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Effects of microRNA156 on Flowering Time and Plant Architecture in Medicago sativa

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

EFFECTS OF microRNA156 ON FLOWERING TIME AND PLANT ARCHITECTURE IN *MEDICAGO SATIVA*

(Thesis format: Monograph)

by

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ABSTRACT

MiR156 regulates plant biomass production through regulation of members of *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes. In this study, I investigated function of miR156 in *Medicago sativa* (alfalfa). Alfalfa plants overexpressing alfalfa miR156 and *Lotus japonicus* miR156 were generated, and the miR156 cleavage targets were validated. *In silico* analysis showed that some alfalfa sequence reads (~ 60 bp) are similar to miR156 precursors but the hairpin secondary structure could not be produced from these sequences. Of the five predicted target *SPLs* genes, three (*SPL*6, *SPL*12 and *SPL*13) contain miR156 cleavage sites and their expression was downregulated in transgenic alfalfa overexpressing miR156. These transgenic alfalfa genotypes had reduced internode length, enhanced shoot branching, and elevated biomass. Although alfalfa miR156 had little effect on nodulation and flowering time, *L. japonicus* miR156 reduced nodulation and delayed flowering time (up to 50 days). Our observations imply that miR156 could be employed in improving alfalfa biomass.

Keywords: miR156, *SPL* genes, flowering time, shoot branching, biomass production.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
AGO	Argonaute
Al^{3+}	Aluminum
ANOVA	Analysis of variance
APS	Amonium persulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
CBC	Nuclear cap-binding complex
cDNA	Complimentary DNA
CTAB	Hexadecyltrimethylammonium bromide
CTP	Cytosine triphosphate
DCL	Dicer-Like
DDL	Dawdle
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DEPC	Diethylpyrocarbonate
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
EST	Expressed sequenced tags

Fe(III)EDTA	Ethylenediaminetetraacetic acid iron(III) sodium salt
5' RLM-RACE	RNA ligase-mediated 5′ amplification of cDNA ends
GTP	Guanosine triphosphate
GSS	Genome survey sequences
HTGS	High throughput genomic sequences
HYL1	Hyponasty Leaves 1
HEN1	Hua Enhancer 1
HCL	Hydrogen chloride
HPLC	High-performance liquid chromatography
″	Inches
lb	Pound
M	Molar
mg	Milligram
μM	Micromole
μl	Microliter
MIR	microRNA gene
min	Minute
miRNAs	microRNAs
miR156-OE	microRNA overexpression
ml	Milliliter
mRNA	Messenger RNA
MRE	miRNA responsive element
m^7G	7-methylguanosine

NGS	Next Generation Sequencing
nt	Nucleotide
OE	Overexpression
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
%	Percentage
Pol II	RNA polymerase II
Pol III	RNA polymerase III
Poly A	Polyadenylation A
pri-miRNA	Primary microRNA
pre-miRNA	Precursor microRNA
PTGS	Posttranscriptional gene silencing
qRT-PCR	Reverse Transcription qPCR
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
RNAi	RNA interference
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room Temperature
s	Second
SDS	Sodium Dodecyl Sulphate
SE	Serrate

SDN	Small RNA degrading nuclease
siRNAs	Small Interfering RNAs
SPL	SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE
SPL-OE	*SPL* gene overexpression
TAE	Tris-acetate EDTA
TBE	Tris/Borate/EDTA Buffer
TF	Transcription factor
tRNA	Transfer RNA
Tris	Tris-(hydroxylmethyl)-methylamine
TTP	Thymidine triphosphate
2,4-D	2,4-Dichlorophenoxyacetic acid
TY medium	Bactotryptone-Yeast extract medium
U	Unit
UTR	Untranslated region
UV	Ultra-violet
Vol	Volume
w/v	Weight/Volume

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Importance of alfalfa (*Medicago sativa* L.)

Alfalfa (*Medicago sativa* L.) is a forage legume that is widely grown in North America. It is also known as "Queen of Forages" to dairy producers around the world due to its many benefits to modern agriculture.

Among cultivated crops, alfalfa has the deepest rooting system, which is more than 4.6 m in depth compared to 1.5 – 1.8 m of that in maize (*Zea mays* L.) (Putnam et al., 2001). Vigorous and deep rooting systems are a vital solution in dealing with the problem of soil erosion and contamination in surface and ground water. The deep root and perennial nature of alfalfa also improve water use efficiency; that is, the amount of water required for production of a unit of crop yield, which is an important factor in modern farming where water is a limited resource. As it is a perennial crop, alfalfa also provides permanent vegetation on the farm and enhances soil fertility by adding organic matter to the soil. Long-term growing of alfalfa can contribute to the improvement of soil resource and cropping systems in modern farming.

As a legume crop, alfalfa is able to fix a large amount of atmospheric nitrogen (N_2) and thus has the potential to play an important role in the production of biomass for bioenergy. Depletion of fossil fuels and increase in the price of conventional petroleum products have led some to question the sustainability of current energy resources. To deal with the problems of energy crisis and unprofitable farming systems, the use of alternative and more sustainable biofuel sources is crucial. Alfalfa has the potential to fix around 120-540 lb N_2 per acre per year (Putnam et al., 2001), and this ability of alfalfa to fix atmospheric N_2 allows it to grow in a wide range of soil types without a need for N_2

fertilizers, and also provides a source of soil nitrogen that is very useful for subsequent crops in the crop rotation.

1.2 Area of production and biomass yield

Alfalfa is currently grown on about 30 million hectares (74 million acres) worldwide, which is about 3 million hectares less than the growing areas in the 1970s (Cash, 2009). In Canada, it is grown on about 10 million acres at a value of >$2 billion/year which is about one third of the area of alfalfa forage production in the USA (over 25 million acres at $8.1 billion/year) (http://bldg6.arsusda.gov/campbell.html; http://forages.oregonstate.edu/php/fact_sheet_print_legume.php?SpecID=1). The areas of production declined mainly because of the rise in farming costs, emergence of new pests and diseases, and farmers' preference for grain crops. Although alfalfa is a productive perennial forage legume, the relatively high biomass yield of alfalfa is eclipsed by high yielding grasses, such as miscanthus (*Miscanthus* species) and switchgrass (*Panicum virgatum L.*), which do not fix N_2. For instance, alfalfa yields a biomass of 7.18 - 11.26 tons/acre/year (Volenec et al., 2002), which is about half of the biomass from miscanthus (25 tons/acre/year) (Freedom^{TM} Giant Miscanthus, SunBeltBiofuelsLLC.com). However, forage yield of alfalfa is dependent on variety, irrigation and other farming practices (Ludwick, 2000).

To enhance the economic value of alfalfa as a forage and bioenergy crop, varieties that produce greater biomass from fewer harvests are required. Alfalfa is a cross-pollinated crop meaning that plants produced from seeds of the same plant are heterozygous and the

segregating population creates a diverse genotypic variation in alfalfa (Putnam et al., 2001; http://forages.oregonstate.edu/php/fact_sheet_print_legume.php?SpecID=1). In addition, the polyploidy process further complicates the genomic diversity in this plant species. These characteristics hinder the use of natural breeding and hybridization methods to improve alfalfa varieties.

1.3 Advances in alfalfa breeding

Alfalfa breeding efforts began in the USA during the early 20th century (Barnes et al., 1977), and improved alfalfa genotypes were identified under both government and private breeding programs. This led to the release of cultivars such as 'Atlantic' in 1940, 'Ranger' in 1942 and 'Vernal' in 1953 (Barnes et al., 1977). However, efforts towards improvement of alfalfa using classical breeding are still ineffective.

At least 6 pathogenic organisms cause alfalfa diseases: *Colletotrichum trifolii* causes anthracnose, *Clavibacter michiganese* subsp. *insidiosum* induces bacterial wilt, *Fusarium oxysporum* f. sp. *medicaginis* is a causal agent of fusarium wilt, infection of *Verticillium albo-atrum* leads to verticillium wilt, *Phytophthora megasperma* f. sp. *medicaginis* triggers phytophthora root rot and *Aphanomycetes euteiches* is a disease agent of aphanomycetes crown and root rot (Volenec et al., 2002). The same authors showed that most of the newly released alfalfa varieties are tolerant to diseases that occur in a particular region. However, the cultivars with high disease resistance in a particular region often show disease susceptibility in other regions. This indicates the limitation of disease assessment or poor criteria used to determine the level of resistance. For instance,

alfalfa diseases are usually assessed based solely on the aboveground stand performance. This is often unreliable because cluster of 2 or 3 alfalfa plants can be confused as one (Volenec et al., 2002), and hence the data generated for this trait are inaccurate.

Research on alfalfa improvement has also emphasized identification of the cultivars that are high in winter survival rate. Evaluation of the old cultivars that were released in 1950s and those released in the 1990s revealed that breeding programs aimed at producing greater winter hardiness cultivars are not effective (Volenec et al., 2002). In fact, identification of the cultivars with high winter survival rate is difficult because winter is unique in each location, and as such, temperature, extent of snow cover and duration of winter are different among the growing regions.

Acidity is a major problem that interferes with symbiotic interaction between alfalfa and nitrogen-fixing rhizobia (Papa et al., 1999). Toxicity to aluminum (Al^{3+}) is one of the abiotic stresses that hampers alfalfa production worldwide (Passos et al., 2012). Thus, producing alfalfa genotypes that are tolerant to aluminum is one of the major goals in alfalfa breeding. Devine et al. (1976) produced an alfalfa variety (AT-3) that is tolerant to aluminum. Similarly, GA-AT, aluminum tolerant cultivar was developed from selection of the pre-existing cultivars in the USA (Bouton and Radclinffe et al., 1989). Passoss et al. (2012) assessed the degree of toxicity to aluminum among the alfalfa cultivars and observed that most of the alfalfa genotypes showed sensitivity to Al^{3+} stress except Crioula, Victoria and Alpha-200 cultivars, which were tolerant to Al^{3+} at concentration of 4 mg per liter.

Although winter survival and disease resistance are traits of priority in alfalfa breeding, other traits such as forage yield and quality are often ignored. Assessment of the productivity of alfalfa varieties revealed that forage yield of the newly released varieties (1990s) are not higher than those of the early released varieties (1950s) in first and second harvests (Volenec et al., 2002). However, increase in the yields of the new varieties was observed in the fourth harvest, which is usually performed in early September (Volenec et al., 2002). This indicated that alfalfa improvement programs in the late 20th century were directed at breeding for varieties with reduced fall dormancy and greater winter hardiness. A report on a field trial in California showed that yield averages over the three years for the respective alfalfa varieties were 7.18 to 11.26 tons/acre (Putnam et al., 2010) which is far less than the yields from other forage crops. Hence, much remains to be done to achieve the high-yield goal in alfalfa breeding.

Alfalfa is an outcrossing species (Lesins and Lesins, 1979; Volenec et al., 2002). Although alfalfa flowers can attract pollinators for fertilization, this cross-pollinated nature also contributes to gene flow in alfalfa which is grown for seed production. In addition, flowering unleashes accumulation of lignin, and thus reduces the quality of alfalfa forage. While alfalfa breeding efforts are targeted at improving many other agronomic traits, none are focussed on manipulation of flowering time in alfalfa.

Advances in biotechnology provide many opportunities for plant breeders and researchers to use modern biotechnology tools to improve alfalfa traits. The discovery of microRNA has added a new tool to the arsenal of plant geneticists for the genetic improvement of

crops, including alfalfa (Jiao et al., 2010; Macovei et al., 2012; Sunkar et al., 2012; Wang et al., 2012; Zhou and Luo, 2013).

1.4 Origin of plant microRNA

MicroRNAs (miRNAs), approximately 16-26 nucleotides (nt) in length, are sequence-specific regulators of posttranscriptional gene expression in eukaryotes (Voinnet, 2009; Sun, 2012). Since they were first identified in *Caenorhabditis elegans* (Lee et al., 1993), miRNAs have been extensively studied and characterized in plants (Voinnet, 2009; Sun, 2012).

MiRNAs are highly conserved across species within each of the plant and animal kingdoms but no individual miRNA shares sequence similarity between the two lineages (Axtell and Bowman, 2008). Furthermore, the secondary structure of premature miRNA, organization of its genome, and mode of interaction with targets are not identical between the two lineages (Chapman and Carrington, 2007). It was thus hypothesized that plant and animal miRNAs evolved independently from separate ancestors (Voinnet, 2009). On the other hand, the nature of miRNAs between land plants and green algae favor the common origin hypothesis, which states that plant and animal miRNAs evolved from common ancestors but diversification during evolution resulted in sequence differences between plant and animal miRNAs (Sun, 2012). However, the common origin hypothesis is less likely given that none of the miRNAs identified from the single-cell algae (*Chlamydomonas*) are conserved in multicellular plants (Molnar et al., 2007) and newly evolved plant miRNAs are highly dynamic (Sun, 2012).

Although there is no concrete data to support the evolutionary history of the first miRNAs, studies have provided clues for the origin of newly evolved plant miRNAs. Three models have been proposed to explain the origins of plant miRNAs (Voinnet, 2009). First, it is hypothesized that inverted duplication of target genes in the genome produces double stranded (ds) RNA with perfect base pairing. These dsRNAs are subsequently processed by one of the Dicer-Like (DCL) proteins. Eventually, mutations in the dsRNA create non matching segments in the dsRNA structure, hence creating stem-loop regions which are a unique feature of miRNAs (Voinnet, 2009). Secondly, it is proposed that plant miRNAs evolved from transposable elements. It is highly probable that the abundance of transposable elements and their transposition in the plant genome contribute to the emergence of new miRNA genes (Voinnet, 2009). Finally, it is postulated that miRNA genes are derived from spontaneous evolution of random inverted repeats which are wide-spread in the plant genome. Although there is no proof for the spontaneous model, lines of evidence have been obtained to support the other two models of plant miRNA evolution (Allen et al., 2004; Piriyapongsa and Jordan, 2008; Sun, 2012). It was found that 32 miRNA families in *Arabidopsis lyrata* and *Arabidopsis thaliana* share sequence similarities with their targets in the fold-back regions, thereby supporting the model of inverted duplication of target genes in plant miRNA evolution (Fahlgren et al., 2010).

1.5 Biogenesis of plant miRNAs

Although miRNA biogenesis is well understood in animal miRNAs, this process is still less understood in plants. MiRNAs are encoded in many loci in the intergenic regions of

the plant genome (Zhang et al., 2009). Most eukaryotic *miRNA* genes are RNA polymerase II (Pol II) transcription units, but studies showed that some miRNAs are also transcribed by RNA polymerase III (Lee et al., 2004; Faller and Guo, 2008; Voinnet, 2009). In the process of biogenesis, RNA Pol II transcribes primary miRNA (pri-miRNA), which contains many features of RNA poly II transcripts such as 5' cap and 3' poly (A) tail. pri-miRNA with stem-loop structure is stabilized by RNA-binding protein Dawdle (DDL) before it is processed into premature miRNA (pre-miRNA) in the nuclear processing centre (D-body) (**Figure 1**). Conversion of pri-miRNA to pre-miRNA is orchestrated by functions and interactions of DCL proteins, Hyponasty Leaves (HYL1), Serrate (SE) and Nuclear cap-binding complex (CBC). It is believed that the DCL protein also functions in processing of miRNA-miRNA* (~ 21 nt) from pre-miRNA since plants lack a Drosha-like enzyme (Kurihara et al., 2006).

The miRNA-miRNA* duplex with 2 nt overhanging at 3' ends is methylated by Hua Enhancer 1 (HEN1) to protect it from being degraded by Small RNA Degrading Nuclease (SDN) exonuclease. The miRNA-miRNA* duplex is then exported to the cytoplasm by one of the Exportin 5 ortholog HASTY (Jung et al., 2009; Voinnet, 2009). It is unclear which form of miRNAs is exported to the cytoplasm since the locations of miRNA-miRNA* strand separation and loading into RNAi Induced Silencing Complex (RISC) are elusive in plants. Nonetheless, only a single strand becomes a mature miRNA while the other strand (miRNA*) is eventually degraded by an unknown mechanism (Voinnet, 2009).

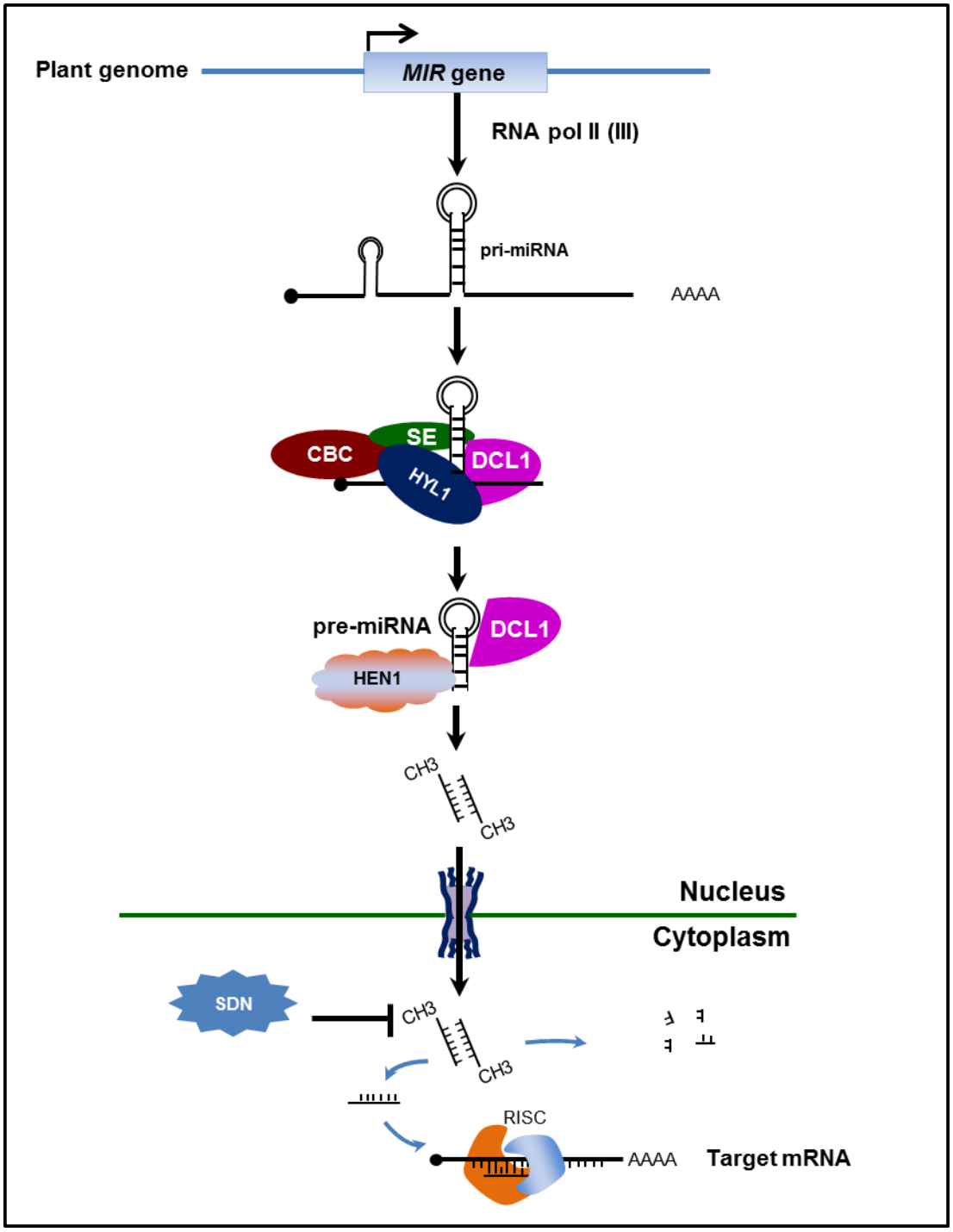


Figure 1. Biogenesis of plant miRNAs. pri-miRNAs are transcribed from endogenous *MIR* genes and processed into mature miRNA to regulate gene expression at posttranscription level. Abbreviations: Dicer-Like; HYL1, Hyponasty Leaves 1; SE, SERRATE; and CBC, Nuclear cap-binding complex. (Others are detailed in text).

1.6 Mechanism and activity of plant miRNAs

Small RNAs are synthesized by RNA dependent RNA polymerase from exogenous RNAgenome or derived from transcription of inverted-repeat encoded in endogenous loci (Sun, 2012). Upon dicing and modifying by effector proteins, the small RNAs can be engaged in transcriptional gene regulation by modifying chromatin activity in the nucleus, or they are exported to the cytoplasm to induce posttranscriptional gene expression (PTGS) (Phillips, 2008).

During biogenesis, plant miRNAs share similarity with animal miRNAs as miRNAs in the two kingdoms are excised from stem-loop precursors (Voinnet, 2009; Sun, 2012). In terms of target recognition and function, however, plant miRNAs resemble small interfering RNA (siRNA) as both entities employ perfect or near-perfect complementary modes to scrutinize their targets (Rhoades et al., 2002). In contrast, animal miRNAs use imperfect complementary mode to scan the targets, and as such, an individual animal miRNA regulates dozens of targets due to the relaxed base-pairing requirement with the targets (Voinnet, 2009). Nonetheless, plant miRNAs must be perfectly or near perfectly complementary to their targets to regulate gene expression (Rhoades et al., 2002; Gandikota et al., 2007). Generally, plant miRNAs recognize their targets in the region of miRNA responsive elements (MRE) which are located in the coding regions (Bartel, 2004; Jones-Rhoades and Bartel, 2004), 5′ untranslated region (UTR) (Allen et al., 2005; Chiou et al., 2006) or 3′ UTR (Rhoades et al., 2002; Sunkar and Zhu, 2004) of the targets.

MiRNA induced PTGS is accomplished by the functions and interactions of Argonaute (AGO) proteins in RISC and other unknown factors (Voinnet, 2009; Sun, 2012). The

miRNA itself has no ability to cleave mRNAs or interfere with translation of the targets but it plays roles in scanning the appropriate target. To be functional, mature miRNA is incorporated into RISC containing one member of the AGO protein family. The AGO protein contains a small RNA-binding PAZ domain, and PIWI domain with catalytic residues which confer endonucleolytic activity to that RISC (Voinnet, 2009). Upon binding to the appropriate target, RISC induces cleavage of the target transcripts (Voinnet, 2009). As cleavage usually occurs in the middle of the target transcripts, it was suggested that RISC cleaves the target transcripts in the region where miRNA and targets are complementary. However, recent studies have shown that cleavage sites can also be found beyond these miRNA complementary regions (Kim et al., 2012; Wang et al., 2014).

In addition to cleaving the target, plant miRNAs can also regulate gene expression through translational inhibition of the target (Sun, 2012) though it was previously thought that this mechanism was only exploited by animal miRNAs. Although the mechanism of translational repression is unknown, it was suggested that RISC presumably interferes with 7-methylguanosine (m^7G) cap functions in recruiting eukaryotic translation initiation factor 4E (eIF4E) during translation (Pillai, 2005). Gandikota et al. (2007) observed that sequence binding between miR156 and miR157 and their targets, *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* 3 (*SPL*3) gene at MRE region renders translational repression rather than degradation of the transcript (Gandikota et al., 2007). Similarly, Chen, (2004) showed that miR172 regulates the expression of floral homeotic gene, *APETALA2*, through translational inhibition of the transcript. It is thus evident that plant miRNAs regulate gene expression through cleavage or translational repression of

the targets. It is also believed that plant miRNAs employ both mechanisms to regulate gene expression; nevertheless, prevalence of one mechanism based on the position or degree of base paring between plant miRNAs and their targets is unpredictable based on current knowledge in the field.

1.7 Identification and annotation of plant miRNAs

Initially, the discovery of a large number of animal miRNAs inspired researchers to search for miRNAs in the model plant *Arabidopsis*. Since plant miRNAs were identified in *Arabidopsis* in 2002 (Llave et al., 2002; Reinhart et al., 2002), miRNA research has become one of the hottest fields in plant biology (Sun, 2012). To deal with a growing number of plant miRNAs, researchers suggested uniform criteria for their annotation and characterization (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2008). Distinction between miRNAs and siRNAs in plants is narrow as both entities are similar in their origins and modes of operation. The formation of stem-loop structures and excision of miRNA-miRNA* duplex from pre-mature miRNA is the only step that differentiates miRNAs from siRNA, and hence these features are used to annotate new miRNAs (Meyers et al., 2008).

Currently, more than 3070 plant miRNAs are deposited in miRNA databases (Sun, 2012). These miRNAs are identified from 43 plant species which include 10 species from monocotyledons, 28 from dicotyledons, two from conifers, two from moss and one from green algae (Sun, 2012). Several approaches have been used to identify plant miRNAs. Direct cloning was the first approach employed in identification of plant miRNAs. In this method, small RNAs (~80 – 300 bp) are directly cloned and sequenced. However, it is

not easy to identify which RNA sequences are miRNAs since there are many species of small RNAs in plants. During the last decade, intensive research in miRNAs provided a better understanding of their mechanism and biogenesis. This together with the guidelines proposed for miRNA annotation (Ambros et al., 2003) enabled a direct cloning approach which is successfully used to identify miRNAs in plants. Using the cloning approach, more than 100 miRNAs were initially identified from *Arabidopsis* (Llave et al., 2002; Reinhart et al., 2002). Although cloning is a time-consuming process, the possibility of false positives, cost and technology demands are lower than deep sequencing technology.

The development of next generation sequencing technology has largely expanded the number of plant miRNAs in databases. Deep sequencing became a powerful approach in miRNA research because it is capable of producing millions of sequence reads in a single experiment, and hence, it can recover a substantial fraction of lower abundance miRNAs (Jones-Rhoades, 2012). Moreover, deep sequencing is more straightforward than traditional sequencing technique in analyzing the potential miRNAs (Rajagopalan et al., 2006). Since it was first employed in identification of plant small RNAs in 2005, deep sequencing technology (both 454 and Illumina) has been extensively used in the field of plant miRNAs (Lu et al., 2005; Fahlgren et al., 2007; Zhou et al., 2010; Jones-Rhoades, 2012).

Identification of miRNAs in *Arabidopsis* led to the discovery that many miRNAs are highly conserved across plant species (Jones-Rhoades and Bartel, 2004; Sunkar et al., 2005; Zhang et al., 2005). This provided a powerful approach to identify new miRNAs

using known miRNAs from other plants. Through identifying homologus sequences in EST databases, Zhang et al. (2005) annotated a total of 481 miRNAs from a wide range of plant species. Recently, Li et al. (2012) developed a web server (SoMART) for identification and characterization of plant miRNAs and their target genes. Likewise, Muñoz-Mérida et al. (2012) developed a web server (Semirna) to search for plant miRNAs with high level of accuracy using target sequences. Although experimental validation is needed, the bioinformatics approach is straightforward and demands lower cost.

1.8 Identification and annotation of targets of plant miRNAs

Identification of miRNA targets is a challenging but necessary step to understand the function of miRNAs and miRNA-mediated gene regulation. MiRNAs play important roles in almost all biological and metabolic processes in plants (Voinnet, 2009; Sun, 2012), which indicates that miRNAs regulate these developmental stages by targeting one or more genes controlling these processes.

To be considered as a putative target, it was suggested that nucleotide positions 10^{th} and 11^{th} in the miRNA sequence must perfectly match to the target sequence (Joshi et al., 2010). In addition, any mismatch with miRNA should not be more than one in the positions between 2^{nd} and 9^{th} nucleotides of the miRNA sequences (Joshi et al., 2010). However, these criteria should be revised as variations have been found in newly identified miRNAs across plant species. Naya et al. (2010) found that a specific isoform of miRNA156 (miR156) in root apexes of *Medicago truncatula* cleaved non-conserved

target *WD40* genes, where the mismatch between the miR156 and its target was more than four nucleotides.

Several methods have been developed and used to identify targets of plant miRNAs. Plant miRNAs employ sequence complementary mechanism to scan their target transcripts. This provides a sound approach to predict the targets of plant miRNAs using publicly available databases. Currently, four tools are available for prediction of targets of plant miRNAs: miRU (Zhang, 2005), Helper tools (Moxon et al., 2008), TAPIR (Bonnet et al., 2010) and Target-align (Xie and Zhang, 2010). These tools were developed based on available databases such as genome survey sequences (GSS), high throughput genomic sequences (HTGS), genomic sequences and EST deposited in Genbank. Based on sequence complementary to target, Rhoades et al. (2002) predicted the targets of 14 miRNA families and found 49 targets, most of which are involved in developmental patterning or cellular differentiation in *Arabidopsis*.

So far, 5′ RNA ligase-mediated amplification of cDNA ends (5′ RLM-RACE) is the most successful method used to predict the cleavage targets of plant miRNAs *in vivo*. Plant miRNAs regulate gene expression through the cleavage of the target transcripts. The miRNA cleavage generates 3′ end products with 5′ monophosphates that can be detected using 5′ RLM-RACE in which RNA adapters are ligated to the cleavage products followed by RNA reverse transcription and PCR amplification (Llave et al., 2002). Other approaches such as bioinformatics, transcript profiling and sequencing of 3′ end targets are not frequently used because they may produce false results in many experiments (German et al., 2008; Kawashima et al., 2008; Voinnet, 2009). To validate the targets of

plant miRNAs, the outcomes from any approaches must be supported by experimental evidences such as cloning, expression analysis and characterization of the changes in plant morphological traits.

1.9 Plant miRNAs regulate members of transcription factors

The majority of plant miRNAs regulate the genes encoding for transcription factor (TF) though some are also involved in regulation of other genes functional in plant immune response, gibberellins signalling and fungal toxin. It is unclear why miRNAs preferentially target TFs but these two types of regulators share common actions in regulation of their target genes.

Transcription factors (TFs) and miRNAs are the largest families of gene regulatory molecules in multicellular organisms (Hobert, 2004; 2008). While TFs regulate gene expression at the transcriptional level, miRNAs act at the posttranscriptional level. However, these regulators are similar in terms of pleiotropic effects, requirement for effector complexes and degree of accessibility to their binding sites in the targets. Individual TF and miRNA regulate dozens of target genes, and a number of individual genes are found to be regulated by many TFs and miRNAs (Hobert, 2008; Joshi et al., 2010). Although TFs are functional proteins, they still require cofactors and enhancers to bind to the target DNA sequences and operate the functions. Similarly, miRNAs employ RISC to scan the target sequences and regulate gene expression. Hence, both TFs and miRNAs require combinatorial and cooperative activity from effector complexes. In addition, both regulators are restricted to their target binding sites. Degree of

accessibility of TFs to binding sites largely depends on the coverage of nucleosome on the DNA sequences whereas messenger ribonucleoprotein particle (mRNPs) and secondary structure of mRNA restrict the binding of miRNAs to their targets (Hobert 2004; 2008).

Studies have shown that most plant miRNA targets are TFs that play crucial roles in plant development. Of the 46 genes predicted to be targets of new miRNAs in rice, 16 encode transcription factors (Sunkar et al., 2005). Likewise, Zhang et al. (2009) found that 12 out of 26 miRNA families in maize regulate transcription factor genes. In soybean (*Glycine max*), Joshi et al. (2010) identified 603 targets for 78 miRNAs in which 174 are transcription factors. Recently, Sun, (2012) summarized a list of miRNAs and validated targets with their known functions in plants. The functions of most of these plant miRNAs targets are involved in cell fate and embryonic development, leaf morphogenesis, shoot branching, root branching, flowering time, signal transduction, response to biotic and abiotic stresses (Sun, 2012).

1.10 The plant miRNA regulated root symbiosis

In plants, root symbiosis depends on a complex dialogue between rhizobia and plant roots (Bazin et al., 2012). Successful symbiotic interactions require the repression of pathogenic defense genes in plants. Analysis of genome-wide small RNA in *M. truncatula* uncovered a number of small RNAs that are spatially enriched in nodule meristematic tissues, vascular bundles and zones of bacterial infection (Lelandais-Brière et al., 2009). Overexpression of miR166a, which targets class III HD-ZIP transcription

factors, reduced the number of nodules and lateral roots in *M. truncatula* roots (Boualem et al., 2008; Bazin et al., 2012). Regulation of transcription factor CCAAT-binding family (MtHAP2-1) by miR169a delayed the processes of nodulation and affected nodule growth (Combier et al., 2006; Boualem et al., 2008). Moreover, miR171 regulates transcription the factors MtNSP2 in *M. truncatual* and LjNSP2 in *L. japonicus* that are involved in Nod-factors signalling for root symbiosis (De Luis Margarit, 2010; Branschied et al., 2011). Overexpression of miR156 also caused a reduction in nodule numbers in *L. japonicas* (Wang et al., 2014).

In contrast, miRNAs involved in regulation of plant defense responses promote root symbiosis. For instance, Li et al. (2010) demonstrated that transgenic soybean (*Glycine. max*) expressing miR482, miR1512 and miR1515 significantly increase the number of nodules while root length and later root density are not altered. The authors also showed that miR482 cleaves two predicted targets; GSK3-like protein kinase (encoding regulator of plant immunity) and TIR-NBS-LRR encoding disease resistant protein. The cleavage of TIR-NBS-LRR by miR482 is also found in *M. truncatula* roots and seedlings (Jagadeeswaran et al., 2009; Devers et al., 2011). It is therefore evident that root symbiosis in legumes is orchestrated, at least in part, by miRNA-dependent gene regulation.

1.11 The miR156 regulated *SPL* genes control plant development

SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) genes are plant specific transcription factors (Yamasaki et al., 2004; Birkenbihl et al., 2005) that play important

roles throughout the different stages of plant development (Martin et al., 2010a; Yu et al., 2010; Gandikota et al., 2007; Manning et al., 2006). MiR156 regulates 10 out of the 16 members of *SPL* genes in Arabidopsis, whereas it targets 11 members of the 19 *SPLs* in rice (Xie et al., 2006; Nonogaki, et al., 2010). Structure and functions of most of these *SPL* genes are well documented in *A. thaliana* (Cardon et al., 1997; 1999).

SPL genes are structurally diverse, and encode putative transcription factors. The distinguished characteristic of *SPL* family members is the SBP-box encoding SBP-domain that functions in interaction with DNA (Cardon et al., 1999). This SBP-box encodes a conserved protein domain with a length of 76 amino acids. Apart from this domain, *SPL* genes are highly variable in genomic organization, transcript size, position of introns, size of exons and amino acid sequences of the proteins. For instance, *Arabidopsis SPL*3 encodes 131 amino acids whereas the peptide size of *SPL*12 is 927 amino acids (Cardon et at., 1999). Numeric suffixes of the *SPL* genes merely indicate the chronology of their isolation from the same plant species.

Regulation of members of *SPL* genes by miR156 is observed in many plant species, including *Arabidopsis* (Xing et al., 2010), rice (Jiao et al., 2010), potato (Bhogale et al., 2014), maize (Mica et al., 2006) and tomato (Manning et al., 2006; Zhang et al., 2011). In plants, miR156 induces a range of phenotypic variations through regulation of these *SPL* genes. These variations include plant height, number of shoots, leaf morphology, trichome density, stem thickness, biomass production, grain yield and stress responses (Sunkar et al., 2012; Zhou and Luo, 2013). Recently, Padmanabhan et al. (2013) showed that *SPL*6 is a novel positive regulator in Nucleotide Binding-Leucine Rich Repeat (NB-

LRR) receptor-mediated plant innate immunity in *Arabidopsis.* In their work, the authors merely addressed the role of *SPL*6 in the plants infected by *Pseudomonas syringae* but the relationship between miR156 and *SPL*6 was not documented. According to Martin et al. (2010a; 2010b), *Arabidopsis* overexpressing *SPL*13 that is mutated in the region complementary to miR156-conserved sequence over-accumulate the miRNA-resistant transcripts, and show a delay in the production of vegetative leaves. Hence, miR156-targeted *SPL*13 is a negative regulator of emergence of vegetative leaves at the cotyledon stage (Martin et al., 2010a; 2010b).

Regulatory networks between miR156 and *SPL* genes are also critical for the production of lateral organs and transition from vegetative to reproductive phases. *Arabidopsis* mutants with loss-of-function of *SPL*9 and *SPL*15 showed a delay in juvenile-to-adult phase transition and a shortened plastochron (Schwarz et al., 2008). In addition, these mutants produced more shoot branches and altered inflorescence structure compared to wild type. These phenotypes are similar to the transgenic *Arabidopsis* overexpressing miR156b with downregulated *SPL*9 and *SPL*15 transcripts (Schwarz et al., 2008). Moreover, Shikata et al. (2009) found that the production of lateral organs and shoot maturation in the reproductive stage are regulated by miR156 targeted *SPL*2, *SPL*10 and *SPL*11. The authors demonstrated that transgenic plants expressing a dominant repressor for *SPL*2, SPL10 and SPL11 and loss-of-function mutants for *spl2, spl10* and *spl11* showed similar phenotypes, suggesting that these *SPL* genes are functionally redundant in the control of such morphological traits (Shikata et al., 2009). Likewise, *SPL*3, *SPL*4 and *SPL*5 function redundantly in controlling flowering time in *Arabidopsis*. Wu and Poethig, (2006) found that overexpression of miR156 reduces the transcripts levels of *SPL*3, *SPL*4

and *SPL*5 with a prolonged vegetative stage and delayed flowering time. In contrast, *Arabidopsis* overexpressing *SPL*3, *SPL*4 and *SPL*5 with mutated miR156 recognition region accumulate high levels of these *SPL* transcripts and show an early flowering phenotype, thereby validating the regulatory roles of these *SPL* genes in the vegetative to reproductive phase transition and flowering time (Nonogaki, 2010).

Effects of miR156/SPL-gene regulation were also found in many other plant species, including *Oryza sativa* (Xie et al., 2006), *Lotus japonicus* (Wang et al., 2014), *Brassica napus* (Wei et al., 2010), *Panicum virgatum* (Fu et al., 2012), and *Solanum tuberosum* ssp. *andigena* (Bhogale et al., 2014). For instance, expression of *O. sativa* miR156 in switchgrass (*P. virgatum*) enhanced apical dominance and delayed flowering time leading to an increase in biomass production in the transgenic switchgrass with the degree of morphological alterations in the transgenic plants largely dependent on the level of miR156 expression (Fu et al., 2012). Furthermore, a significant increase in biomass production and a two-month delay in flowering time were observed in transgenic *L. japonicus* overexpressing miR156 (Wang et al., 2014).

Apart from plant growth and development, effects of miR156/SPL-gene regulatory network are also observed in plant secondary metabolism. Constitutive expression of *Arabidopsis* miR156b in *B. napus* enhanced levels of the carotenoids lutein and β carotene in the seed (Wei et al., 2010). Moreover, increased carotenoid content was also found in *Arabidopsis* activation-tagged mutant, *sk156.* The *sk156* mutant was developed by incorporating four CaMV35S enhancers in the T-DNA insert to enhance the expression of miR156b. Enhanced expression of miR156b results in downregulation of

*SPL*15 and increase in the amount of carotenoids (Wei et al., 2012). Besides, overexpression of miR156 augments the accumulation of anthocyanin in *Arabidopsis* (Gou et al., 2011). These authors demonstrated that miR156-targeted *SPL*9 reduces the anthocyanin accumulation by regulating the downstream anthocyanin biosynthetic genes. It is thus obvious that miR156 plays crucial roles in many aspects of plant development and metabolism through regulation of the target *SPL* genes.

1.12 Scope of the research project

MiR156 is reported to cleave transcripts of target *SPL* genes, which in turn causes delayed flowering time, enhanced shoot branching, and increased seed carotenoid content (Gandikota et al., 2007; Wei et al., 2010; Fu et al., 2012; Wei et al, 2012). Since miR156 is evolutionarily and functionally conserved, I hypothesized that these effects would also be observed in alfalfa. To test my hypothesis, an *in silico* search was first conducted to determine the number of loci encoding miR156 paralogs in alfalfa. Alfalfa plants overexpressing miR156 (miR156-OE) were generated. The miR156 cleavage of *SPL* targets was then determined using the 5'-RACE technique. Alfalfa plants with RNAi-silenced target *SPL* genes (SPL-RNAi) were generated. It was expected that target *SPL* gene transcripts would be downregulated in SPL-RNAi alfalfa plants. Furthermore, SPL overexpression alfalfa plants (SPL-OE) were generated. Finally, the effects of miR156 on alfalfa were investigated by observing and comparing changes in morphological traits among miR156-OE and empty vector control.

The effects of miR156 on root architecture and nodulation have been observed in the model legume *Lotus japonicus* (Li et al., 2010; Wang et al., 2014). Hence, I also expected altered root morphology in miR156-OE, SPL-OE, and SPL-RNAi plants. The impact of miR156 on nodulation was evaluated by inoculating alfalfa with *Sinorhizobium meliloti* tagged with *Lac Z*. The number of nodules was counted and nodule development was analyzed through β-galactosidase activity as described in Yano et al., (2006). Finally, I expected that enhanced shoot branching and nodulation would improve alfalfa plant as a forage and biofuel crop.

1.13 Hypothesis and Objectives

I hypothesize that miR156 controls flowering time, shoot branching and nodulation in alfalfa by regulating target *SPL* genes. The objectives of this study are:

- To generate miR156 overexpression alfalfa genotypes
- To identify miR156-targeted *SPL* genes in alfalfa
- To investigate the effects of miR156/SPL on flowering time and plant architecture in alfalfa
- To study the impact of miR156/SPL on nodulation in alfalfa

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Wild type (WT) *M. sativa* ecotype N442 was obtained from Dr. Dan Brown (AAFC-London).WT and transgenic alfalfa plants generated in this study were maintained in a greenhouse at 25-27°C with a photoperiod of 16 hours per day.

2.2 *In silico* search for miR156 paralogs in *M. truncatula* and *M. sativa* genomes

To determine the number of loci encoding miR156 paralogs in *M. truncatula* and *M. sativa* genomes, an *in silico* search was conducted using publicly available sequences and guidelines from a previous research article (Meyers et al., 2008). First, all miR156 precursor sequences from different plant species were retrieved from miRBase (Griffiths-Jones et al., 2008). These precursors were used to conduct a BLAST search against *M. truncatula* and *M. sativa* sequences in the NCBI database. In addition, the precursor sequences were also used in a BLAST search against Next Generation alfalfa RNA sequence reads by Dr. Ryan Austin (AAFC-London). The *M. truncatula* and *M. sativa* sequences that were similar to the precursors (expectation, e-value: 1e-34) were extracted and used to predict RNA secondary structure using miRTour (Milev et al., 2011). Only the sequences that were predicted to produce hairpin secondary structures were considered as potential miR156 precursors.

2.3 Primer design

Gene-specific sequences were retrieved from the sequence database of the closely related species, *M. truncatula*, and primers used in this study were initially designed based on *M. truncatula* sequences. To predict miR156-target *SPL* genes, gene-specific primers were designed in the regions flanking miR156 binding sequences. Primers flanking full length *M. truncatula SPL* sequences were used to isolate the corresponding *M. sativa SPL* genes to generate the overexpression constructs (SPL-OE). To obtain SPL fragments for RNAi constructs, *M. sativa SPL* genes were first isolated and sequenced. The *SPL* sequences were then aligned and the primers unique to each *SPL* gene were designed. Using these primers, unique *M. sativa SPL* sequences (250 bp) were amplified to generate SPL-RNAi constructs specific to the 3'-end of each gene. All of these primers were designed using OligoCalc (Oligonucleotide Properties Calculator) available at http://www.basic. northwestern.edu/ biotools/OligoCa lc.html.

To test the expression of *SPL* regulated downstream genes, *AP3* (*APETALA3*), and *FT* (*FLORAL LOCUS T*), I searched for gene-sequences in the *M. truncatula* databases and gene-specific primers were designed as described above for use in alfalfa.

To design primers for quantitative real-time RT-PCR (qRT-PCR), *M. sativa* gene sequences were first isolated using primers that were designed based on *M. truncatula* sequences. The *M. sativa SPL* sequences were then used to design gene-specific primers for qRT-PCR using primer BLAST (http://www.ncbi.nlm .nih.gov/tools/primer-blast/) and IDT (UNFOLD) website (https://www.idtdna.com/UNA Fold?) to ensure the primers were neither self-complementary nor produce secondary structures. All the primers used in this study are listed in **Table** 1**.**

Table 1. List of primers used in this study

Name	Sequence (5' to 3')	Product size (bp)[α]	Target gene
SPL6-R2 (Inner)	TGAGCTTGTCATCAATGGTGT	142	*SPL*6
SPL6-R3 (Outer)	TGAAACAGATCAACAGTGCCA	327	*SPL*6
SPL12-R2 (Inner)	CGAGAACGAGATACAGGCACT	181	*SPL*12
SPL12-R3 (Outer)	CTGAACAGCTGAAAGTCTTGA	583	*SPL*12
SPL13-R2 (Inner)	CCATGGACTCAAAGCTATTGT	134	*SPL*13
SPL13-R3 (Outer)	TGCCTTGTGATCCATCAGAAC	604	*SPL*13
SPL10-R2	GACCAAGATCAGGGACAACCT	170	*SPL*
SPL10-R3	TATAATGCCTCCTTCCCTGCT	515	*SPL*
SPL3-R2	GTATGAGGACATGAAAGAAAC	151	*SPL*
SPL3-R3	GGGACATTTGATCACTACTCT	543	*SPL*
RNAiMsSPL6-F2	CACCCATATCAAACTGGAAGAGGAC	246	*SPL*6
RNAiMsSPL6-R	TCGTTCATGTTTATTCGCAGAGCT	246	*SPL*6
RNAiMsSPL12-F2	CACCACAGGTCTAGAAGATCCAA	250	*SPL*12
RNAiMsSPL12-R2	CGAGAACGAGATACAGGCACT	250	*SPL*12
RNAiMsSPL13-F2	CACCTGACAACAACAATCCCTCA	249	*SPL*13
RNAiMsSPL13-R2	CCATGGACTCAAAGCTATTGT	249	*SPL*13
OEMsSPL6-F	CACCATGGAATCTTGGAGTTATAT	1429	*SPL*6
OEMsSPL6-R	TTACACGTTTGAGAAACAACAATC	1429	*SPL*6
OEMsSPL6-F2	CACCATGGADTCTTGGAGYYAYR	1429	*SPL*6
OEMsSPL6-R2	TYAMRYRKTWGRRAAACARY	1429	*SPL*6
OEMsSPL12-F2	CACCATGGAGTGKAAYGYRAAAKCT	1387	*SPL*12
OEMsSPL12-r2	TYARTYCARYTGRYYRSAMGRRA	1387	*SPL*12
OEMsSPL12-F	CACCATGGAGTGGAACGTGAAATC	1387	*SPL*12
OEMsSPL12-R	TTAATCCAGCTGGTTGCAA	1387	*SPL*12
OEMsSPL13-F	CACCATGGAGTGGAATTTGAAAGC	1135	*SPL*13
OEMsSPL13-R	CTATTCCCATTGATAGGGAAATAGT	1135	*SPL*13
Ms-SPL6Fq1	CTCGGCCGATACATCAAACTG	191	*SPL*6
Ms-SPL6Rq1	CCTCTGTTCAACACCATTGACG	191	*SPL*6
Ms-SPL12Fq1	CCTCAGCCTGAAGCAGTGAA	174	*SPL*12
Ms-SPL12Rq1	CTTGCTGTTGGGCATGTCTG	174	*SPL*12
MsSPL13rtpcr-F	CACTTCATAGCCAAACCACACCTCTT	170	*SPL*13
MsSPL13rtpcr-R	AGGGCTGCATAAGAGACATTGAATGA	170	*SPL*13
WD1-R1 (Inner)	ACAAGTGTGCAACTGCTGTT	160	*WD*1
WD1-R2 (Outer)	AACGGACTAGTAAAGAGACA	160	*WD*1
WD2-R1 (Inner)	TGTCCTCATACGTAGTTCGA	165	*WD*2
WD2-R2 (Outer)	AAGCCATGAAATGAACAGCT	165	*WD*2
WD3-R1 (Inner)	AGTAGGAGCTACATGCCCAA	170	*WD*3
WD3-R2 (Outer)	TCCTTGGAAACCACCAGCAA	170	*WD*3
WD4-R1 (Inner)	TCATCCTCTACATCCTCCAT	160	*WD*4
WD4-R2 (Outer)	ATATTCATCGACGTCAGACT	160	*WD*4

Table 1. List of primers used in this study (continued)

Name	Sequence (5' to 3')	Product size(bp) [α]	Target gene
WD5-R1 (Inner)	CAAAGCTAACACCTGGTCGA	175	*WD*5
WD5-R2 (Outer)	AATTTCAATGGCATTGGTGA	175	*WD*5
WD6-R1 (Inner)	CTAAATACCTAGGTGAATCGT	160	*WD*6
WD6-R2 (Outer)	TCAATAATCACGGTACCAATA	160	*WD*6
Mt2139F11-156bF	CACCACTCCACATAGTTTCCAGTTTTG	298	MtmiR156b
Mt2139F11-156bR	GTAGTAGGGAGTAGCGGTGATCTTGC	298	MtmiR156b
Ms_156Fq3	TGTTCCCATTCATCACCTCCAA	80	MsmiR156
Ms_156Rq3	AGGGAGTAGCGGTGATCTTG	80	MsmiR156
Lj-MIR156b-Fq	TCTGATGAAGGAGCTGAACAACAC	95	LjmiR156
Lj-MIR156b-Rq	AATAAGCGCCAAAGCAACAGTAG	95	LjmiR156
BA-Acc1-F	GATCAGTGAACTTCGCAAAGTAC	91	Acetyl CoA
BA-Acc1-R	CAACGACGTGAACACTACAAC	91	Acetyl CoA
BA-Acc2-F	GATCAGTGAACTTCGCAAAGTAC	154	Acetyl CoA
BA-Acc2-R	GAGGGATGCTGCTACTTTGATG	154	Acetyl CoA
18SrRNA-F	AAGGAATTGACGGAAGGGCACCA	120	18SrRNA
18SrRNA-R	TAAGAACGGCCATGCACCACCA	120	18SrRNA
Lj-bactin-Fq	GCATTGTTGGTCGTCCTCGT	79	Actin
Lj-bactin-Rq	TGTGCCTCATCCCCAACATA	79	Actin
MtAP3F2	TCTGGTGCCAATTTCGGTGG	88	*AP*
MtAP3R2	GAGGGGACTAATCTCACCGC	88	*AP*
MtFTa1F2	ACCTAGCCCAAGTAACCCCA	120	*FT*
MtFTa1R2	GTGGGTCGTGGCCTTTCATA	120	*FT*
MtFTa2F1	GCGTAAATGACCCAACAGCC	123	*FT*
MtFTa2R1	GCCGCATTAGTTGCTGGAAT	123	*FT*
MtFTb1F1	TCCAGCGACTACAGGGACAA	85	*FT*
MtFTb1R1	CTGTGTGCCTACAAGGTTGC	85	*FT*
pJET-F	CGACTCACTATAGGGAGAGCGGC		pJET vector
pJET-R	AAGAACATCGATTTTCCATGGCAG		pJET vector
35S-F3	CAATCCCACTATCCTTCGCAAGACCC		35S Promoter
M13 Forward	GTAAAACGACGGCCAGT		Universal
pJET1.2	CGACTCACTATAGGGAGAGCGGC		pJet vector
NOS-R2	ATAATCATCGCAAGACCGGCAAC		pBI121 vector
pMDC32 control	TCATCAGGCGGGCAAGAATGTGAATA		pMDC32 vector
pHELLGATE12intron	TAGACACACGAAATAAAGTAATCA		Phellgate vector
miR156-probe	TGACAGAAGAGAGTGAGCACCCTGTCTC	21	miR156

[α] PCR products for Outer and Inner primers are based on the primer combination with the primers from FirstChoice® RLM-RACE Kit (Ambion, USA). PCR product for cloning vectors are not shown.

2.4 Primer test

Since most of the primers used in this study were initially designed based on *M. truncatula* sequences, the specificity of these primers in *M. sativa* was tested using PCR and sequencing approaches. Using these primers, *SPL* gene-specific sequences were amplified from *M. sativa* genomic DNA. Following PCR amplification, standard electrophoresis was used to analyze PCR products in which 10 µl of the amplified products were run on a 1% agarose gel and visualized under UV light. PCR products with expected sizes were then isolated and purified using a gel purification kit (Qiagen, Canada) and cloned into pJET1.2/blunt cloning vector according to the manufacturer's instructions (Fermentas, Canada). Colonies were screened by colony PCR with gene-specific primers and positive clones were sequenced using M13 forward primer**.**

2.5 Isolation of miR156 precursors

This experiment was performed with help from Lisa Amyot (Technician) and Ying Wang (M.Sc. student) in Dr. Hannoufa's laboratory. To isolate miR156 paralogs in alfalfa, total RNA was extracted from shoots of wild type *M. sativa* (N442) using Trizol reagent. The cDNA was synthesized using SuperScript^{TM}III First-Strand Synthesis System for RT-PCR (Life Technologies) following the manufacturer's instruction. A fragment of the gene sequence encoding miR156 precursor (~300 bp) was then amplified from the alfalfa cDNA template using gene specific primers (Mt2139F11-156bF and Mt2139F11-156bR) (Table 1) that were designed based on *M. truncatula* miR156 sequence (Accession: CU019603). The isolated gene sequences were confirmed by sequencing and used to search for paralogs in publicly available *M. sativa* databases. Similarly, a precursor from

L. japonicus was isolated using the primers that were designed based on *LjmiR156* gene sequence (Wang et al., 2014). Both *M. sativa* miR156 (MsmiR156) and *L. japonicus* miR156 (LjmiR156) precursors were used to overexpress in alfalfa**.**

2.6 Generation of vector constructs

Cloning of miR156 precursors into overexpression vectors was also conducted with help from Lisa Amyot and Ying Wang in Dr. Hannoufa's laboratory. To generate MsmiR156 and LjmiR156 overexpression constructs (MsmiR156-OE and LjmiR156-OE), fragments of the respective miR156 precursors were amplified from the cDNA template using platinum® Taq DNA Polymerase High Fidelity (Life Technologies). PCR products with expected sizes (~300 bp) were purified using a gel purification kit (Qiagen, Canada) and cloned into pENTR/D-TOPO entry vector (Invitrogen). The entry clones were verified by colony PCR with M13 forward and gene specific primers (reverse: mt2-139F11-R for MsmiR156 and Lj-MIR156b-Rq for LjmiR156) (Table 1) and confirmed by sequencing. The cloned MsmiR156 was then sub-cloned into the pBINPLUS expression vector downstream of 35S promoter between the *BamHI* and *SacI* restriction sites (insert replaced the *GUS* gene in the vector). The cloned LjmiR156 was sub-cloned into pBI121 vector replacing the GUS gene as described in Wang et al. (2014). Expression clones for MsmiR156 and LjmiR156 were checked by colony PCR using a 35S promoter primer (forward) and gene-specific reverse primers (Table 1) and confirmed by sequencing. The expression clones were then transferred to *Agrobacterium tumefaciens* (LBA4404) competent cells using the freeze thaw method. Again positive clones were confirmed by

colony PCR using respective primers (35S promoter and gene-specific primers, Table 1). The generated *A. tumefaciens* strains were then used for alfalfa transformation. *A. tumefaciens* strains harboring empty vectors were used to generate the empty vector control alfalfa**.**

To study the functions of miR156 target *SPL* genes in alfalfa, the *SPL* genes were manipulated by overexpression and downregulation in alfalfa. Transgenic alfalfa overexpressing SPL-OE and SPL-RNAi constructs were generated, and used to compare with the miR156-OE and empty vector control.

To generate SPL overexpression constructs (SPL-OE), total RNA was extracted and cDNA was synthesized as described above. Then, full-length *SPL* genes were amplified from the cDNA template using platinum® Taq DNA Polymerase High Fidelity (Invitrogen). PCR conditions were 1 cycle at 98˚C for 30 sec, then 35 cycles at 98˚C for 30 sec, 55˚C for 45 sec, 72˚C for 1 min followed by final extension at 72˚C for 10 min. PCR products with expected sizes (1.3 - 1.5 kb) were purified using a gel purification kit (Qiagen, Canada) and confirmed by sequencing prior to cloning into pENTR/D-TOPO entry vector (Invitrogen). The entry clones were checked by colony PCR with 35S promoter primer (forward) and gene specific primer (reverse) and confirmed by sequencing. The positive entry clones were then cloned into gateway destination vector (pMDC 32: overexpression under 35S promoter) via an LR reaction. The LR reaction was performed using equal amount of entry clone and destination vector (1:1) and 1 unit of LR clonase (Invitrogen). The LR reaction was incubated overnight at room temperature

and then transformed into *E.coli* competent cells using the heat shock method. Positive destination clones were screened by PCR as described above.

To generate SPL-RNAi constructs, approximately 250 bp fragments unique to each *SPL* gene were isolated using respective primers**.** PCR conditions were 1 cycle at 98˚C for 30 sec, then 35 cycles at 98˚C for 30 sec, 55˚C for 45 sec, 72˚C for 30 sec followed by final extension at 72˚C for 10 min. PCR products with expected sizes were purified using gel purification kit (Qiagen, Canada) and confirmed by sequencing. These fragments were then cloned into pENTR/D-TOPO entry vector, and subsequently cloned into Gateway destination vector (pHellgate12: hpRNA expression under 35S promoter) and positive clones were analyzed and confirmed as mentioned above.

2.7 Plant transformation

MiR156 overexpression, SPL overexpression, and RNAi and empty vector control constructs were transformed into alfalfa according to Tian et al. (2002) with slight modifications. The ingredients and types of media used in tissue cultures are detailed in **Appendix A**.

Alfalfa leaves and petioles (~5 cm) from WT were used in this study by first pre-culturing them for 2 days on basal SH2K medium in a growth chamber (26˚C). The explants were then infected with *A. tumefaciens* cells suspended in liquid co-cultivation medium (basal SH2K except plant tissue culture agar, 20 µM acetosyringone) by soaking the explants in the cell suspension for 10 min. The explants were then blot dried on sterile filter paper and transferred to plates supplemented with co-cultivation medium (pre-culture medium,

20 µM acetosyringone). To facilitate *A. tumefaciens* infection, the plates were wrapped with foil and incubated for 5 days in a growth chamber (26˚C). Following co-cultivation, the infected explants were transferred to callus induction medium (basal SH2K medium, 300 mg/l timentin) and incubated for 2 weeks. The transformed calli were then transferred to callus induction medium containing the appropriate antibiotics; hygromycin was used for SPL overexpression construct (pMDC32 vector) whereas kanamycin was used for miR156 overexpression (pBINPLUS and pBI121 vectors) and RNAi construct (pHellgate12 vector). During the first week of selection, the antibiotics were used at concentration of 50 mg/l, but the amount was increased to 75 mg/l in the following weeks.

To induce embryos, the calli were transferred to embryo induction medium supplemented with the appropriate antibiotics (75 mg/l) and incubated for 6-8 weeks at 26˚C. During these periods, the media were changed every 2 weeks to ensure the media were fresh to facilitate embryo development. Green embryos were then subsequently transferred to embryo germination and plant development media containing the appropriate antibiotics at a concentration of 75 mg/l. Following plant development, agar media were carefully removed and tissue culture plantlets were transferred to 4″ square plastic pots filled with moistened soil media (Promix, Mycorrhizae) and covered with a magenta box for a week. These tissue culture plantlets were kept in a growth room at 22°C, 70 % relative humidity with a photoperiod of 16 hours per day. Finally, acclimatized plants were transferred to larger clay pots filled with soil media (Promix, Mycorrhizae) and kept in a greenhouse.

2.8 Screening of alfalfa transformants

Prior to characterization, alfalfa transformed with overexpression, RNAi and empty vector constructs were screened for the presence of the appropriate transgenes in the genome. To screen transgenic alfalfa, DNA samples were extracted from alfalfa leaves using a modified CTAB buffer [2% CTAB, 1.4 M NaCl, 20 mM ETDA, 100 mM Tris, pH 8.0]. To extract DNA, leaf samples were ground in 1.5 ml tubes using disposable pestles. To the homogenized samples, 0.5 ml CTAB buffer and 0.5 ml chloroform were added and centrifuged at 3000 rpm for 5 min. The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with isopropanol and subsequently washed with 70 % ethanol. The DNA pellet was then re-suspended in water and used directly for PCR.

The presence of transgenes in miR156 overexpression alfalfa genotypes was confirmed by PCR using a 35S promoter primer (forward) and MsmiR156 and LjmiR156 gene specific reverse primers; Mt2139F11-156bR and Lj-MIR156b-Rq (**Table 1**). Transgenic plants transformed with empty vectors (pBINPLUS and pBI121) were genotyped using 35S promoter and vector specific primers (NOS-R2) (**Table** 1).

Similarly, SPL overexpression alfalfa genotypes (SPL-OE) were screened by PCR using a 35S promoter and gene-specific primers whereas the empty vector control plants were confirmed using a combination of 35S promoter and pMDC32 vector-specific primers (**Table** 1).

SPL RNAi alfalfa genotypes were confirmed using PCR as described above. To ensure *SPL* genes were transformed in the correct orientations, i.e., the two *SPL* fragments were present in opposite orientations flanked by intron sequences, SPL-RNAi alfalfa were

further genotyped using pHellgate12 intron primer and gene-specific forward primer (**Table** 1). PCR conditions were 94˚C for 3 min, then 35 cycles at 94˚C for 1 min, 55˚C for 30 sec, 72˚C for 30 sec followed by final extension at 72˚C for 10 min.

2.9 Detection of miR156 cleavage products

The cleavage of alfalfa *SPL* genes by miR156 was verified using 5' rapid amplification of cDNA end (5'-RACE) (Llave et al., 2002). The experiment was conducted using the FirstChoice® RLM-RACE Kit according to the manufacturer's instruction (Ambion, USA) with slight modifications. First, total RNA was extracted from the shoots of transgenic alfalfa (miR156-OE) using Trizol reagent and the quality of RNA was verified using NanoVue spectrophotometer and by gel electrophoresis on 1% agarose gel to ensure RNA integrity. Total RNA was directly ligated to the 5' RLM-RACE adaptor sequence. The adaptor ligated RNA was then reverse transcribed using M-MLV Reverse Transcriptase and random decamers. The miR156-cleaved *SPL* transcripts were amplified using Outer 5'RLM-RACE PCR with outer adaptor sequence specific (forward primer supplied with the kit) and gene specific (reverse) primers. The PCR products were then diluted with water (1:10 dilution), and proceeded to Inner 5'RLM-RACE PCR (nested) using inner adaptor sequence-specific (forward primer supplied with the kit) and gene-specific (reverse) primers. PCR conditions were 1 cycle at 94˚C for 3 min, then 35 cycles at 94˚C for 30 sec, 50˚C for 45 sec, 72˚C for 30 sec followed by final extension at 72˚C for 10 min. PCR products with expected sizes (~ 250 bp) from Inner 5' RLM-RACE PCR were purified using a gel purification kit (Qiagen, Canada) and cloned into pJET1.2/blunt cloning vector (Fermentas, Canada). Positive colonies were screened by colony PCR with

gene-specific primers as well as by digesting with *Hind*III and *Xho*I restriction enzymes that each cuts once in the pJET1.2/blunt cloning vector sequence. Finally, at least 20 positive clones for each *SPL* RACE reaction were subjected to sequencing using pJET1.2/blunt sequencing primer.

2.10 Propagation of alfalfa by stem cuttings

To characterize alfalfa genotypes, the plants were propagated by stem cuttings. Prior to vegetative propagation by stem cutting, alfalfa plants were cut back and allowed to grow for one to two months to ensure all plant materials were at the same stage. In this study, vermiculite mixed with sand was used as growing media for alfalfa propagation. For each alfalfa plant genotype, at least 3 biological replicates were used in the propagation. For each replicate, about 3 to 4 stems containing the same number of nodes were cut and planted into a 4″ square plastic pot filled with the moistened growing media. The pots were covered and kept in the greenhouse for 3 weeks to allow rooting from the cut-stem. During the rooting period, the plants were watered once a week to ensure the media was not dried out. The plants were then thinned and only one stem was kept in each pot for further characterization.

2.11 Morphological characterization of alfalfa

To characterize the phenotypic variations in alfalfa, the plants generated from stem-cuttings were used in this study. All of these plants were maintained in greenhouse and watered as required. The plants were characterized at 40 days as well as at 4 months after

cutting. The phenotypes included in the characterization were number of main branches, lateral branches, plant height, internode length, number of nodes, stem diameter, trichome density and root length.

The branches directly emerging from the soil were considered as main branch while those that were on the main branch or above ground were counted as lateral branches. The longest stem in each biological replicate was used to measure plant height and the length of internode. In addition, the number of nodes in each replicate were also counted on the three longest stems. To represent stem size in each replicate, the base of the stem was cut and the diameter was measured under the microscope using 5 fold magnification. The appearances of trichome in the leaves were checked under the microscope using 100 fold magnification. The root length was measured using one month-old alfalfa cutting.

2.12 Nodulation test

Alfalfa plants generated by stem cuttings were used for nodulation test. To ensure the plant materials are uniform, i.e, growing at the same developmental stage, alfalfa plants were cut back and allowed to grow for 4-5 weeks. Prior to stem-cutting, the growing media (vermiculite mixed with sand) was sterilized by autoclaving. The alfalfa stems containing the same number of nodes were then cut and planted into a 4″ square plastic pot containing the media moistened with sterilized distilled water and allowed to set root for 2 weeks.

To test nodulation in alfalfa, *Sinorhizobium meliloti* Sm1021 (courtesy of Dr. Michael Udvardi, Noble Foundation, Oklahoma) carrying the pXLGD4 plasmid with the

hemA::LacZ reporter was used in this study. The *S. meliloti* were cultured on TY medium containing tryptone, yeast extract, 10 mM $CaCl_2$ and agar for 2 days at 28˚C. A single colony was then inoculated in liquid TY medium and incubated at 28˚C until the culture reached cell density of 10^8 cells/ml. The cell culture was then pelleted and re-suspended in sterilized distilled water. One milliliter of the bacterial suspension was used to inoculate into each pot containing rooted alfalfa stem, and the pot was covered with clean transparent plastic cover. The plants were then kept in the greenhouse and watered with distilled water once a week. The total number of nodules from each stem was counted two weeks after inoculation with the *S. meliloti*.

2.13 Quantitative real-time RT-PCR

The transcript levels of *miR156,* target *SPL* and potential downstream genes were analyzed by quantitative real-time RT-PCR (qRT-PCR) using CFX96 Touch™ Real-Time PCR Detection System. Prior to conducting real-time PCR experiment, the PCR assay was tested to ensure optimal annealing temperature, efficiency and specificity of the reaction were established. To achieve reliable data in qRT-PCR, the guidelines for the minimum information for publication of quantitative real-time PCR experiments (MIQE) were also followed (Bustin et al., 2009, Taylor, 2010).

After RNA extraction as described earlier (sections 2.5), the RNA was treated with Turbo DNAse I (Ambion, USA) to eliminate any genomic DNA contamination in the sample. The DNAase was then removed using DNAase inactivation reagents (Ambion, USA) as per the manufacturer's instructions. The revere transcription reaction was performed

using 900 ng total RNA and qScript^{TM} cDNA SuperMix (Quanta Bioscience, Canada) following the manufacturer's instruction.

To determine the efficiency of the assay, serial dilutions of the pooled cDNA (cDNA mixed from different samples) was used in the reaction to generate a standard curve. The cDNA was diluted with sterilized distilled water (1:3) and 2 µl of the diluted cDNA was used in the PCR reaction. A total volume of 10 µl containing 0.2 µM of each forward and reverse primer, 1X PerfeCta SYBR Green FastMix, 2 µl cDNA was used in each qRT-PCR reaction. For each alfalfa plant genotype, at least three biological and three technical replicates were tested. The PCR was performed in two steps; 95˚C for 3 min followed by 44 cycles at 95˚C for 10 sec and 58˚C for 30 sec. To test transgenic alfalfa overexpressing miR156, CoA carboxylase 1 and acetyl CoA carboxylase 2 (Alexander et al., 2007) were used as reference genes in the reaction. To analyze the SPL-RNAi alfalfa plant genotypes, the transcript levels of target *SPL* genes were normalized to 18S ribosomal RNA and β-actin genes in qRT-PCR. Finally, transcript levels of the target genes were analyzed using relative quantification by comparative Ct ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen,2001).

2.14 Small RNA blot

2.14.1 Isolation of small RNA

Small RNA was extracted from young shoots (one week after cut-back) of empty vector control, *M. sativa* miR156 overexpression (MsmiR156-OE) and *L. japonicus* miR156 overexpression (LjmiR156-OE) alfalfa plants using *mir*VanamiRNA isolation kit (Life

Technologies™) according to the manufacturer's instruction. The RNA quality and integrity was confirmed as described above.

2.14.2 Probe synthesis

To obtain a good quality probe, a highly purified (HPLC grade) primer was specifically designed based on miR156 sequence. The probe used in this study was synthesized using *mir*Vana™miRNA probe construction kit (Ambion, USA) according to the manufacturer's instruction with slight modifications. First, 10 μM of miR156 oligonucleotide template was mixed with 1X T7 promoter primer, 1X DNA hybridization buffer and the mixture was heated at 70˚C for 5 min, and incubated at room temperature for an additional 5 min to allow hybridization to occur. To this hybridized oligonucleotides, a mixture containing 10 X klenow reaction buffers, 10X dNTP mix, 2 units Exo-Klenow was added and incubated for 30 min at 37˚C.

To transcribe the probe, 2 μl of the above reaction was mixed with 10X transcription buffer, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 1 mM UTP (Digoxigenin-11-dUTP alkali labile, Germany), 2 units of T7 polymerase and the reaction was incubated for 30 min at 37˚C. To remove non-transcribed DNA template, 1 unit of DNaseI was added and incubated for additional 10 min at 37˚C.

2.14.3 Separation and blotting of the small RNAs

To prepare 10% polyacrylamide gel, 8 M of urea was dissolved in 0.5X TBE [40 mMTris-Cl, pH 8.3, 45 mM boric acid, 1 mM EDTA], 30 % acrylamide (19:1,

acrylamide: bis acrylamide) and filled the volume to 15 ml with DEPC-treated water. Then, 10 % ammonium persulfate (APS) and 15 µl Temed were added the gel mixture and immediately poured into gel casting frame (BioRAD). The gel was pre-run in 0.5X TBE buffer at 150 Volt for 1 hr at 4˚C. The samples were prepared by mixing 3 µg small RNA with bromophenol blue loading dye and denatured at 65˚C for 10 min. The RNA was then immediately chilled on ice and loaded into the gel that was pre-run at 4˚C. The RNA was separated on the gel using electrophoresis at 120 Volt for about 2 hrs.

Prior to blotting the separated RNA, nylon membrane, whatman paper and sponges were moistened in 0.5X TBE buffer. Then, transfer sandwich was built on the black side of cassette by adding sponge, 3 pieces of whatman paper, gel, membrane, another 3 pieces of whatman paper, sponge and covered with white side of the cassette. The sandwich cassette was transferred to BioRad Trans-Blot cell filled with pre-chilled 0.5X TBE buffer. The blotting was performed using constant current at 90 mA for 1 hr at 4˚C. The cassette was then disassembled, and the membrane was trimmed and marked. The blotted RNA in the membrane was UV cross-linked up and down, and the membrane was baked at 80˚C for 2 hr prior to hybridization with probe.

2.14.4 Hybridization

The blotted RNA in the membrane was hybridized with the miR156 probe prepared using mirVana^{TM}miRNA probe construction kit. The membrane was placed in a hybridization bottle, and 20 ml of pre-warned hybridization buffer (Ultrahyb$^{®}$, Ambion, USA) was added to the bottle. It was then pre-hybridized in hybridization chamber with constantly rotating bottle for 1 hr at 45˚C. The probe was denatured by boiling 10 µl of the probe in

boiling water (95˚C) for 5 min, and immediately chilled on ice for 2 min. The pre-hybridization buffer was discarded and 5 ml fresh hybridization buffer was added to the hybridization bottle. The probe was then immediately added to the hybridization bottle, and the bottle was incubated for 19 hr in the hybridization chamber kept at 45˚C.

To wash the hybridization bottle, the hybridization buffer was discarded and the bottle was washed twice each with 25 ml low stringency buffer [0.3 M NaCL, 30 mM sodium citrate, pH 7.0, 0.1% SDS] for 5 min at room temperature. The low stringency buffer was then discarded and 30 ml of high stringency buffer [15 mM NaCL, 1.5 mM sodium citrate, pH 7.0, 0.1% SDS] preheated at 50˚C was added to the bottle and incubated for 15 min in the hybridization chamber kept at 45˚C. Washing with high stringency buffer was also repeated twice as above.

2.14.5 Immunological detection

All steps in the detection processes were performed in a hybridization chamber kept at room temperature. To detect mature miR156, the high stringency buffer was removed and 50 ml washing buffer [maleic acid buffer (100 mM maelic acid, 150 mM NaCl, pH 7.5), 0.3% Tween 20] was added to the bottle incubated for 5 min. The wash buffer was then removed and the membrane was blocked with 30 ml blocking solution [10X blocking stock (100 mM maleic acid, 150 mM NaCl, pH 7.5, 10% blocking reagent: maleic acid buffer (1:10)] for 30 min. Then, the blocking solution was removed and the bottle was incubated with 10 ml antibody solution [Anti-Digoxygenin-AP, Roche, USA: blocking solution (1:5000)] for another 30 min. Again, the antibody solution was discarded and the bottle was washed twice each with 50 ml washing buffer for 15 min.

After removing the washing buffer, the membrane was equilibrated with 30 ml detection buffer (0.1 M Tris-HCL, 0.1 M NaCl, pH 9.5) for 5 min. The membrane was then removed from the hybridization bottle and placed on a plastic film with RNA side facing up. Then, 1 ml CDP star (Roche, Germany) that was diluted to 1:100 with detection buffer was immediately applied on the membrane until the entire surface was evenly soaked. The membrane was mounted in X-ray cassette and incubated at room temperature for 5 min. The excess CDP star was then squeezed out and the membrane was exposed to X-ray film in the dark room for 10 min.

2.14.6 Membrane stripping and re-blotting

As loading control in the small RNA gel blot, a primer specifically designed based on U6 gene was used in this study. To remove the blotted miR156 probe, the membrane was thoroughly washed twice in sterilized distilled water. The membrane was then soaked in 100 ml stripping buffer [50% deionized formamide, 5% SDS, 0.5 M $NaCl_2$, pH 7.5] and incubated for 2 hrs in the hybridization chamber set at 80˚C. Again, the membrane was washed twice in 2X SSC for 5 min and hybridized with U6 probe. Hybridization, washing and detection were subsequently performed as described above.

2.15 Statistical analysis

In this study, both t-test and ANOVA were used to analyze the data. To conduct ANOVA, CoStat (Statistics Software, version 6.4) was used. For each alfalfa plant genotype, at least 3 biological and 3 technical replicates were used in characterization. The difference of the means among the control and different transgenic alfalfa were tested

using one way ANOVA with Duncan's statistical test or t-test, and the results were shown in bar graphs.

3 RESULTS

3.1 Potential miR156 precursors in *M. truncatula* and *M. sativa*

Currently, 15 members of the miR156 family (miR156a – miR156o) are deposited in the miRBase database (Griffiths-Jones et al., 2008). These miR156 precursors were identified in different plant species, and many of them are found in single plant species. For instance, 10 members of miR156 (miR156a – miR156i) are found in *Arabidopsis* and 11 members are found in *M. truncatula*. I searched for the paralogs of all of these miR156 members in publicly available sequences of *M. sativa.* Using NCBI database and guidelines for miRNA annotation (Griffiths-Jones et al., 2008), no paralogs of miR156 precursors were identified in *M. sativa*. The precursors from miRBase were also used to search and align with Next Generation alfalfa RNA sequencing reads (Austin,unpublished). Only short miR156-like sequences (~60 bp) were found in the search, and thus hairpin structures could not be predicted in these short sequences (data not shown). Further analysis, including direct cloning and sequencing of small RNAs are needed to determine if these short sequences are miR156 precursors in alfalfa.

To clone alfalfa miR156 (MsmiR156), I extended the *in silico* search for miR156 precursors to publicly available genomic sequences of *M. truncatula*, and found 11 members of miR156 precursors in this plant species (**Table 2**). I then used miRTour software to predict RNA secondary structure and found that hairpin structures could be produced from all of the 11 sequences (data not shown).

A paralog of miR156 precursor was isolated from alfalfa using the primers designed based on *M. truncatula* miR156d precursor. Sequencing and BLAST results showed that

Table 2. *M. truncatula* genomic loci encoding miR156 precursors

Query precursors[α]	*M. truncatula* sequence hit in BLAST search[β]	*M.truncatula* accessions[δ]	**P**osition hit
miR156a	AGCCAUGAAUCAGUCCGAGAUGACAGAAGAGAGAGAGCACACCCACCUGAUUACAAGUAACAAAAAACUACCAACUAUC AACAUUUUGUCUACUAUGAAGAAAAUCACAUGUCUAGACUAUAGUUACGAAUGGAAGAUACAUUCAAUGAUGUUUAUGA AAAUUUGAGACGGUCGAGGACUAUAGUUACUCUAUCGCAUACAUUUAAUGACAAAAAAAAUUGUGUACUCUUAUUUAUU UUCAAUACAUCUUCAUUCAAUUUCUAUGCAAGAAUCAAAAUGCAGUAUAAAAAAUUGCAUUCAAUAUCAUGGUAAAAAA AUUGUUCAAAAAAAGUAAAUAUCAUGGUAAAAAAAUCAUUAGAUUUUGCAAUAAGUAAGAGUCAAGUUUUACCUUGUGA GGAAUUCCUCCCAACAGAAUCAUGGGAGCUCUUUCUUCUUCUCUCAUUGUGCCCUGCCAGUCUCC	AC151743.28/ AC151915.8	87629-88088/ 56762-57221
miR156b	GGAGGTGACAGAAGAGAGTGAGCACACATGGTACTTTCGTGTATGATGTTTCATTCTCGAAGCTATGTGTGCTCACTCT CTATCTGTCACCCCATCACCAT	AC152551.33/ AC157537.16	14882-14927/ 25095-25140
miR156c	TGACAGAAGAGAGTGAGCACACATGGTGTTTTCTTGCACGATTATGTTTCCTGCTTGAAGCTATATGTGCTTACTCTCT ATCTGTCACCCCACCACCAT	CR956434.13	12594-12634
miR156d	GAAATTGACAGAAGAGAGTGAGCACATAGACACTCGGTATAGATGTATATCGTTGCCTTTGCGTGCTCACTCATCTTTC TGTCAAATTC	CU019603.9/ AC133571.47	39724-39756/ 32582-32614
miR156e	GCATATCACTCCTTTGTGCTCTCTATGCTTCTGTCATCACCTTCATCCTCCTGTTGACAGAAGATAGAGAGCACAGATG ATGATATGCAT	AC165438.8	135596-135686
miR156f	TGTTTGTTGACAGAAGATAGAGAGCACATCTGAACAAACACAAAAGACATTGTGTTTGAACAGTTGTGTGCTCTCTGCT CTTCTGCCAACA	CT961057.10	39524-39556
miR156g	TAAGTAAGGTTGTTGACAGAAGATAGAGGGCACTAAGGATGATATGCATACACATATATATACAACATGGAGGAGGAGC TTAATTGCATTTCATTTCCTTTGTGCTCTCTAGACTTCTGTCATCACCT	AC174377.13	60219-60277
miR156h	TGATGCTGTTGACAGAAGATAGAGAGCACAGATGATGAAATGCATGGAAGGCAATGGCATCTCATTCCTTTGTGCTCTT TATTCTTCTGTCATCA	AC144375.14	85721-85815

Table 2. *M. truncatula* genomic loci encoding potential miR156 precursors (Continued)

Query Precursors[α]	***M.*** *truncatula* sequence hit in BLAST search[β]	***M.*** *truncatula accessions*[δ]	**P**osition hit
miR156i	GATATTGACAGAAGAGAGTGAGCACATGCTGCAGTGATTGTATGATAGCATACAATTCTTGGTGCGTGCTCACTTCTCT TTCTGTCATCTT	AC122160.21/ AC169120.12/ AC159537.24	**1**04800-104832/ 37396-37428/ 87060-87092
miR156j	ACAUAGAAAUUGACAGAAGAGGGUGAGCACAAAAAAAGUCUUUAGUAUAUAAUGUUUAUACCAUUAUUUUUGUGUGCUCA UACUCUUCUGACAAUUUUCAAUG	No Information	

[α:] All miR156 precursors (miR156a-o) were used as query sequences in BLAST search but only MtmiR156a-j are shown.
[β] Only *M. truncatula* sequences are shown since no similar precursors were found in *M. sativa* sequences.
[δ] *M. truncatula* accessions in NCBI databases.

the isolated sequence was similar to the member of miR156d precursors from *M. truncatula* and other plant species. In addition, the sequence contained mature miR156 sequence, and produced the secondary structure (hairpin) as in typical miR156 precursors (**Figure 2**). Based on these results, it was evident that the isolated sequence was miR156 precursor and was designated as MsmiR156.

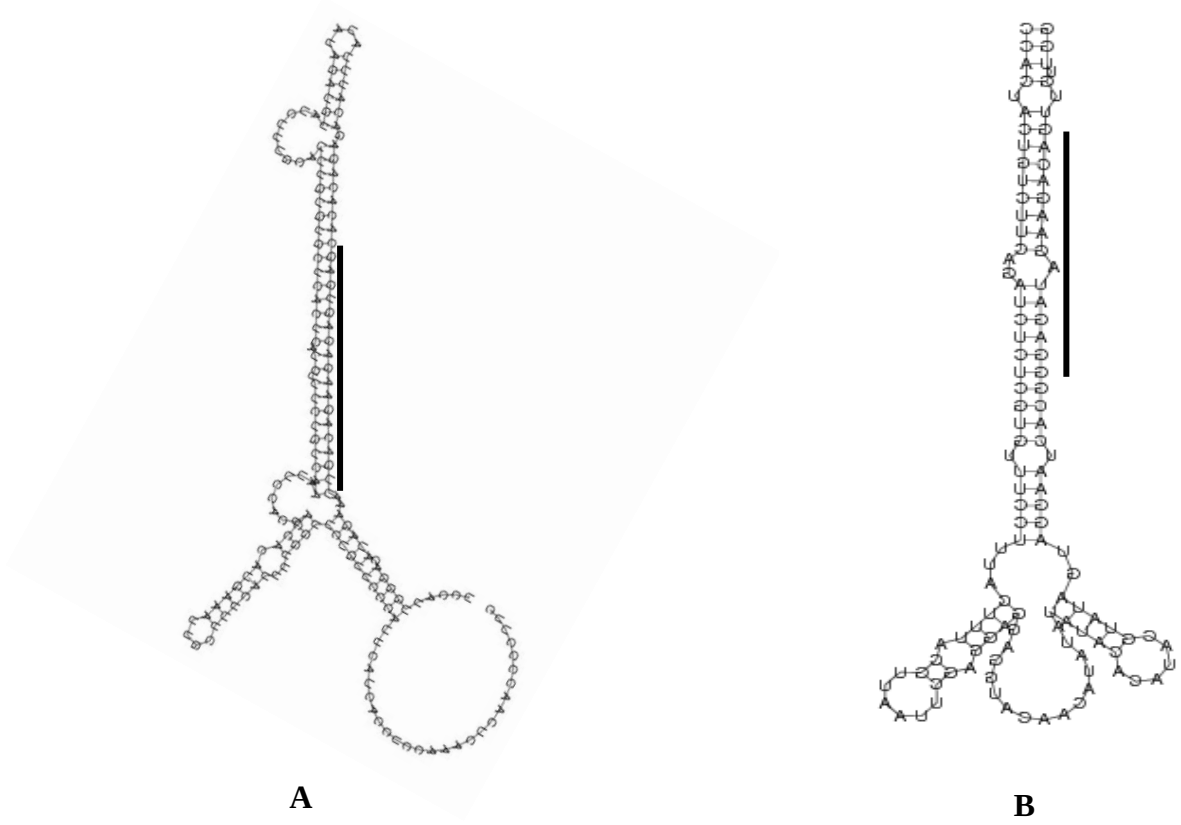
3.2 Sources of gene-specific primers

In silico analysis revealed that no alfalfa public database contained sequences of homologs for *SPL* and *WD*40. Hence, the homologs of these genes were retrieved from the *M. truncatula* database (**Table 3**), and *SPL* and *WD40* gene-specific primers were initially designed based on these *M. truncatula* gene accessions. Details of nomenclature of alfalfa *SPLs* and number of clones analyzed in 5' RACE (see below) are summarized in **Table 3**.

Likewise, homologs of *AP*3 and *FT* genes were obtained from *M. truncatula* database and gene-specific primers were designed based on these accessions: *AP*3; MTR_5g016710**,** *FTa*1; HQ721813, *FTa*2; HQ721814, *Fb*1; HQ721815, *FTb*2; HQ721816.

3.3 Prediction of miR156 targets in alfalfa

A search for *SPL* homologs in *M. truncatula* genomic sequences revealed 10 *SPL* gene accessions. However, only five contained sequences that were similar to the mature miR156 sequence, and thus these *M. truncatula SPL* accessions were used to design gene-



A **B**

Figure 2. Prediction of secondary structure of MsmiR156 using miRTour. **A**. Hairpin structure of MsmiR156; **B**. Hairpin structure of *M. truncatula* miR156 (MtrmiR156). The black lines indicate mature miR156 sequences.

Table 3. *M. truncatula* gene accessions, nomenclature of alfalfa homologs and number of clones analyzed.

M. truncatula accessions	Similarity in NCBI BLAST[α]	Nomenclature of *SPL* homolog	No. of clone analyzed[β]	Sequencing result[δ]
XM_003614178	*G. max SPL*6	*M. sativa SPL*6	20	*SPL*
XM_003601719	*G. max SPL*12	*M. sativa SPL*12	20	*SPL*
XM_003602747	*G. max SPL*13	*M. sativa SPL*13	20	*SPL*
XM_003593569	*G. max SPL*1	*M. sativa SPL*1	30	Unknown
XM_003625188	*G. max SPL*9	*M. sativa SPL*9	30	Unknown
XM_003638731	Uncharacterized	*M. sativa WD*40_1	20	*WD*40
XM_003594338	Uncharacterized	*M. sativa WD*40_2	20	*WD*40
XM_003621683	Uncharacterized	*M. sativa WD*40_3	20	Unknown
XM_003598247	Uncharacterized	*M. sativa WD*40_4	20	Unknown
XM_003598255	Uncharacterized	*M. sativaWD*40_5	20	Unknown
XM_003598248	Uncharacterized	*M. sativa WD*40_6	20	Unknown

[α]: Uncharacterized; uncharacterized proteins in NCBI. [β]: Number of clones sequenced and analyzed in 5' RACE. [δ]: Unknown; sequencing results showed no sequence similarity (e value: 1e-34) in NCBI BLAST search.

specific primers. The cleaved sites of the five *SPL* homologs in alfalfa were investigated using 5' RACE and DNA sequencing approaches. Expected products were obtained from each *SPL* gene in 5' RLM-RACE PCR (**Figure 3).** The PCR products from Inner 5' RLM-RACE PCR were cloned into pJET1.2/blunt cloning vector and positive clones were screened using restriction enzymes (*Hind*III and *Xho*I), and restriction digestion showed the presence of the expected inserts in the cloning vector (**Figure 4**).

Of the five *SPL* genes, only three (*SPL*6, *SPL*12 and *SPL*13) contained miR156 cleavage sites based on 5'-RACE analysis. Among the 20 clones, 15 of the cleaved sites in *SPL*6 were found after the 11^{th} nucleotide of the complementary miR156 (**Figure 5A)**. In contrast, two cleavage sites were identified for *SPL*12 and *SPL*13 transcripts, respectively. In *SPL*12, 2 cleavage sites were found after the 10^{th} nucleotide and 11 cleavages were found after the 11^{th} nucleotide of miR156 (**Figure 5B**). In *SPL*13, however, cleavages were observed after the 9^{th} (9 cleavages) and 10^{th} (6 cleavages) nucleotides of the miR156 sequences (**Figure 5C**).

Two predicted *SPLs* (*SPL*1 and *SPL*9) isolated from alfalfa showed a lack of sequence similarity to other *SPL* genes even though the primers for PCR were designed based on *M. truncatula SPL* gene-specific sequences containing miR156 cleaved sites. To validate the 5' RACE results for these two *SPLs*, 10 clones from the 5' RLM-RACE PCR products of each of the two *SPL* genes were sequenced, but none revealed miR156 cleavage sites, confirming the earlier data, and hence these two genes were excluded from further analysis.

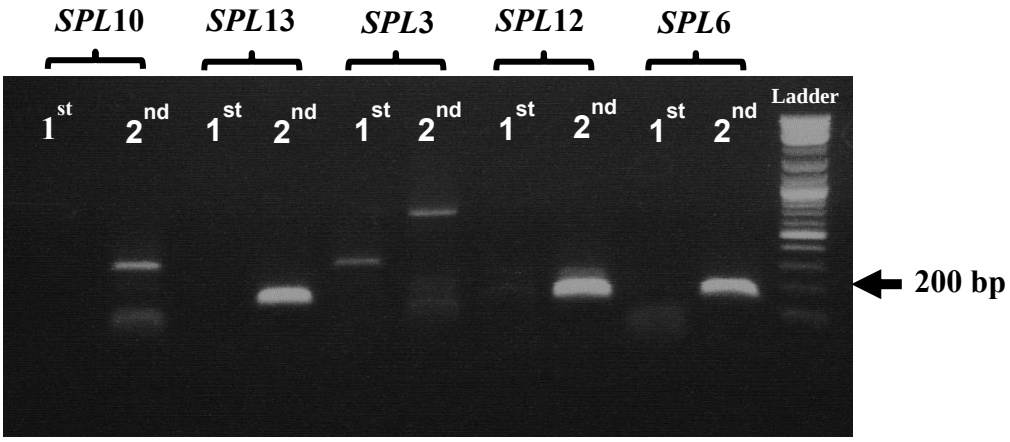


Figure 3. Visualization of 5' RLM-RACE PCR products in agarose gel. 1st refers to outer PCR products whereas 2nd represents inner PCR products from each *SPL* gene. Ladder; HyperLadder II DNA ladder.

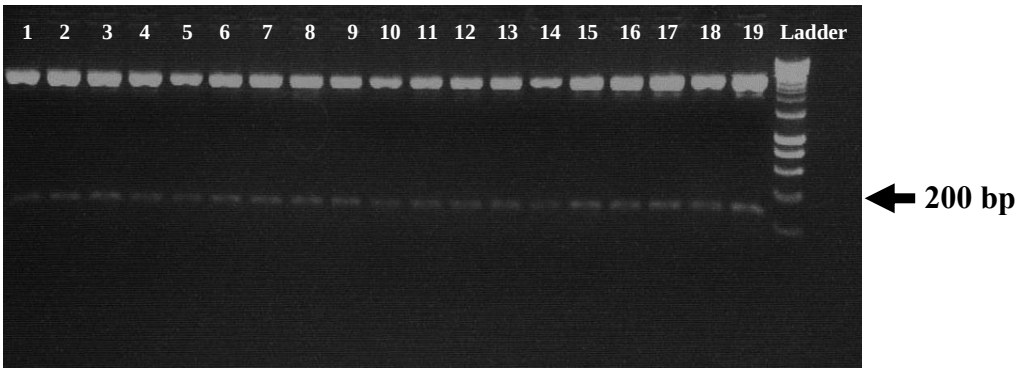


Figure 4. Screening for *SPL*6 positive clones by digesting with *Hind*III and *Xho*I restriction enzymes. Lane 1-19 refers to the clone number for *SPL*6. Ladder; HyperLadder II DNA ladder.

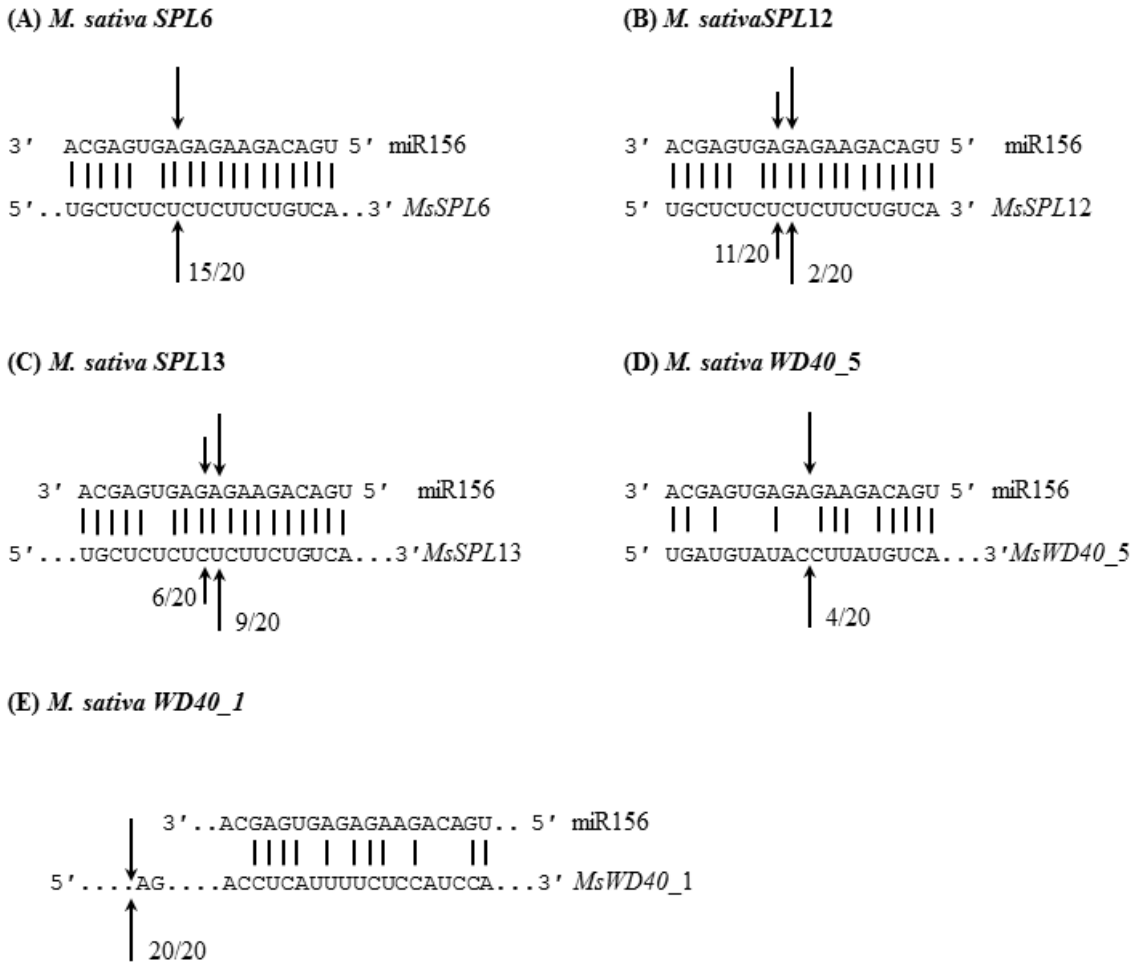


Figure 5. Validation of the miR156 cleavage sites in *SPL* and *WD40* gene transcripts in miR156-OE plants. Complementary miR156 sequences and a fragment of target *SPL* sequences are shown. Arrows indicate the 5′ termini of cleaved target mRNA. Denominators refer to the number of clones sequenced whereas the nominators represent the number of clones cleaved at a particular site.

MiR156 is also known to cleave non-conserved transcripts of *WD*40 genes in *M. truncatula* (Naya et al., 2010). Using 5' RLM-RACE PCR, I found that miR156 cleaved the transcripts of two members of *WD*40, *WD40*_1 and *WD40_5*, in alfalfa. As in *SPL* gene transcripts, the cleavage sites in *WD*40_5 were observed in the middle of the complementary miR156 binding site but those in *WD40_1* were found beyond this region (**Figure 5D and E**).

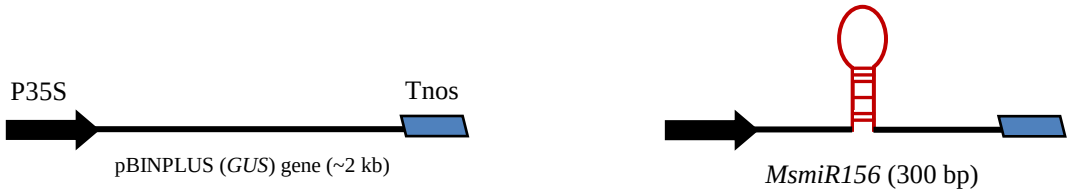
3.4 Generating alfalfa plants that overexpress *MsmiR156*

The precursor sequence of *MsmiR156* gene (Section 3.1) was cloned into pBINPLUS vector for expression under 35S promoter, and transferred to alfalfa to generate miR156 overexpression genotypes (MsmiR156-OE) (**Figure 6**).

3.4.1 Genotyping of alfalfa plants transformed with *MsmiR156*

To ensure the presence of the 35S::MsmiR156 transgene in the transformed plants, tissue culture-derived alfalfa plantlets were genotyped by PCR using 35S promoter (forward) and miR156 gene-specific (Ms_156Rq3; reverse) primers (**Table 1**). PCR products with expected sizes were obtained from 26 independent transgenic alfalfa plants indicating that these plants contained the transgene (data not shown). Plants transformed with the empty vector control were also tested using 35S promoter (forward) and pBI121 vector-specific (NOS-R2; reverse) primers (data not shown).

A



B

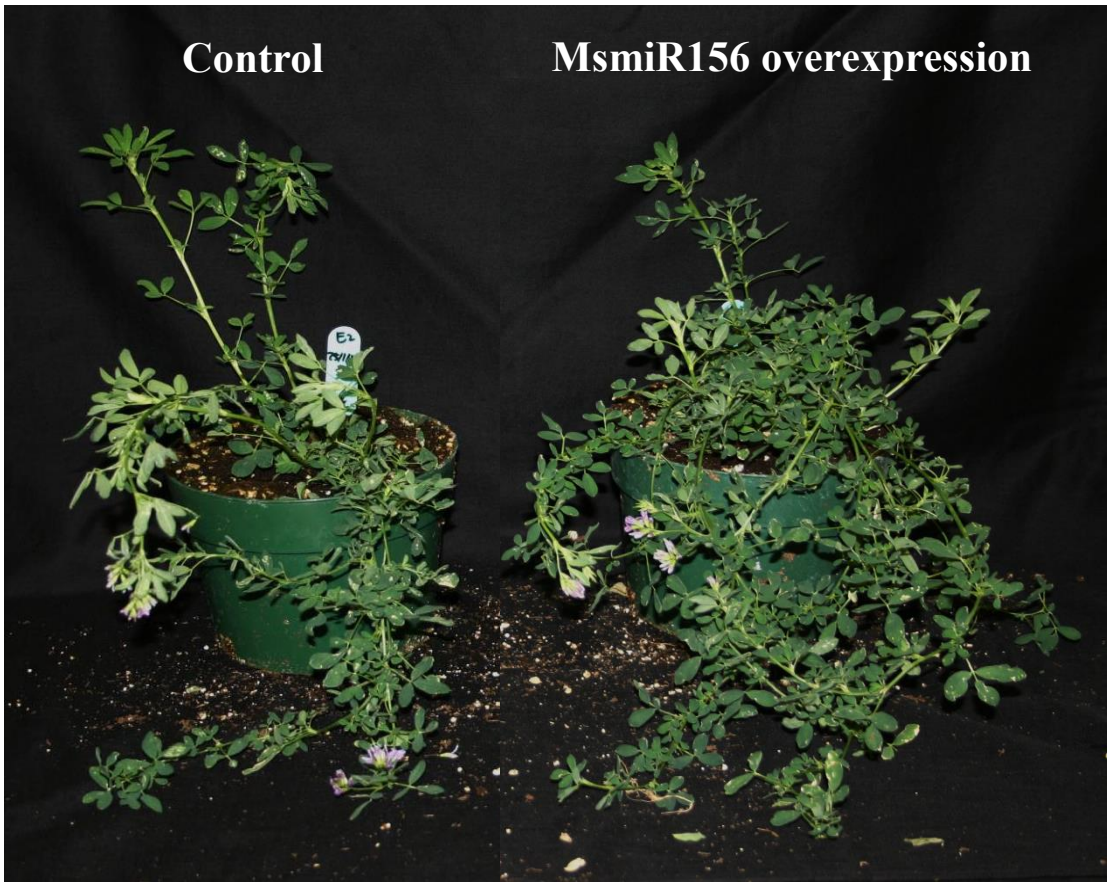


Figure 6. Schematic diagrams of vector constructs and transgenic alfalfa expressing respective constructs (Empty vector pBINPLUS and MsmiR156-OE). **A.** Empty vector pBINPLUS containing *GUS* gene and overexpression construct carrying a *MiR156* gene insert. **B.** Transgenic alfalfa plant carrying a MsmiR156 transgene (right), and empty vector control (left). The plants are 4-month-old.

The above results merely indicated the presence of miR156 precursor (~ 285 bp) in alfalfa. To ensure that the *MsmiR156* was expressed and the precursor was processed to mature miR156 (5'-UGACAGAAGAGAGUGAGCACA-3') in transgenic alfalfa, small RNA gel blot was performed using a *miR156* gene-specific probe. Using this approach, the mature miR156 sequence (20 nt) was detectable in transgenic as well as empty vector control alfalfa (**Figure 7**). It was also found that band intensity of the mature sequence in MsmiR156-OE plants was stronger than that in empty vector control indicating that the transcript level of miR156 in MsmiR156-OE alfalfa was higher than that in the control plant.

3.4.2 Expression profiles of *MsmiR156* and target *SPL* genes in transgenic alfalfa

Prior to analyzing gene expression, efficiency of qRT-PCR amplification was tested to ensure it is between 95 – 100 % for all the primers used in this study (data not shown). Additionally, qRT-PCR products were visualized in agarose gel to ensure the efficiency and specificity of the reactions (data not shown). To quantify the fold of gene expression, transcript levels of genes of interest were determined and normalized to two reference genes (*acetyl CoA carboxylase 1* and *acetyl CoA carboxylase 2*) (Alexander et al., 2007) and expressed as relative to the empty vector control.

5' RACE analysis revealed that miR156 cleaves transcripts of three *SPL* genes (*SPL*6, *SPL*12 and *SPL*13) in alfalfa shoots. To confirm these results, the transcript levels of miR156 as well as those of the putative target *SPL* genes were determined using qRT-PCR. I found that at least one of the 3 *SPL* genes was downregulated in all alfalfa plants

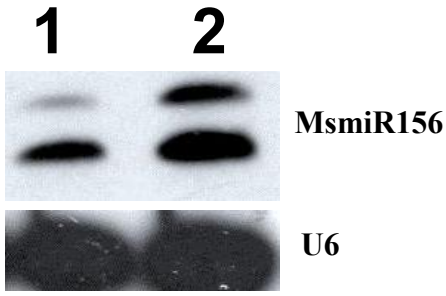


Figure 7. Detection of mature miR156 in transgenic alfalfa using small RNA gel blot. Lane 1: empty vector control; lane 2: MsmiR156-OE alfalfa. Small RNAs (3 μg) were equally loaded in each lane and hybridized with *miR156* gene specific probe. Small RNA U6 gene was used as loading control.

overexpressing MsmiR156 except in the plant genotype A4c (**Figure 8**), where none of the *SPL*s were silenced. As expected, the higher the transcript levels of miR156, the more *SPL* genes were downregulated (**Table 4**). Based on the transcripts levels of MsmiR156, transgenic plants were assembled into three groups. Transgenic alfalfa with low expression of MsmiR156 (10-999 relative transcript level) included A5c, A5b, A3b, A3, A4a, A4c, A4b, A2b, A2a, A19b and A2d. The plants with medium expression of MsmiR156 (1000-15000 relative transcript level) were A2e, A11a, A20, A16b, A3c, A8 and A8a. The plants with transcript levels ranging from 15100-400000 were considered as high expression group including A13, A19c, A16a, A11, A20a, A17, A19 and A6b alfalfa genotypes (**Figure 8 and Table 4**).

In the low expression group, the regulation of a specific *SPL* gene by MsmiR156 was unpredictable. For instance, I found that the transcript levels of *SPL*12 and *SPL*13 were lower in A5c alfalfa genotype in which the level of MsmiR156 was the lowest among the transgenic plants. However, only the transcript of *SPL*6 was affected in A3a genotype which contains higher level of MsmiR156 transcripts. Moreover, the transcripts of all three *SPL* genes were not affected in A4c genotype in which the level of MsmiR156 was higher than that in A5c as well as A3a genotypes.

In the medium expression group, the regulation of alfalfa *SPL* genes by MsmiR156 was more predictable. In this group, the transcript levels of *SPL*12 and *SPL*13 were downregulated in almost all alfalfa genotypes. It was observed that the transcript levels of *SPL*12 in A8a and *SPL*13 in A3c alfalfa plant genotypes were not different from empty vector control. However, the effect of MsmiR156 on *SPL*6 transcripts was not

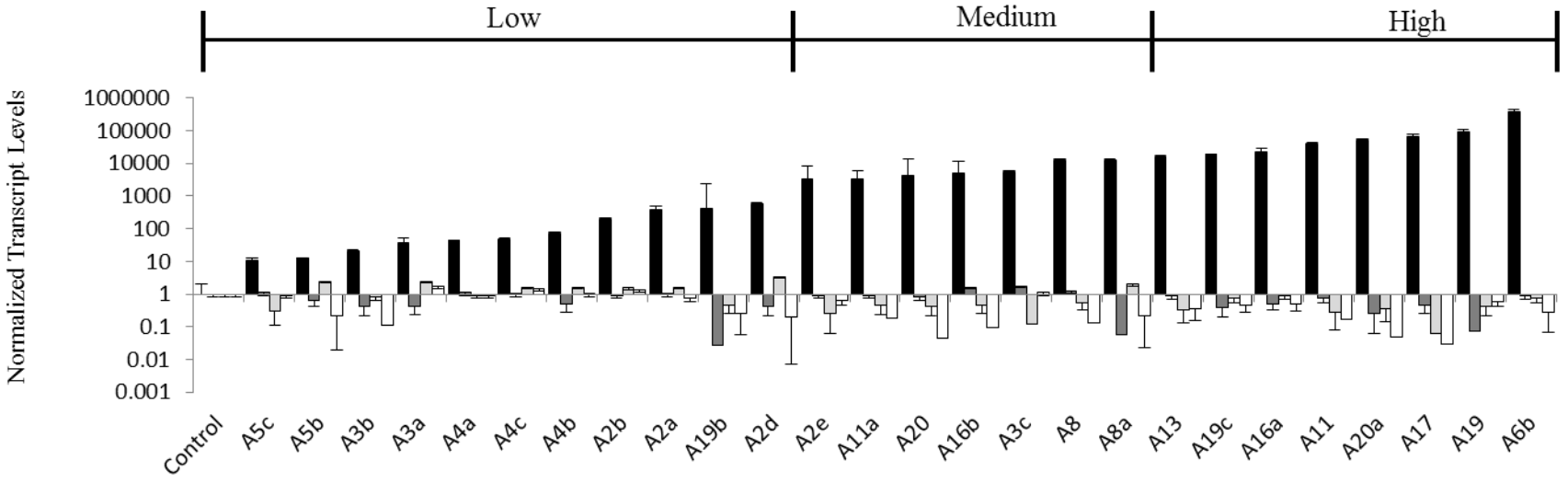


Figure 8. Transcript levels of *MsmiR156* and three target *SPL* genes in all MsmiR156-OE alfalfa genotypes. Low expression; 10 – 999 normalized transcript levels, Medium; 1000 – 1499 and High; 1500 – 400000. The name of transgenic alfalfa genotype consists of prefix capital letter (Alfalfa)-callus number-suffix small letter (refer to different plants from the same callus). Each bar represents the mean values from three experiments for each independent alfalfa genotype. Error bars indicate standard error. ■ MsmiR156, ■ *SPL*6 ■ *SPL*12 and □ *SPL*13.

Table 4. Summary of transcript levels of *MsmiR156* and target *SPL* genes

No.	Alfalfa genotype	MsmiR156	*SPL*6[α]	*SPL*12[α]	*SPL*13[α]
1	A6b	High	⇊	⇊	⇊
2	A19		⇊	⇊	⇊
3	A17		⇊	⇊	⇊
4	A20a		⇊	⇊	⇊
5	A11		⇊	⇊	⇊
6	A16a		⇊	⇊	⇊
7	A19c		⇊	⇊	⇊
8	A13		⇊	⇊	⇊
9	A8a		⇊	—	⇊
10	A8		—	⇊	⇊
11	A3c		—	⇊	—
12	A16b		—	⇊	⇊
13	A20		⇊	⇊	⇊
14	A11a		⇊	⇊	⇊
15	A2e*		⇊	⇊	⇊
16	A2d		⇊	—	⇊
17	A19b		⇊	⇊	⇊
18	A2a		—	—	⇊
19	A2b		⇊	—	—
20	A4b		⇊	—	—
21	A4c		—	—	—
22	A4a*		—	⇊	⇊
23	A3a		⇊	—	—
24	A3b*		⇊	⇊	⇊
25	A5b		⇊	—	⇊
26	A5c		—	⇊	⇊
27	Control*	Low	—	—	—

Transcript level of MsmiR156

[α]: ⇊ reduced transcript levels, — : No difference transcript level among control and transgenic plants.
*Plant genotypes were inclided in morphological characterization.

obvious in A16b, A3c and A8 genotypes though these transgenic plants had relatively higher levels of MsmiR156 transcript.

In the high expression group, the three putative target *SPL* genes were downregulated by MsmiR156. This indicates that higher levels of miR156 are required to regulate many members of *SPL* genes in alfalfa. However, it is still impossible to relate the level of MsmiR156 and the extent to which the target genes are downregulated.

3.5 Phenotypic characterization of MsmiR156 overexpression alfalfa

To investigate the phenotypic effects of MsmiR156 overexpression, select alfalfa genotypes (low and medium expression groups) were propagated by vegetative cuttings. Plants for the high expression group were not included in the phenotypic characterization because generation of these transgenic plants was delayed during the tissue culture processes. Phenotypes including number of branches, plant height, flowering time, appearance of trichomes, root length and nodule numbers were determined.

3.5.1 MsmiR156 affects shoot branching

The effect of MsmiR156 on shoot branching was studied using A2e, A3b and A4a alfalfa genotypes. The number of main branches as well as lateral branches were characterized in forty-day and four-month old alfalfa cuttings. The effect of MsmiR156 overexpression was observed for forty-day old cuttings but it was more obvious at the later stage of plant development (four-month old). Transgenic alfalfa plants that overexpress MsmiR156

A

B

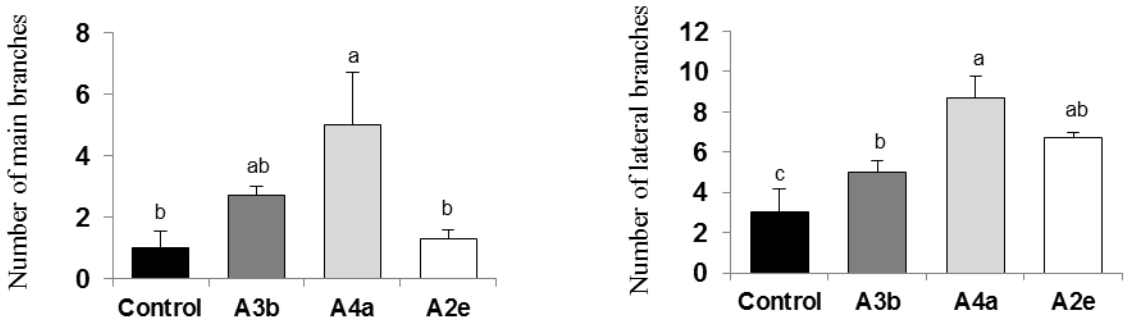
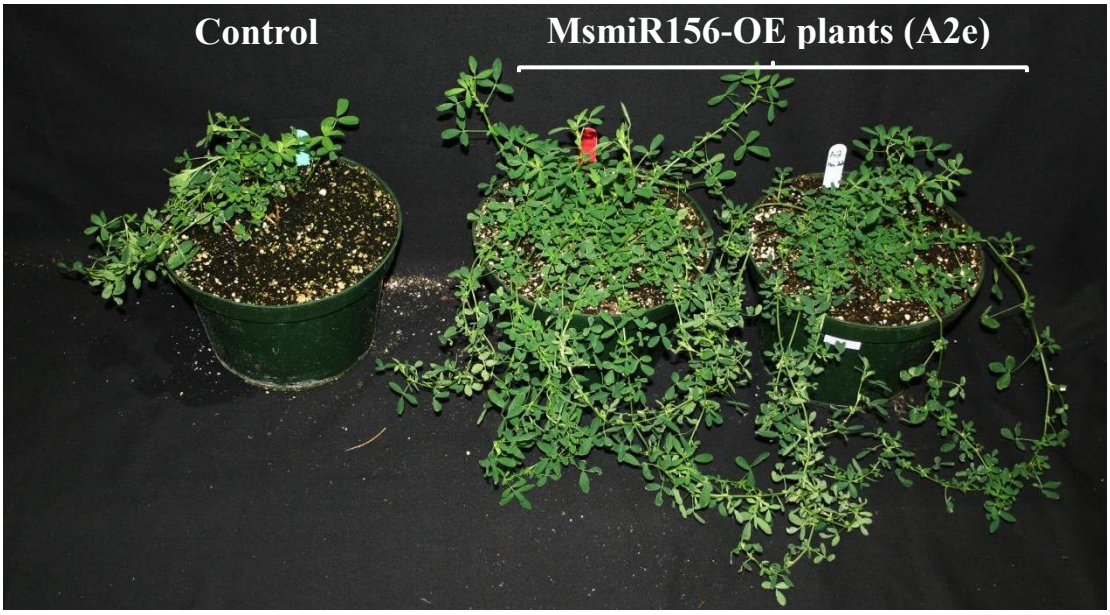


Figure 9. Effect of MsmiR156-OE on branch number in forty-day old alfalfa plants. **A**; Phenotypes of forty-day old MsmiR156-OE alfalfa plants compared to empty vector control. **B**. Number of main branches (left) and lateral branches (right) in transgenic and control plants. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

A



B

Figure 10: (Description on page 64)

C

D

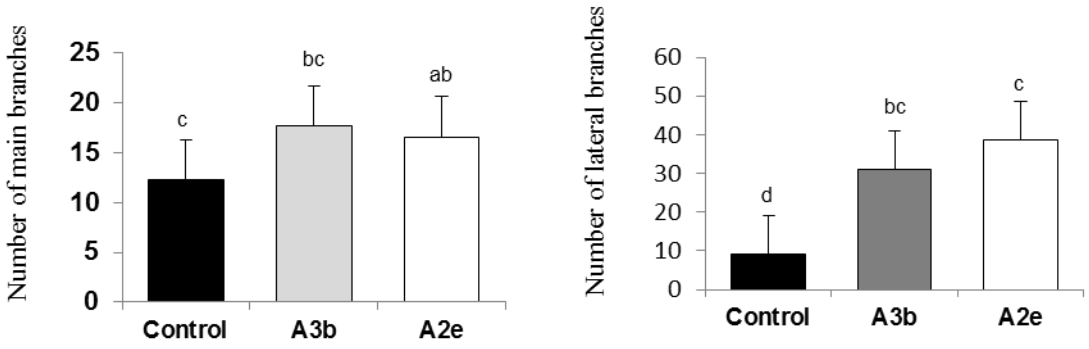


Figure 10. Effect of MsmiR156-OE on number of branches in four-month old alfalfa plants. **A**, **B** and **C;** Phenotypes of A2e, A3b and A4a alfalfa cuttings. **D.** Number of main branches (left) and latera branches (right). Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

showed enhanced shoot branching compared to the empty vector control (**Figure 9 and 10**), however, the transcript level of MsmiR156 was not proportional to the extent of branching; i.e. number of branches. For instance, shoot branches in A2e were less numerous than those in A3b and A4a although the transcript level of MsmiR156 in A2e alfalfa genotype was higher than that in the other two plants. In addition, the number of main branches in A2e was not significantly different from that of the empty vector control in both forty-day and four-month old cuttings. However, the numbers of lateral branches in the three alfalfa genotypes increased by 3-4 folds compared to control. This indicates that overexpression of MsmiR156 promoted lateral shoot branching in alfalfa.

3.5.2 MsmiR156 has a modest effect on plant height

All the plants propagated by stem cuttings continued to grow, and plant heights continued to increase throughout the vegetative stage. Based on the phenotypes of A2e, A3b and A4a transgenic plants, overexpression of MsmiR156 had only a minor effect on plant height in alfalfa. At 40 days post cutting, the plant heights in A2e and A3b genotypes were not significantly different from the control though a significant increase in height was found in A4a (**Figure 11**). At the later stage of plant development; i.e. four months post cutting, however, the plant heights in all MsmiR156-OE alfalfa genotypes were not significantly different from those in empty vector control (data not shown).

3.5.3 MsmiR156 affects internode length and stem thickness

Overexpression of MsmiR156 affected internode length and number of nodes on the stem

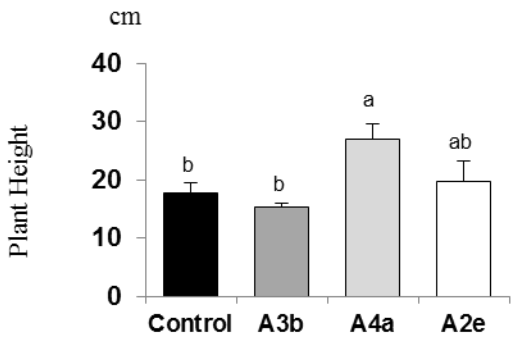


Figure 11. Effect of MsmiR156-OE on plant height in forty-day old alfalfa plants. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

alfalfa, especially at the later stage of plant development (four months post cutting). Overexpression of MsmiR156 reduced the internode lengths by 1-2 folds in A2e and A3b genotypes compared to the control (**Figure 12**). Reducing internode length increased the number of nodes on the stems, thereby producing more branches in the transgenic alfalfa. I also observed a significant reduction in the stem thickness of alfalfa that overexpresses MsmiR156. This reduction in in stem size is shown for both A2e and A3b in **Figure 13**.

3.5.4 MsmiR156 affects trichome density on leaves

Trichomes are hairy appendages or fine outgrowth produced on the plants. In *Arabidopsis*, overexpression of miR156 enhanced leaf trichome production (Schwarz et al., 2008). In this study, increased trichome production was also observed in leaves of transgenic alfalfa overexpressing MsmiR156 as manifested by the higher number of trichomes, and in some cases longer hairs compared to the control (**Figure 14**). Enhanced trichome density is an advantage for protection from pests and insects that hinder alfalfa production worldwide.

3.5.5 MsmiR156 affects flowering time

The effect of miR156 on flowering time was observed in many plant species, including *Arabidopsis*, switchgrass, *L. japonicus* (Zhou and Luo, 2013; Wang et al., 2014). Here, I found that overexpression of MsmiR156 also affected flowering times in alfalfa. Transgenic alfalfa started flowering about three months after vegetative propagation by stem cutting. Compared to the empty vector control, flowering was delayed for 2-5 days

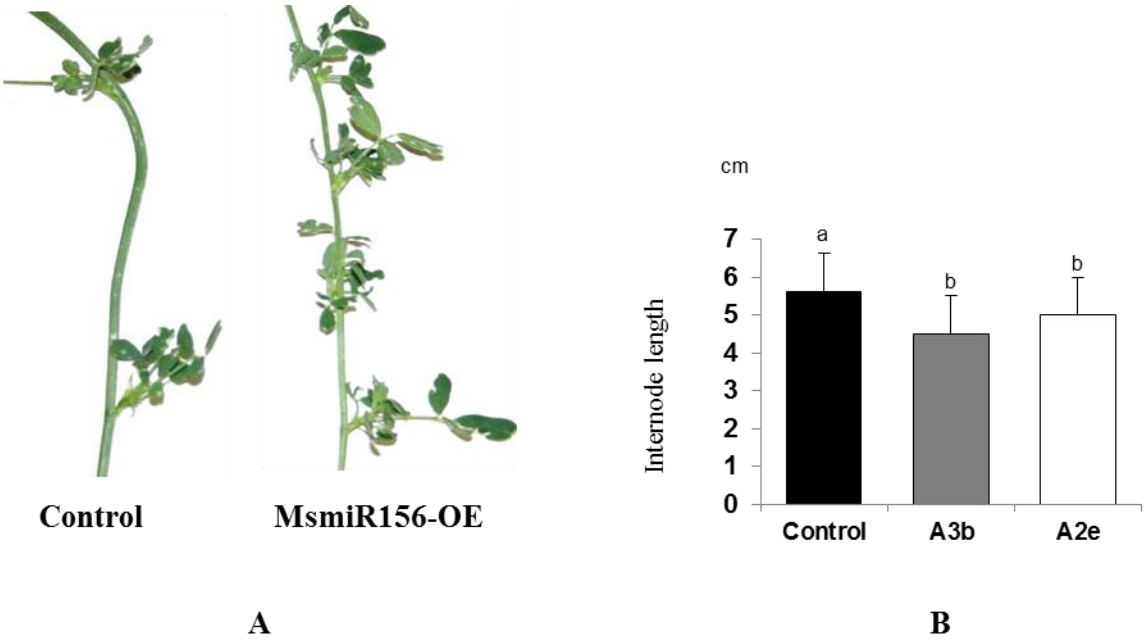


Figure 12. Effect of MsmiR156 on internode length in alfalfa. **A.** Phenotype showing reduced internode length in MsmiR156-OE compared to empty vector control. **B**. Quantitative analysis of internode length. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

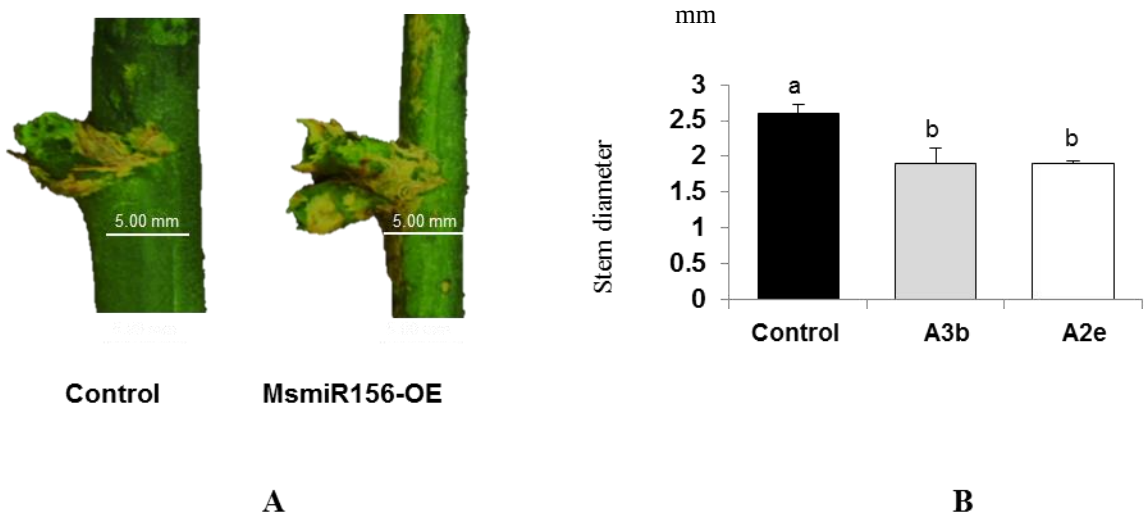


Figure 13. Effect of MsmiR156 on stem thickness in alfalfa. **A.** Phenotype showing reduced stem thickness in MsmiR156-OE compared to empty vector control. **B**. Quantitative analysis of stem thickness. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

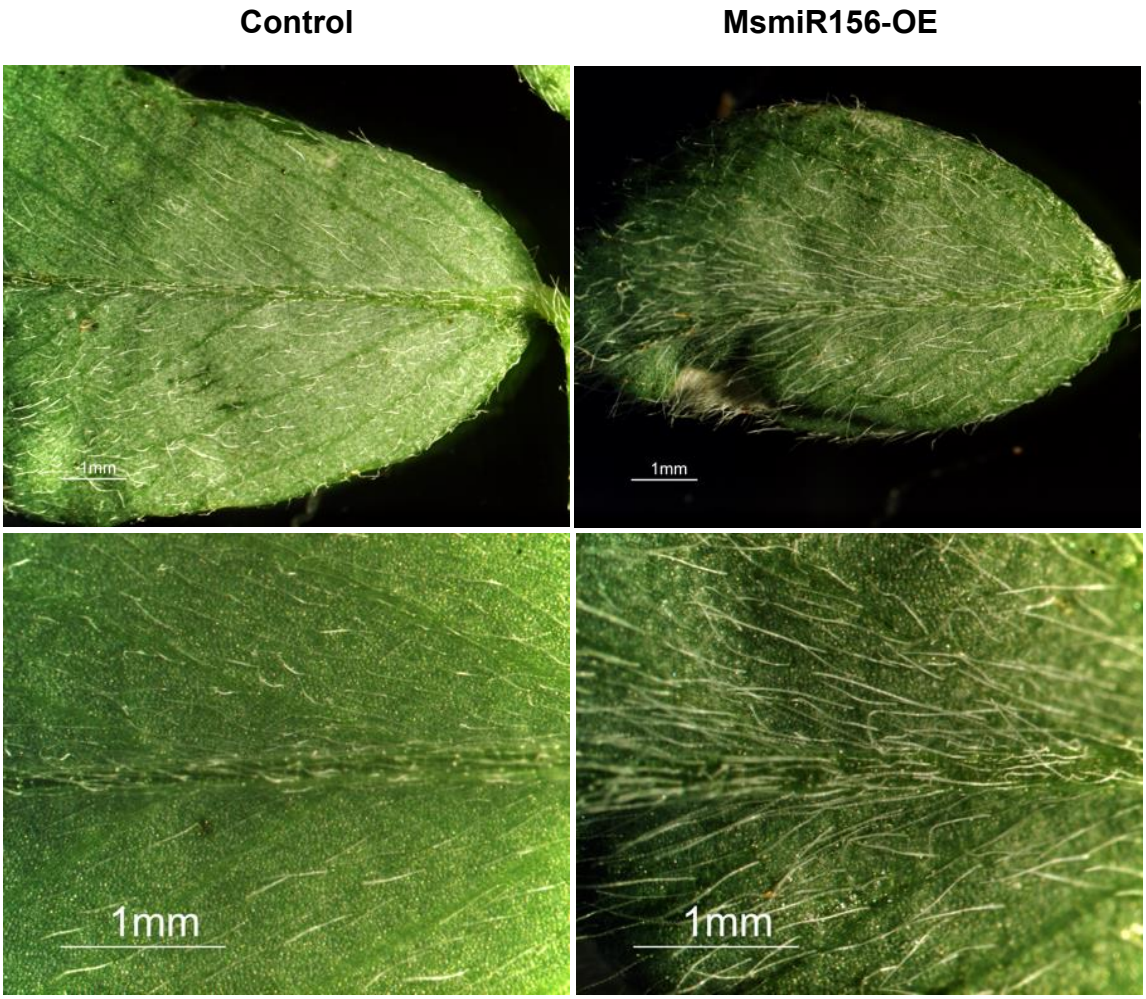


Figure 14. Effect of MsmiR156 on trichome density in alfalfa leaves**.** Trichome texture on the leaves was examined under the microscope using 100 fold magnification.

in transgenic alfalfa; A2e, A3b and A4a. This indicated that overexpression of miR156 prolonged vegetative phase leading to enhanced shoot branching and node production in transgenic plants. In the reproductive phase, the transcript levels of all three *SPL* genes were downregulated (**Figure 15**), indicating that one or more of *SPL*6, *SPL*12 and *SPL*13 are required to control these morphological traits in alfalfa. In addition to *SPL* genes, key regulators of flowering times such as *APETALA 3* (*AP3*) and *FLORAL LOCUS T* (*FTa2*) were also downregulated in transgenic plants overexpressing MsmiR156 (**Figure 16)**. This may indicate that miR156 regulates *SPL* genes which in turn can affect the expression of downstream genes such as *AP3* and *FTa2*.

3.5.6 MsmiR156 affects biomass production

The effect of miR156 on shoot branching was observed in many plant species (Schwarz et al., 2008; Wei et al., 2010; Fu et al., 2012). To investigate the effect of MsmiR156 on forage yield of alfalfa, A2e, A3b and A4a transgenic plants were vegetatively propagated and allowed to grow for three months. The plants were then harvested by cutting all the aboveground tissues, and dried in an oven (60˚C) for 5 days. Dry weights were then measured. Alfalfa plants overexpressing MsmiR156 had up to 2.5-fold increase in in forage yield (**Figure 17**). Although biomass was increased in A2e and A3b, it was not significantly different from the control. However, about 2.5 fold increases in forage yield was observed in A4a genotype, indicating that miR156 is a novel biomass regulator in alfalfa.

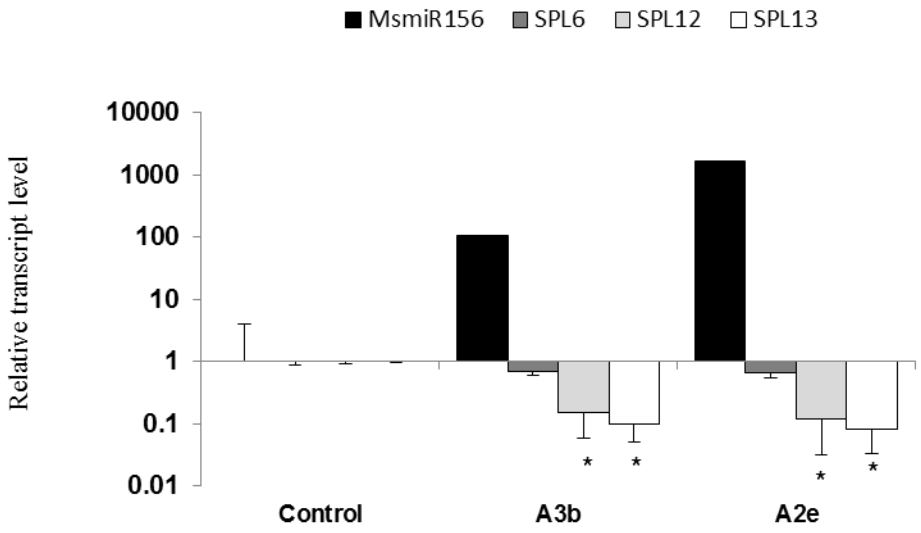


Figure 15. Transcript levels of *MsmiR156* and target *SPL* genes in four-month old alfalfa cutting (Control, A2e and A3b)**.** Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, * $p < 0.05$). Error bar indicates standard error.

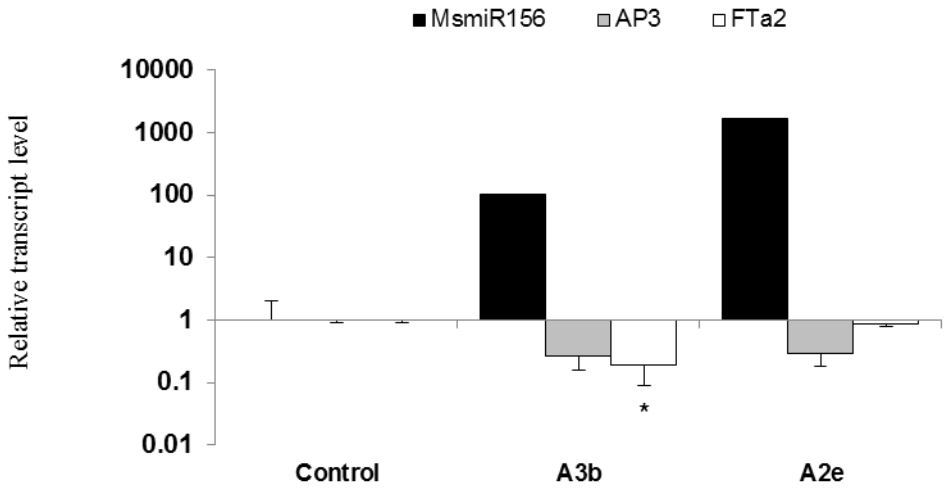


Figure 16. Transcript levels of *MsmiR156* and downstream target genes in four-month old alfalfa cutting (Control, A2e and A3B). Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, * $p < 0.05$). Error bar indicates standard error.

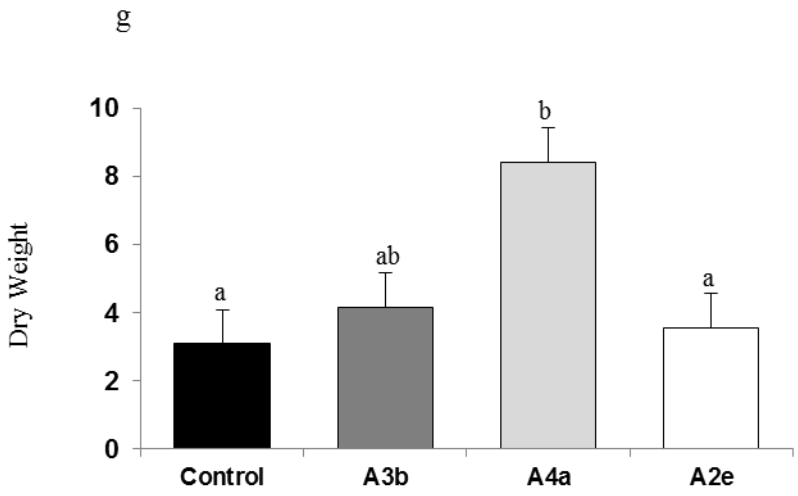


Figure 17. Effect of MsmiR156 on forage yield in alfalfa. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

3.5.7 MsmiR156 has no effect on root length and nodulation

To investigate the effect of miR156 on root length and nodulation, transgenic plants were propagated using sterilized vermiculite mixed with sand media. Two weeks after cutting, the plants were inoculated with *S. meliloti* (Sm1021) and the root length and nodule numbers were analyzed in the following week. I found no significant effect of MsmiR156 on root length in alfalfa (**Figure 18**). In addition, the number of nodules in MsmiR156 overexpression plants was not significantly different from that in the control plants (**Figure 18**).

3.6 Generating alfalfa plants that express *LjmiR156*

To compare the effect of endogenous miR156 (MsmiR156) with that of exogenous miR156, a gene encoding the *L. japonicus miR156* precursor (LjmiR156) was also overexpressed in alfalfa. This gene was isolated from *L. japonicus* and confirmation was carried out as described in Section (3.1). The isolated gene sequence was predicted to produce a secondary structure as shown in **Figure 19**. The precursor was then cloned into pBI121 vector and subsequently transformed to generate LjmiR156 overexpression alfalfa (LjmiR156-OE) (**Figure 20**).

3.6.1 Genotyping of alfalfa plants transformed with *LjmiR156*

To ensure the presence of the transgene (35S::LjmiR156), transgenic plants were screened by PCR using 35S promoter (forward) and gene-specific (LjmiR156bRq: reverse) primers (**Table 1**). In total, 15 transgenic plants showed expected PCR products

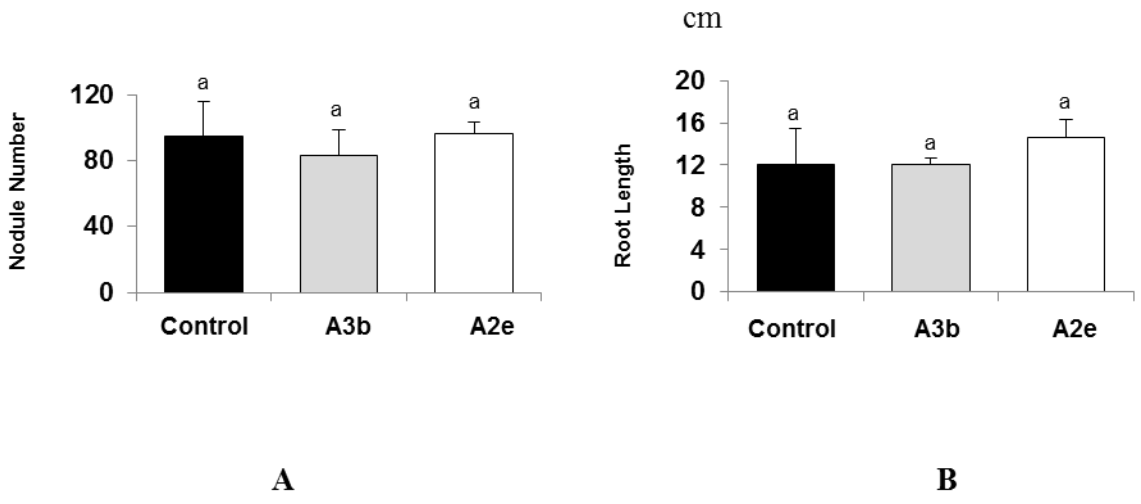
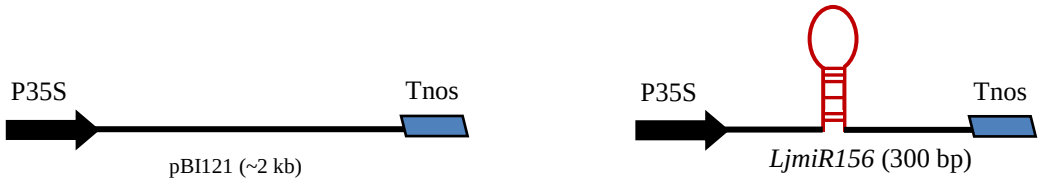


Figure 18. Effect of MsmiR156 on nodule numbers and root length in alfalfa. **A.** Nodule number **B**. Root length. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

Figure 19. Prediction of the secondary structure of LjmiR156 using miRTour. **A**. Hairpin structure of LjmiR156. **B**. Hairpin structure of MtrmiR165. The black lines indicate the mature miR156 sequences.

A



B

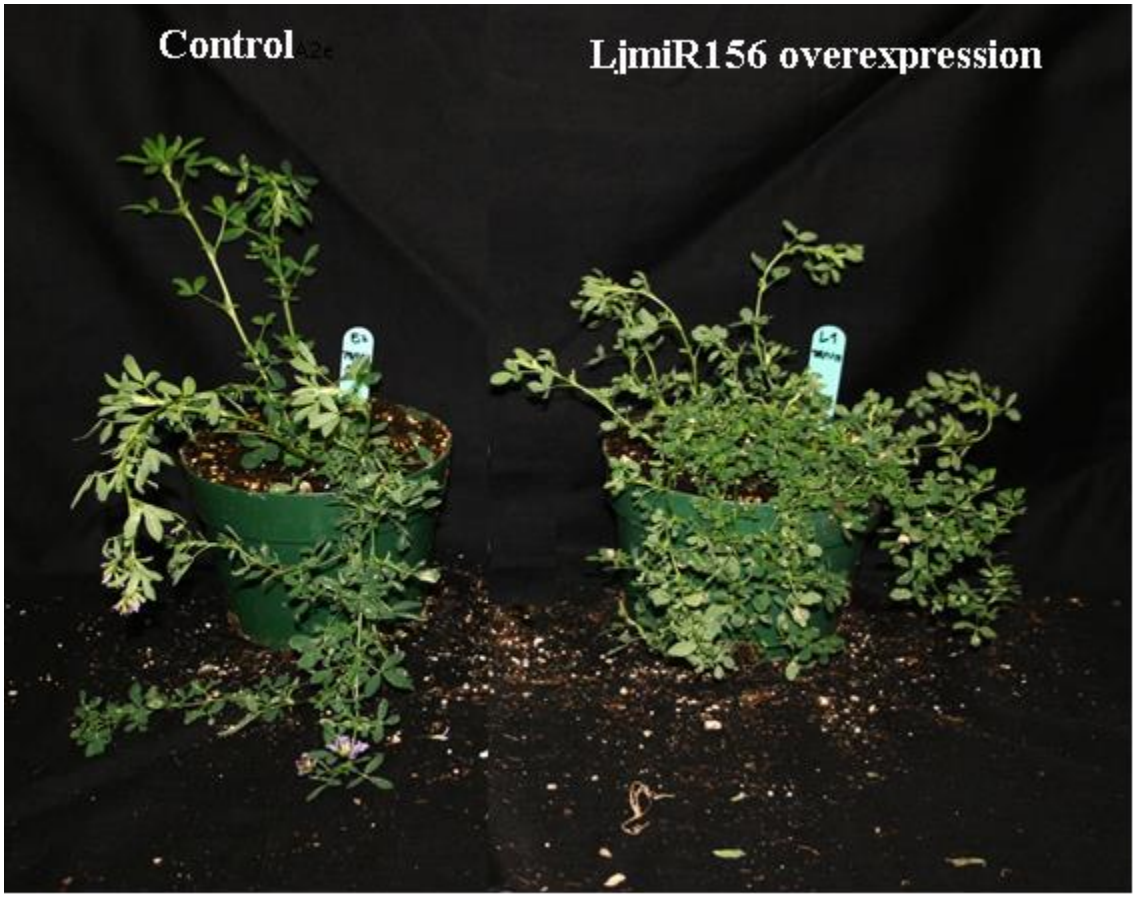


Figure 20. Schematic diagrams of vector constructs and transgenic alfalfa expressing empty vector pBI121 and LjmiR156-OE. **A.** Empty vector PBI121, and overexpression construct carrying a *LjmiR156* gene insert. **B.** Four month old alfalfa plants transformed with either pBI121 control (left) or LjmiR156-OE construct (right).

(data not shown), and the presence of the mature miR156 sequence (20 nt) was confirmed in these plants using small RNA gel blot (**Figure 21**).

3.6.2 Monitoring of transgene and target gene expression

To confirm 5' RACE result (Section 3.3; Figure 5 A, B and C), transcript levels of *LjmiR156* and target *SPL* genes were monitored in LjmiR156 alfalfa plants using qRT-PCR.

Using gene-specific primers for *LjmiR156* transgene, no amplification was obtained from empty vector control (**Figure 22**), and as such, only normalized transcript levels of *LjmiR156* are shown in **Figure 23**.

Based on the transcript levels of LjmiR156, the transgenic plants were assembled into three groups; low expression (L15, L28, L11b, L27, L12), medium (L25, L21, L1b, L9, L20) and high expression (L8b, L1d, L1f, L1c, L1e). As in MsmiR156 transgenic alfalfa, at least one *SPL* gene was downregulated in all transgenic plants encoding 35S::LjmiR156 transgene except for one plant genotype (L11b) (**Figure 24 and Table 5**). Contrary to MsmiR156 transgenic plants, a similar pattern of expression of *SPL*6 and *SPL*13 was observed in all LjmiR156 transgenic plants except for L15 in which only *SPL*13 was reduced. However, the type and number of regulated *SPL* genes did not correlate to the level of *LjmiR156* expression.

In the low expression group, all three *SPL* genes were downregulated in L28 and L27 in which normalized transcript levels of *LjmiR156* gene were among the lowest in the transgenic plants. However, none of the predicted targets were affected in L11b alfalfa

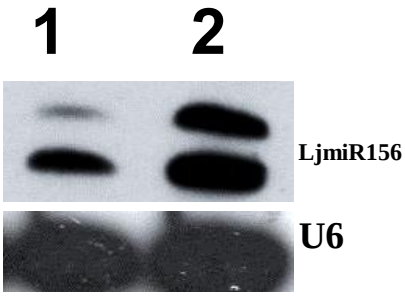


Figure 21. Small RNA gel blot showing mature LjmiR156 in alfalfa. Lane 1: empty vector control; lane 2: LjmiR156-OE alfalfa. Small RNAs (3 μg) were equally loaded in each lane and hybridized with *miR156* gene specific probe. Small RNA U6 gene was used as loading control in the blot.

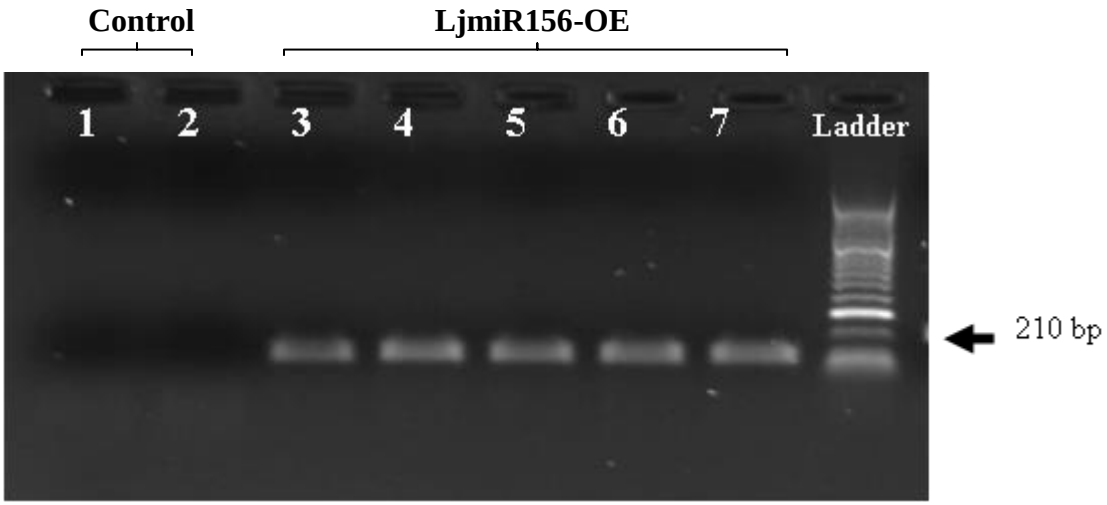


Figure 22. Visualization of qRT-PCR results for LjmiR156 transgene gene in LjmiR156 genotypes. Lanes 1 to 7 refer to Control 1, Control 2, L1a, L1b, L1c, L1d, and L1e. Ladder: DNA HyperLadder II.

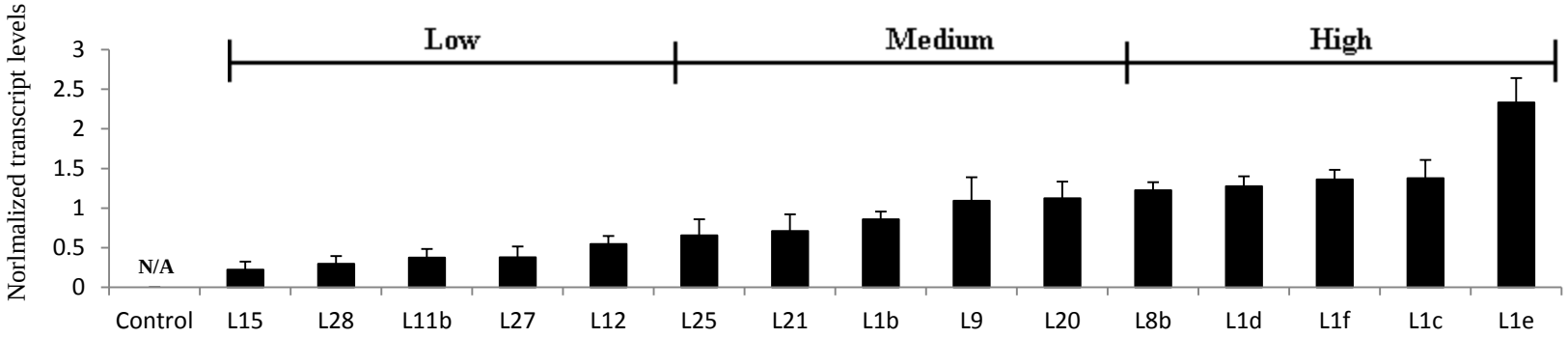


Figure 23. Transcript levels of *LjmiR156* gene in independent LjmiR156 overexpression alfalfa. The name of transgenic alfalfa genotype consists of prefix capital letter (Lotus)-callus number-suffix small letter (refer to different plants from the same callus). Each bar represents the mean values from three experiments for each independent alfalfa genotype. Error bars indicate standard error.

■ LjmiR156; N/A: not amplified

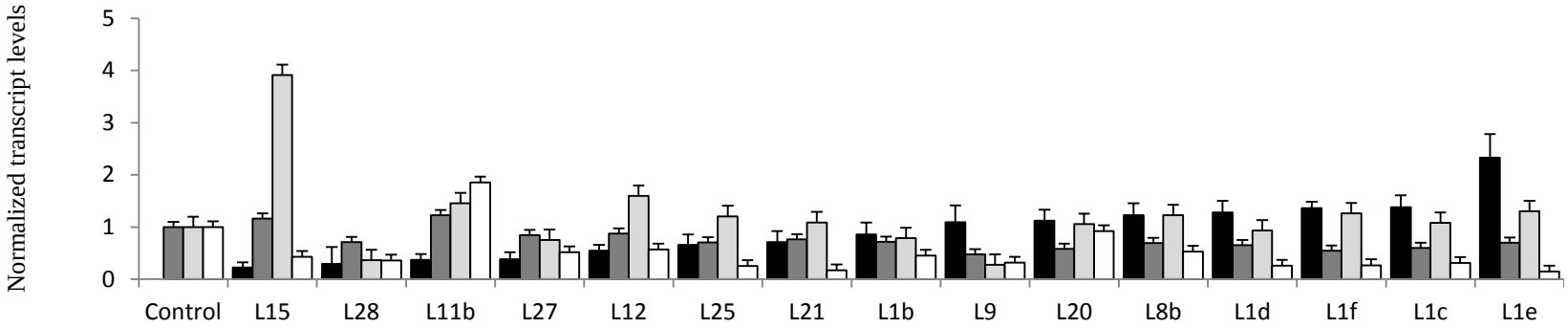


Figure 24. Transcript levels of *LjmiR156* and target genes in independent LjmiR156 overexpression alfalfa. Each bar represents the mean values from three experiments for each independent alfalfa genotype. Error bar indicates standard error. ■ LjmiR156, ■ *SPL*6 ■ *SPL*12 and □ *SPL*13. N/A: not amplified**.**

Table 5. Summary of transcript levels of *LjmiR156* and target *SPL* genes

No.	Alfalfa genotype	LjmiR156	*SPL*6[α]	*SPL*12[α]	*SPL*13[α]
1	L1e*	High	⇊	—	⇊
2	L1c*		⇊	—	⇊
3	L1f		⇊	—	⇊
4	L1d		⇊	⇊	⇊
5	L8b		⇊	—	⇊
6	L20		⇊	—	⇊
7	L9		⇊	⇊	⇊
8	L1b		⇊	⇊	⇊
9	L21		⇊	—	⇊
10	L25		⇊	—	⇊
11	L12		⇊	—	⇊
12	L27		⇊	⇊	⇊
13	L11b		—	—	—
14	L28		⇊	⇊	⇊
15	L15		—	—	⇊
16	Control	Low	—	—	—

Transcript level of LjmiR156

[α]: ⇊ reduced transcript levels, — : No significant difference in transcript level between control and transgenic plants. *These plant genotypes were included in morphological characterization.

genotypes which contained approximately equal levels of LjmiR156 transcript. One (*SPL*13) and two (*SPL*6 and *SPL*13) targets were downregulated in L15 and L12 genotypes, respectively.

In the medium and high expression groups, *SPL*6 and *SPL*13 were regulated in all alfalfa genotypes, but reduced levels of *SPL*12 were observed in two alfalfa genotypes in the medium expression (L9 and L1b) group, and only in L1d in the high expression group. These transcript levels were monitored at the early stage of vegetative phase (about 6 weeks after cutting). At the late stage of the vegetative phase (beginning of reproductive phase), all three *SPL* members were downregulated in LjmiR156 transgenic alfalfa (high expression group) (Section 3.7.4).

3.7 Phenotypic characterization of LjmiR156 overexpression alfalfa

Only L1c and L1e genotypes of LjmiR156 overexpression alfalfa plants were selected for further morphological characterization as described for MsmiR156 (Section 3.5).

3.7.1 LjmiR156 enhances shoot branching

The main and lateral branches in LjmiR156 overexpression alfalfa plants were counted at 40-day post cutting and at the early reproductive stage (4-month-post cutting). Like in the case of MsmiR156 overexpression alfalfa, an increase in the number of shoot branches was observed at 40-day-old cuttings but a more prominent effect on branching was observed at the late stage of plant development (beginning of reproductive phase) (**Figure 25 and 26**). At this stage, the numbers of main branches and lateral branches were

A

B

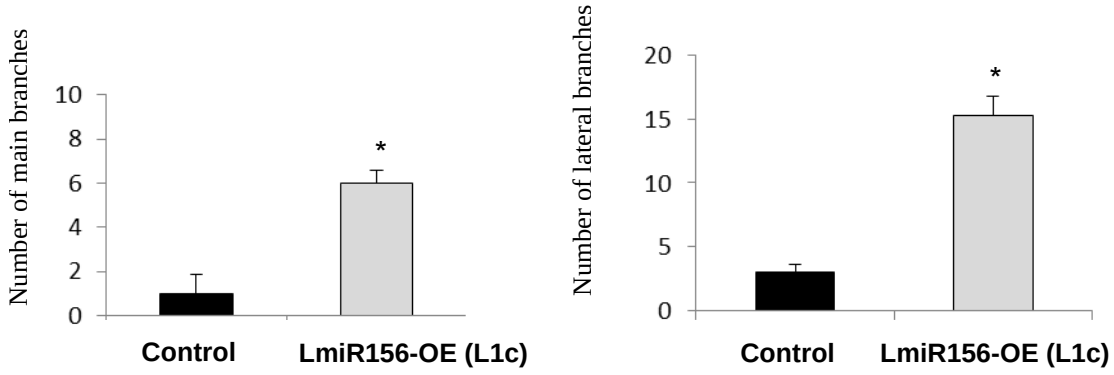


Figure 25. Effect of LjmiR156-OE on branch number in forty-day old alfalfa plants. **A**; Phenotypes of forty-day old LjmiR156-OE alfalfa plants (right) and an empty vector control plant (left). **B**. Number of main branches (left) and lateral branches (right) in LjmiR156-OE and control plants. Each bar represents the mean values from three biological replicates (t-test, *$p < 0.05$). Error bar indicates standard error.

A

B

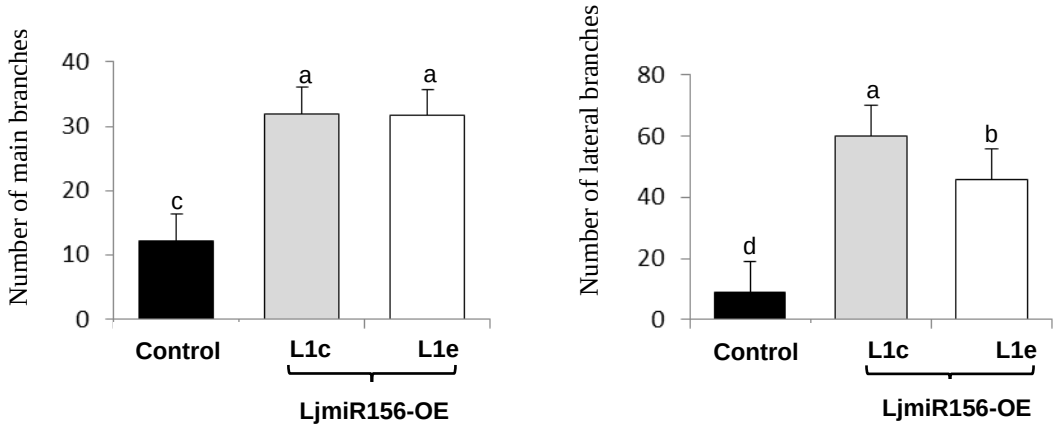


Figure 26. Effect of LjmiR156-OE on branch number in four-month old alfalfa plants. **A**; Phenotypes of forty-day old LjmiR156-OE alfalfa plants (right) and an empty vector control plant (left). **B**. Number of main branches (left) and lateral branches (right) in LjmiR156-OE and control plants. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

significantly increased in the LjmiR156 overexpression plants compared to the empty vector control. The number of the lateral branch was always higher than the number of the main branch. At forty days after cutting, about 4-6 main branches and 13-15 lateral branches were produced in the LjmiR156 overexpression alfalfa whereas only 1-2 main branches and 3-5 lateral branches were found in empty vector control. At the early stage of reproductive phase, about 4-5 fold increase in the numbers of branch was found in the transgenic plants. It is thus obvious that expression of *LjmiR156* significantly increases shoot branching in alfalfa.

3.7.2 LjmiR156 has minor effect on plant height

At forty days after cutting, no significant difference in plant heights was found between the miR156 overexpression plants and the empty vector control (data not shown**)**. As the plants continued to grow, the height in the control plants increased, but that in miR156 overexpression did not. At four-month after cutting, a significant reduction in plant height was found in L1c compared to the control (**Figure 27**).

3.7.3 LjmiR156 affects internode length, stem thickness and trichome density

The effects of LjmiR156 on internode length and stem thickness in alfalfa were similar to those of MsmiR156 (Section 3.5.3 and 3.5.4). However, the effect of LjmiR156 was more prominent. For instance, average internode lengths in LjmiR156 plants were 2 to 3 cm while a longer length was observed in MsmiR156 alfalfa genotypes (3.5 to 4 .5 cm) (**Figure 12 and 28**). In a particular internode length, more nodes were thus produced in

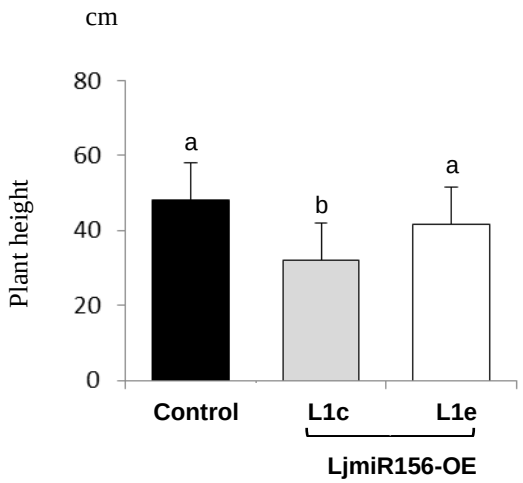


Figure 27. Effect of LjmiR156-OE on plant height in four-month old alfalfa plants**.** Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

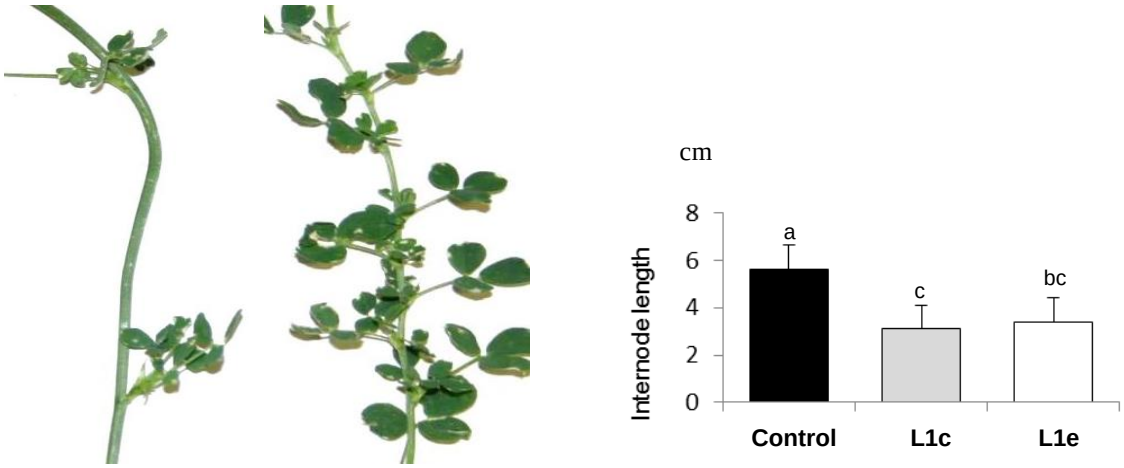


Figure 28. Effect of LjmiR156 on internode length in alfalfa. **A.** Phenotype showing reduced internode length in LjmiR156-OE plant compared to empty vector control. **B**. Quantitative analysis of internode length. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

LjmiR156 alfalfa genotypes. Furthermore, a significant reduction in stem thickness was also observed in LjmiR156 expression plants (**Figure 29**).

As in MsmiR156 overexpression alfalfa, enhanced trichome density and hair length were observed for LjmiR156 expression plants compared to the empty vector control (**Figure 30**).

3.7.4 LjmiR156 affects flowering time

Unlike MsmiR156 overexpression alfalfa, a significant delay in flowering time was found in LjmiR156 expression alfalfa genotypes. Contrary to empty vector control, flowering was delayed for up to 50 days in L1c and L1e LjmiR156 alfalfa. These alfalfa genotypes started flowering when they were 4 to 4 ½ months old whereas empty vector plant flowered about 3 months after vegetative propagation by stem cutting. This suggested that prolonged vegetative phase would contribute to a significant increase in the number of branches in the miR156 overexpression plants. Moreover, I found that the transcript levels of all three *SPL* genes were downregulated at this stage of plant development, indicating that *SPL*6, *SPL*12 and *SPL*13 may be functionally redundant in controlling these morphological traits in alfalfa (**Figure 31**). Like MsmiR156 overexpression alfalfa plants, key regulators of flowering times such as *APETALA 3* (*AP3*) and *FLORAL LOCUS T* (*FTa2*) were also downregulated in transgenic plants expressing LjmiR156 (**Figure 32).**

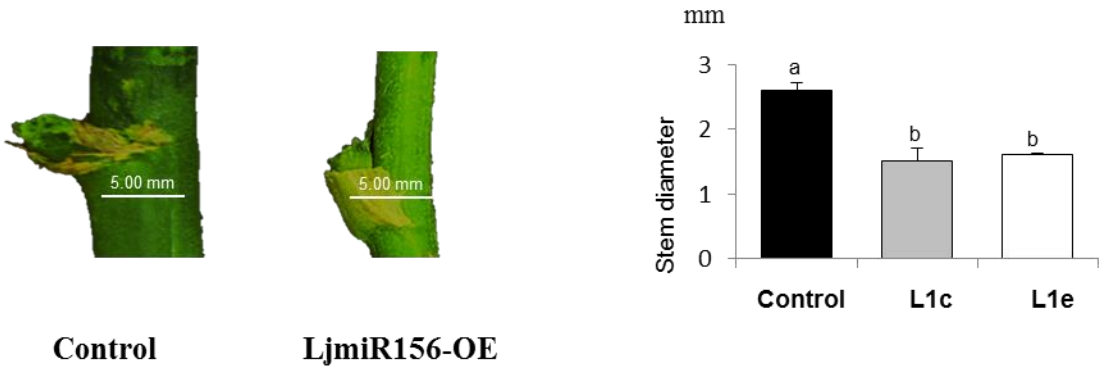


Figure 29: Effect of LjmiR156 on stem thickness in alfalfa. **A.** Phenotype showing reduced stem thickness in LjmiR156-OE compared to empty vector control. **B**. Quantitative analysis of stem thickness. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

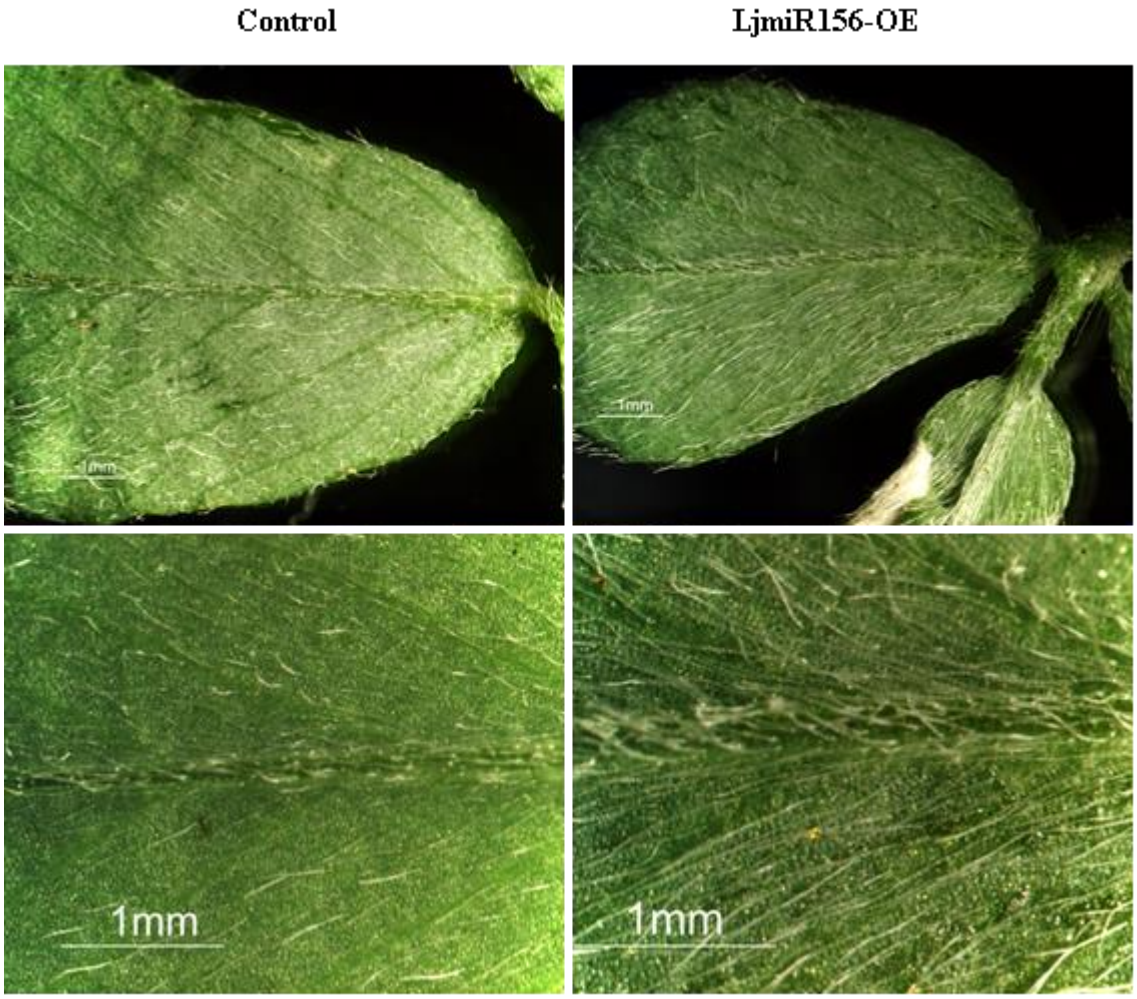


Figure 30: Effect of LjmiR156 on trichome density in alfalfa leaves. Trichome texture on the leaves was examined under the microscope using 100 fold magnification.

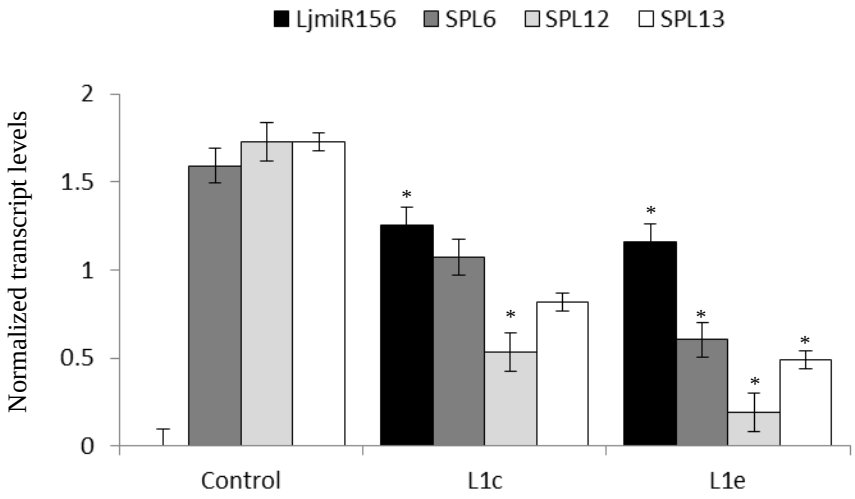


Figure 31. Transcript levels of *LjmiR156* and target *SPL* genes in four month old alfalfa cuttings. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, * $p < 0.05$). Error bar indicates standard error.

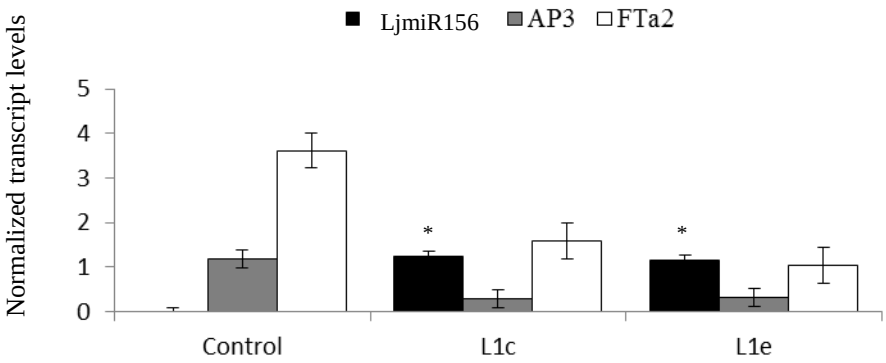


Figure 32. Transcript levels of *LjmiR156, AP3 and FTa2* genes in four-month old alfalfa cuttings (Control, L1c and L1e)**.** Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, * $p < 0.05$). Error bar indicates standard error.

3.7.5 LjmiR156 affects biomass production

The effect of LjmiR156 on alfalfa forage yield was also investigated as described for MsmiR156 (Section 3.5.6). Augmented biomass production was observed in transgenic alfalfa expressing LjmiR156 (**Figure 33**). Both L1c and L1e alfalfa genotypes showed increased forage yield compared to control. This result highlights the potential role that miR156 could play in breeding for improved forage yield in alfalfa.

3.7.6 LjmiR156 affects nodulation but has no effect on root length

The effects of LjmiR156 on nodulation and root length were investigated as described for MsmiR156 alfalfa (Section 3.5.7). Expression of LjmiR156 had no effect on root length (**Figure 34A**), but it caused a range of reductions in nodule numbers in the selected alfalfa genotypes (**Figure 34B**). Although nodulation was reduced in L1a, L1b, L1e, L1f and L1g alfalfa, the reduction in nodule number was not significantly different from the empty vector control. In L1c and L1d, however, the numbers of nodule were significantly reduced. This suggests that MsmiR156 and LjmiR156 have varying effects on nodulation in alfalfa.

3.8 Generating SPL-RNAi alfalfa

To characterize the functions of each target *SPL* gene (*SPL6*, *SPL12* and *SPL13*), alfalfa was transformed with RNAi constructs (**Appendix B**) of each of the 3 *SPL* genes (SPL-RNAi) as described in the Methods section.

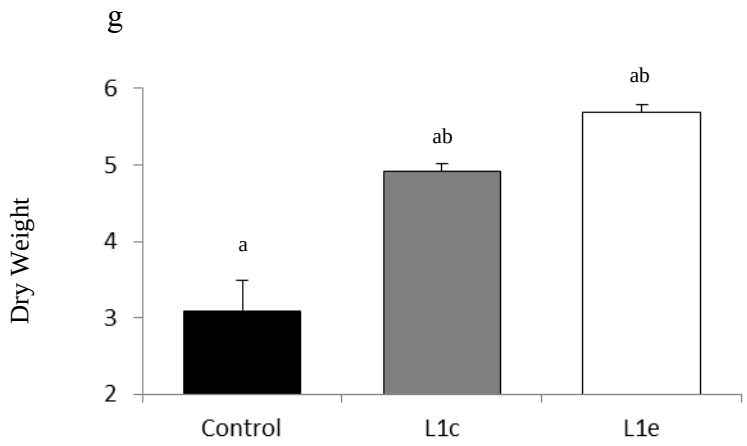


Figure 33. Effect of LjmiR156 on forage yield in alfalfa. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

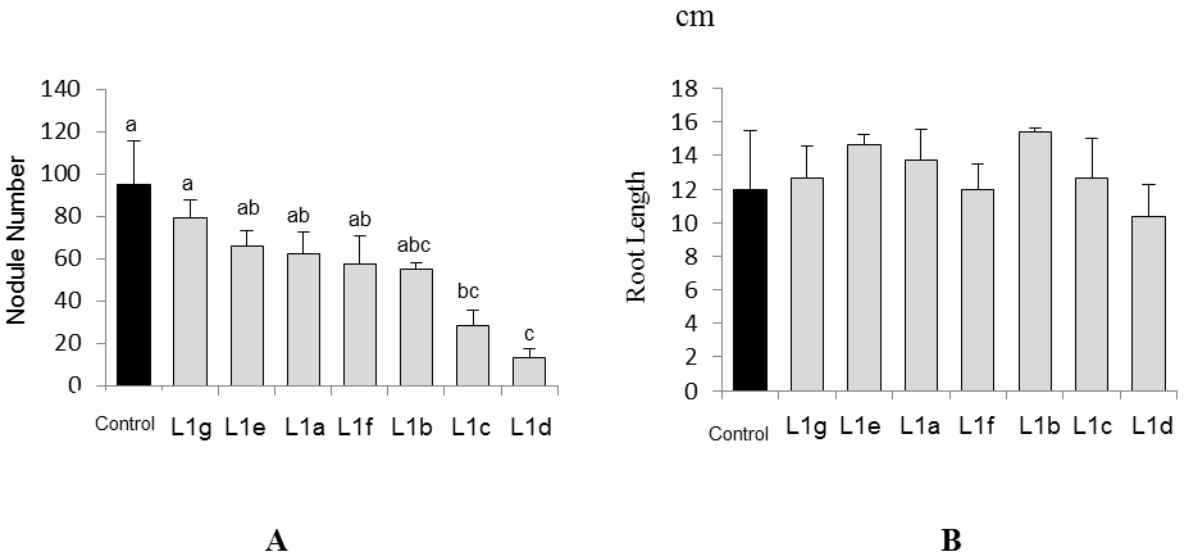


Figure 34. Effect of LjmiR156 on root length and nodulation in alfalfa**. A.** Effect on nodule number; **B**. Effect on root length. Each bar represents the mean values from three biological replicates (One Way ANOVA, Duncan's test, $p < 0.05$). Error bar indicates standard error.

3.8.1 Genotyping of SPL-RNAi alfalfa plants

To confirm the presence of the transgene (35S::SPL-Intron-SPL), transformed plants were genotyped by PCR using 35S promoter (forward) and *SPL* gene-specific reverse primers (Table 1). With these primer pairs, only sense fragments (35S::SPL) were detected. To ensure the presence of the anti-sense fragment (Intron-SPL), the transformed plants were also genotyped using intron-specific and gene-specific forward primers (Table 1). Transgenic plants harboring 35S::SPL-Intron-SPL transgene showed expected PCR products from the two PCR reactions (data not shown). For empty vector control, 35S promoter (forward) and pHellgate12 vector-specific (reverse) primers were used in the PCR. Positive transgenic plants with expected PCR products were selected for further analysis.

3.8.2 Monitoring of target gene expression in SPL-RNAi alfalfa

It is expected that transcript levels of target *SPL* genes are downregulated in respective SPL-RNAi alfalfa. The transcript level of each of *SPL* gene was analyzed in SPL6-RNAi, SPL12-RNAi and SPL13-RNAi alfalfa genotypes using qRT-PCR as described above. In the SPL-RNAi alfalfa, the expression of acetyl CoA carboxylase genes was not uniform across the plant genotypes. Hence, two other reference genes (18S rRNA and β-actin) were used to normalize the transcript level.

Of the 30 SPL6-RNAi alfalfa genotypes, *SPL*6 transcript was lower in five relative to the empty vector control (**Figure 35**). Among the 40 SPL12-RNAi alfalfa genotypes, 17 plants were found with dwonregulation of target *SPL*12 (**Figure 36**). Finally, only 3

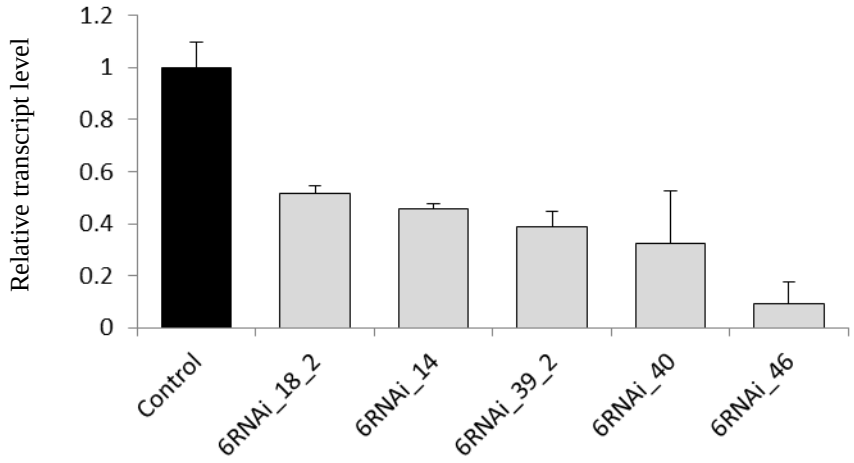


Figure 35. Transcript levels of *SPL*6 in independent SPL6-RNAi alfalfa genotypes. Each bar represents the mean values from three experiments for each independent alfalfa genotype. Error bar indicates standard error.

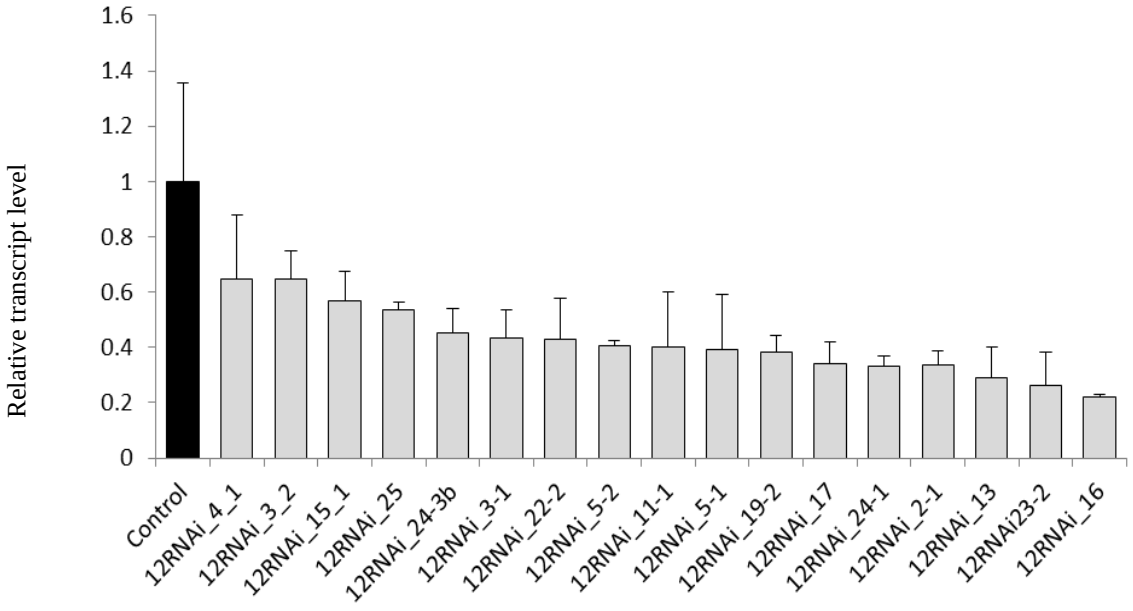


Figure 36. Transcript levels of *SPL*12 in independent SPL12-RNAi alfalfa genotypes. Each bar represents the mean values from three experiments for each independent alfalfa genotype. Error bar indicates standard error.

SPL13-RNAi alfalfa plants (total 30 plants screened) had a silenced *SPL*13 (**Figure 37**). The SPL-RNAi alfalfa genotypes with reduced expression of target *SPL* genes were selected for further characterization.

3.9 **Generating SPL overexpression alfalfa**

To validate the functions of target *SPL* genes, I also overexpressed each of the three target *SPL* genes in alfalfa. To generate *SPL* overexpression alfalfa, full length *SPL* gene sequences were isolated from alfalfa. Complete sequences of alfalfa *SPL*6 and *SPL*13 were obtained and cloned into Gateway destination vector (pMDC32: overexpression under 35S promoter) (**Appendix C**). The complete *SPL*12 sequence was isolated from *M. truncatula* and cloned into the Destination vector. Finally, SPL overexpression alfalfa (SPL-OE) were generated through *A. tumefaciens* transformation.

To date, SPL6-OE and SPL13-OE transgenic plants have been obtained but SPL12-OE alfalfa is still in progress. The presence of transgenes (35S::SPL) in SPL6-OE and SPL13-OE alfalfa plants was confirmed using PCR, and positive plants were selected for further analysis.

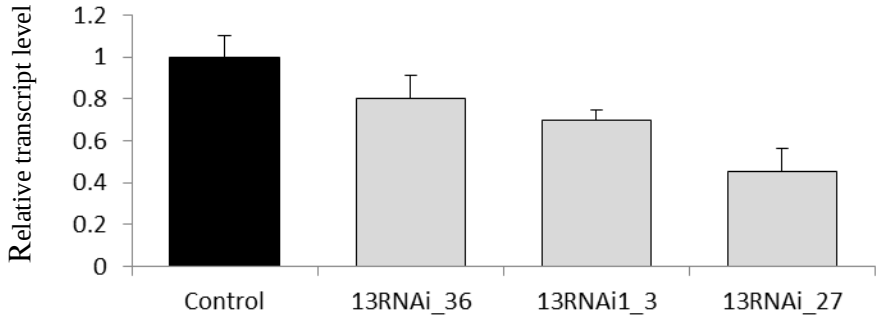


Figure 37. Transcript levels of *SPL*13 in independent SPL13-RNAi alfalfa genotypes. Each bar represents the mean values from three experiments for each independent alfalfa genotype. Error bar indicates standard error.

4 DISCUSSION

4.1 Research overview

MicroRNAs regulate plant development in annual and perennial plants (Rubinelli et al., 2013; Zhou and Luo, 2013), and miR156 has been shown to affect biomass production in plants, including a bioenergy crop; e.g. switchgrass (Chuck et al., 2011; Fu et al., 2012). Thus, I hypothesized that miR156 could also be used in forage yield improvement in alfalfa. Studies have demonstrated that miR156 regulates target *SPL* genes and enhances leaf initiation, shoot branching, and inhibits floral transition in *Arabidopsis*, *B. napus* and *P. virgatum* (Zhou and Luo, 2013, Wei et al., 2010). Given the conserved nature of miR156 function in plants, I also hypothesized that overexpression of miR156 in alfalfa would affect floral transition, plant architecture, and biomass production by regulating target *SPL* genes.

To investigate my hypothesis, an *in silico* search was first conducted to determine the number of miR156 precursors available in alfalfa genome sequence databases. To study the function of miR156 in alfalfa, transformation was carried out with miR156 homologs of alfalfa and *L. japonicus*. The genes targeted by miR156 for cleavage were predicted using 5' RLM-RACE and sequencing approaches. Then, the abundance of miR156 and target *SPL* transcripts was monitored using qRT-PCR. To characterize the phenotypes of alfalfa, transgenic plants were vegetatively propagated by stem cuttings. The phenotypes characterized include number of shoot branches, plant height, stem diameter, internode length, flowering time, biomass, root length and nodulation. The phenotypic changes were then related to the molecular data by analyzing the transcript abundance of miR156,

target *SPLs* and downstream genes. Finally, the potential effects of miR156 on alfalfa improvement are discussed.

4.2 Identification of miR156 and its targets in alfalfa

In silico analysis revealed no miR156 precursors in publicly available alfalfa databases. Indeed, the number of miRNAs obtained in *in silico* analysis depends on the conservation of the miR156 sequences, their structures, the number of previously known miRNAs and the sequences deposited in public databases (Zhang et al., 2006). Next Generation sequence reads of alfalfa obtained by Dr. R. Austin (AAFC; unpublished data) contained short-miR156 like sequences (~ 60 bp). However, it was unclear if these reads were miR156 precursors or other small RNA sequences, since none of them was long enough to produce the secondary structure that is a unique feature which differentiates miRNA from other small RNA species. To unravel the genomic loci encoding miR156 paralogs, complete genome sequencing of alfalfa would be required. In this study, a precursor of MsmiR156 was isolated from alfalfa by PCR and used to conduct functional characterization in this plant. This indicates that miR156 is also encoded in the alfalfa genome though the number of miR156 loci in this plant's genome remains unknown.

Many approaches have been developed to identify cleavage targets of miRNAs, including a modified 5' RACE method that is generally accepted as the most direct approach (Voinnet, 2009). In this study, the bioinformatics analysis revealed 5 *M. truncatula SPL* gene accessions which contain miR156 binding sites. However, 5' RACE showed that only 3 paralogs (*SPL*6, *SPL*12 and *SPL*13) are the targets of miR156 in alfalfa. This supports previous findings that miR156 targets different *SPL* paralogs across

plant species (Xie et al., 2006; Xing et al., 2010). For instance, miR156 affects 10 members of *SPL* in *Arabidopsis*, 11 in *O. sativa* and 2 in *L. japonicus* (Xie et al., 2006; Xing et al., 2010; Wang et al., 2014). However, it is highly probable that more members of miR156-targeted *SPL* genes will be found in alfalfa, as well as *L. japonicus,* once genomes are fully sequenced and published.

4.3 Function of miR156 in alfalfa

Identification of miRNAs across plant species revealed conserved as well as nonconserved (unique) miRNA families (Jones-Rhoades, 2012). Among these, miR156 is known as a conserved family that is identified in many plant species (Jones-Rhoades, 2012, Sun, 2012). Further research in miRNA revealed that miR156 regulates *SPL* paralogs, which function in diverse aspects of plant development (Xie et al., 2006; Martin et al., 2010a; Wei et al., 2010; Fu et al., 2012). Functions of miR156-regulated *SPL* genes have been described for *A. thaliana, G. max, O. sativa, Ricinus communis, Manihot escunlenta, Phaseolus vulgaris, Populus trichocarpa, Solanum lycopersicum, Pinustaeda, Physcomitrella patens* and *Vitis vinifera* (Sun, 2012).

It is well accepted that plant miRNAs exploit both transcript cleavage and translational repression mechanisms to regulate target gene expression (Voinnet, 2009). In other words, the use of a single mechanism, either cleavage or translational inhibition, is not observed in plant miRNA-mediated gene regulation. Using a miRNA-action deficient (*mad*) mutant in *Arabidopsis*, Broderen et al. (2008) demonstrated that miRNA is involved in degradation as well as translational inhibition of the targets. It is now evident that miR156 regulates *SPL*3 gene expression using both cleavage and translation

inhibition mechanisms (Wu and Poethig, 2006; Gandikota et al., 2007). In this study, I found that miR156 regulates alfalfa *SPL* gene expression using a cleavage mechanism. Yet, experiments are needed to unravel the translational inhibition of alfalfa *SPL* transcripts by miR156.

Plant miRNAs employ perfect or near-perfect sequence complementary modes to scan their targets. According to Joshi et al., (2010), nucleotide positions 10 and 11 in miRNA must perfectly match to the target sequence. Also, any mismatch should not be more than 1 in the positions between 2 and 9 in the miRNA. In this study, only one mismatch, which is in the 14^{th} position, was found between miR156 and target alfalfa *SPL* transcripts. Furthermore, it was observed that miR156 binding sites in the alfalfa *SPL* genes are located in coding regions of the corresponding transcripts. The binding sites of plant miRNAs are commonly found in the coding regions, but binding sites in the 5' UTR or 3' UTR of the target genes are also found (Bartel, 2004; Sunkar and Zhu, 2004; Chiou et al., 2006).

The miR156 binding sites among the *SPL* paralogs are not identical. In this study, the number of cleavage and cleaved sites in each alfalfa *SPL* transcript was confirmed through sequencing. Cleavage sites in the alfalfa *SPL* transcripts were observed between the 9 and 11 nucleotide positions in the miR156 binding region. The miR156 cleavage sites on *SPL* transcripts are commonly found in these regions (Mica et al., 2006), but recent studies showed that cleavage beyond these regions was also possible (Kim et al., 2012; Wang et al., 2014). For instance, the cleavage of *SPL*3 transcripts was also observed beyond the miR156 binding sites in *Arabidopsis* (Kim et al., 2012). These

results suggest that prediction of the cleaved sites based solely on bioinformatics analysis is unreliable, and experimental evidence is therefore required to validate the miRNA targets.

Apart from *SPL* genes, miR156 is known to regulate non-conserved *WD*40, which functions in signal transduction, transcription regulation, and apoptosis in eukaryotes (Naya et al., 2010). In this study, cleavage sites of miR156 were also found in *M. sativa WD*40 transcripts, thereby showing that miR156 is a conserved family, regulating target paralogs that are conserved across plant species. However, contrary to the cleavage in *SPL* transcripts, at least 4 mismatches were found between miR156 and target *WD*40 binding sequences. Furthermore, cleavage sites in one of the *WD*40 transcripts (WD40_1) were also found beyond the miR156 binding sites. So far, miR156/SPL gene networks are extensively studied in plants, but research on the miR156-mediated *WD*40 gene regulation is relatively rare. Naya et al. (2010) showed that a specific isoform of miR156 cleaves non-conserved *WD*40 targets in *M. truncatula* root apexes. However, the authors merely predicted *WD*40 as targets of miR156 of isoforms, and no experiment was conducted to validate the prediction. Hence, my 5'RACE analysis is an additional piece of evidence that confirms *WD*40 genes as targets of miR156 in *M. sativa*.

4.4 MiR156-mediated gene regulation in alfalfa

This study revealed that both MsmiR156 and LjmiR156 regulate the target *SPL* genes but the two miR156s exhibit different patterns of regulation. To further validate the targets of miR156, the abundance of *SPL*6, *SPL*12 and *SPL*13 transcripts were monitored in both MsmiR156 and LjmiR156 overexpression alfalfa genotypes. The transcript of the three

SPL genes were reduced in miR156 overexpression alfalfa, thereby reinforcing the finding that these alfalfa *SPL* genes are regulated by miR156. However, in some overexpression plants, not all three *SPL* genes are downregulated. For instance, the transcript levels of all three *SPL* genes are reduced in some alfalfa genotypes (A3b and L28), but only one or two are downregulated in other genotypes (A3c, A8, L15 and L1e). It was noticed that relatively high abundance of MsmiR156 (high expression group) is required to silence all three alfalfa *SPL* genes. However, the number of regulated *SPL* genes is not obviously related to the abundance of LjmiR156 transcripts. These regulation patterns might reflect the origin of the two miR156s overexpressed in alfalfa. Although mature sequences of MsmiR156 and LjmiR156 are identical, the pre-mature state shows differences in nucleotide sequences. While it is true that mature sequences (~ 21 nt) are functional miRNAs, it is also obvious that miRNAs are regulated at different stages; transcription, processing and activity (Voinnet, 2009).

A feedback mechanism would explain the difference in patterns of gene regulation between MsmiR156 and LjmiR156. Studies showed that some miRNAs regulate the expression of genes (e.g. DCL and AGO) involved in miRNA processing and activity (Xie et al., 2003; Vaucheret et al., 2004). Xie et al. (2003) and found that the abundance of DCL1, the main enzyme involved in the processing of the pre-mature plant miRNAs is regulated by miR162 in *Arabidopsis*. Similarly, it was also found that the level of effector protein (AGO) is controlled by miR168 in *Arabidopsis* (Vaucheret et al., 2004). Regulation of these effector proteins would then impact the function of miRNAs. Besides the feedback of key enzymes that function in the processes of miRNA biogenesis, the activity of plant miRNA is also regulated by other miRNA targeted genes. For instance,

the abundance of miR172 is limited by miR156-targeted *SPL*13 during the early stages of plant development (Nonogaki, 2010). The accumulation of miR156 resistant *SPL*13 increased the level of miR172, which in turn downregulated the *SCHNARCHZAPFEN (SNZ)* gene resulting in a delay in the emergence of vegetative leaves (Martin et al., 2010a; 2010b). In my study, variations in phenotypes along with differences in the type of downregulated *SPL* genes are observed between MsmiR156 and LjmiR156 overexpression alfalfa. It is highly possible that the difference in the accumulation of the corresponding targets is a consequence of feedbacks of unique MsmiR156 and LjmiR156 overexpressed in alfalfa.

In addition to feedback, the possibility of a silencing mechanism during the processing of mature miR156 should not be overlooked. Transgene silencing is commonly found in transgenic research. This occurs when homologous genes are introduced to overexpress in plants (Velten et al., 2012). In this study, both MsmiR156 and LjmiR156 were introduced independently under the 35S promoter in alfalfa. However, it was found that the abundance of MsmiR156 is lower than that of LjmiR156 (small RNA blot). Compared to LjmiR156 which was from *L. japonicus*, the chance of silencing for MsmiR156 (alfalfa miR156) is likely to be higher, contributing to the difference in the abundance of MsmiR156 and LjmiR156 in the transgenic plants.

Compared to control, however, the levels of MsmiR156 and LjmiR156 are increased in miR156 overexpression plants. The relatively high level of MsmiR156 reduced the transcript abundance of the three target *SPL* genes. Ori et al. (2007) found that a gradient of miR319 expression generated an opposing gradient of its target (*LANCEOLATE*, *LAN*)

in tomato meristem. However, it is highly unlikely that abundance of the target transcript is always related to the levels of the miR156. Regardless of the high level of LjmiR156, not all target *SPL* transcripts, in particular *SPL*12, are reduced in some alfalfa genotypes. This suggests that LjmiR156 might use a translational inhibition mechanism rather than cleavage to regulate gene expression. A change in floral transition in LjmiR156 overexpression alfalfa is likely to be the consequence of the translational inhibition of the alfalfa *SPL* transcripts by miR156. Further research to investigate miR156-mediated gene regulation should explain the mechanism of miR156/SPL networks in alfalfa.

Many factors are involved in the miR156-mediated *SPL* gene regulations in plants. One study showed that the expression of miR156-targeted *SPL*3 is temperature dependent in which the level of *SPL*3 mRNA was mainly reduced at 16˚C (Kim et al., 2012). Moreover, plant developmental stages mediate the regulation of target genes by miR156. *SPL* genes are plant-specific transcription factors that whose expression varies temporally or spatially during plant development (Cardon et al., 1999). Hence, regulation of each *SPL* gene is observed at different stages of plant development (Xing et al., 2010). Plant species is also a factor that modulates miR156-mediated gene regulation. Effects of miR156 are investigated in many plants but a single, common pattern of miR156-mediated *SPL* gene expression is not observed across all plant species (Xie et al., 2006; Xing et al., 2010). In addition to *SPL*6 and *SPL*13, *SPL*12, which is closely related to *G. max SPL*12, is also the target of miR156 in alfalfa. In *Arabidopsis*, however, *SPL*12 is not the target of miR156 (The *Arabidopsis* Information Resource, TAIR), thereby explaining that miR156 targets different *SPL* paralogs in different plants.

Regulatory networks of miR156 and *SPL* genes affect the expression of downstream target genes. Fu et al. (2012) showed that *SPL* genes affect the expression of downstream genes, such as glutathione transferase, molecular chaperone and RNA-binding proteins in switchgrass (*P. virgatum*). Likewise, *SPL* genes regulate the expression of genes that function in floral development in *L. japonicus* (Wang et al, 2014). In *M. truncatula*, *FTa* genes are key regulators of flowering time, and in *Arabidopsis*, floral development is initiated upon induction of *AP1* by *FT* and other genes (Abe et al., 2005; Wigge et al., 2005; Yeoh et al., 2011). In this study, overexpression of MsmiR156 as well as LjmiR156 affect the expression of *AP3* and *FTa2* genes, and result in a delay in flowering time in miR156 overexpression plants. This further supports the notion that miR156/SPL networks induce phenotypic changes in plants by regulating downstream target genes.

I propose a model to explain the miR156/SPL-gene regulatory network in alfalfa (**Figure 38**), whereby overexpression of miR156 regulates three *SPL* genes (*SPL*6, *SPL*12 and *SPL*13) by transcript cleavage. I also found that miR156 cleaves *WD*40 gene transcripts; nevertheless, evidence from other experiment such as real-time qPCR to determine the transcript levels of the *WD40* genes is lacking at this time. Regulation of *SPL* and potentially *WD40* genes affects the expression of potential downstream genes such as *AP*3 and *FTa*2. However, it is still unclear how miR156 regulates these downstream genes in alfalfa.

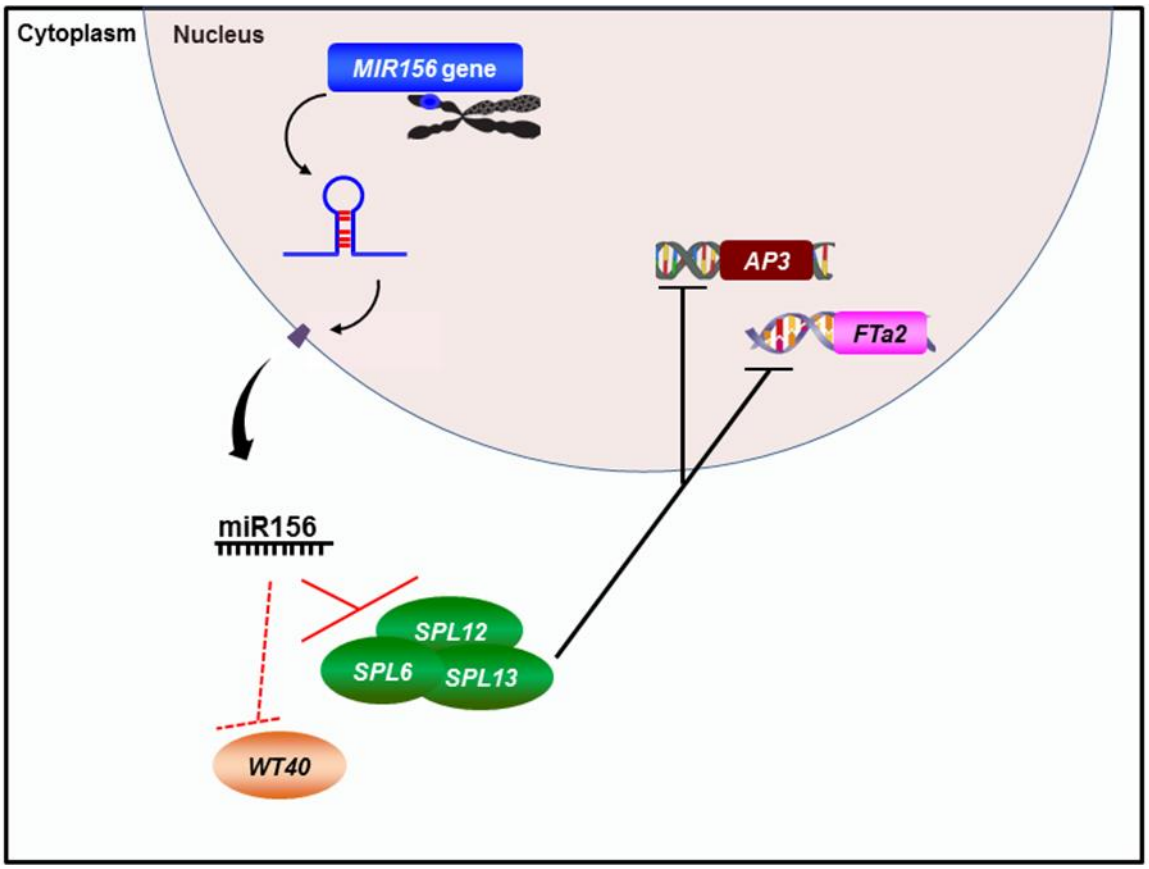


Figure 38. Overview of miR156-meidated gene regulation in alfalfa. Solid black or red lines with a perpendicular bar indicate repression of validated target genes and downstream genes expression. Dash line refers to regulation of potential target gene.

4.5 Overexpression of miR156 inhibits flowering in alfalfa

The combined effect of miR156, *SPL*s and other downstream genes controls a number of phenotypes during plant development. Floral transition is inhibited by high abundance of miR156 in plants (Fu et al., 2012). A decrease in miR156 expression enhances the expression of floral activated genes and promotes floral initiation in the reproductive stage (Wang et al., 2009; Chuck et al., 2011; Fu et al., 2012). This suggests that an increase in the abundance of miR156 prevents flowering in the plants. Overexpression of MsmiR156 delayed flowering time 2-5 days compared to control alfalfa plants. In fact, the relative abundance of MsmiR156 in overexpression plants was much higher than that in the control, but only up to a five-day delay in flowering time is observed in A2e, A3b and A4a alfalfa genotypes. It appears that genes involved in floral transition are less sensitive to miR156 compared to those controlling branch numbers and other lateral organs (Fu et al., 2012). If this hypothesis is true, a more significant effect of miR156 on flowering time will likely be observed in high expression group alfalfa, which was not included in the characterization. However, a dose-dependent effect of MsmiR156 on flowering time is not found in the alfalfa genotypes characterized so far in this study.

Contrary to MsmiR156, the effect of LjmiR156 is more obvious on flowering time, which was delayed for up to 50 days compared to the control. This observation is similar to the finding in transgenic switchgrass overexpressing *Corngrass1* (*Cg1*) miRNA (a miR156 family member) in which floral initiation is not observed after 2 years of growth (Chuck et al., 2011). Likewise, Fu et al. (2012) demonstrated that complete inhibition of floral transition is found in switchgrass overexpressing miR156. In addition, miR156 mediated-floral inhibition is also observed in rice and maize (Xie et al., 2006; Chuck et al., 2007).

Gandikota et al. (2007) and Kim et al. (2012) demonstrated that miR156-targeted *SPL*3 prevents early flowering in *Arabidopsis*. Furthermore, overexpression of miR156 coupled with a reduction in the transcript levels of *SPL*3, *SPL*4 and *SPL*5 inhibited floral transition in *Arabidopsis* (Wu and Poethig, 2006). These results indicate that miR156-mediated floral inhibition is a general phenomenon in plants. Delayed flowering time is advantageous in many crops. In particular, delayed flowering in alfalfa allows for flexibility in harvest times. Moreover, alfalfa is a cross pollinated crop, and hence the risk of cross pollination between transgenic and/ or non-transgenic plants will be reduced by delaying flowering (Fu et al., 2012). Also, inhibition of flowering can overcome the problem of transgene flow, which is one of the hottest issues for framers regarding transgenic alfalfa. An additional benefit is that the quality of forage will be improved because undesirable secondary metabolites, such lignin, are reduced if the transition from vegetative to reproductive phase is delayed (Fu et al., 2012). So far, alfalfa breeding programs are mainly focused on breeding for resistant and high-yield varieties; efforts toward manipulation of flowering time to improve forage quality are still lacking. Thus, overexpression of miR156 in alfalfa results in beneficially delayed flowering, and these plants would be a suitable candidate for field trial testing for improved alfalfa varieties.

4.6 Overexpression of miR156 improves shoot branching, biomass production and trichome density in alfalfa

A delay in the vegetative to reproductive phase transition impacts the production of lateral organs in plants (Shikata et al., 2009; Fu et al., 2012). MiR156 prolongs the vegetative phase and enhances shoot branching in many plant species (Zhou and Luo,

2013). According to Wei at al. (2010), constitutive expression of AtmiR156b enhances the number of flowering shoots in *B. napus* though a significant effect on seed weight is not observed in the transgenic plants. In *Arabidopsis*, Schwarz et al. (2008) demonstrated that miR156 increases vegetative shoot branching by downregulating the target *SPL*9 and *SPL*15. In addition to annual species, Rubinelli et al. (2013) recently showed that overexpression of *Cg1* miRNA promotes axillary meristem outgrowth in poplar tree. In the present study, a similar effect on shoot branching is also found in alfalfa overexpressing miR156. The increase in the number of shoot branches is observed in MsmiR156 as well as LjmiR156 transgenic alfalfa. The effect of miR156 on branching is found in 40 day-old alfalfa generated from vegetative propagation, but it is more visible in the reproductive phase, which is about 3 to 4 months after cutting.

Enhanced shoot branching improves biomass production in forage crops. Fu et al. (2012) showed that low and moderate levels of miR156 are required to enhance biomass production in transgenic switchgrass. Overexpression of *Cg1* miRNA and miR156 enhances vegetative shoot branching and leaf initiation in switchgrass but the high abundance of the miRNAs significantly reduces biomass production (Chuck et al., 2011; Fu et al., 2012). This is due to the thinner stem, smaller leaves, and dwarfed phenotype of the transgenic plants (Chuck et al., 2011; Fu et al., 2012). Likewise, a significant increase in biomass production is not found in all alfalfa genotypes overexpressing miR156 though shoot branching was enhanced in these plants. However, a significant increase in biomass is observed in A4a genotype, in which stem diameter is slightly larger than that of other miR156 overexpression genotypes. Although significant increases in dry weight

masses are not found in A2e, A3b, L1c and L1e genotypes, the biomass production is improved compared to the control plant.

Apart from shoot branching and leaf initiation, miR156 also regulates the production of other lateral organs. MiR156 controls trichome appearance, leaf structure, floral buds, male and female floral organs, seed shape and grain yield (Zhou and Luo, 2013). Schwarz et al. (2008) showed that overexpression of miR156 promotes the production of trichome texture in *Arabidopsis*. Enhanced trichome texture is also found in transgenic alfalfa overexpressing miR156. This is an advantage for alfalfa as trichomes can offer protection against pest and insect attack, which is considered one of the main obstacles to alfalfa production worldwide.

4.7 Overexpression of miR156 reduces internode length, plant height and stem thickness

This study shows that overexpression of miR156 in alfalfa causes many positive effects, but also some adverse ones. While miRNA enhances shoot branching, forage and grain yield, it also interferes with the development of many plant organs (Rubio-Somoza and Weigel, 2011; Zhou and Luo, 2013). High abundance of *Cg1* miRNA and miR156 reduces internode length and plant height in transgenic poplar and switchgrass, respectively (Chuck et al., 2011*;* Fu et al., 2012; Rubinelli et al., 2013). A significant decrease in internode length was found in both MsmiR156 and LjmiR156 transgenic alfalfa. However, increase in the numbers of nodes is found on the stem in line with the phenotypes found in transgenic switchgrass (Fu et al., 2012). In contrast, Rubinelli et al.

(2013) showed that the numbers of node produced are not significantly different among *Cg1* miRNA overexpression lines and wild type poplar tree.

It is unknown how miRNA controls plant heights but it seems that the effect of miRNA on height is also a general phenomenon. *Cg1* miRNA and miR156 have similar effects on plant height in of poplar and switchgrass, though these miRNAs are not in the same family (Chuck et al., 2011; Fu et al., 2012; Rubinelli et al., 2013). In alfalfa, a decrease in plant height is found in LjmiR156 alfalfa compared to control. In contrast, a slight increase in plant height is observed in MsmiR156 overexpression alfalfa at 40-days old but these height differences slowly disappear as the plants continue to grow.

Regarding stem diameter, there was a marked contrast between miR156 overexpressing alfalfa and the control. Overexpression of mi156 significantly reduced stem thickness in alfalfa. A significant decrease in stem diameter is also observed in transgenic switchgrass overexpressing miR156 (Fu et al., 2012). In poplar, however, no significant difference in trunk diameter is observed among *Cg1* miRNA overexpression line and wild type (Rubinelli et al., 2013), indicating that *Cg1* miRNA regulates phenotypes other than stem thickness.

4.8 MiR156 regulates root development and symbiotic interaction in alfalfa

In addition to regulation of plant development in the aerial parts (Wei et al., 2012, Fu et al., 2012), miR156 also affects root development (Rubio-Somoza and Weigel, 2011). In alfalfa, I observed that the root generating capacity of miR156 overexpression plants was better than that of empty vector control; nevertheless, a detailed characterization is

needed to confirm this finding. In this study, I expressed miR156s in alfalfa under 35S promoter, thus miR156 would be expected to be expressed in the roots and affect metabolic and developmental pathways involved in generating root systems in new alfalfa cuttings.

Auxin signaling, which plays an important role during root development (Overvoorde et al., 2010), is regulated by a number of plant miRNAs (Rubio-Somoza and Weigel, 2011). For instance, networks of miR164 and target NAM/ATAF/CUC 1(NAC1) genes regulate lateral root initiation while miR167-Auxin response factor 8(ARF8) (Mallory et al., 2004; Guo et al., 2005) and miR393-Auxin signaling F-Box 3(AFB3) affect the emergence and elongation of the lateral root (Vidal et al., 2010). In my study, overexpression of miR156 showed no effect on alfalfa root length; nevertheless further study will be required to investigate the effect of miR156 on lateral root development.

MiRNAs have also been reported to influence root symbiosis with nitrogen fixing rhizobia. While some miRNAs promote root symbiosis, others interfere with this process. MiRNAs that regulate plant defense response genes enhance the infection of rhizobia and increase nodulation in roots (Jagadeeswaran et al., 2009; Li et al., 2010; Devers et al., 2011). To initiate nodule development, rhizobia must receive root exudates (e.g.phenolic compounds) to induce the biosynthesis of the Nod factor that triggers cell differentiation and cell division to produce nodule primordium at the infection site in the root. Infection threads then develop and penetrate into root hairs (Yano et al., 2006; Bazin et al., 2012). According to this process, downregulation of defense genes would be required for successful root symbiosis. As evidence, regulation of the genes encoding

GSK3-like protein kinase (a regulator of plant immunity) and the TIR-NBS-LRR encoding disease resistant protein by miR482, increase the number of nodules in soybean (Li et al., 2010). However, overexpression of LjmiR156 causes a range of reductions in nodule number in alfalfa, but no effect of MsmiR156 on nodulation is observed in this plant. It is unclear if LjmiR156 downregulates class III HD-ZIP or CCAAT-binding family transcription factors that promote the process of nodulation and nodule growth in plants. Studies showed that miR169a regulates CCAAT-binding transcription factors and affects nodulation in *M. truncatula* (Combier et al., 2006; Boualem et al., 2008). This indicates that the miR156-mediated regulatory network entailed in root symbiosis remains to be investigated in alfalfa.

4.9 Functional characterization of miR156-targeted genes in alfalfa

Research in this study showed that downregulation of *SPL*6, *SPL*12 and *SPL*13 results in a variation of phenotypic changes in alfalfa. This suggests that these *SPL* genes control such plant phenotypes as shoot branching, internode length, stem diameter, flowering and nodulation. However, it is unclear if these phenotypes are redundantly controlled by the three *SPL* genes or whether morphological characters are controlled by a unique *SPL* gene.

To uncover the function of each *SPL* gene, alfalfa plants with overexpressed or RNAi-silenced *SPL* genes (*SPL6, SPL12* and *SPl3*) were generated. Expression analysis showed that the three target *SPL* genes are downregulated in respective RNAi alfalfa genotypes. These alfalfa plants were then vegetatively propagated by stem cutting. However, the RNAi plants are still too immature to characterize their phenotypes. Likewise, molecular

and morphological characterizations are needed in *SPL* gene overexpression alfalfa. At this point, it is still too early to conclude how the three *SPLs* are contributing to the phenotypic changes observed in miR156 overexpression alfalfa. Similarly, the functions of miR156-target *WD40* genes are currently inconclusive, although this group of regulatory genes are known mainly for their role in regulating secondary metabolism in plants (Pang et al., 2009).

4.50 Perspectives on the use miR156 in alfalfa improvement

Alfalfa breeding and improvement programs have been extensively expanded in recent years, especially in North America. Some are directed at breeding for stress-resistant varieties, while others are targeted at increasing forage yield and nutritive values; however progress in alfalfa breeding programs is still limited (Lamb et al., 2006). Although many programs are structured for improving important agronomic traits, none are focused on the manipulation of flowering, which is one of the traits that impact forage quality and gene flow in this cross-pollinated crop.

Although alfalfa is known as a forage crop for feeding animals, it is also grown for seed production. While flowering and pollination are completely necessary in the latter, they are not required in the former. In addition, the onset of flowering could reduce forage quality and nutritive value of alfalfa due to accumulation of lignin. Manipulation of flowering time would thus improve alfalfa forage quality. In this study, overexpression of miR156 delays the onset of flowering in alfalfa. While overexpression of MsmiR156 causes a minor delay in flowering time (2-5 days), LjmiR156 delays flowering up to 50 days compared to control. Thus, transgenic alfalfa overexpressing miR156 coupled with

delayed flowering time will be an ideal forage cultivar in the future. Besides improving the forage quality, it will also allow for flexibility in harvest times throughout the growing season. Depending on the purpose of the production (forage or seed), however, the choice between MsmiR156 and LjmiR156 will be required.

Increased forage production requires increased number of shoot branches in the plants to increase biomass yield. For more than a century, scientists and plant breeders have been trying to improve alfalfa forage yield but progress on this agronomic trait is also limited. Ludwick (2000) reported that alfalfa yields 24 ton/acre provided that a combination of 508 lb N_2 fertilizer and 151 inches water per acre are supplied. However, such an amount of investment will definitely risk losing profits for farmers. Evaluation of the performance of the alfalfa cultivars released over the five decades showed no significant improvement in alfalfa breeding towards augmenting forage yield (Lamb et al., 2006). Here, I found that overexpression of MsmiR156 as well as LjmiR156 significantly increases the number of shoot branches in alfalfa. As expected, improved biomass production is also found in the transgenic alfalfa with increased levels of miR156, suggesting that this study provides a key to overcome the limitations encountered in forage yield improvement in alfalfa.

Alfalfa production is always hindered by biotic and abiotic stresses. Breeding for stress tolerant varieties is thus one of the main goals in alfalfa research. Most of the varieties released in the late 1970s were tolerant to diseases commonly occurring in alfalfa (Volenec et al., 2002). Furthermore, the new varieties have greater winter hardiness (Volenec et al., 2002), and were tolerant to abiotic stress (Passos et al. (2012). Enhanced

trichome texture in miR156 overexpression alfalfa will further enhance the ability of this plant to counter insect pests.

As a legume, alfalfa can form a symbiotic interaction with nitrogen fixing rhizobia. Hence, N_2 is gained in exchange for carbohydrate as a result of this symbiosis. According to Putnam et al. (2001), alfalfa is able to fix 120 to 540 lb N_2 per acre per year. Successful symbiotic interaction produces functional nodules in the plant roots. In this study, it was found that overexpression of LjmiR156 causes a range of reductions in nodule numbers in the transgenic alfalfa. However, no significant impact for MsmiR156 on nodulation was observed. In addition, no effect for either MsmiR156 or LjmiR156 could be detected on root length. Given that the nodule number is an indicator of nitrogen fixing ability, MsmiR156 would be favored over LjmiR156. However, further research to determine the amount of N_2 fixed in each alfalfa genotype is needed prior to approving the superiority of MsmiR156 over Ljmi156 regarding N_2 fixing ability.

Besides nodulation, plant height in MsmiR156 overexpression alfalfa is also higher than that in LjmiR156. During the early stage of plant development (40 days old), an increase in plant height was also observed in MsmiR156 overexpression alfalfa compared to control. An increase in plant height is an advantage under field conditions because the plants are able to compete for natural sunlight. This suggests that MsmiR156 overexpression would perform better under natural field conditions. However, stem sizes are smaller in MsmiR156 as well as LjmiR156 overexpression alfalfa compared to control. Potential effects of MsmiR156 and LjmiR156 on alfalfa are summarized in **Table 6**. Future research should focus on the advantages and disadvantages of

manipulation of MsmiR156 and LjmiR156 to produce superior alfalfa cultivars, especially research to uncover and understand the miR156-SPL gene regulatory network.

Table 6. Summary of the effects of MsmiR156 and LjmiR156 on alfalfa

Plant phenotype	MsmiR156-OE	LjmiR156-OE
Plant height	Enhanced	No effect
Shoot branching	Enhanced	Enhanced
Internode length	Reduced	Reduce
Number of nodes on stem	Enhanced	Enhanced
Stem thickness	Reduced	Reduced
Trichome density	Enhanced	Enhanced
Root length	No effect	No effect
Root generating capacity	Enhanced	Enhanced
Nodulation	No effect	Reduced
Flowering delay	2-5 days	Up to 50 days
Biomass production	Improved	Improved

5. CONCLUSION AND PROSPECTS FOR FUTURE RESEARCH

Research in this study was designed to investigate the effect of miR156 on shoot branching, flowering time, and nodulation in alfalfa. *In silico* analysis revealed no miR156 precursors in existing alfalfa databases. Next Generation alfalfa reads contained short miR156-like sequences but it could not be concluded that these sequence reads were miR156 precursors since no miR156 features are found in the short reads. However, a precursor of miR156 was isolated from this plant by PCR.

The potential targets of miR156 were identified using 5' RACE and sequencing approaches. Three *SPL* genes (*SPL*6, *SPL*12 and *SPL*13) were found to be targeted by miR156 in alfalfa. Furthermore, I found that miR156 cleaves the transcripts of *WD*40 genes in alfalfa. To validate *SPL* genes as targets of miR156, the abundance of *SPL* transcripts was monitored using qRT-PCR. It was observed that the three *SPL* genes were downregulated in MsmiR156 and LjmiR156 overexpression alfalfa genotypes though the types of downregulated *SPL* genes were not identical in all alfalfa genotypes. Although a high abundance of MsmiR156 was required to reduce the transcripts of all three *SPL* genes, miR156 dose-dependent *SPL* gene expression was not observed in LjmiR156 alfalfa genotypes. This suggests that in addition to transcript cleavage, translational inhibition by miR156 may be more prevalent in some LjmiR156 overexpression alfalfa genotypes.

Overexpression of miR156 improves the yield of alfalfa forage, as manifested by the fact that miR156 enhances the number of shoot branches compared to control. These phenotypes were observed in as early as 40 day old alfalfa cuttings generated from

vegetative propagation. The effect of miR156 on shoot branching is more visible as the plants continue to grow. Contrary to MsmiR156 alfalfa, more enhanced shoot-branching is observed in LjmiR156 alfalfa, indicating the different effects of the two miR156s used to express in alfalfa. As expected, enhanced shoot branching is coupled with improved biomass production in miR156 overexpression alfalfa. Furthermore, a high abundance of miR156 improved trichome texture in this plant, which may have a positive impact on the plant's tolerance to insect pests.

Study on the effects of miR156 also revealed that miR156 prolongs the vegetative phase by delaying the onset of flowering in alfalfa. A difference in the effects of MsmiR156 and LjmiR156 on flowering time was observed. While overexpression of MsmiR156 causes a minor delay (2-5 days), LjmiR156 significantly delays flowering time; i.e. up to 50 days. Investigation of the expression of genes involved in floral development showed that *AP*3 and *FTa2* genes were downregulated in MsmiR156 and LjmiR156 overexpression genotypes.

It was observed that overexpression of miR156 significantly reduces internode length and stem thickness in alfalfa. The effect of miR156 on these phenotypes partly impacts on the weight of the dry mass. I found that the effect of miR156 on plant height is slightly variable. Overexpression of MsmiR156 increases height in some alfalfa genotypes during the early stage of plant development, but the difference diminishes gradually as the plants continue to grow. Furthermore, a reduction in plant height was observed in LjmiR156 overexpression alfalfa genotypes compared to the control.

Investigation of the effect of miR156s on underground plant parts showed that both MsmiR156 and LjmiR156 had no effect on root length. However, while MsmiR156 had no effect on nodulation, overexpression of LjmiR156 caused a range of reductions in nodule numbers. However, it is unclear how miR156 regulates nodulation, and thus analysis of the miR156/SPL-gene regulatory network should be investigated.

In this study, it was observed that miR156 regulates three target *SPL* genes and induce a number of phenotypic traits in alfalfa. However, it is unclear if these *SPL* targets are functionally redundant in controlling such morphological traits in this plant, and what roles other targets, e.g. WD40, play in determining these traits. Future research will uncover the functions of *SPL*3, *SPL*12 and *SPL*13 by comparing the molecular and morphological characters among miR156 overexpression, RNAi and SPL overexpression alfalfa. To this end, improved alfalfa cultivars can be generated by manipulating the desirable *SPL* genes that regulate only desirable agronomic traits.

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Appendix A

Composition of Alfalfa Transformation Media*

Basal SH2K Medium

Component	Amount/l	Final concentration
10x Schenk and Hildebrandt salt	3.2 g	
Nicotinic acid	5 mg	
Pyridoxine HCL	0.5 mg	
Thiamine HCL	5 mg	
Myo-inositol	200 mg	
Potassium sulfate	4.35 g	50 mM
Proline	0.288 g	25 mM
Kinentin (10 mg/ml)	40 µl	2.14 µM
2,4-D (100 mg/ml)	40 µl	18.12 µM
Sucrose,	30 g	3% (w/v)
Adjust pH to 5.8		
Plant tissue culture agar,	8 g	
Thioproline (100 mg/l)	530 µl	53 mg/l

Co-cultivation medium

Component	Amount/l	Final concentration
Basal SH2K medium plus:		
Acetosyringone (10 mM)	2 ml	20 Mm

Callus Induction medium

Component	Amount/l	Final concentration
Basal SH2K medium plus:		
Timentin (300 mg/ml)	1 ml	300 mg/l

Callus Induction with antibiotics (RNAi constructs with pHellgate12 vector)

Component	Amount/l	Final concentration
Basal SH2K medium plus:		
Timentin (300 mg/ml)	1 ml	300 mg/l
Kanamycin (100 mg/ml) (first 10 days)	500 µl	50 mg/l
(after 10 days)	750 µl	75 mg/l

Callus Induction with antibiotics (Overexpression constructs with pMDC32 vector)

Component	Amount/l	Final concentration
Basal SH2K medium plus:		
Timentin (300 mg/ml)	1 ml	300 mg/l
Hygromycin (50 mg/ml) (first 10 days)	1 ml	50 mg/l
(after 10 days)	1.5 ml	75 mg/l

Embryo Development medium (BOi2Y)

Component	Amount/l	Final concentration
10x Blade's Stock with myo-inositol	100 ml	
Yeast extract	2 g	
Sucrose	30 g	3% (w/v)
Plant TC agar (Sigma A7921)	8 g	
pH to 5.8 with HCl		
Timentin (300 mg/ml)	1 ml	300 mg/l
Appropriate antibiotics		75 mg/l

Embryo Germination medium (½ MSO)

Component	Amount/l	Final concentration
MS Basal Salts	2.165 g	
10X MS-modified vitamins	100 ml	
Glycine (10 mg/ml)	100 µl	1 mg/l
Sucrose	30 g	3% (w/v)
Plant TC agar (Sigma A7921)	8 g	
pH to 5.8 with KOH		
Timentin (300 mg/ml)	1 ml	300 mg/l
Appropriate antibiotics		75 mg/l

Plant development medium (MSO)

Component	Amount/l	Final concentration
MS Basal Salts	4.33 g	
10X MS-modified vitamins	100 ml	
Glycine (10 mg/ml)	100 µl	
Sucrose	30 g	
Plant TC agar (Sigma A7921)	8 g	
Timentin (300 mg/ml)	1 ml	300 mg/l
Appropriate antibiotics		75 mg/l

10x SH modified vitamins with myo-inositol

Component	Amount/2l	Final concentration
Nicotinic acid	100 mg	5 mg/l
(B_6) pyridoxine HCl (10 mg/ml)	1 ml	0.5 mg/l
(B_1) thiamine HCl	100 mg	5 mg/l
Myo-inositol	4 g	200 mg/l

10x Blade's stock with myo-inositol

Component	Amount/2l	Final concentration
$MgSO_4$-$7H_2O$	700 mg	35 mg/l
$MnSO_4$-H_2O	88 mg	4.4 mg/l
$Ca(NO_3)_2$-$4H_2O$	6.94 g	347 mg/l
NH_4NO_3	20 g	1000 mg/l
KNO_3	20 g	1000 mg/l
KH_2PO_4	6 g	300 mg/l
KCl	1.3 g	65 mg/l
H_3BO_3	32 mg	1.6 mg/l
$ZnSO_4$-$7H_20$	30 mg	1.5 mg/l
KI	16 mg	0.8 mg/l
Fe(III)EDTA	720 mg	3.6 mg/l
Nicotinic acid	10 mg	0.5 mg/l
(B_6) pyridoxine HCl (10 mg/ml)	200 µl	0.1 mg/l
(B_1) thiamine HCl (10 mg/ml)	200 µl	0.1 mg/l
Glycine (10 mg/ml)	4 ml	2 mg/l
Myo-inositol	2 g	100 mg/l

10X MS-modified vitamins

Component	Amount/2l	Final concentration
Nicotinic acid	10 mg	0.5 mg/l
(B_6) pyridoxine HCl (10 mg/ml)	1 ml	0.5 mg/l
(B_1) thiamine HCl (10 mg/ml)	2 ml	1 mg/l
Myo-inositol	4 g	100 mg/l

Note: All antibiotics were added after autoclaving the other components.

* Modified and optimized by Lisa Amyot (Technician, AAFC, London)

Appendix B

SPL6-RNAi construct

Insert Description: *SPL6* gene specific primers were designed based on *M. truncatula* genome accession (XM003614178). These primers were used to amplify SPL sequence (250 bp) from alfalfa (*M.sativa*). The amplified sequences were confirmed by sequencing and the fragment was finally cloned into pHELLGATE12 destination vector to generate SPL6-RNAi construct.

DNA Sequence (SPL6-RNAi)

attR1 and attR2 regions Reverse repeats of SPL6 (250 bp) Flanking Intron

GAAGGATCTTACTAAGAGTCATTTCATTTGGAGAGGACACGCTCGAGACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCCATATCAAACT
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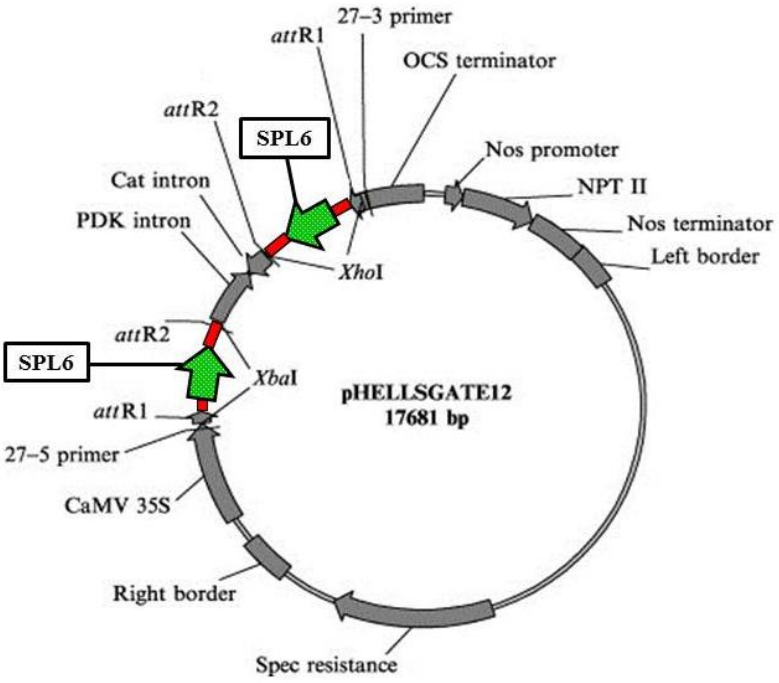
Map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attR2
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Cat intron
NPT II
PDK intron
Nos terminator
Left border
XhoI
attR2
SPL6
XbaI
pHELLSGATE12
17681 bp
attR1
27–5 primer
CaMV 35S
Right border
Spec resistance

SPL12-RNAi construct

Insert Description: *SPL12* gene specific primers (RNAiMsSPL12-F2-CACCACA GG TCTAGAAGATCCAA and RNAiMsSPL12-R2-CGAGAACGA GATA CAGGCACT) were designed based on *M. truncatula* genome accession (XM003601719). These primers were used to amplify SPL12 (250 bp) sequence from alfalfa (*M.sativa*), and the fragment was finally cloned into pHELLGATE12 destination vector to generate SPL12-RNAi construct.

DNA Sequence (SPL12-RNAi)

attR1 and attR2 regions Reverse repeats of SPL12 (250 bp) Flanking Intron

CGAAACGCAGTCATTTCATTTGGAGAGGACACGCTCGAGACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCACAGGTCTAGAAGATCCAA
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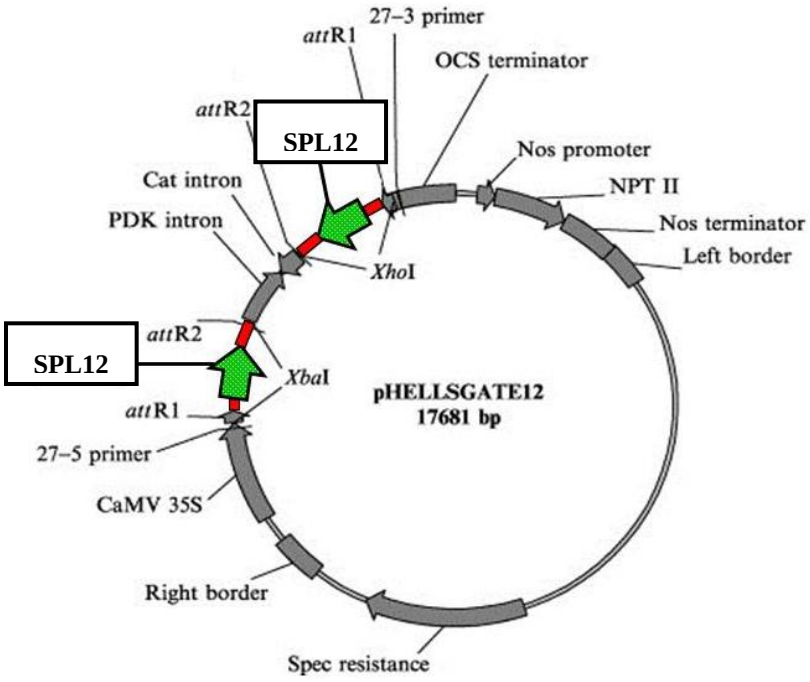
Map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NPT II
PDK intron
Nos terminator
Left border
XhoI
attR2
SPL12
XbaI
pHELLSGATE12
17681 bp
attR1
27–5 primer
CaMV 35S
Right border
Spec resistance

SPL13-RNAi construct

Insert Description: *SPL13* gene specific primers (RNAiMsSPL13-F2-CACCTGACAACAACAATCCCTCA and RNAiMsSPL13-R2-CCATGGACTCAA AGCTATTGT) were designed based on *M. truncatula* genome accession (XM003602747). These primers were used to amplify SPL13 (249 bp) sequence from alfalfa (*M.sativa*), and the fragment was finally cloned into pHELLGATE12 destination vector to generate RNAi construct.

DNA Sequence (SPL13-RNAi)

attR1 and attR2 regions Reverse repeats of SPL13 (250 bp) Flanking Intron

CTTTAGCAGTTCATTTCATTTGGAGAGGACACGCTCGAGACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCTGACAACAACAATCCCTCA
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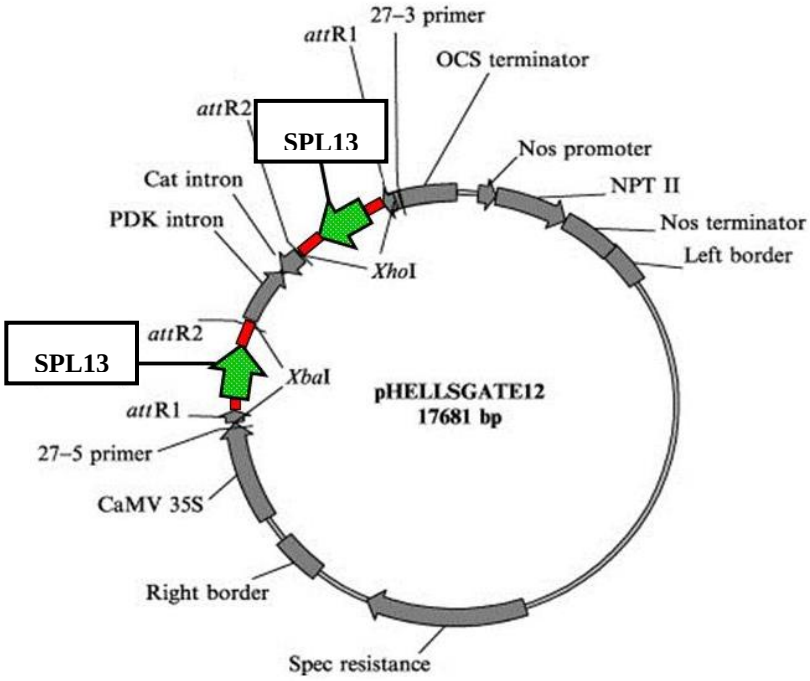
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attR2
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Nos promoter
Cat intron
NPT II
PDK intron
Nos terminator
Left border
XhoI
attR2
SPL13
XbaI
pHELLSGATE12
17681 bp
attR1
27–5 primer
CaMV 35S
Right border
Spec resistance

Appendix C

SPL6 overexpression construct (SPL6-OE)

Insert Description: *SPL6* gene specific primers were designed based on *M. truncatula* genome accession (XM003614178). These primers were used to amplify respective full length sequence from alfalfa (*M.sativa*). The alfalfa SPL sequences were confirmed by sequencing and the fragment were then cloned into pMDC32 destination vector to generate SPL6 overexpression construct.

Peptide sequence (*SPL6*)

MESWSYIPEERSYLFSDEMDFSLDAFMRSRKPLVEWENKSSCNFERDGFNSDREVVKSMEFVDLGFPMESSRTVRGSVQTSSCELDSNNSSKRENSSIHVIAF
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LQRVEKQRNSVLVKWENEDCCFSNV

DNA sequence (SPL6-OE)

35S Promoter **attR1 and attR2 regions** **SPL6 full length sequence**

ACCGGCGTTCCCAAACCAACCGGTTCTTCAAGGCCAGTGAATTGAATGTGATATCTCACTGACGTAAGGGATGAACGCACATTCCACTATTCCTTCGCAAGAC
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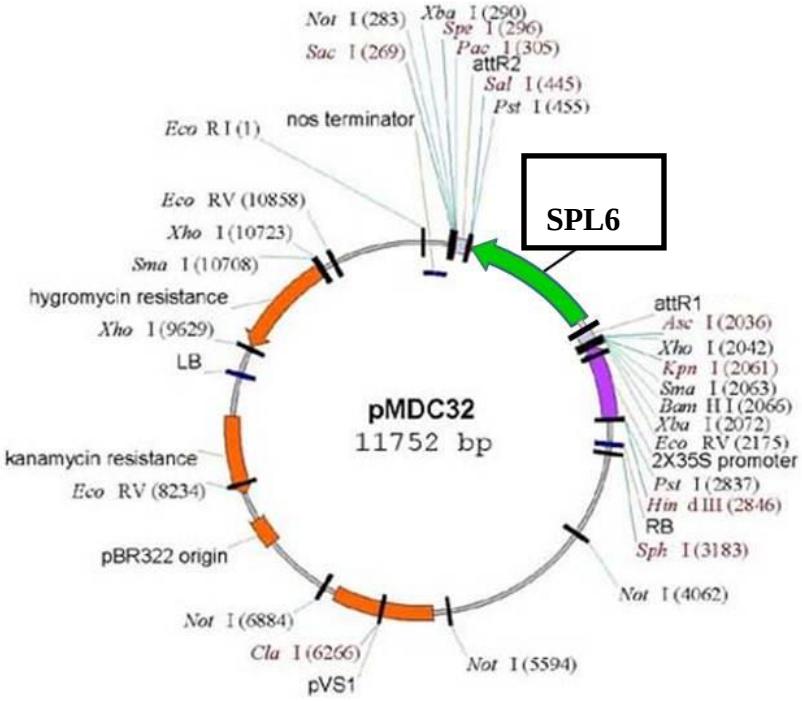
Map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Eco RV (10858)
Xho I (10723)
Sma I (10708)
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Xho I (9629)
LB
attR1
Asc I (2036)
Xho I (2042)
Kpn I (2061)
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Pst I (2837)
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Not I (4062)
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Cla I (6266)
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SPL13 overexpression construct (SPL13-OE)

Insert Description: *SPL13* gene specific primers (OEMsSPL13-F-CACCATGGAGTGG AATTTGAAAGC and OEMsSPL13-R-CTATTCCCATTGATAGGGAAATAGT) were designed based on *M. truncatula* genome accession (XM003602747). These primers were used to amplify SPL13 (1135 bp) sequence from alfalfa (*M.sativa*), and the fragment was finally cloned into pMDC32 destination vector to generate SPL13 overexpression construct.

Peptide sequence

MEWNLKAPSWDLGGIEEATLPNIETMEESNRFGVYKMKGEFSVDLKLGQVGNSATDQSPLPLSNDAVVVSKIATPTSSSGSSKRARAMNNATLTVSCLVDGCN
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QMVQQPHSMSLMQPLGLSLHGNNSFESMEGVLVPNGSESDHCSSLYNMGSDGSQGNDAPQLFPYQWE

DNA sequence (SPL13-OE)

35S Promoter attR1 and attR2 regions SPL13 full length sequence

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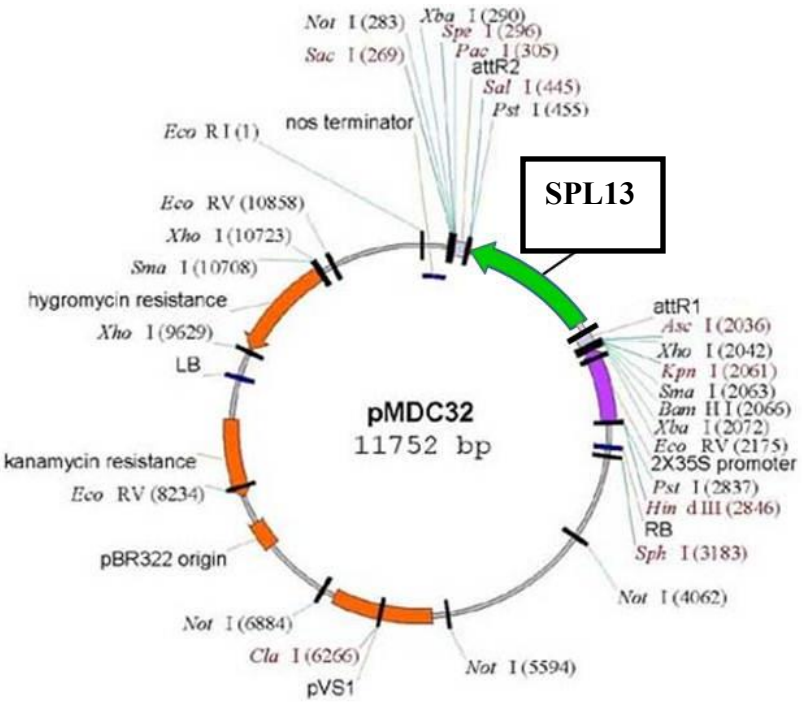
ACAGACGCACACACCTGATAATGGTTTGAATCAAATGGTGCAGCAGCCTCATTCAATGTCTCTTATGCAGCCCTTGGGACTGAGTCTGCATGGTAACAATAGC
TTTGAGTCCATGGAAGGAGTGTTGGTCCCAAATGGGAGTGAGAGTGATCATTGTTCCTCGTTGTATAACATGGGTTCTGATGGATCACAAGGCAATGATGCAC
CTCAACTATTTCCCTATCAATGGGAATAGAAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTGGGTTCGATAATTTCTTAATTAACTAGTTTCTAGA
GCGGGCCGCCCACCGCGGTGGGAGCTCGAATTTCCCCGATCGTTTCAAACATTTGGGCAATAAAGTTTCTTAGATGGATCTGCTGGCGTCTTTGGCGAATGAT
TTAATCCAATTATTAAACTGT

Map


Not I (283)
Xba I (290)
Spe I (296)
Sac I (269)
Pac I (305)
attR2
Sal I (445)
Pst I (455)
Eco R I (1)
nos terminator
SPL13
Eco RV (10858)
Xho I (10723)
Sna I (10708)
hygromycin resistance
Xho I (9629)
LB
attR1
Asc I (2036)
Xho I (2042)
Kpn I (2061)
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kanamycin resistance
Eco RV (8234)
pBR322 origin
Not I (4062)
Not I (6884)
Cla I (6266)
Not I (5594)
pVS1

SPL12 overexpression construct (SPL12-OE)

Insert Description: *SPL12* gene specific primers (OEMsSPL12-F-CACCATGGAGTGG AACGTGAAATC and OEMsSPL12 R-TTAATCCAGCTGGTTGCAA) were designed based on *M. truncatula* genome accession (XM003614178). These primers were used to amplify SPL12 (1314 bp) sequence from *M. truncatula* and the fragment was finally cloned into pMDC32 destination vector to generate SPL12 overexpression construct.

Peptide sequence

MEWNVKSPGQWDWENLFFLNSKAAETHRLQSTDWSMEEDREINVGMLIPSGGSGYSVSKLMHASSSRSSKSASNNSSSNEDSKTSMLTQ
EGSPDNSTGKKESSKGDPIETSPAAEPLLTLKLGKRLYFEDVSTGSHSKKASSSAVPLLCGKKGKSSSQNMLNPSCQVEGCGLDLSFAK
DYHRKHRICDSHSKSPVVVVAGLERRFCQQCSRFHDLSEFDDKKRSCRRRLSDHNARRRKPQPEAVKLNPSALSSSPYDGRQAMGPFAF
PKNTSNLAWPDMPNSKLPQTKDFMLKTPKNFNKIVTMLSDDSSGHFISKGKGTNIAVPGLEDPNTLSDPSATQDVNRALSLLSTNSWGA
YDTKPPSFVHSNRTTGTPEYATAQRSPFSSPEYWHTDQHQASSSACISFSGYHNSNRYQDFRLFSEPYESSFPCNQLD

DNA sequence (SPL12-OE)

35S Promoter **attR1 and attR2 regions** **SPL12 full length sequence**

ACCGGCGTTCCCAAACCAACCGGTTCTTCAAGGCCAGTGAATTGAATGTGATATCTCACTGACGTAAGGGATGAACGCACATTCCACTATTCCTTCGCAAGAC
CCTTCCTCTATATAAGGAAGTTCATTTCCATTGGAGAGGACCTCGACTCTAGAGGATCCCCGGGTACCCGGGCCCCCCCCTCGAGGCGCGCCAAGCTATCAAA
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TTCAAAAGCAGCAGAAACTCACAGGTTACAATCTACTGATTGGAGTATGGAGGAAGATCGAGAAATCAATGTTGGGATGTTGATTCCATCTGGTGGTAGCGGC
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TAAAAGATTATACTTTGAGGATGTTAGCACTGGAAGTCATTCCAAGAAAGCCTCTTCCTCTGCAGTTCCTCTTTTGTGCGGAAAGAAAGGTAAATCGAGCAGT
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AATCGCCTGTGGTGGTAGTAGCTGGTTTGGAGCGTCGATTTTGCCAGCAATGTAGCAGGTTCCATGATCTCTCAGAGTTTGATGATAAAAAAAGAAGCTGCAG
ACGCCGTCTTTCAGATCACAATGCAAGGCGTCGCAAACCTCAGCCTGAAGCAGTGAAATTGAATCCATCAGCTCTCTCTTCGTCTCCCTATGATGGGAGGCAA
GCAATGGGCCCATTTGCTTTTCCAAAGAATACTTCAAATTTAGCATGGCCAGACATGCCCAACAGCAAGCTCCCCCAAACCAAAGATTTTATGCTCAAAACTC
CAAAAAACTTCAACAAGATTGTCACTATGCTTTCTGATGATTCAAGTGGCCACTTTATATCCAAAGGCAAAGGAACCAATATTGCTGTCCCAGGTCTAGAAGA
TCCAAATACCTTGTCTGATCCAAGTGCTACACAAGATGTTAACCGTGCTCTCTCTCTTCTGTCAACCAATTCATGGGGTGCATATGATACCAAGCCCCCCTCC
TTCGTACACTCGAATCGGACAACCGGCACCCCTGAGTATGCAACTGCTCAGCGCTCACCTTTTTCGTCACCGGAATATTGGCACACTGATCAACATCAGGCCA
GCTCCAGTGCCTGTATCTCGTTCTCGGGTTACCACAATAGCAATCGCTATCAAGACTTTCGGCTGTTCAGCGAACCCTATGAGTCAAGTTTCCCTTGCAACCA
GCTGGATTAAAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTGGTTCGATAATTCTTAATTAACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCT
CGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGT
TAAGCATGTAATAATTAACATGTAATGCATGACGTTATTATGAGATGGGTTTTTATGATTAGAGTCCCCGCAATTATACATTTATACGCGATAGAACAATATA
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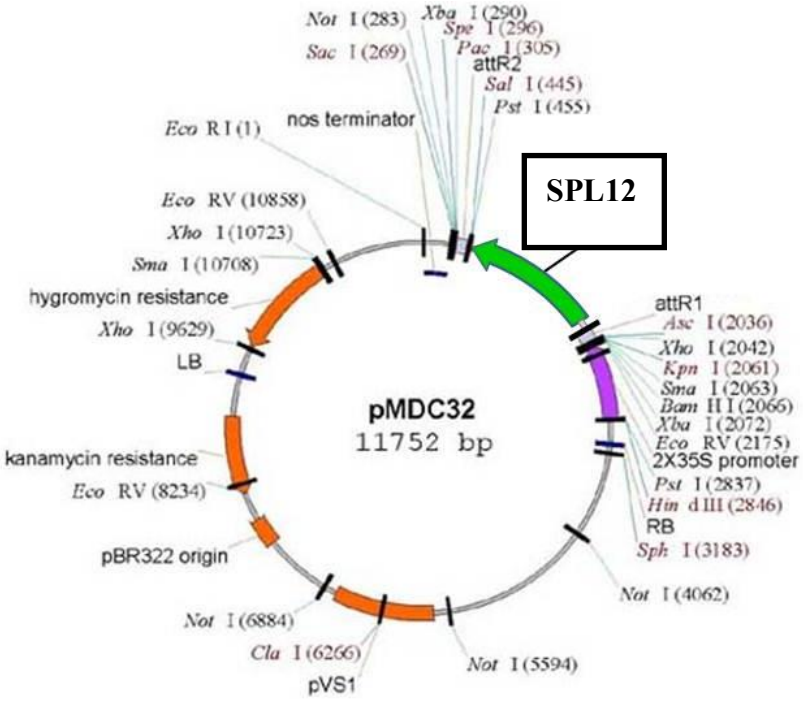
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CURRICULUM VITAE

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2012-2014

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2008-2010

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2010-2012

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Poster and Oral presentation

Aung, B., Amyot, L., Keyghobadi, N. and Hannoufa, A. (2012). Characterization and Target Validation of miR156 in *Medicago sativa***.** Canadian Society of Plant Biologists (CSPB) Eastern Regional Meeting and Plant Development Workshop, Wilfrid Laurier University, Waterloo, ON, Canada.

Aung, B., Gruber, M., Amyot, L., Keyghobadi, N. and Hannoufa, A. (2013). Functional characterization of microRNA156 in *Medicago sativa.* Fourth Annual Biology Graduate Research Forum 2012, University of Western Ontario, London, ON, Canada.

Aung, B., Gruber, M., Amyot, L., Keyghobadi, N. and Hannoufa, A. (2013). Overexpression of microRNA156 leads to enhanced forage yield in alfalfa. Canadian Society of Plant Biologists (CSPB) Eastern Regional Meeting and Plant Development Workshop, The University of Toronto Mississauga, ON, Canada.