0.062**	78.1%	9.98±0.75**	67.7%
WT(L*er*)	2.121±0.138		1.539±0.076		12.62±0.87	
as2-101	1.697±0.095**	80.0%	0.954±0.109**	65.8%	8.72±0.72**	69.1%

[a]Double 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(L*er*).

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(L*er*). 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 (L*er*) (G) and *as2-101* (H). Red arrows show the aborted embryos, yellow arrows show the normal growing seeds. Scale bar = 100μm.


A
Seed number per siliques
60
50
40
30
20
10
0
**
**
**
WT(Col)
35S:miR156
35S:SPL2SRDX
WT(Ler)
as2-101
B
C
D
E
F
G
H

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(L*er*), 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 L*er*), 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(L*er*) 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

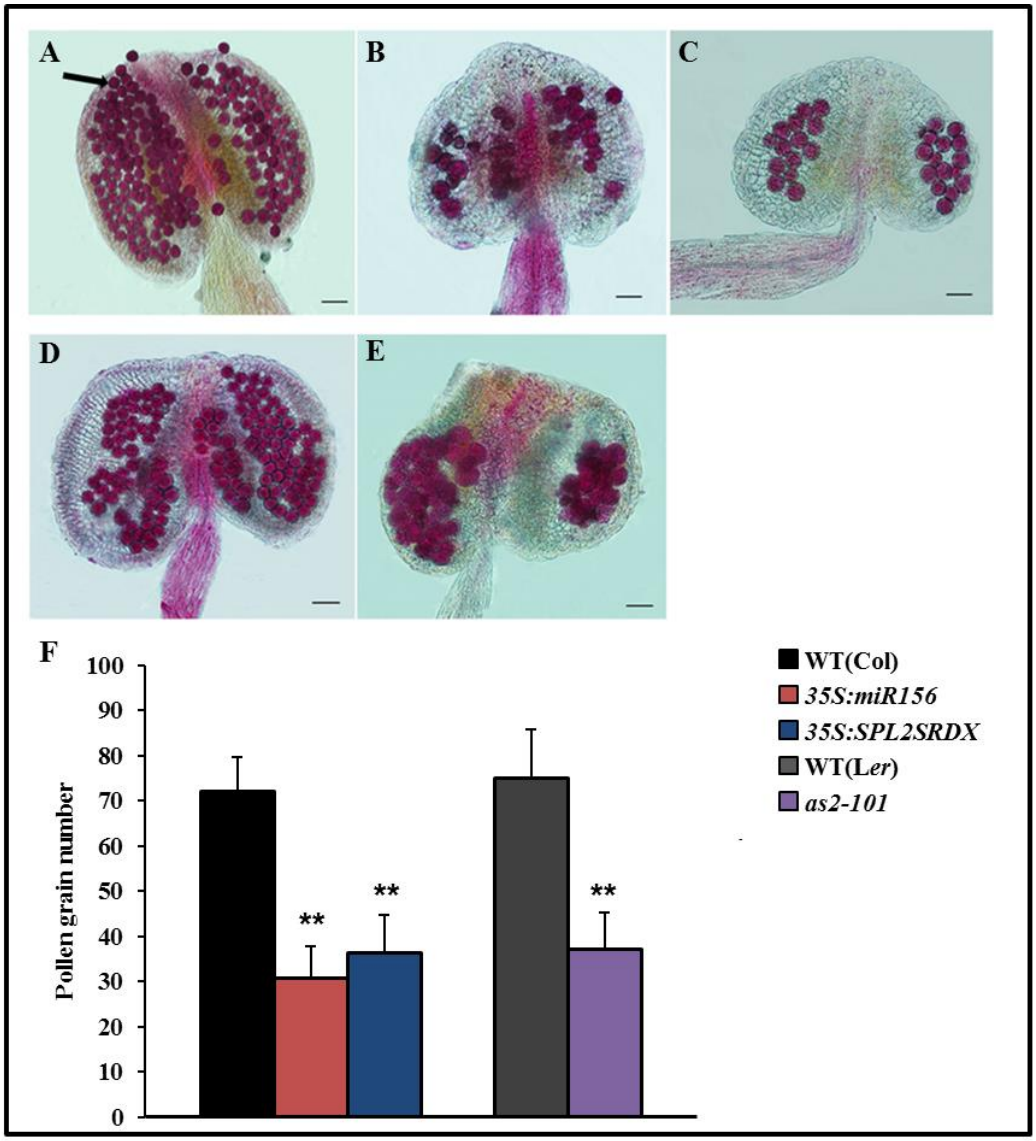
To further investigate the factors causing the sterile phenotypes that were observed in the mutants, I examined the pollen grains in all the genotypes. I used the Alexander staining method to examine the viability of pollen grains in the anthers (Alexander, 1969). In the Alexander staining method, the 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(L*er*) and *as2-101*

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

[a]Double 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(L*er*).

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 (L*er*) (D), and *as2-101* (E). Scale bar = 50μm. 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(L*er*). Double asterisks show significant difference at $P < 0.01$.

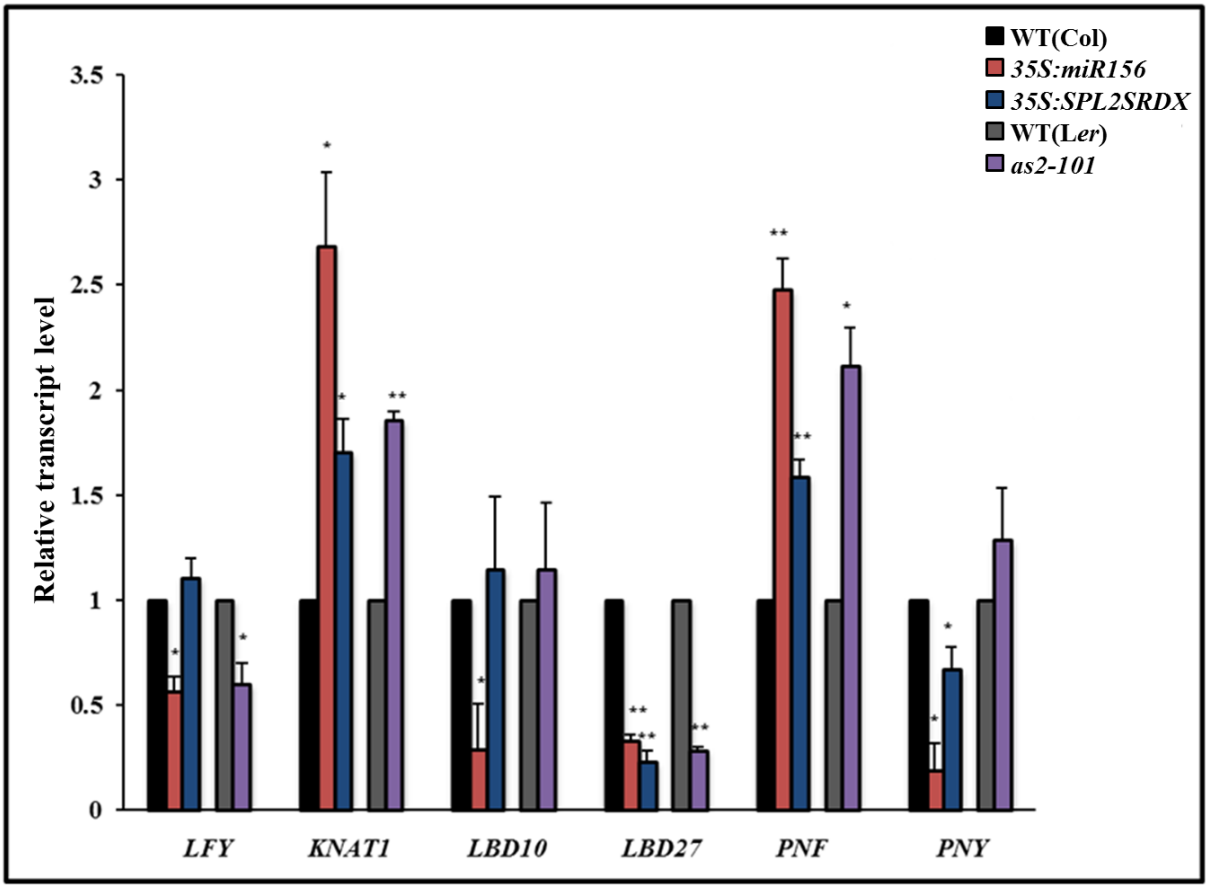

A
B
C
D
E
F
Pollen grain number
100
90
80
70
60
50
40
30
20
10
0
**
**
**
WT(Col)
35S:miR156
35S:SPL2SRDX
WT(Ler)
as2-101

mutants was dramatically decreased. When compared with WT(Col) and WT(L*er*) 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(L*er*) (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(L*er*) (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(L*er*). 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 (L*er*) (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(L*er*). Single asterisk shows significant difference at $P < 0.05$, double asterisks show significant difference at $P < 0.01$.


WT(Col)
35S:miR156
35S:SPL2SRDX
WT(Ler)
as2-101
Relative transcript level
3.5
3
2.5
2
1.5
1
0.5
0
LFY
KNAT1
LBD10
LBD27
PNF
PNY

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 L*er*). 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
AS2-1	CTCTAGTACCAAAT	SPL2
AS2-2	AATTAGTACTAGTC	SPL2
AS2-3	ACACGGTACAAATT	SPL2
AP1-1	CCTGCGTACAAATC	SPL3[1]
AP1-2	GGTCCGTACAATGT	SPL3[1]
FUL-1	AGTCCGTACGGTAC	SPL3[1]
FUL-2	GTACGGTACGGAAA	SPL3[1]
LFY	GAACGGTACGGTAT	SPL3[1]
MiR398c	TCGCAGTACACAAT	SPL7[2]
TCL1-1	ATGGTGTACCTATA	SPL9[3]
TCL1-2	ATCATGTACCGTTC	SPL9[3]
TCL1-3	TCATCGTACCACAT	SPL9[3]
TRY-1	GTAGGGTACTTTAT	SPL9[3]
TRY-2	GAAGCGTACTTTAT	SPL9[3]
TRY-3	CATACGTACTATAT	SPL9[3]
DFR-2	GTTACGTACCACAC	SPL9[4]
DFR-3	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*.

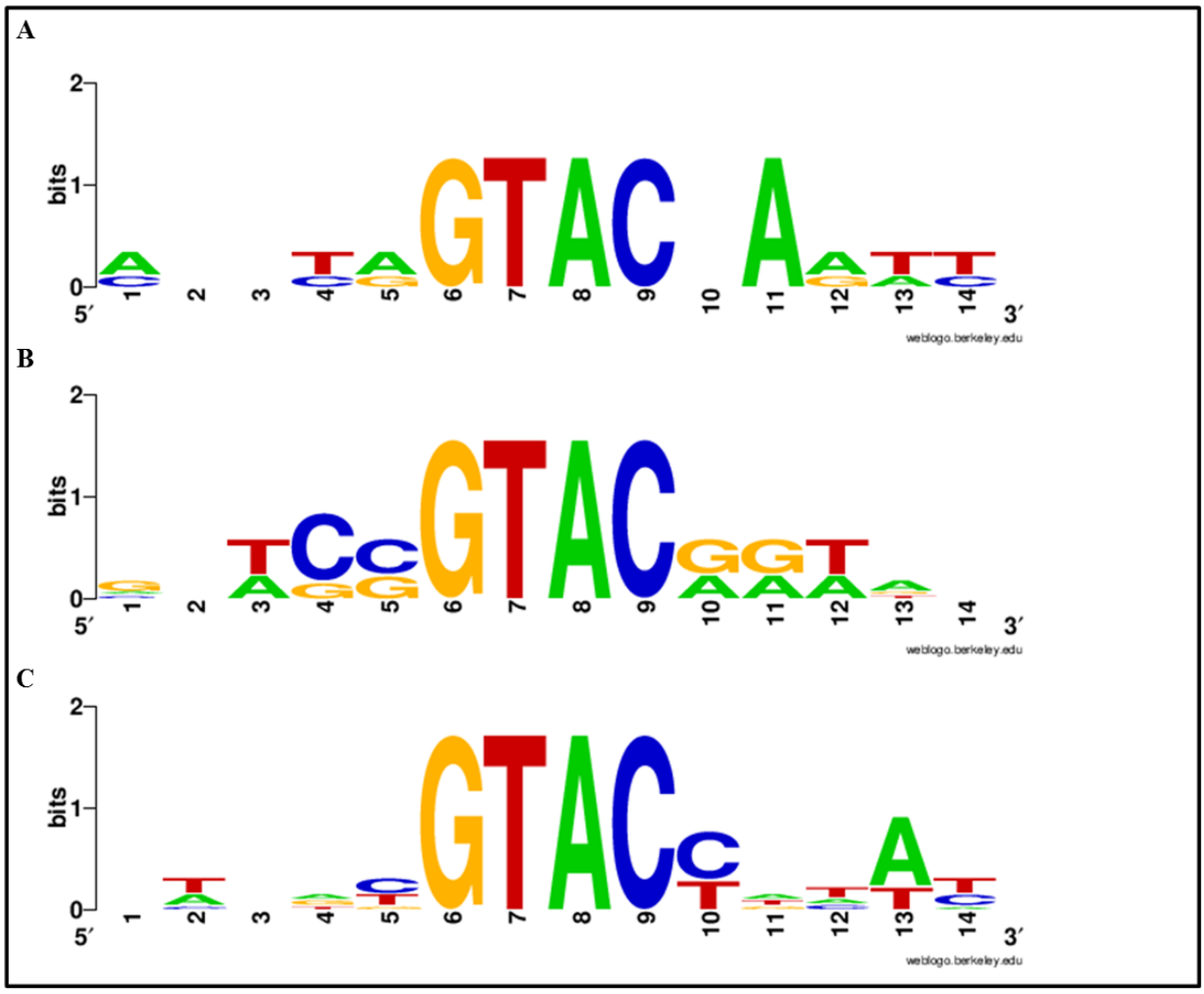
4.5 Diversity of SPL Binding Sites in Arabidopsis

In Arabidopsis, several genes have been identified as direct targets of SPLs. For example, SPL3 can bind to specific regions that contain GTAC in *AP1*, *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.


A
bits
2
1
0
5′
1
2
3
4
5
6
7
8
9
10
11
12
13
14
3′
weblogo.berkeley.edu
B
bits
2
1
0
5′
1
2
3
4
5
6
7
8
9
10
11
12
13
14
3′
weblogo.berkeley.edu
C
bits
2
1
0
5′
1
2
3
4
5
6
7
8
9
10
11
12
13
14
3′
weblogo.berkeley.edu

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(L*er*) 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 *KNAT1* (Guo et al., 2008b), which is a negative regulator of cell division (Truernit and Haseloff, 2008). To mediate regulation of *KNAT1*, 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 *KNAT1* 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 *BEL1-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*.

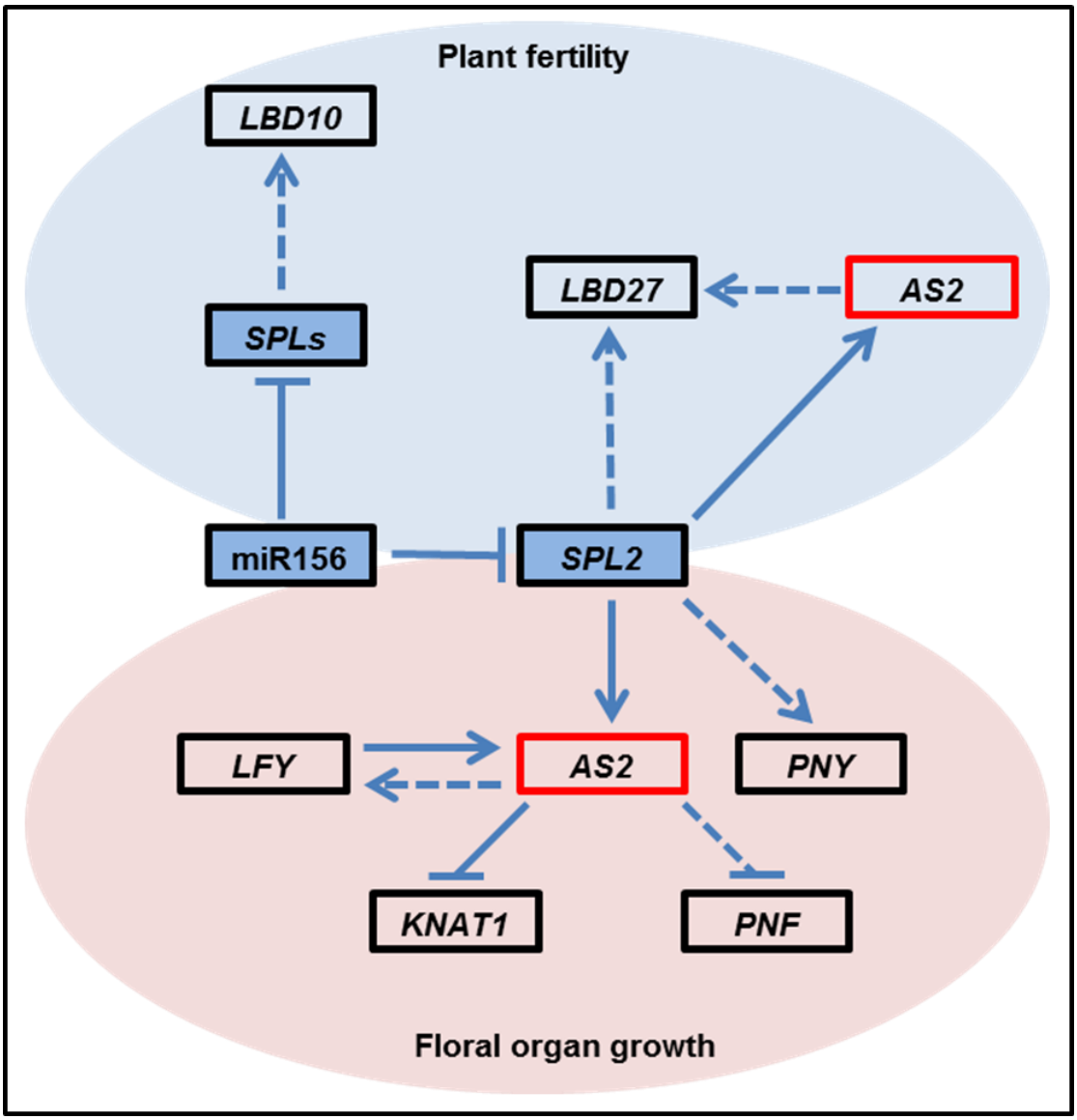
Previous research showed *LBD10* and *LBD27* were crucial for maintaining plant fertility in Arabidopsis (Kim et al., 2015). My analysis revealed that *LBD27* was regulated by miR156/SPL2 pathway, as well as AS2 (Figure 14). Moreover, the genomic sequence of *LBD27* contains both SBP-domain and LBD core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


Plant fertility
LBD10
SPLs
LBD27
AS2
miR156
SPL2
LFY
AS2
PNY
KNAT1
PNF
Floral organ growth

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*.

REFERENCES

Abel, S., Oeller, P.W., and Theologis, A. (1994). Early auxin-induced genes encode short-lived nuclear proteins. Proc Natl Acad Sci USA 91, 326-330.

Alexander, M.P. (1969). Differential staining of aborted and nonaborted pollen. Stain Technol 44, 117-122.

Allen, G.C., Flores-Vergara, M.A., Krasynanski, S., Kumar, S., and Thompson, W.F. (2006). A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. Nat Protoc 1, 2320-2325.

Ambros, V. (2004). The functions of animal microRNAs. Nature 431, 350-355.

Ariizumi, T., Hatakeyama, K., Hinata, K., Inatsugi, R., Nishida, I., Sato, S., Kato, T., Tabata, S., and Toriyama, K. (2004). Disruption of the novel plant protein NEF1 affects lipid accumulation in the plastids of the tapetum and exine formation of pollen, resulting in male sterility in *Arabidopsis thaliana*. Plant J 39, 170-181.

Aung, B., Gruber, M.Y., and Hannoufa, A. (2015a). The microRNA156 system: A tool in plant biotechnology. Biocat Agric Biotech, DOI: 10.1016/j.bcab.2015.08.002.

Aung, B., Gruber, M.Y., Amyot, L., Omari, K., Bertrand, A., and Hannoufa, A. (2015b). MicroRNA156 as a promising tool for alfalfa improvement. Plant Biotechnol J 13, 779-790.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-297.

Beemster, G.T.S., Fiorani, F., and Inzé, D. (2003). Cell cycle: The key to plant growth control? Trends Plant Sci 8, 154-158.

Bellusci, F., Musacchio, A., Stabile, R., and Pellegrino, G. (2010). Differences in pollen viability in relation to different deceptive pollination strategies in Mediterranean orchids. Ann Bot 106, 769-774.

Berger, F. (2008). Double-fertilization, from myths to reality. Sex Plant Reprod 21, 3-5.

Birkenbihl, R.P., Jach, G., Saedler, H., and Huijser, P. (2005). Functional dissection of the plant-specific SBP-domain: overlap of the DNA-binding and nuclear localization domains. J Mol Biol 352, 585-596.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7, 1513-1523.

Budar, F., and Pelletier, G. (2001). Male sterility in plants: occurrence, determinism, significance and use. C R Acad Sci III 324, 543-550.

Byrne, M.E., Simorowski, J., and Martienssen, R.A. (2002). *ASYMMETRIC LEAVES1* reveals *KNOX* gene redundancy in Arabidopsis. Development 129, 1957-1965.

Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterisation of the Arabidopsis SBP-box genes. Gene 237, 91-104.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 94, 4223-4228.

Chen, X. (2004). A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. Science 303, 2022-2025.

Chen, X. (2005). MicroRNA biogenesis and function in plants. FEBS Lett 579, 5923-5931.

Cheng, Y., and Zhao, Y. (2007). A role for auxin in flower development. J Integr Plant Biol 49, 99-104.

Cheng, Z.J., Wang, L., Sun, W., Zhang, Y., Zhou, C., Su, Y.H., Li, W., Sun, T.T., Zhao, X.Y., Li, X.G., Cheng, Y., Zhao, Y., Xie, Q., and Zhang, X.S. (2013). Pattern of auxin and cytokinin responses for shoot meristem induction results from the regulation of cytokinin biosynthesis by AUXIN RESPONSE FACTOR3. Plant Physiol 161, 240-251.

Chuck, G., Cigan, A.M., Saeteurn, K., and Hake, S. (2007). The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. Nat Genet 39, 544-549.

Chung, C.T., and Miller, R.H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acid Res 16, 3580

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. Plant Physiol 133, 462-469.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139, 5-17.

Dong, X., Nou, I.S., Yi, H., and Hur, Y. (2015). Suppression of *ASKbeta* (*AtSK32*), a Clade III Arabidopsis *GSK3*, leads to the pollen defect during late pollen development. Mol Cells 38, 506-517.

Du, T., and Zamore, P.D. (2005). MicroPrimer: the biogenesis and function of microRNA. Development 132, 4645-4652.

Feng, X.L., Ni, W.M., Elge, S., Mueller-Roeber, B., Xu, Z.H., and Xue, H.W. (2006). Auxin flow in anther filaments is critical for pollen grain development through regulating pollen mitosis. Plant Mol Biol 61, 215-226.

Ferreira e Silva, G.F., Silva, E.M., Azevedo Mda, S., Guivin, M.A., Ramiro, D.A., Figueiredo, C.R., Carrer, H., Peres, L.E., and Nogueira, F.T. (2014). MicroRNA156-targeted SPL/SBP box transcription factors regulate tomato ovary and fruit development. Plant J 78, 604-618.

Gendrel, A.V., Lippman, Z., Martienssen, R., and Colot, V. (2005). Profiling histone modification patterns in plants using genomic tiling microarrays. Nat Methods 2, 213-218.

Goldberg, R.B., De Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science 266, 605-614.

Gong, W., Gottlieb, S., Collins, J., Blescia, A., Dietz, H., Goldmuntz, E., McDonald-McGinn, D.M., Zackai, E.H., Emanuel, B.S., Driscoll, D.A., and Budarf, M.L. (2001). Mutation analysis of *TBX1* in non-deleted patients with features of DGS/VCFS or isolated cardiovascular defects. J Med Genet 38, E45.

Gou, J.Y., Felippes, F.F., Liu, C.J., Weigel, D., and Wang, J.W. (2011). Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-targeted SPL transcription factor. Plant Cell 23, 1512-1522.

Guo, A.Y., Zhu, Q.H., Gu, X., Ge, S., Yang, J., and Luo, J. (2008a). Genome-wide identification and evolutionary analysis of the plant specific SBP-box transcription factor family. Gene 418, 1-8.

Guo, M., Thomas, J., Collins, G., and Timmermans, M.C.P. (2008b). Direct repression of *KNOX* loci by the ASYMMETRIC LEAVES1 complex of Arabidopsis. Plant Cell 20, 48-58.

Hay, A., and Tsiantis, M. (2010). *KNOX* genes: Versatile regulators of plant development and diversity. Development 137, 3153-3165.

Horner, H.T., and Palmer, R.G. (1995). Mechanisms of genic male sterility. Crop Sci 35, 1527-1535.

Hu, Y.,Xie, Q.,Chua, N. H. (2003). The Arabidopsis auxin-inducible gene *ARGOS* controls lateral organ size. Plant Cell 15, 1951-1961.

Husbands, A., Bell, E.M., Shuai, B., Smith, H.M., and Springer, P.S. (2007). LATERAL ORGAN BOUNDARIES defines a new family of DNA-binding transcription factors and can interact with specific bHLH proteins. Nucleic Acids Res 35, 6663-6671.

Islam, M.T., Ferdous, A.S., Najnin, R.A., Sarker, S.K., and Khan, H. (2015). High-throughput sequencing reveals diverse sets of conserved, nonconserved, and species-specific miRNAs in jute. Int J Genomics 2015.

Iwakawa, H., Iwasaki, M., Kojima, S., Ueno, Y., Soma, T., Tanaka, H., Semiarti, E., Machida, Y., and Machida, C. (2007). Expression of the *ASYMMETRIC LEAVES2* gene in the adaxial domain of Arabidopsis leaves represses cell proliferation in this domain and is critical for the development of properly expanded leaves. Plant J 51, 173-184.

Iwakawa, H., Ueno, Y., Semiarti, E., Onouchi, H., Kojima, S., Tsukaya, H., Hasebe, M., Soma, T., Ikezaki, M., Machida, C., and Machida, Y. (2002). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana*, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. Plant Cell Physiol 43, 467-478.

Iwasaki, M., Takahashi, H., Iwakawa, H., Nakagawa, A., Ishikawa, T., Tanaka, H., Matsumura, Y., Pekker, I., Eshed, Y., Vial-Pradel, S., Ito, T., Watanabe, Y., Ueno, Y., Fukazawa, H., Kojima, S., Machida, Y., and Machida, C. (2013). Dual regulation of *ETTIN* (*ARF3*) gene expression by AS1-AS2, which maintains the DNA methylation level, is involved in stabilization of leaf adaxial-abaxial partitioning in Arabidopsis. Development 140, 1958-1969.

Jiao, Y., Wang, Y., Xue, D., Wang, J., Yan, M., Liu, G., Dong, G., Zeng, D., Lu, Z., Zhu, X., Qian, Q., and Li, J. (2010). Regulation of *OsSPL14* by OsmiR156 defines ideal plant architecture in rice. Nat Genet 42, 541-544.

Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57. 19-53.

Jun, J.H., Ha, C.M., and Fletcher, J.C. (2010). BLADE-ON-PETIOLE1 coordinates organ determinacy and axial polarity in Arabidopsis by directly activating *ASYMMETRIC LEAVES2*. Plant Cell 22, 62-76.

Kanrar, S., Bhattacharya, M., Arthur, B., Courtier, J., and Smith, H.M.S. (2008). Regulatory networks that function to specify flower meristems require the function of homeobox genes *PENNYWISE* and *POUND-FOOLISH* in Arabidopsis. Plant J 54, 924-937.

Karimi, M., Depicker, A., and Hilson, P. (2007). Recombinational cloning with plant gateway vectors. Plant Physiol 145, 1144-1154.

Keta, S., Iwakawa, H., Ikezaki, M., Semiarti, E., Kojima, S., Machida, Y., and Machida, C. (2012). Roles of the *ASYMMETRIC LEAVES2* gene in floral organ development in *Arabidopsis thaliana*. Plant Biotech 29, 1-8.

Kim, M.J., Kim, M., Lee, M.R., Park, S.K., and Kim, J. (2015). *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*)*10* interacts with *SIDECAR POLLEN/LBD27* to control pollen development in Arabidopsis. Plant J 81, 794-809.

Klein, J., Saedler, H., and Huijser, P. (1996). A new family of DNA binding proteins includes putative transcriptional regulators of the *Antirrhinum majus* floral meristem identity gene *SQUAMOSA*. Mol Gen Genet 250, 7-16.

Koncz, C., and Schell, J. (1986). The promoter of *TL-DNA GENE 5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204, 383-396.

Kropat, J., Tottey, S., Birkenbihl, R.P., Depège, N., Huijser, P., and Merchant, S. (2005). A regulator of nutritional copper signaling in *Chlamydomonas* is an SBP domain protein that recognizes the GTAC core of copper response element. Proc Natl Acad Sci USA 102, 18730-18735.

Kurihara, Y., and Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through DICER-LIKE 1 protein functions. Proc Natl Acad Sci USA. 101, 12753-12758.

Lal, S., Pacis, L.B., and Smith, H.M. (2011). Regulation of the *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* genes/microRNA156 module by the homeodomain proteins PENNYWISE and POUND-FOOLISH in Arabidopsis. Mol Plant 4, 1123-1132.

Lännenpää, M., Jänönen, I., Hölttä-Vuori, M., Gardemeister, M., Porali, I., and Sopanen, T. (2004). A new SBP-box gene *BpSPL1* in silver birch (*Betula pendula*). Physiol Plant 120, 491-500.

Lease, K.A., Wen, J., Li, J., Doke, J.T., Liscum, E., and Walker, J.C. (2001). A mutant Arabidopsis heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. Plant Cell 13, 2631-2641.

Lee, H.W., Kim, N.Y., Lee, D.J., and Kim, J. (2009). *LBD18*/*ASL20* regulates lateral root formation in combination with *LBD16*/*ASL18* downstream of *ARF7* and *ARF19* in Arabidopsis. Plant Physiol 151, 1377-1389.

Leshem, Y., Johnson, C., Wuest, S. E., Song, X., Ngo, Q. A., Grossniklaus, U., Sundaresan, V. (2012). Molecular characterization of the glauce mutant: A central cell-specific function is required for double fertilization in Arabidopsis. Plant Cell 24, 3264-3277.

Li, A., Zhang, Y., Wu, X., Tang, W., Wu, R., Dai, Z., Liu, G., Zhang, H., Wu, C., Chen, G., and Pan, X. (2008). DH1, a LOB domain-like protein required for glume formation in rice. Plant Mol Biol 66, 491-502.

Li, S., Yang, X., Wu, F., and He, Y. (2012). HYL1 controls the miR156-mediated juvenile phase of vegetative growth. J Exp Bot 63, 2787-2798.

Li, X.M., Sang, Y.L., Zhao, X.Y., and Zhang, X.S. (2013). High-throughput sequencing of small RNAs from pollen and silk and characterization of miRNAs as candidate factors involved in pollen-silk interactions in maize. PloS One 8.

Liang, X., Nazarenus, T.J., and Stone, J.M. (2008). Identification of a consensus DNA-binding site for the *Arabidopsis thaliana* SBP domain transcription factor, AtSPL14, and binding kinetics by surface plasmon resonance. Biochemistry 47, 3645-3653.

Lin, W.C., Shuai, B., and Springer, P.S. (2003). The Arabidopsis LATERAL ORGAN BOUNDARIES-domain gene *ASYMMETRIC LEAVES2* functions in the repression of *KNOX* gene expression and in adaxial-abaxial patterning. Plant Cell 15, 2241-2252.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402-408.

Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002). Cleavage of *SCARECROW-LIKE* mRNA targets directed by a class of Arabidopsis miRNA. Science 297, 2053-2056.

Lord, E.M., and Russell, S.D. (2002). The mechanisms of pollination and fertilization in plants. Annu Rev Cell Dev Bi 18, 81-105.

Luo, L., Ando, S., Sasabe, M., Machida, C., Kurihara, D., Higashiyama, T., and Machida, Y. (2012). Arabidopsis ASYMMETRIC LEAVES2 protein required for leaf morphogenesis consistently forms speckles during mitosis of tobacco BY-2 cells via signals in its specific sequence. J Plant Res 125, 661-668.

Matsumura, Y., Iwakawa, H., Machida, Y., and Machida, C. (2009). Characterization of genes in the *ASYMMETRIC LEAVES2*/*LATERAL ORGAN BOUNDARIES* (*AS2*/*LOB*) family in *Arabidopsis thaliana*, and functional and molecular comparisons between *AS2* and other family members. Plant J 58, 525-537.

May, P., Liao, W., Wu, Y., Shuai, B., Richard McCombie, W., Zhang, M.Q., and Liu, Q.A. (2013). The effects of carbon dioxide and temperature on *microRNA* expression in Arabidopsis development. Nat Commun 4.

Meinke, D.W., and Sussex, I.M. (1979). Embryo-lethal mutants of *Arabidopsis thaliana*. A model system for genetic analysis of plant embryo development. Dev Biol 72, 50-61.

Millar, A.A., and Waterhouse, P.M. (2005). Plant and animal microRNAs: similarities and differences. Funct Integr Genomic 5, 129-135.

Mizukami, Y. (2001). A matter of size: Developmental control of organ size in plants. Curr Opin Plant Biol 4, 533-539.

Moreno, M.A., Harper, L.C., Krueger, R.W., Dellaporta, S.L., and Freeling, M. (1997). *LIGULELESS1* encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. Gene Dev 11, 616-628.

Nemhauser, J.L., Zambryski, P.C., and Roe, J.L. (1998). Auxin signaling in Arabidopsis flower development?: Commentary. Curr Opin Plant Biol 1, 531-535.

Niesenbaum, R.A. (1999). The effects of pollen load size and donor diversity on pollen performance, selective abortion, and progeny vigor in *Mirabilis jalapa* (Nyctaginaceae). Am Journal Bot 86, 261-268.

Nodine, M.D., and Bartel, D.P. (2010). MicroRNAs prevent precocious gene expression and enable pattern formation during plant embryogenesis. Genes Dev 24, 2678-2692.

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Mutations in *FIE*, a WD polycomb group gene, allow endosperm development without fertilization. Plant Cell 11, 407-415.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature 425, 257-263.

Preston, J.C., and Hileman, L.C. (2013). Functional evolution in the plant *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene family. Front Plant Sci 4, 80.

Rast, M.I., and Simon, R. (2012). Arabidopsis *JAGGED LATERAL ORGANS* acts with *ASYMMETRIC LEAVES2* to coordinate *KNOX* and *PIN* expression in shoot and root meristems. Plant Cell 24, 2917-2933.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002). MicroRNAs in plants. Genes Dev 16, 1616-1626.

Riese, M., Höhmann, S., Saedler, H., Münster, T., and Huijser, P. (2007). Comparative analysis of the SBP-box gene families in *P. patens* and seed plants. Gene 401, 28-37.

Rubin, G., Tohge, T., Matsuda, F., Saito, K., and Scheible, W.R. (2009). Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. Plant Cell 21, 3567-3584.

Ryan, P.T., Ó'Maoiléidigh, D.S., Drost, H.G., Kwaśniewska, K., Gabel, A., Grosse, I., Graciet, E., Quint, M., and Wellmer, F. (2015). Patterns of gene expression during Arabidopsis flower development from the time of initiation to maturation. BMC Genomics 16, 488.

Saito, H., Oikawa, T., Hamamoto, S., Ishimaru, Y., Kanamori-Sato, M., Sasaki-Sekimoto, Y., Utsumi, T., Chen, J., Kanno, Y., Masuda, S., Kamiya, Y., Seo, M., Uozumi, N., Ueda, M., and Ohta, H. (2015). The jasmonate-responsive GTR1 transporter is required for gibberellin-mediated stamen development in Arabidopsis. Nat Commun 6, 6095.

Schnable, P.S., and Wise, R.P. (1998). The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci 3, 175-180.

Schuster, J., Knill, T., Reichelt, M., Gershenzon, J., and Binder, S. (2006). BRANCHED-CHAIN AMINOTRANSFERASE4 is part of the chain elongation pathway in the biosynthesis of methionine-derived Glucosinolates in Arabidopsis. Plant Cell 18, 2664-2679.

Schwarz, S., Grande, A.V., Bujdoso, N., Saedler, H., and Huijser, P. (2008). The microRNA regulated SBP-box genes *SPL9* and *SPL15* control shoot maturation in Arabidopsis. Plant Mol Biol 67, 183-195.

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C., and Machida, Y. (2001). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. Development 128, 1771-1783.

Serivichyaswat, P., Ryu, H.S., Kim, W., Kim, S., Chung, K.S., Kim, J.J., and Ahn, J.H. (2015). Expression of the floral repressor miRNA156 is positively regulated by the AGAMOUS-like proteins AGL15 and AGL18. Mol Cells 38, 259-266.

Shikata, M., Koyama, T., Mitsuda, N., and Ohme-Takagi, M. (2009). Arabidopsis SBP-box genes *SPL10*, *SPL11* and *SPL2* control morphological change in association with shoot maturation in the reproductive phase. Plant Cell Physiol 50, 2133-2145.

Shu, K., Wu, Y., Yang, W., and Xie, Q. (2014). Concurrent deficiency of gibberellins and abscisic acid causes plant male sterility. J Genet Genomics 41, 601-604.

Shuai, B., Reynaga-Pena, C.G., and Springer, P.S. (2002). The lateral organ boundaries gene defines a novel, plant-specific gene family. Plant Physiol 129, 747-761.

Steiner-Lange, S., Unte, U.S., Eckstein, L., Yang, C., Wilson, Z.A., Schmelzer, E., Dekker, K., and Saedler, H. (2003). Disruption of *Arabidopsis thaliana MYB26* results in male sterility due to non-dehiscent anthers. Plant J 34, 519-528.

Ter-Avanesian, D.V. (1978). The effect of varying the number of pollen grains used in fertilization. Theor Appl Genet 52, 77-79.

Tran, D., El-Maarouf-Bouteau, H., Rossi, M., Biligui, B., Briand, J., Kawano, T., Mancuso, S., Bouteau, F. (2013). Post-transcriptional regulation of GORK channels by superoxide anion contributes to increases in outward-rectifying K(+) currents. New Phytol 198, 1039-1048.

Truernit, E., and Haseloff, J. (2008). *Arabidopsis thaliana* outer ovule integument morphogenesis: Ectopic expression of *KNAT1* reveals a compensation mechanism. BMC Plant Biol 8, 35.

Usami, T., Horiguchi, G., Yano, S., and Tsukaya, H. (2009). The *more and smaller cells* mutants of *Arabidopsis thaliana* identify novel roles for *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* genes in the control of heteroblasty. Development 136, 955-964.

Wang, H., and Wang, H. (2015). The miR156/SPL module, a regulatory hub and versatile toolbox, gears up crops for enhanced agronomic traits. Mol Plant 8, 677-688.

Wang, J.W., Czech, B., and Weigel, D. (2009). MiR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell 138, 738-749.

Wang, J.W., Schwab, R., Czech, B., Mica, E., and Weigel, D. (2008). Dual effects of miR156-targeted *SPL* genes and *CYP78A5*/*KLUH* on plastochron length and organ size in *Arabidopsis thaliana*. Plant Cell 20, 1231-1243.

Wang, J.W., Wang, L.J., Mao, Y.B., Cai, W.J., Xue, H.W., and Chen, X.Y. (2005). Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. Plant cell 17, 2204-2216.

Wang, Y., Wang, Z., Amyot, L., Tian, L., Xu, Z., Gruber, M.Y., and Hannoufa, A. (2015). Ectopic expression of *miR156* represses nodulation and causes morphological and developmental changes in *Lotus japonicus*. Mol Genet Genomics 290, 471-484.

Wei, S., Gruber, M.Y., Yu, B., Gao, M.J., Khachatourians, G.G., Hegedus, D.D., Parkin, I.A.P., and Hannoufa, A. (2012). Arabidopsis mutant *sk156* reveals complex regulation of *SPL15* in a *miR156*-controlled gene network. BMC Plant Biol 12, 169.

Weigel D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., Meyerowitz, E.M. (1992). LEAFY controls floral meristem identity in Arabidopsis. Cell 69:843-859.

Wen, M., and Jetter, R. (2009). Composition of secondary alcohols, ketones, alkanediols, and ketols in *Arabidopsis thaliana* cuticular waxes. J Exp Bot 60, 1811-1821.

Wu, G. (2013). Plant microRNAs and development. J Genet Genomics 20, 217-230.

Wu, G., and Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by *miR156* and its target *SPL3*. Development 133, 3539-3547.

Wu, G., Lin, W.C., Huang, T., Poethig, R.S., Springer, P.S., and Kerstetter, R.A. (2008). KANADI1 regulates adaxial-abaxial polarity in Arabidopsis by directly repressing the transcription of *ASYMMETRIC LEAVES2*. Proc Natl Acad Sci USA 105, 16392-16397.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., and Poethig, R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell 138, 750-759.

Wu, L., Zhou, H., Zhang, Q., Zhang, J., Ni, F., Liu, C., and Qi, Y. (2010a). DNA methylation mediated by a microRNA pathway. Mol Cell 38, 465-475.

Wu, Y., Yan, J., Zhang, R., Qu, X., Ren, S., Chen, N., and Huang, S. (2010b). Arabidopsis FIMBRIN5, an actin bundling factor, is required for pollen germination and pollen tube growth. Plant Cell 22, 3745-3763.

Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A., and Carrington, J.C. (2005). Expression of Arabidopsis *miRNA* genes. Plant Physiol 138, 2145-2154.

Xing, S., Rosso, M.G., and Zachgo, S. (2005). *ROXY1*, a member of the plant glutaredoxin family, is required for petal development in *Arabidopsis thaliana*. Development 132, 1555-1565.

Xing, S., Salinas, M., Hohmann, S., Berndtgen, R., and Huijser, P. (2010). MiR156-targeted and nontargeted SBP-box transcription factors act in concert to secure male fertility in Arabidopsis. Plant Cell 22, 3935-3950.

Xing, S., Salinas, M., Garcia-Molina, A., Höhmann, S., Berndtgen, R., and Huijser, P. (2013). *SPL8* and miR156-targeted *SPL* genes redundantly regulate Arabidopsis gynoecium differential patterning. Plant J 75, 566-577.

Xu, B., Li, Z., Zhu, Y., Wang, H., Ma, H., Dong, A., and Huang, H. (2008). Arabidopsis genes *AS1*, *AS2*, and *JAG* negatively regulate boundary-specifying genes to promote sepal and petal development. Plant Physiol 146, 566-575.

Xu, H., Knox, R.B., Taylor, P.E., and Singh, M.B. (1995). *BCP1*, a gene required for male fertility in Arabidopsis. Proc Natl Acad Sci USA 92, 2106-2110.

Xu, L. (2003). Novel *as1* and *as2* defects in leaf adaxial-abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and *2* and *ERECTA* functions in specifying leaf adaxial identity. Development 130, 4097-4107.

Yamaguchi, A., Wu, M.F., Yang, L., Wu, G., Poethig, R.S., and Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of *LEAFY*, *FRUITFULL*, and *APETALA1*. Dev Cell 17, 268-278.

Yamaguchi, N., Yamaguchi, A., Abe, M., Wagner, D., and Komeda, Y. (2012). LEAFY controls Arabidopsis pedicel length and orientation by affecting adaxial-abaxial cell fate. Plant J 69, 844-856.

Yamasaki, H., Hayashi, M., Fukazawa, M., Kobayashi, Y., and Shikanai, T. (2009). *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7* is a central regulator for copper homeostasis in Arabidopsis. Plant Cell 21, 347-361.

Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., Aoki, M., Seki, E., Matsuda, T., Nunokawa, E., Ishizuka, Y., Terada, T., Shirouzu, M., Osanai, T., Tanaka, A., Seki, M., Shinozaki, K., and Yokoyama, S. (2004). A novel zinc-binding motif revealed by solution structures of DNA-binding domains of Arabidopsis SBP-family transcription factors. J Mol Biol 337, 49-63.

Yasuor, H., Abu-Abied, M., Belausov, E., Madmony, A., Sadot, E., Riov, J., and Rubin, B. (2006). Glyphosate-induced anther indehiscence in cotton is partially temperature dependent and involves cytoskeleton and secondary wall modifications and auxin accumulation. Plant Physiol 141, 1306-1315.

Yu, L., Patibanda, V., and Smith, H.M.S. (2009) A novel role of *BELL1*-like homeobox genes, *PENNYWISE* and *POUND-FOOLISH*, in floral patterning. Planta 229, 693-707.

Yu, N., Niu, Q.W., Ng, K.H., and Chua, N.H. (2015). The role of miR156/SPLs modules in Arabidopsis lateral root development. Plant J 83, 673-685.

Yu, N., Cai, W.J., Wang, S., Shan, C.M., Wang, L.J., and Chen, X.Y. (2010). Temporal control of trichome distribution by microRNA156-targeted *SPL* genes in *Arabidopsis thaliana*. Plant Cell 22, 2322-2335.

Zgurski, J.M., Sharma, R., Bolokoski, D.A., and Schultz, E.A. (2005). Asymmetric auxin response precedes asymmetric growth and differentiation of *asymmetric leaf1* and *asymmetric leaf2* Arabidopsis leaves. Plant Cell 17, 77-91.

Zhang, B., Pan, X., Cobb, G.P., and Anderson, T.A. (2006a). Plant microRNA: a small regulatory molecule with big impact. Dev Biol 289, 3-16.

Zhang, S., Liu, Y., and Yu, B. (2014). PRL1, an RNA-binding protein, positively regulates the accumulation of miRNAs and siRNAs in Arabidopsis. PLoS Genet 10.

Zhang, S., Xie, M., Ren, G., and Yu, B. (2013). CDC5, a DNA binding protein, positively regulates posttranscriptional processing and/or transcription of primary microRNA transcripts. Proc Natl Acad Sci USA 110, 17588-17593.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., and Chua, N.H. (2006b). *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc 1, 641-646.

Zhao, M., Yang, S., Chen, C.Y., Li, C., Shan, W., Lu, W., Cui, Y., Liu, X., and Wu, K. (2015). Arabidopsis BREVIPEDICELLUS interacts with the SWI2/SNF2 chromatin remodeling ATPase BRAHMA to regulate *KNAT2* and *KNAT6* expression in control of inflorescence architecture. PLoS Genet 11.

Zhao, Q., Yu, D., Chang, H., Guo, X., Yuan, C., Hu, S., Zhang, C., Wang, P., Wang, Y. (2013). Regulation and function of Arabidopsis *AtGALK2* gene in abscisic acid response signaling. Mol Biol Rep 40, 6605-6612.

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	TGACACGTGTGCGAGAATCA	CAAGCAGCTGTCCCTGAAGA
unknown	AT5G22580	TGCTTAGACAAGGATTCACTCA	ACGGGGAAATCGAGGAGAAC
ATDSEL	AT4G18550	AGGTATGGCCCAACAAGACG	GTTTCCAAACATCAACACTTGACA
unknown	AT1G80120	GTTGTCCTCATGGACGCTCA	TTCCTTGCCCCGAAACTACC
unknown	AT1G31250	ATCGAGGCTAGGGAAGGCAA	GGAGGTATCGAAAGAGAAGCGT
unknown	AT1G47395	CTCATCACACTTCTTCACTCGG	CACGGAAGCACGGTCAAAAC
AST91	AT1G23090	ACGAGTGGTCATGGCAAACA	AGCTTCGGCTTCTTCATGCT
CASPL2A2	AT3G14380	ACTGAGAAATCGGCAGAGGC	ACGCCATTAGCACTCACCAA
ATSDI1	AT5G48850	GAAGAACCCGGAGATGGCAA	CTTGGAACAACGAGGACGGA
unknown	AT3G21460	GAGCCCGGCAATTGTAGAGA	CCCTCCAACAAAAACCGCAG
GAMMA-TIP1	AT2G36830	CAGATGAAGCCACCCGTCC	GAGACAGCGACGAAGAGTCC
unknown	AT3G27025	ATGTCCAAGACATCCGCACA	CAGGGTCTGTACCAAGCGTC
SAUR31	AT4G00880	ACAAGCGACTTCACCATCCA	TGCCATGGTTTCTGTCTCTCC
unknown	AT3G62960	CCTGAGCCCTCAGACCAAAA	TTCTCGATCTCACGGCAGTC

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

DELTA-TIP1	AT3G16240	AGTTGCCTTTGGTTCCTTTGATGA	CTAGTCCCGGTGTATCAAGAGCAG
unknown	AT5G62720	GTCTCCTATTGGCTAAGAAAACAGA	GTTCCGGCGAAGAAGAGGTA
UMAMIT30	AT4G01450	CGTTATAGCGGGAGTGGTTG	AGATCCAATAACGCTTCCCA
GLC	AT1G65450	CGTACCAAGAATCTCCACCGT	CGCTGGTTTCTTCAGATGGG
unknown	AT5G22390	GCCGAGAACAGCAGTCTTCA	CTTCGTGGTTCCCACTCCTC
MYB86	AT5G26660	AGCTTATAGAACCTCCAAGAACCA	GGACAGAACTCCAACAGCCA
unknown	AT4G18425	TGGAGATCAAAGTTGACGAAGG	CGAGAACGGTTCCTGTTGGT
RNS3	AT1G26820	ACGTACAAAGTTTCGCCCAAGATT	GCATCCACCGGTTTTGTAGTT
PXG4	AT1G70670	TGCAGAGACATGTCGCTTTTT	GCTGCTGAGACCTATGTTTATGAA
BCAT4	AT3G19710	AGTGGGAAGAGTTGGCATTCA	CCCTGGCCATACTGAAGAACA
unknown	AT1G54095	TTTGTGGTACGGCGGTGATT	ACCTCGCCGGCAAATAAAGT
ATFRO1	AT1G01590	CGTCACCCGAGAAGAGAAAAC	GCCCGAGGATTGGTGAGATG
unknown	AT1G58170	TGTAACCGGCCAAGATTCGT	TGTGCCCTTCCCACCATTTT
unknown	AT1G70000	AGTCTCTGTGTTGTTTTGGTCA	ACGGCAAATTACTGGAAAAGTC
unknown	AT2G37880	ACTCCGACGAATCCAACCAC	GTAGTCGACGGCGGAAGTAG
unknown	AT3G62040	CCCTTTGAGCATCGGAATCA	ACCCATAACCTTGAGACCAGC
AGP6	AT5G14380	AATCTCCATCACCCACTGCC	AGATGATTTAGGTGCGGCGG
unknown	AT4G10290	GCTCCTGAGGTTCCCACTG	TAACCCCGAGTTCAGCAAGC
ATMC9	AT5G04200	GGCAGTCCTTGACCACTTGA	CCTGACACCCGCTCATCAAT
unknown	AT1G67870	TGTGGAGTCATCAGGAGGCT	GTCCGGCTGCTCTCAAAGAA

unknown	AT3G01240	GGGAAACCTGAGCTAGAAAAACC	CAACGTTTTCACCTCAGATTTCAA
ATGPX7	AT4G31870	ACCCTTCAATGGTTTCGCTG	GCAGCCCTTGCATAGACAC
unknown	AT3G50280	TTGTGTCAAGCTCTCCTCGG	TCACAAGTACGTCGGGCAAT
ATACA1	AT3G52720	AAAGGGAACCACACAGCACA	GACATTGACCTCACCTTGCC
ATWRKY23	AT2G47260	CCATGCACACGAACCAACAC	TTGGTCAACGCGTAAGTCAGA
unknown	AT1G29720	AGGAGAGCTGTCGGATGGAA	AACTCGCGGTTTCCTTGACT
LFY	AT5G61850	TGAAGAAGCAACAGCAGCAG	GGATGCTCCCTCTGTCTCTC
KNAT1	AT4G08150	GATCGGGAACTCAAGAACCA	TTGTAATGCAACTCCCACCA
LBD10	AT2G23660	CTTAAGTGGGGATGGCTTCA	GCGTTGATCAGGTGGAAGAT
LBD27	AT3G47870	GGCAAGGAGATTCTGACCAA	TTCCACTCGCTGATGATTTG
PNY	AT5G02030	AGTCCTCTCACCGCACAGAT	CAGCGTTGTCGTTGTTGTCT
PNF	AT2G27990	CTCAGCAGCAGCATCAGAAC	AGGGCCTTGTGGGTCTCTAT
SPL2	AT5G43270	TTGGGACTTCCAGTGACTCTGGCTTC	CATCAAACTCAGAGAGACAGTGGAACC
SPL4	AT1G53160	GAGAAGGATCAGGTCGGAGAGGAAT	CTGTGGTCGCTTGAATGATGAGTTT
SPL6	AT1G69170	CCACCGTACAAGTAGACTCGTGAG	GAGATTTTGGTTGGGTTGGGTGA
SPL10	AT1G27370	GTGGGAGAATGCTCAGGAGGC	GAGTGTGTTTGATCCCTTGTGAATCC
UBC21	AT5G25760	CTGAGCCGGACAGTCCTCTTAACTG	CGGCGAGGCGTGTATACATTTGTG
PP2AA3	AT1G13320	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT

Appendix 1B. Primers used in cloning

Primer	Gene	Sequence (5’ to 3’)
pSPL2:SPL2-GFP-5’	*SPL2*	CACCATGGCATATGCGAAAAAGTCTA
pSPL2:SPL2-GFP-3’	*SPL2*	GTTATAAAACTGGTTCAAGCTGAAGTA

Appendix 1C. Primers used in screening of transformed plants

Primer	Gene	Sequence (5’ to 3’)
SPL2-5’	*SPL2*	TTTCCGATACCGAGCACAATAG
GFP-3’	*GFP*	GGTAATGGTTGTCTGGTAAAAGG

Appendix 1D. Primers used in ChIP-qPCR

Gene	Sequence (5’ to 3’)	
	Forward	Reverse
AS2-I	CTCTCCGTTTCCTTAGCCTC	AGAAAAGAGGAAGGCATAGA
AS2-II	CTCCTTAGAAACTCGCAACT	TGAGGCTCTCGATGGGCAAA
AS2-III	CTTCCTTCTCTTCTCTCTTC	GATATAGAGGAACTTGTAG
EIF4A1	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 (½MS)	Sucrose	10
medium (pH=5.7)	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 *35S:miR156* 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	AT1G67870*	AT4G33790*
	AT1G37130*	AT5G10770*	AT3G19710*
	AT5G56540*	AT4G26530*	AT2G47880*
	AT3G62410*	AT2G18328*	AT2G36830*
	AT4G24780*	AT1G20070*	AT3G52720*
	AT4G04020*	AT1G64360*	AT4G00780*
	AT1G64770*	AT5G14740*	AT5G04200*
	AT1G04250*	AT3G01345	AT2G40100*
	AT4G30450*	AT5G37300*	AT1G70670*
	AT2G30766	AT5G48850*	AT4G30460*
	AT1G13650*	AT3G24460*	AT1G76680*
	AT3G56290*	AT3G21550*	AT5G22580*
	AT2G27420*	AT1G57750*	AT2G32160*
	AT3G16240*	AT1G67860	AT5G23750*
	AT2G15020*	AT1G66725	AT1G07440*
	AT3G62960*	AT5G63850*	AT3G48340*

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	

[a]Genes have at least one GTAC in the 2kb upstream of the translation start codon are indicated by asterisk

[b]Genes 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.

ctctctctatctctatatatcgacacaccataaaagttgttctttctctccgtttccttagcctctgcatctgggcgattttctct
ttctatctccctacaccttagggagtactgatcctaaactcatcatctttctatgccttcctcttttctttcacttattttctttt
ctatttttttctttcttactttgcgtaaaactagcgagtcaaaaataaagacacttgcatgatcctaatataatattatatattg
taattagagaaagagagagaagagaagaaagaaagtggcagtagattcttatggattttggtataaagaaacatgaaaaaatgggt
ttgaccgaagaaactttgaggacggtaacttcattatcaacttttcgactgtatttatattttagcttttgtcaaaccgtatcata
taatattaatgcgaattactagtagagagtataataatagtaataacaaaatgcttggttactgtatgtaaatccctagacaaaaa
gaataagaataaaaagagcaaaattcttcatcccaagatccatcaggagagtcagagtcagaccccatctctcatttcccatctct
ccttttcacatttatttccttttttttctttctagatacttttttatttgaaatttaatactaataactatacatttattgttat
tattgttgttagagtctatgctagggtccaagggttcatcccaatatcccttcttctccttagaaactcgcaacttcttcacatgg
aaatcaattccctctagtaccaaatcctattactattgaaggtatactctatagttatttatttagttaagtatatatattaaaaa
taaataacattaattagtactagtcttatcatatttattagctcattttcttataaaatactaatataataggaaatttaagcaca
agatgagctttgcccatcgagagcctcaaaacccaaaagtctcttcttcttcacgagatccaatcttttgctttttagtaattaat
tacatcttcttccttctcttctctcttcttcttccctctctcatacctacctataagcaacacaaagaacaccattgatcccatct
gttttgttccctatatttcgttaatccatccatacgcattccaactacacggtacaaatttccatctctttttaatttgtttttat
aatgattttaaatctctcaggttgtctctagactcttaggtctagttaccaaaatttgactagcaatcatatagctacaagttcct
ctatatcatatagctacacatatctctaggtctgaaatgatcttttatctcccaagcatatattatatatttggatgcatacctttt
atgtttgactttcgaaaagcaccttcatgttactcatttttcctctatttgctttcaacagttgacaagaaggttggatcagtat
atataagtatacatatacacttatgtgtgtgtgtagtgtatgtgcatatatagctcaccaaatatctttaatactactaattgcaa
agtttctcttatttttacagctttttgtatgcaaccccaaatagcagcttaaagaaagcagagaggatcttctttttatttcccctct
gagcaacagaagccattattcaaagaaaggcttctttaatttactcgcaggtaaaagctttcttattctatagagattgaacaact
ttttttttttttaattgacaacaataaagattgagcaacttctctacattatttctttctttatttctcacttaatttcatatt
tgattacaaaaacagataatgtaatatctccaaaatcacaattgatttcataatatctttttttgtttccttataccttctacctc
caaaaactaaattcaaaaacccttatttctctcaattttcaatggcggctttgtgtagggagagggaaaagagaaaaaattgaaaa
actccattttcaagtcattaaaATGGCATCTTCTTCAACAAACTCACCATGCGCCGCTTGCAAATTCCTCCGGCGAAAATGTCAAC
CGGAATGTGTATTCGCGCCCTATTTCCCACCGGACCAGCCACAAAAATTCGCAAACGTTCACAAAGTGTTTGGAGCAAGTAACGTG
ACAAAGCTCCTCAACGAGCTTCACCCTTCACAACGTGAAGACGCAGTGAACTCTTTGGCCTATGAAGCCGACATGCGCCTCCGTGA
CCCTGTCTACGGCTGCGTCGGCGTCATCTCTCTCCTCCAACATCAGCTTCGTCAGCTTCAGATAGATCTCAGCTGTGCTAAATCTG
AGCTCTCTAAGTACCAAAGCCTCGGTATCCTCGCCGCCACTCATCAGAGTCTTGGCATCAACTTACTCGCCGGAGCAGCAGATGGA
ACAGCCACCGCCGTGAGAGACCACTATCACCACCACCAGTTTTTTCCTAGAGAACAAATGTTTGGTGGCTTGGATGTTCCGGCCGG
TAACAACTACGACGGTGGGATTCTTGCCATTGGACAGATCACTCAGTTTCAGCAGCCGAGAGCCGCCGCTGGAGATGATGGTCGCC
GTACTGTTGATCCGTCTTGA

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.

aattctaccataaccgatataaaagtatttaaaccgactaattaagcttcacaaatttcggtttgtactttattata
ttgggccctattattattctctgagcttttgcgtctcaccaaaagacagagatcatcaggtgcgttagtgattacgt
aggcatctaatgaacggcagggattagtcaaacttattaatgggcctaatctttggcccatcgttttccctcgattc
ctgtcacacaaaaaactcctagctcttcctctacctacacaagctaaatacatatttttgcttatcctaagcatca
tgattatgttttgccctctccagcttttttcttcaatggcaacagattctaagaaagtctcttgaggctaaaatcaaa
gcatttttttgttgaagatagatagcaacttgctgcttcttcatactagctagttaccttcttcttactcaatgtgtt
ttgcttcgtttcaaggattcttcatatcacttgtggaacaattacatgattaaacattcaacatagagagatagatg
tgttaataagtaaagacattttcagataaaacgttcttatcagtcactttattcttctaatatcctcgttgtaatcg
ggaaaatgctttgtaacgtcaaaaagcaataaaagttgcaggagaaagaaagctttggaaaagaaaataaaataaat
gaagaatattttctttgctagtcacaaaataaatgaagaatttatgttcctaatttcccactagatatttgtttatt
tatttttgccaaaatcaagttaagacaatgagctaagtgttggaaaaccttgtccgagccaaaagagtaaaaagaaa
gggaataaaggggtaaaaccggaaatccgaaaaagaaaaggagaagatttccaaaggagaaaaccctaaagacggag
tatataaacaaggtaacgcgttttctctcagcctctttcggatattccaccagtctctcgcaatcttcgctcttctc
tttgctctctctctctcaacgcggttcagatccgagtttgggagattcaagctccctgaaaaaagccctttactctc
gcttaccaactttgctccaggttcgcatcctatcggattgtctatctattatctcgatgtaacttttacgattcatg
attgtttctttacttttggattgttctgatttgtttttttgtgttttccgatagatttggtttcaactttgttgagt
tgatttggaagttttgatttttcgttacttcaagatcgtttttttttgctgtggaagtgctttcgtttttgttctctt
tattagattctgagctgctgatgatgacgattattaaatttaggatctacacatctgtagatttgttgatgggtttg
tagattttgttacggctaggttagtctctcaatagaatgaaatgaatcgtcttcaaagctcagatgttcttgtctta
acactcacttggttttgtctgattgaatgtttttttttgtaatttggagaaataggttgagtggtgtctatgtttaa
tagataaccatatgtgcctttatacattttctccgcacatcatctgaggcagatattaatgcttgttttcctctctt
tatggacttcgagtttggctttcttgtcacatttcctcctttgcaatcatattattaacgatatcaaaataggtgtt
gtctctcttcctttgatatgaaatctgattgtttttgttctttttatgtagc

Curriculum Vitae

Name	Zhishuo Wang
Post-secondary Education and Degrees:	Western University, London, Ontario, Canada September 2013- present Master of Science Candidate
	Yantai University, Yantai, Shandong, China September 2008- June 2012 Bachelor of Science,
Honors and Awards:	Western Graduate Research Scholarship September 2013- August 2015
Related Work Experience:	Teaching Assistant The University of Western Ontario September 2013- December 2015
Research Affiliate Program:	Agriculture and Agri-Food Canada January 2015- November 2015

Publications:

Wang, Y., **Wang, Z.**, Amyot, L, Tian, L., Xu, Z., Gruber, M. Y. and Hannoufa, A.

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

Wang, Z., Wang, Y., Kohalmi, SE., Amyot. L. and Hannoufa, A. (2015). "SQUAMOSA PROMOTER BINDING PROTEIN LIKE 2 controls floral organ development and plant fertility by directly activating *ASYMMETRIC LEAVES 2* in *Arabidopsis thaliana*." Submitted to *Plant Mol Biol* (Oct 2015).

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.