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Characterization of SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL), and its role in drought stress tolerance in Medicago sativa (alfalfa).

Alexandria K. Hanly, *The University of Western Ontario*

Supervisor: Hannoufa, Abdelali, *The University of Western Ontario*
Co-Supervisor: Karagiannis, Jim, *The University of Western Ontario*
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Abstract

Traditional breeding involving *Medicago sativa* (alfalfa), has resulted in minimal yield increases. Moreover, extreme environmental conditions threaten to further limit production. Strategies that make use of molecular tools – such as small non-coding RNA, miR156 – represent an innovative means by which to influence tolerance to abiotic stress. miR156 functions, at least in part, through the SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) family of transcription factors. In this study, the role of SPL9 in regulating alfalfa development and drought tolerance is evaluated. Examination of alfalfa plants with RNAi-mediated *SPL9* (*SPL9*-RNAi) showed that plant height, stem thickness, and internode length are positively regulated by SPL9, whereas shoot branching is negatively regulated. *SPL9*-RNAi alfalfa also had enhanced tolerance to drought mediated by elevated anthocyanin content and expression of DIHYDROFLAVONOL 4-REDUCTASE (DFR), an enzyme involved in anthocyanin biosynthesis. Thus, manipulation of SPL9-mediated downregulation of *DFR* may represent one strategy to improve drought tolerance in alfalfa.

Keywords

alfalfa, drought, gene regulation, miR156, SPL, abiotic stress tolerance

Summary for Lay Audiences

Alfalfa is an important forage crop that is used mainly as feed for ruminant animals. While breeding programs have produced winter hardy alfalfa varieties that are capable of growth in harsh Canadian climates, further yield improvements have been limited. Furthermore, climate change is resulting in prime agricultural areas exhibiting extreme weather events such as drought. Drought can limit the growth of alfalfa and cause toxic levels of reactive oxygen species (ROS) to accumulate within the plant. ROS can cause damage to DNA, lipids, and proteins but molecules like anthocyanins, which have stress reducing antioxidant activity, can mitigate ROS accumulation.

Novel molecular tools that can be used to alter alfalfa to promote the induction of desired traits are highly sought after. One such tool, miR156, is a small RNA molecule that influences the expression of a family of proteins – called SQUAMOSA PROMOTER BINDING PROTEIN LIKE or SPL proteins – that are important regulators of development and stress tolerance. In this study, the role of one SPL protein (SPL9) was investigated by comparing alfalfa plants with reduced levels of *SPL9* to plants expressing normal levels of *SPL9*. These studies revealed that SPL9 positively regulates plant height, stem thickness, and internode length and negatively regulates branching. Interestingly, the plants with reduced *SPL9* levels were also more tolerant to drought in that they maintained growth, had reduced leaf senescence, and had enhanced relative water content while under water-deficit conditions. Importantly, the plants with reduced *SPL9* levels also exhibited increased anthocyanin accumulation and effected *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) transcript levels, an enzyme involved in anthocyanin biosynthesis. This suggested that the drought tolerance exhibited by plants with reduced levels of SPL9 were at least partly due to the SPL9-mediatd negative regulation of *DFR*. Taken together, these results indicate that the manipulation of SPL9 can be used as a potential molecular strategy to improve drought tolerance in alfalfa.

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List of Abbreviations

A	absorbance
ABA	abscisic acid
ABTS•+	2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation
ADF1	ACTIN-DEPOLYMERIZING PROTEIN1
At	*Arabidopsis thaliana*
bHLH	basic helix-loop-helix
CAT1	CATALASE1
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CRISPR	clustered regularly interspaced short palindromic repeats
Dc	*Dendrobium catenatum*
DFR	DIHYDROFLAVONOL 4- REDUCTASE
DSB	double stranded break
dsRNA	double stranded RNA
DW	dry weight
EDTA	Ethylenediaminetetraacetic acid
eIF4A	ELONGATION INITIATION FACTOR4A
eIF4E	ELONGATION INITIATION FACTOR4E

EV	empty vector
FTW	fresh turgid weight
FW	fresh weight
FW_{t0}	fresh weight at 0 minutes
FW_{tn}	fresh weight at n minutes
g	gram
gDNA	genomic DNA
GSH	GLUTATHIONE SYNTHETASE
HR	homology-directed repair
hrs	hours
LB	Luria-Bertani
min	minute
miRNA	microRNA
miR156	microRNA156
mRNA	messenger RNA
Ms	*Medicago sativa*
Mt	*Medicago truncatula*
NHEJ	non-homologous end joining
NLS	nuclear localization signal
NTC	no template control

OE	overexpression
PAM	protospacer-adjacent motifs
PAP1	PRODUCTION OF ANTHOCYANIN PIGMENT1
PP2C	PROTEIN PHOSPHATASE TYPE 2C
RAR	repeat and anti-repeat region
RCAR	REGULATORY COMPONENT OF ABA RECEPTOR1
RCF	relative centrifugal force
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	reactive oxygen species
SBP	squamosal promoter binding domain
sgRNA	single guide RNA
siRNA	short interfering RNA
SnRK2	SNF1-RELATED PROTEIN KINASE2
Sp	*Streptococcus pyrogenes*
SPL	SQUAMOSA PROMOTER BINDING PROTEIN LIKE
SPL9	SQUAMOSA PROMOTER BINDING PROTEIN LIKE9
stRNA	small temporal RNA
TC	tissue culture
TT8	TRANSPARENT TESTA8

TTG1	TRANSPARENT TESTA GLABRA1
Zm	*Zea mays*

Chapter 1

1 Introduction

1.1 The need for crop improvement

The explosive growth of human populations in recent centuries is expected to continue for at least the next several decades (Godfray et al., 2010; Tilman et al., 2011). With this growth, comes increased demand for resources such as water and energy. The same is true with respect to our collective requirement for food. Tilman et al. (2011) estimate that the global demand for crop production will double by 2050.

Agricultural strategies employed to meet future food demands include extensification and sustainable intensification (Godfray et al., 2010; Tilman et al., 2011). Extensification is achieved by expanding the agricultural land available, but this comes at great environmental costs (Tilman et al., 2011). Cultivating new land inevitably results in the destruction of natural ecosystems, thereby impacting biodiversity and greenhouse gas emissions (Tilman et al., 2011). Sustainable intensification by improving the output of existing agricultural lands greatly reduces ecological impacts compared to extensification practices (Tilman et al., 2011). The application of fertilizers to soil can increase the yield of crops (Seufert et al., 2012), but can also result in the pollution of surrounding water sources by runoff, which has been linked to some cancers in humans (Weyer et al., 2001).

Abiotic stress can limit plant growth significantly (Daryanto et al., 2016). Furthermore, the continuing escalation of the impacts of climate change will lead to more extreme weather phenomena like drought (Schindler and Donahue, 2006). While drought is a poorly defined term, it invariably involves regions experiencing water deficits (Maybank et al., 1995) and is relevant to countries such as Canada that have seen declining annual precipitation in regions with a history of severe drought events (Schindler and Donahue, 2006). The extent of yield loss due to drought depends on the crop species being examined. For example, field evaluation of wheat and maize between 1980 and 2015 showed a 21% and 40% yield reduction, respectively (Daryanto et al., 2016).

Some breeding programs have been successful in producing crop cultivars with satisfactory performance under water deficient conditions. Haley et al. (2007) developed the wheat variety “Ripper” that outperformed common varieties used in the non-irrigated Colorado (USA) region. A drought tolerant cultivar of maize was also developed by Badu-Apraku and Yallou (2009). On the other hand, yield improvements of *Medicago sativa* (alfalfa) through conventional breeding have been limited (Volenec et al., 2002). Between the 1950s and 1990s there was little yield increase as a result of breeding programs (Volenec et al., 2002). For this reason, there is great interest in developing molecular tools to improve alfalfa yield (Volenec et al., 2002).

1.2 Plant response to drought

When exposed to unfavourable conditions like drought, plants generally respond with two strategies: avoidance or acclimation (Lamaoui et al., 2018). The avoidance strategy involves whole plant morphological changes that minimize water loss (Lamaoui et al., 2018). Remaining plant resources are shunted to important functions and consequently, changes to stomata and transpiration occur (Lamaoui et al., 2018; Schakel and Hall, 1979; Sicher et al., 2012). Drought conditions are often accompanied by decreased atmospheric humidity resulting in changes to leaf turgor and water potential (Lamaoui et al., 2018; Meyer and Boyer, 1972). In response to drought, plants reduce stomatal conductance by closing existing stomata (Lamaoui et al., 2018; Sicher et al., 2012). In this way, water loss as a result of transpiration is minimized and levels of photosynthesis fall (Lamaoui et al., 2018; Sicher et al., 2012). The effects of water deficiency are also minimized by reducing the leaf surface (Schakel and Hall, 1979) and delaying leaf senescence (Rivero et al., 2007).

Plants also respond to drought by altering gene expression to counter water scarcity and provide tolerance in a process called acclimation (Huang et al., 2008). The plant hormone, abscisic acid (ABA), plays an important role in acclimation and can promote the closure of stomata, mitigating water loss from the plant (Bauer et al., 2013). ABA is first released in roots, caused by water-deficient soil (Zhang and Davies, 1989), and positively regulates root growth (Spollen et al., 2000). Enhanced levels of ABA reduce

the production of ethylene and, consequently, increases root growth to support the uptake of any remaining water in the soil (Spollen et al., 2000). ABA is carried by xylem to plant leaves to cause stomata closure and prevent water loss (Zhang and Davies, 1989). Further water loss from young leaves is prevented due to enhanced accumulation of ABA produced in mature leaves during wilting (Zhang and Davies, 1989). SNF1-RELATED PROTEIN KINASE 2 (SnRK2) protein family is responsible for the regulation of these ABA-dependent responses and insensitivity to ABA occurs if *SnRK2* is knocked out (Umezawa et al., 2009). SnRK2 is under constant inactivation as a result of dephosphorylation by PROTEIN PHOSPHATASE TYPE 2C (PP2C) (Umezawa et al., 2009). Activation of ABA-dependent responses occurs when REGULATORY COMPONENT OF ABA RECEPTOR 1 (RCAR) binds to PP2C (Ma et al., 2009) thereby releasing SnRK2 in the presence of ABA (Umezawa et al., 2009).

Even though reactive oxygen species (ROS) are produced during normal aerobic metabolism, their levels increase significantly under stress and can be detrimental to proteins, lipids, and DNA (Apel and Hirt, 2004; Halliwell, 2006). ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals can oxidize DNA bases causing DNA lesions (Dizdaroglu and Jaruga, 2012) or can oxidize cysteine residues causing inactivation of catalytic proteins like protein tyrosine phosphatases (Denu and Tanner, 1998). Vanacker et al. (2006) found that levels of hydrogen peroxide were correlated with levels of lipid peroxidation, the oxidation of lipids resulting in degradation. ROS are also capable of activating ROS sensors, oxidizing unrelated signaling pathways, and modifying transcription factors culminating in altered gene expression (Apel and Hirt, 2004; Choudhury et al., 2013; Desikan et al., 2001; Halliwell, 2006; Vranová et al., 2002). During abiotic stress, ROS production overwhelms cellular defenses to ROS that include antioxidant response (Apel and Hirt, 2004; Choudhury et al., 2013). Antioxidants are capable of converting ROS into more stable forms of oxygen and can operate through enzymatic or non-enzymatic mechanisms resulting in tolerance to oxidative and abiotic stresses (Sarker and Oba, 2018). Catalase is an example of an enzymatic ROS scavenger and catalyzes the conversion of hydrogen peroxide to water (Apel and Hirt, 2004; Choudhury et al., 2013). Non-enzymatic antioxidants include the ascorbate-glutathione cycle, flavonoids, alkaloids and carotenoids (Apel and Hirt, 2004). The ascorbate-

glutathione cycle involves a number of dedicated reactions to detoxify hydrogen peroxide (Apel and Hirt, 2004; Choudhury et al., 2013). In some cases, the activity and expression of these antioxidants and genes related to their biosynthesis increases in response to abiotic stress (Kang et al., 2020; Rüegsegger et al., 1990; Sarker and Oba, 2018; Arshad et al., 2017a).

1.3 Significance of alfalfa to society

Alfalfa is a forage legume crop grown on 3.8 million hectares in Canada (Statistics Canada, 2016). Alfalfa popularity increased in North America once breeding programs included winter hardy alfalfa cultivars from Siberia (Russelle, 2001). Alfalfa is now considered to be the most important forage crop in Western Canada necessitating special attention and tracking of its yield in response to variations in water availability (Attram et al., 2016).

The majority of alfalfa's significance can be attributed to its nutritional value. Although the whole plant is harvested and used as fodder for ruminant animals, the highest nutritional value originates in alfalfa leaves (Radović et al., 2009; Marković et al., 2007). Alfalfa is high in soluble proteins but its nutritional value diminishes with maturity (Marković et al., 2007). In addition, due to the rapid release of soluble proteins, alfalfa forage can also lead to pasture bloat in ruminant animals (Wang et al., 2006). Soluble proteins that are in high abundance in alfalfa are released from plant cells mainly in the ruminant adding to its viscosity which is correlated with ruminant animal gas accumulation (Jonker et al., 2012). Bloat occurs as a consequence of foam, created by free protein degradation in the rumen, which prevents gases from escaping and organs are crushed as it expands, eventually leading to death (Mangan, 1959). Rumen is responsible for degrading cellulose and hemicellulose in ruminant animals making its presence necessary for plant digestion (Aerts et al., 1999; Leschine, 1995).

Proanthocyanidins, if present, slow down the degradation of soluble proteins and therefore reduce bloat incidences from feed (Aerts et al., 1999; Jones and Mangan, 1977; Waghorn et al., 1987). Naturally occurring within legumes, proanthocyanidins, which are capable of binding to proteins in rumen, inhibit protein degradation (Jones and Mangan,

1977; Waghorn et al., 1987). The higher pH of the rumen allows for the association between proanthocyanidins and proteins which disassociate in the low pH of the small intestine to allow for proper protein digestion (Jones and Mangan, 1977). Proanthocyanidins share partial biosynthesis with other flavonoids such as anthocyanins that begin with the phenylpropanoid pathway (Aerts et al., 1999; Dixon et al., 2013). The last shared enzymatic step in the biosynthesis of proanthocyanidins and anthocyanins is the conversion of dihydrokaempferol to leucoanthocyanidins by DIHYDROFLAVONOL 4-REDUCTASE (DFR) (Aerts et al., 1999; Dixon et al., 2013).

In addition to its nutritional value, alfalfa has extensive root structures, which allow it to resist soil erosion that causes soil loss in agricultural fields (Radović et al., 2009). Also, like most legumes, alfalfa roots can form a symbiotic relationship with the Rhizobium family of bacteria (Jones et al., 2007). Rhizobia infect alfalfa roots and form nodules within which rhizobia are capable of fixing atmospheric nitrogen into a form that is usable by alfalfa and other plants (Jones et al., 2007). Through this process, alfalfa can meet 80% of its nitrogen requirements (Hardarson et al., 1988).

Before the invention of the Haber-Bosch process and advent of synthesized nitrogen fertilization practices, legumes like alfalfa were used in rotation with other crops to replenish nitrogen levels in the soil (Bullock, 1992). Crop rotation, dedicating 20-50% of their available agricultural land to the growth of legumes that are incorporated back into the soil to benefit future crops, doesn't provide enough nitrogen to solely support the world's agriculture (Crews and Peoples, 2004). Nitrogen fertilizers have the capability of increasing crop yields (Seufert et al., 2012), but fertilizers containing ammonia directly acidify soils (Crews and Peoples, 2004). Soils with low pH can put plants at risk of metal toxicities, such as aluminum which is only phytotoxic when soil pH is low (Kochian, 1995). Surrounding aquatic environments are also impacted by fertilizer runoff leading to eutrophication and contamination of fresh drinking water becoming a threat to human health (Weyer et al., 2001). To complicate matters, fertilizer use has been linked to the increased release of greenhouse gasses contributing to climate change (Ma et al., 2018; Syakila and Kroeze, 2011). Due to our dependence on fertilizers for agricultural production, agricultural land is the single greatest anthropogenic contributor to nitrous

oxide levels (Syakila and Kroeze, 2011). As such, utilizing alfalfa in crop rotation systems presents an environmentally sustainable alternative to nitrogen fertilizer use to improve soil quality (Ma et al., 2018). In addition, crop rotations have been shown to reduce insect pests, weeds, and plant diseases that plague continuous cropping systems (Felton et al., 1998; Liebman and Dyck, 1993; Tonhasca and Byrne, 1994). Legume-based farming practices were also found to decrease nitrogen leaching from farmland thereby decreasing aquatic-based pollution from agricultural systems (Drinkwater et al., 1998). It has also been suggested that the nitrogen sequestered by legumes is incorporated back into the soil slowly by decaying green manure, can resist the leaching that fertilizers are prone to and can potentially replace fertilizers as a source of nitrogen for subsequent crops (Diekmann et al., 1993).

1.4 Application of miRNAs in plant biotechnology

MicroRNAs (miRNA) were first discovered in *Caenorhabditis elegans* by Lee et al. (1993). The 21-22 nucleotide RNAs, *lin-4* and *let-7*, were found to regulate *C. elegans* development at distinct stages and were therefore called small temporal RNA (stRNA) (Lee et al., 1993; Reinhart et al., 2000). Soon thereafter, additional small RNA molecules of similar sizes were discovered in *C. elegans*, *Drosophila melanogaster*, and humans (Lagos-Quintana et al., 2003; Lau et al., 2001; Lee and Ambros, 2001). These new small RNA molecules were similar in length but were not expressed at distinct temporal stages like stRNAs (Lagos-Quintana et al., 2003; Lau et al., 2001; Lee and Ambros, 2001; Reinhart et al., 2002). As such, the term miRNA was coined and used to refer to any small (~22 nucleotides) non-coding regulatory RNA molecule endogenous to the organism, even if its exact functions were unknown (Lagos-Quintana et al., 2003; Lau et al., 2001; Lee and Ambros, 2001; Reinhart et al., 2002).

Much in the same way as short interfering RNAs (siRNA) involved in RNA interference (RNAi) (Hutvágner and Zamore, 2002), miRNAs are processed from larger precursor RNAs by the dsRNA endoribonuclease, Dicer (Bernstein et al., 2001). Precursor RNA must be between 30 and 500 nucleotides in length to be a functionally mature miRNA product (Elbashir et al., 2001). Mature miRNAs are generally 20-24 nucleotides in length

and maintain terminal phosphate and hydroxyl groups on the 5' and 3' ends, respectively (Elbashir et al., 2001). The smaller mature dsRNA product is processed with a 3' two-nucleotide overhang (Elbashir et al., 2001). This nucleotide overhang is important to miRNA function as demonstrated by Elbashir et al. (2001) who found that dsRNA with the two-nucleotide overhang had better efficiency than blunt end dsRNA. The processed dsRNAs are recruited by an RNA-induced silencing complex (RISC) but only the strand with the least stable 5' end is kept to target complementary genes for cleavage, while the rejected strand with a more stable 5' end is degraded (Elbashir et al., 2001, Khvorova et al., 2003).

Ultimately, the function of miRNAs is to silence target genes at the posttranscriptional level. miRNAs achieve repression of their targets through either transcript cleavage, messenger RNA (mRNA) destabilization, or translational repression (**Figure 1**) (Cannell et al., 2008; Hutvágner and Zamore, 2002; Mathonnet et al., 2007; Olsen and Ambros, 1999; Pillai et al., 2005; Wu et al., 2006). When perfect complementarity exists between the miRNA and its mRNA target, cleavage at the complementary site occurs similar to that of siRNAs (Hutvágner and Zamore, 2002). Destabilization of mRNA with imperfect complementarity to miRNAs occurs through deadenylation or trimming of the 3' poly(A) tail (Wu et al., 2006). Translational repression can occur at or after translational initiation (Cannell et al., 2008; Mathonnet et al., 2007; Olsen and Ambros, 1999; Pillai et al., 2005). miRNA inhibits translational initiation by interfering with the ability of ELONGATION INITIATION FACTOR 4E (eIF4E) to recognize the 7-methylguanylate cap of mRNAs (Mathonnet et al., 2007; Pillai et al., 2005). Olsen and Ambros (1999) also demonstrated that translation can be inhibited after initiation by miRNAs. In these experiments the researchers observed that while mRNA adenylation and association with polyribosomes was unaffected by miRNA, mRNA products were still downregulated (Olsen and Ambros, 1999). The miRNA-mediated repression of the mRNA product did however occur before production of the full protein and was a result of the dissociation of necessary translation proteins from the mRNA template (Petersen et al., 2006). Due to the requirement of near-perfect to perfect complementarity of miRNAs to their mRNA targets, plant miRNAs operate mainly through transcript cleavage (Rhoades et al., 2002).

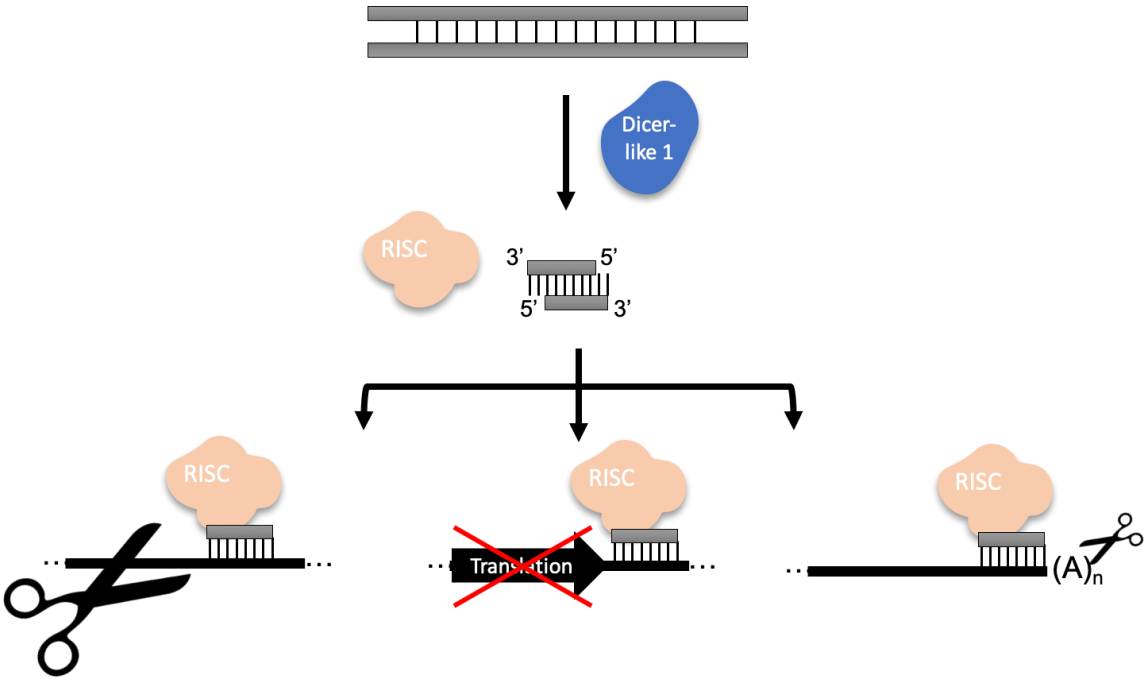

Figure 1. Mechanisms of posttranscriptional repression by miRNAs

Dicer creates short double stranded miRNA with two-nucleotide 3' overhangs. miR156 associates with RISC endonucleases resulting in the silencing of downstream complementary mRNA targets through cleavage, translational repression and mRNA instability. Figure adapted from Bartel and Bartel, 2003; Hutvágner and Zamore, 2002.

The search for *Arabidopsis thaliana* mRNAs with sequence complementary to miRNAs yielded, in most cases, targets that belonged to gene families encoding transcription factors acting in plant development (Rhoades et al., 2002). miR156 is no exception, as it has been implicated in plant development of many species including *A. thaliana* (Schwab et al., 2005; Wu and Poethig, 2006; Wu et al., 2009), *Carica papaya* (papaya) (Xu et al., 2020), *Solanum lycospersicon* (tomato) (Salinas et al., 2012), *Dendrobium catenatum* (Zheng et al., 2019), *Mangifera indica* (mango), *Persea Americana* (avocado), *Macadamia integrifolia* (macadamia) (Ahsan et al., 2019) and *Medicago sativa* (alfalfa) (Aung et al., 2015).

Wu et al. (2009) demonstrated that miR156 is an essential regulator of development in *A. thaliana* and is sufficient to control phase change from the vegetative non-flowering state to the adult reproductive phase. Overexpression (OE) of miR156 in *A. thaliana* resulted in plants with smaller leaves without abaxial trichomes characteristic of juvenile leaves (Wu and Poethig, 2006; Wu et al., 2009). OE in *A. thaliana* also delayed flowering (Schwab et al., 2005; Wu and Poethig, 2006) and decreased apical dominance (Schwab et al., 2005) while the extended juvenile phase of mango, avocado and macadamia plants is at least partly due to enhanced miR156 expression (Ahsan et al., 2019). In fruiting trees such as papaya, miR156 expression is reduced during fruit development (Xu et al., 2020).

It has been proposed that since miRNAs, specifically miR156, are heavily involved in development, it could be utilized as a molecular tool to improve economically important plant species like alfalfa (Aung et al., 2015). In alfalfa, miR156 functions in much the same capacity as in other species in that it regulates plant development, specifically, the transition from vegetative to reproductive phase (Aung et al., 2015). Similar to *A. thaliana* (Schwab et al., 2005; Wu and Poethig, 2006), miR156 OE in alfalfa resulted in plants with delayed flowering (Aung et al., 2015). In addition, miR156 OE resulted in alfalfa with thinner stems, shorter plant height, and reduced internode length, all characteristics of juvenile plants (Aung et al., 2015). Especially significant to crop improvement, miR156 OE in alfalfa resulted in plants with enhanced branching, number of nodes, and root length culminating in an overall increase in biomass yield (Aung et al., 2015). Similarities between alfalfa (Aung et al., 2015) and *A. thaliana* (Schwab et al.,

2005; Wu and Poethig, 2006; Wu et al., 2009) plants overexpressing miR156 further support a conclusion made by Rhoades et al. (2002) that miRNAs and their targets are conserved in flowering plants.

Regulation of plant development is not the only function of miRNAs; they have also been implicated in the response to various abiotic environmental stresses including salinity, drought, hypoxia and UV-B radiation (Rajwanshi et al., 2014). In *Glycine soja* (wild soybean), miR156 negatively regulates responses to aluminum toxicity (Zeng et al., 2012), while miR156 in alfalfa positively regulates responses to salinity (Arshad et al., 2017b) and heat stress (Matthews et al., 2019). In addition, miR156 was upregulated in response to both salinity and water-deficient conditions in *Panicum virgatum* (switchgrass) (Sun et al., 2012), *A. thaliana* (Cui et al., 2014), and *Brassica juncea* (brown seeded mustard) (Bhardwaj et al., 2014). *A. thaliana* plants overexpressing miR156 were able to survive drought and salt treatments that killed control plants and plants with reduced miR156 (Cui et al., 2014). Similarly, introducing constitutive expression of *Zea mays* (maize) miR156 to *Nicotiana tabacum* (tobacco) resulted in seedlings and mature plants with greater tolerance to drought and salinity conditions (Kang et al., 2020). miR156 OE also enhanced tolerance to drought in alfalfa (Arshad et al., 2017a). Alfalfa with miR156 OE had better root growth resulting in reduced shoot water loss, enhanced biomass yield, and better recovery from drought exposure than control empty vector (EV) plants (Arshad et al., 2017a). Feyissa et al. (2019) confirmed miR156 regulation of alfalfa plant water status by observing increased root length and biomass in miR156 OE alfalfa plants that resulted in enhanced leaf water potential. Enhanced stomatal conductance and increased accumulation of antioxidants were also observed, culminating in miR156 OE alfalfa plants that were able to survive drought better than EV plants demonstrating the usefulness of miR156 as a tool for alfalfa improvement (Arshad et al., 2017a).

1.5 *SPL*s as downstream targets of miRNA

Individual miRNAs often have multiple mRNA targets – usually belonging to the same gene family – and target domains that are weakly conserved in the encoded proteins

(Rhoades et al., 2002). Proteins belonging to the SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) family contain a Squamosa Promoter Binding Protein (SBP) domain of approximately 76 amino acids with an acidic N-terminus, a zinc-finger-like domain, and a nuclear localization signal (NLS) (Klein et al., 1996; Yamasaki et al, 2004). The SBP domain is required for SPL proteins to bind and regulate downstream genes having promoters with the consensus TNCGTACAA sequence (Cardon et al., 1999). As a result of their regulatory nature, and since abundance of miRNAs decreases over the lifetime of the plant, the expression of the miR156-targeted *SPL*s increases (Ahsan et al., 2019; Wu and Poethig, 2006, Wu et al., 2009, Xu et al., 2020, Zheng et al., 2019). The expression of some *SPL*s can even be undetectable at early stages of development when miRNA expression is highest (Wu and Poethig, 2006). At later stages of development, such as ripening in fruiting plants, *SPL* expression increases (Salinas et al., 2012; Xu et al., 2020). In alfalfa, at least seven *SPL*s, namely *MsSPL2*, *MsSPL3*, *MsSPL4*, *MsSPL6*, *MsSPL9*, *MsSPL12*, and *MsSPL13*, are targeted for transcript cleavage by miR156 (Aung et al., 2015; Gao et al., 2016).

Extensive characterization of the function of the miR156/*SPL* gene regulatory network has been conducted in many plant species (**Figure 2**), but there is variation in the number of *SPL*s with miR156 complementarity. There are 17 *SPL*s in *A. thaliana*, 11 of which are silenced by miR156 (Addo-Quaye, 2008; Cardon et al., 1999; Guo et al., 2008; Shikata et al., 2009; Wang and Wang, 2015; Wu and Poethig, 2006). In papaya and tomato, seven of 14 (Xu et al., 2020) and ten of 16 (Salinas et al., 2012) *SPL*s have miR156 complementarity, respectively. *SPL* genes in land plants have evolved from common ancestors and those with miR156 complementarity can be grouped into four phylogenetic clades in *A. thaliana* based on the amino acid sequences of their SBP domains: *AtSPL6/13/17*, *AtSPL9/15*, *AtSPL2/10/11*, and *AtSPL3/4/5* (Guo et al., 2008). A phylogenic comparison of these *SPL*s revealed that homologous *SPL*s in alfalfa can also be grouped into the same clades, and have high nucleotide and amino acid sequence complementarity to that of *AtSPL*s (Gao et al., 2016). Wu et al. (2009) speculated that single loss of function *spl* mutant plants did not show strong changes in phenotype due to redundancy in function within the *SPL* clades. In general, *spl* mutants from the same clade have similar phenotypes and deviated from wild type (WT) control plants in similar

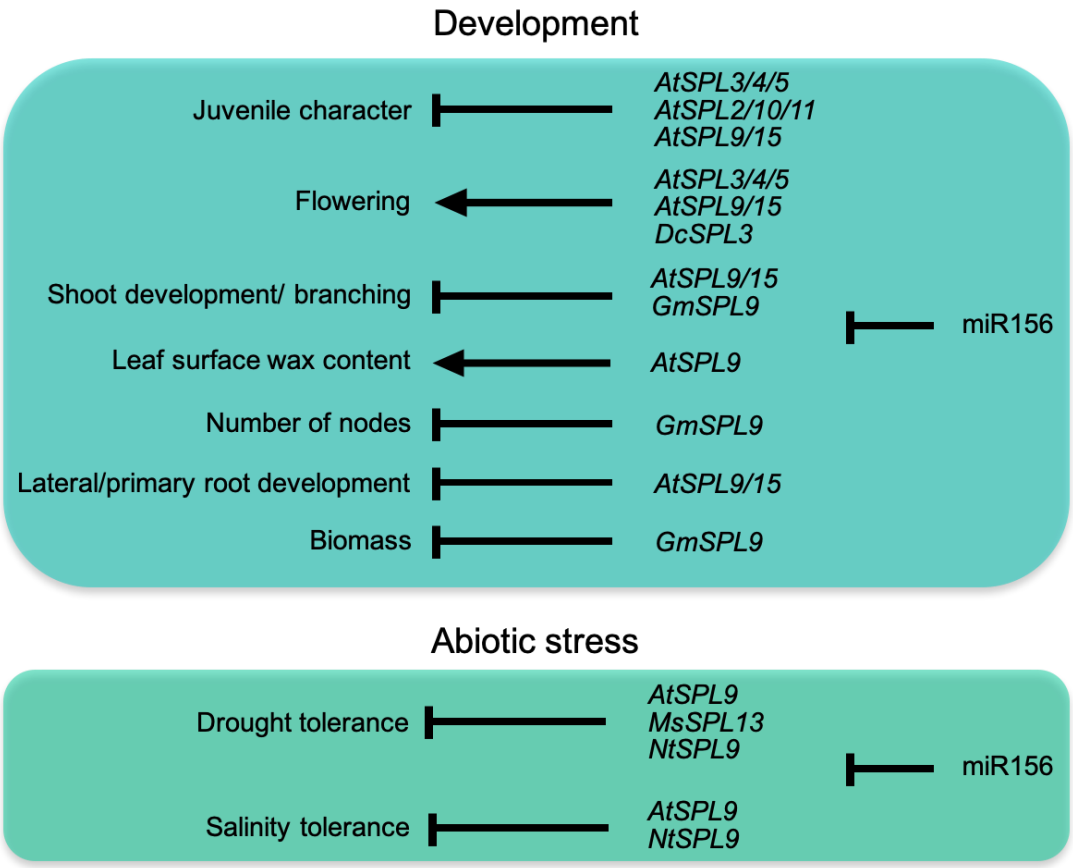

Figure 2. Overview of miR156-mediated regulation of plant development and stress tolerance

*SPL*s have miR156 complementary regions, and through their regulation miR156 controls the transcription of downstream genes resulting in changes to abiotic stress tolerance and developmental traits (Arshad et al., 2017a; Bao et al., 2019; Cui et al., 2014; Feyissa et al., 2019; Kang et al., 2020; Li et al., 2019; Schwarz et al., 2008; Shikata et al., 2009; Wu and Poethig, 2006; Yu et al., 2015; Zheng et al., 2019).

traits, supporting redundancy in SPL functions (Shikata et al., 2009; Wu and Poethig, 2006). *A. thaliana* plants overexpressing *SPL3*, *SPL4*, and *SPL5* showed early flowering and enhanced adult phenotype (Wu and Poethig, 2006). Early flowering and enhanced adult phenotypes are opposite to those observed for miR156 OE in the same study, which is consistent with the fact that miR156 silences expression of specific *SPL*s (Wu and Poethig, 2006). However, *DcSPL3* is the only *SPL* implicated in flowering in *D. catenatum*, a species of orchid (Zheng et al., 2019). A temporal expression analysis of *D. catenatum* revealed that only *DcSPL3* was upregulated in mature leaves and stems (Zheng et al., 2019). In the *AtSPL2/10/11* clade, these *SPL*s impact shoot development specifically reducing cauline leaf width and trichome abundance (Shikata et al., 2009). Although *AtSPL2*, *AtSPL10* and *AtSPL11* operate redundantly, knocking down *SPL10* alone resulted in plants with altered leaves but trichome abundance was not effected to the same extent as double mutants (Shikata et al., 2009). Much like the redundancy observed in *AtSPL2/10/11* (Shikata et al., 2009) and *AtSPL3/4/5* (Wu and Poethig, 2006), Schwarz et al. (2008) observed redundancy between *AtSPL9* and *AtSPL15*, with the two proteins having 75% similarity in their amino acid sequences. The expression of both *SPL9* and *SPL15* increased over time, much like the temporal regulation of other *SPL*s, but *SPL9* expression was higher than that of *SPL15* (Schwarz et al., 2008). *SPL9* and *SPL15* single knock-out mutants as well as a double *spl9/spl15* mutant were developed and it was observed that while the single mutants displayed changes in shoot development, flowering time, and juvenile character, some changes were more extreme in the double mutants (Schwarz et al., 2008). The *spl9/spl15* double mutants also had more lateral root development than the respective single mutants (Yu et al., 2015). Therefore, it was concluded that SPL9 and SPL15 have redundant functions in regulating vegetative phase transition, the transition from the juvenile to adult phase, in addition to lateral root development in *A. thaliana* (Schwarz et al., 2008; Yu et al., 2015). The involvement of *SPL9* in the miR156-mediated regulation of phase transition is not observed in all species (Ahsan et al., 2019). For example, Ahsan et al. (2019) found that *SPL9* expression did not increase with maturity in mango, avocado or macadamia plants.

In addition to affecting vegetative phase transition, SPL9 also plays other roles within plants. SPL9 was found to negatively regulate primary root length and lateral root

development in *A. thaliana* (Yu et al., 2015). In addition, mutated *SPL9* in *Glycine max* (soybean) resulted in plants with enhanced branching as well as increased node number and dry weight (Bao et al., 2019). Li et al. (2019) demonstrated that miR156 downregulates *SPL9* to control surface stem and leaf wax synthesis. OE of miR156 and reduction of *SPL9* resulted in decreased total wax content in stems while reduction in miR156 availability and overexpression of *SPL9* resulted in increased total wax content in stems and leaves with a higher density of epicuticular wax in *A. thaliana* (Li et al., 2019).

Lastly, SPL9 is also implicated in miR156-mediated abiotic stress tolerance. For example, *SPL9* is downregulated in response to salinity and drought stress in *N. tabacum* (Kang et al., 2020). Additionally, mutant *A. thaliana* plants in which *SPL9* was made insensitive to miR156-mediated regulation were found to be sensitive to drought and salinity stress (Cui et al., 2014). The negative regulation of abiotic stress tolerance by SPL9 was attributed to its negative regulation of anthocyanin biosynthesis through interaction with the transcription activating complex of *DFR* (Cui et al., 2014).

In comparison to the model organism *A. thaliana*, minimal investigation of *SPL* function has been performed in alfalfa. Specifically, *MsSPL8* has been investigated for its role in shoot branching (Gou et al., 2018). Down-regulating *SPL8* in transgenic alfalfa plants resulted in increased branching and consequently enhanced biomass yield (Gou et al., 2018). In addition to vegetative development, drought tolerance related studies have also been investigated in alfalfa (Arshad et al., 2017a; Feyissa et al., 2019; Gou et al., 2018). Both *MsSPL8* and *MsSPL13* are involved in alfalfa drought response and down-regulating *MsSPL8* or *MsSPL13* resulted in alfalfa that were less susceptible to drought (Arshad et al., 2017a; Feyissa et al., 2019; Gou et al., 2018).

1.6 Impact of the miR156-*SPL9*-*DFR* pathway on anthocyanin biosynthesis

Anthocyanins are responsible for the red pigment observed in some plant tissues (Nakatsuka et al., 2007). While originally thought to serve as a visible deterrent to herbaceous insects, contradictory results from different studies have decreased confidence

in this theory (Coley and Aide, 1989; Costa-Arbulú et al., 2001; Schaefer and Rolshausen, 2006). Instead, Schaefer and Rolshausen (2006) coined the "defence indication hypothesis" that states that anthocyanin content correlates with the biosynthesis of compounds with known anti-insect functions, thereby decreasing the number of insects that would utilize plants displaying anthocyanins. While the possibility that anthocyanins can deter herbivorous insects is disputed, it is commonly accepted that anthocyanins are produced in response to abiotic stress (Chalker-Scott, 1999; Schaefer and Rolshausen, 2006). Their antioxidant ability to scavenge ROS has been demonstrated with many different anthocyanin compounds (Azuma et al., 2008; Kähkönen and Heinonen, 2003) that is impacted by the position of hydroxyl and methoxy substituents (Ali et al., 2016; Rice-Evans et al., 1996).

As part of the phenylpropanoid pathway anthocyanin biosynthesis is tightly regulated (Chaves-Silva et al., 2018), in part through control of *DFR* transcription (Gonzalez et al., 2008; Gou et al., 2011). *DFR* is transcribed when activated by the binding of the *DFR* transcription activation complex to its promoter region (Gou et al., 2011). The *DFR* transcription activation complex is made of a ternary complex of proteins containing members of the MYB protein family, basic helix-loop-helix (bHLH) factors, and TRANSPARENT TESTA GLABRA1 (TTG1), a WD40 repeat-containing protein (Gonzalez et al., 2008; Gou et al., 2011). SPL9 can compete with TRANSPARENT TESTA8 (TT8), a bHLH protein, for binding to PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1) to prevent the assembly of the complex and subsequent *DFR* transcription and biosynthesis of anthocyanins (**Figure 3**) (Gou et al., 2011). Although the regulation of anthocyanin biosynthesis was confirmed in *A. thaliana*, Feyissa et al. (2019) also observed an increase in anthocyanin pigments in alfalfa with miR156 OE confirming a similar interaction is present. In addition, Gupta et al. (2019) speculated that miR156 in soybean targets *SPL9* to control anthocyanin biosynthesis through related biosynthesis genes such as *DFR*.

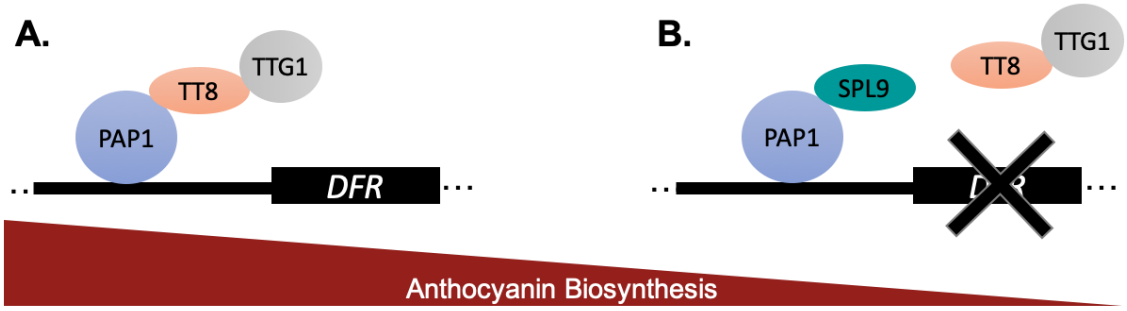

Figure 3. SPL9 interrupts the *DFR* transcription activating complex to regulate anthocyanin biosynthesis.

A. In the presence of PAP1, TT8, and TTG1, *DFR* transcription is initiated. DFR is an enzyme involved in the biosynthesis of anthocyanins therefore the *DFR* transcription activating complex regulates anthocyanin production. **B.** SPL9 competes with TT8 for binding to PAP1 thus interrupting the transcription activating complex and decreasing anthocyanin biosynthesis. Adapted from Gou et al. (2011).

1.7 Molecular tools for gene characterization

Both RNAi and clustered regularly interspaced short palindromic repeats (CRISPR)/ *Streptococcus pyogenes* CRISPR associated proteins (Cas) have been used by scientists to knock-out or knock-down genes to elucidate function (Arshad et al., 2017a; Bao et al., 2019; Feyissa et al., 2019). While arguments have been made regarding which method is superior, they can be complementary to each other for a more complete characterization of gene function and for biotechnological applications (Taylor and Woodcock, 2015).

RNAi utilizes mechanisms analogous to that of miRNAs to knock-down transcripts for genes of interest for characterization using siRNA (Bartel and Bartel, 2003). Delineation between RNAi and miRNA is fine, as such, past literature has used the terms almost interchangeably (Llave et al., 2002; Reinhart et al., 2002). Nevertheless, some differences exist between miRNAs and siRNA that allow for miRNA to be distinguished for annotation (Ambros et al., 2003). First, miRNAs are encoded by endogenous genes in different loci than the targets they regulate, whereas siRNAs originate from the targets they regulate (Bartel and Bartel, 2003). siRNAs are also processed from larger RNA duplexes whereas miRNAs are generally a result of the processing of RNA hairpins (Bartel and Bartel, 2003). Lastly, miRNAs are more highly conserved between organisms than siRNAs (Bartel and Bartel, 2003). As a result of their similarities to miRNAs, siRNAs can be used to regulate the expression of desired genes with some specificity (Karimi et al., 2007).

CRISPR and Cas proteins can be employed to knock-out genes by introducing repair mutations into a genome from double-stranded breaks (DSB) (Cong et al., 2013; Feng et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013; Zhang et al., 2017). Cas9 is a nuclease owing its specificity to the complementarity between a single guide RNA molecule (sgRNA) and its target, directly adjacent to protospacer-adjacent motifs (PAM) (Cong et al., 2013; Feng et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013; Zhang et al., 2017). Mutations are introduced through non-homologous end joining (NHEJ), or homology-directed repair (HR) at the site of the DSB (Shan et al., 2013; Xie and Yang, 2013, Zhang et al., 2017). Unlike previously popular

DSB exploiting techniques, to change the target of Cas9, only the identity of the sgRNA is altered, negating protein reprograming (Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013; Zhang et al., 2017). When designing sgRNAs, structural parameters must be considered. These include, but are not limited to, off target complementarity, GC content, and secondary structure (Doench et al., 2014; Doench et al., 2016; Hsu et al., 2013; Hua Fu et al., 2014; Liang et al., 2016; Ma et al., 2015).

1.8 Proposed research

The use of molecular tools to improve yields are essential for crops like alfalfa, which have seen little recent improvements through traditional breeding programs (Volenec et al., 2002). miR156 is a potential molecular tool to improve alfalfa yield due to its ability to regulate plant development (Aung et al., 2015). For example, OE of miR156 results in plants with increased biomass (Aung et al., 2015). Like other miRNAs, miR156 imparts its developmental control by reducing the expression of its complementary target genes (Gao et al., 2016). One such target, *SPL9*, has also been shown to control developmental traits in *A. thaliana* (Schwarz et al., 2008; Yu et al., 2015). Therefore, this study investigates the potential role of SPL9 in miR156-mediated alfalfa development through altering *SPL9* expression and evaluating changes in phenotypic character relative to WT plants at multiple points in development.

In alfalfa, OE of miR156 not only alters traits related to development (Aung et al., 2015), but also enhances tolerance to drought stress (Arshad et al., 2017a; Feyissa et al., 2019). Investigating traits related to drought stress in transgenic alfalfa with altered levels of *SPL9* could thus illuminate its involvement in miR156-mediated drought tolerance. Therefore, in this study, *SPL9*-RNAi alfalfa is compared to WT in its ability to grow, its plant water status, and its ROS scavenging capabilities in response to drought stress. The possibility that drought tolerance is impacted by reduced anthocyanin biosynthesis (via SPL9-mediated prevention of *DFR* transcription) is also examined by monitoring anthocyanin content and the transcription of *DFR* in *SPL9*-RNAi and WT alfalfa in response to drought. Determining the role, if any, of SPL9 in drought response will

further illustrate the mechanism by which miR156 achieves enhanced drought tolerance in alfalfa.

1.9 Hypothesis and objectives of proposed research

Rhoades et al. (2002) found that miRNA complementary target sites in protein families are conserved across flowering plants. It is thus unsurprising that Gao et al. (2016) demonstrated that miR156 OE results in *SPL9* downregulation in alfalfa due to miR156 complementarity. Therefore, the hypothesis that miR156-mediated traits are achieved by targeting *SPL9* in alfalfa was investigated.

Arshad et al. (2017a) and Feyissa et al. (2019) demonstrated that alfalfa plants with miR156 OE were more tolerant to drought than control plants and furthermore, that SPL13 was targeted by miR156 to regulate some of the drought tolerance traits in these plants. However, all of the drought tolerance characters of the miR156 OE alfalfa were not accounted for in plants with altered expression of SPL13 (Arshad et al., 2017a; Feyissa et al., 2019). Due to the involvement of SPL9 in the inhibition of *DFR* expression by disrupting its transcription activating complex (Gou et al., 2011), Cui et al. (2014) found that, in *A. thaliana*, *SPL9* is silenced by miR156 to induce anthocyanin biosynthesis and promote drought stress tolerance. A similar mechanism of tolerance could be present in alfalfa and therefore, the second hypothesis of this study is that the miR156-*SPL9*-*DFR* pathway mediates the regulation of anthocyanin biosynthesis, thereby influencing drought stress tolerance in alfalfa.

The objectives of this study were as follows:

1. To develop transgenic alfalfa maintaining OE of *SPL9* impervious to miR156 (*SPL9m*-OE), RNAi mediated *SPL9* knock down alfalfa and CRISPR/Cas9-mediated *SPL9* silenced (*SPL9*-CRISPR) alfalfa.
2. To evaluate phenotypic and stress response traits in *SPL9* transgenic alfalfa.
3. To investigate the role of the miR156-*SPL9*-*DFR* pathway in drought tolerance.

In this way, insight into the role of SPL9 in plant development and drought tolerance, as well as a foundation for the future development of novel molecular strategies that might be used to influence drought tolerance in alfalfa, is provided.

Chapter 2

2 Materials and Methods

2.1 Alfalfa propagation and growth conditions

Alfalfa (*Medicago sativa* L.) N.4.2.2 clone (Badhan et al., 2014) was used as the source of all plant material. Alfalfa plants were maintained under greenhouse conditions (16-hour light/8-hour dark, 56% relative humidity, 23°C) for the duration of the experiments. Genotypes were propagated using stem cuttings of at least two nodes that were grown in Oasis Rootcubes® (Oasis Growing Solutions, Kent, OH) for four weeks. Rooted cuttings were then transferred to BX Mycorrhizae (PRO-MIX®, Smithers-Oasis North America, Kent, OH) soil and allowed to grow for at least five weeks in 15.24 cm pots before being used in characterization and drought stress experiments. Stock plants and those maturing for experimental use were watered twice per week.

2.2 Generation of *SPL9* transgenic alfalfa plants

2.2.1 *SPL9*-RNAi

A construct for the RNAi-mediated silencing of *SPL9* (*SPL9*-RNAi) was made previously in the Hannoufa lab by Banyar Aung (Aung, 2014) and subsequently used in *Agrobacterium*-mediated alfalfa transformation by Qing Shi Mimmie Lu. A 300 bp fragment of *Medicago sativa* was amplified using primers SPL9-RNAi-F and SPL9-RNAi-R (**Appendix A**), which were specific to the *SPL9* protein coding sequence (**Appendix B**). This blunt-end fragment was cloned into the vector pENTR (Invitrogen, Carlsbad, CA) using the pENTR/D-TOPO Cloning Kit (Invitrogen). The vector was transferred to *Escherichia coli* (TOP10) (Thermo Fisher Scientific, Waltham, MA) by heat shock (Froger and Hall, 2007) and positive transformants were selected by growth on 50 µg mL^{-1} kanamycin Luria-Bertani (LB) media (5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 10 g L^{-1} Bacto-tryptone, 15 g L^{-1} bacterial agar). Plasmid DNA was extracted from individual colonies using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific)

and the presence of the correct insert was confirmed by Sanger sequencing. Transformants containing the *MsSPL9* insert with no sequence errors were used in an LR reaction according to the Gateway LR Clonase II Enzyme Mix protocol (Invitrogen) with pHELLSGATE12 (Helliwell and Waterhouse, 2003) as the destination vector. Positive transformants were again selected on 50 µg mL^{-1} kanamycin LB plates isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and plasmid DNA was analyzed by Sanger sequencing. pHELLSGATE12:*MsSPL9* vector was then transferred to *Agrobacterium tumefaciens* (GV3101) (Holsters et al., 1980) by heat shock (Höfgen and Willmitzer, 1988) and the resulting strain was used in the transformation of alfalfa N.4.4.2 germplasm (Badhan et al., 2014).

2.2.2 *SPL9m*-OE

The *M. sativa SPL9* (*MsSPL9*) protein coding region was synthesized by Bio Basic Inc. with seven point mutations within the miR156 complementary region (*MsSPL9m*) (**Figure 4)**. Each mutation changes a single nucleotide, conserving the MsSPL9 amino acid sequence, while introducing mismatches to the miR156 complementary region to prevent complementary binding and subsequent cleavage. Schwab et al. (2005) determined that endogenous miRNAs can tolerate up to five mismatches before preventing cleavage of its target. Seven mismatches were chosen in the *MsSPL9m* construct to guarantee inhibition of miR156 function. The restriction digestion sites, *Asc*I and *Pac*I, were also synthesized flanking the *MsSPL9m* sequence for ease of cloning. The *MsSPL9m* sequence was cloned into pMDC32 (Curtis and Grossniklaus, 2003), a vector containing the cauliflower mosaic virus (CaMV) 35S promoter, using T4 DNA ligase (Invitrogen). Positive transformants were selected on 50 µg mL^{-1} kanamycin LB plates and plasmid DNA was extracted and analyzed by Sanger sequencing to validate the sequence. Plasmid DNA was transferred to *Agrobacterium tumefaciens* (EHA 105) (Hood et al., 1993) by heat shock (Höfgen and Willmitzer, 1988) and the resulting strain was used to transform alfalfa N.4.4.2 germplasm (Badhan et al., 2014).

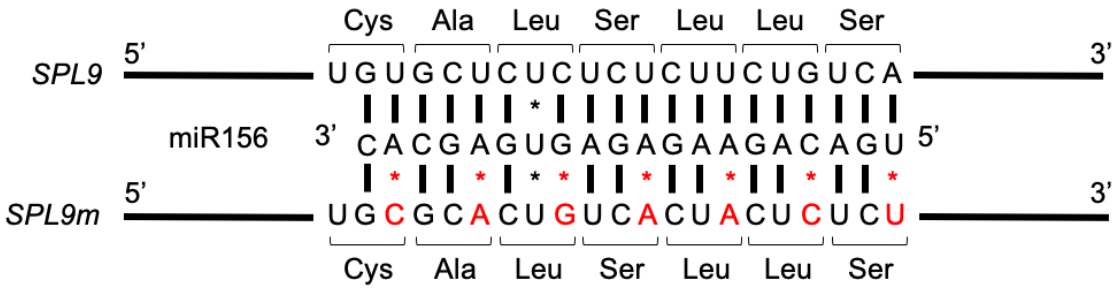

Figure 4. Mutations introduced to *SPL9* to prevent miR156 complementarity

Seven point mutations (red) were introduced to the *SPL9* coding sequence within the region complementary to miR156 to produce *SPL9m*. Each point mutation chosen conserves the SPL9 amino acid sequence. Asterisks indicates mismatches between miR156 and the mRNA sequence.

2.2.3 *SPL9*-CRISPR

The sgRNA, a 20 nt sequence that flanks a PAM sequence, was designed using the online tool CRISPR-P 2.0 (Liu et al., 2017). To narrow down the list of potential sgRNAs, guides found outside the *Medicago truncatula SPL9* sequence or located within *MtSPL9* introns were eliminated. To be chosen, the sgRNA had to have a maximized ON-target score (Doench et al., 2014), a minimized OFF-target score (Doench et al., 2016; Hsu et al., 2013), a minimized number of OFF-targets, and GC content between 30-80% (Doench et al., 2014; Liang et al., 2016). When considering its secondary structure, the chosen sgRNA also had to have less than seven consecutive base pairs with the scaffold sequence, less than 12 total base pairs with the scaffold sequence, less than six internal base pairs, a repeat and anti-repeat region (RAR), a stem loop 2, and a stem loop 3 (Liang et al., 2016). Only one guide met the aforementioned criteria (**Appendix B**). The chosen sgRNA was synthesized by Bio Basic Inc. with a *Medicago truncatula U6* promoter (*MtU6*). The *MtU6*:sgRNA insert was amplified by PCR using the primers MtU6-F and Scaffold-R (**Appendix A**). Amplified *MtU6*:sgRNA insert was cloned into pFGC5491-Cas9 (Meng et al., 2017) according to the In-Fusion Cloning (Takara Bio Inc., Shinga, Japan) protocol. The vector was transferred to *E. coli* (TOP10) (Thermo Fisher Scientific) by heat shock (Froger and Hall, 2007) and the presence of the insert was confirmed by Sanger sequencing of plasmid DNA. Plasmid DNA was then transferred to *A. tumefaciens* (EHA 105) (Hood et al., 1993) by heat shock (Höfgen and Willmitzer, 1988) and the resulting strain was used in the transformation of alfalfa N.4.4.2 germplasm (Badhan et al., 2014).

2.2.4 Transformation of *Agrobacterium tumefaciens*

Plasmids extracted from *E. coli* and whose sequences were validated by Sanger sequencing, were transferred to *A. tumefaciens* (EHA 105) (Hood et al., 1993) by heat shock according to Höfgen and Willmitzer (1988). A total of 200 ng of plasmid was added to thawed *A. tumefaciens* and kept on ice for 5 min. The bacterial mix was transferred to liquid nitrogen for 5 min and incubated at 37°C for 5 min. Cells were then incubated at 28°C for 2 hrs with shaking at 200 rpm. The supernatant was removed after

cells were spun down for 2 min at 6300 RCF. Cells were resuspended in LB and plated onto LB-agar medium containing 50 µg mL^{-1} of rifampicin and vector-specific selection.

2.2.5 *Agrobacterium tumefaciens*-mediated alfalfa transformation

Alfalfa transformation by *A. tumefaciens* (EHA105) (Hood et al., 1993) was performed using a modified protocol from Tian et al. (2002). Tissue culture material was allowed to grow at 26°C under a 16 hr/8hr light/dark schedule for all stages. Using *M. sativa* N4.2.2 (Badhan et al., 2014) as germplasm, transformation progressed in seven stages: preculture, co-cultivation, callus induction, callus selection, embryo induction, embryo germination, and plant development.

The preculture stage consisted of growing the *A. tumefaciens* culture and preparing the germplasm for the co-cultivation stage. A 5 mL starter culture of *A. tumefaciens* containing the construct of interest was grown for one day before being added to 100 mL LB containing 20 µM acetosyringone. Both the starter and larger cultures were grown at 28°C with shaking at 200 rpm. Simultaneously, approximately 0.8 cm fragments of alfalfa leaves and petioles were incubated for two days in Basal SH2K media (3.2 g L^{-1} Schenk and Hildebrandt Basal Salt Mixture [Sigma-Aldrich, Oakville, Canada], 0.5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} [B_6] pyridoxine HCl, 0.5 mg L^{-1} [B_1] thiamine HCl, 20 mg L^{-1} myo-inositol, 4.35 g L^{-1} K_2SO_4, 0.288 g L^{-1} proline, 3% w/v sucrose, 2.14 µM kinetin, 18.12 µM 2,4-dichlorophenoxyacetic acid, 8 g L^{-1} plant tissue culture [TC] agar, and 53 mg L^{-1} thioproline).

The co-cultivation stage followed in which explant fragments were soaked in the *A. tumefaciens* culture for 10 min, blot dried on sterile filter paper, placed on Basal SH2K media supplemented with 20 µM acetosyringone, and incubated for five days in the dark. After rinsing in Basal SH2K media, the tissue was transferred to Basal SH2K media supplemented with 300 mg L^{-1} timentin to induce callus formation for two weeks. Positive calli were selected by transferring calli to Basal SH2K media supplemented with 300 mg L^{-1} timentin and antibiotics specific to the construct being selected; 50 µg mL^{-1} hygromycin B, 10 µg mL^{-1} glufosinate ammonium, and 50 µg mL^{-1} kanamycin were used to select for *SPL9m*-OE, *SPL9*-CRISPR, and *SPL9*-RNAi, respectively. After 10 days,

calli were transferred to Basal SH2K media supplemented with 300 mg L^{-1} timentin and increased concentrations of antibiotics specific to the construct being selected for one week; 75 µg mL^{-1} hygromycin B, 15 µg mL^{-1} glufosinate ammonium, and 75 µg mL^{-1} kanamycin to select for *SPL9m*-OE, *SPL9*-CRISPR, and *SPL9*-RNAi, respectively.

Embryo induction was then initiated by transferring calli to embryo induction media (3.5 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 0.44 mg L^{-1} $MnSO_4 \cdot H_2O$, 34.7 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 100 mg L^{-1} NH_4NO_3, 100 mg L^{-1} KNO_3, 30 mg L^{-1} KH_2PO_4, 6.5 mg L^{-1} KCl, 0.16 mg L^{-1} H_3BO_3, 0.15 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.08 mg L^{-1} KI, 0.36 mg L^{-1} Fe(III) ethylenediaminetetraacetic acid [EDTA], 0.05 mg L^{-1} nicotinic acid, 0.01 mg L^{-1} [B_6] pyridoxine HCl, 0.01 mg L^{-1} [B_1] thiamine HCl, 0.2 mg L^{-1} glycine, 10 mg L^{-1} myo-inositol, 2 g L^{-1} yeast extract, 3% w/v sucrose, 8 g L^{-1} plant TC agar, 300 mg L^{-1} timentin, pH 5.8) and transferring to fresh media every two weeks. The same concentration of antibiotics used in the second callus selection phase was also used in the embryo induction media.

Elongated embryos were kept for the embryo germination stage. Embryos were transferred to embryo germination media (2.165 g L^{-1} Murashige and Skoog [MS] Basal Salt Medium [Sigma-Aldrich], 0.05 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} [B_6] pyridoxine HCl, 0.1 mg L^{-1} [B_1] thiamine HCl, 20 mg L^{-1} myo-inositol, 1 mg L^{-1} glycine, 3% w/v sucrose, 8 g L^{-1} plant TC agar, 300 mg L^{-1} timentin, 75 µg mL^{-1} hygromycin B, pH 5.8) and kept on this media until a well-formed cotyledon was observed. The same concentration of antibiotics used in the second callus selection phase was also used in the embryo germination media. The media was renewed every two weeks

Germinated embryos were transferred to embryo germination media supplemented with an additional 2.165 g L^{-1} of MS Basal Salt Medium (Sigma-Aldrich) for rooting. Generally, it took 4-6 weeks for roots to form in the germination media. Once formed, excess media was rinsed from rooted plants, which were then transferred to BX Mycorrhizae (PRO-MIX®, Smithers-Oasis North America) soil in 10.16 cm pots and placed in the greenhouse (16-hour light/8-hour dark, 56 relative humidity, 23°C). For the first week after transfer, a small jar covered the plant to increase the humidity around the plant. The jar was gradually lifted as the week progressed. After one month, plants were

transferred to 22.86 cm pots and could be used to propagate alfalfa for experiments. To identify potential *SPL9*-RNAi and *SPL9m*-OE plants, qRT-PCR was performed and *SPL9* transcript levels of potential transgenic alfalfa and WT plants were compared. The presence of the *MtU6* promoter and *SpCas9* genes was assessed by PCR of genomic DNA (gDNA) to identify potential *SPL9*-CRISPR plants.

2.3 cDNA synthesis and qRT-PCR analysis

To extract RNA from alfalfa, 100 mg of alfalfa tissue was ground with a mortar and pestle in liquid nitrogen. The tissue was transferred to a Precellys Lysing Kit (Bertin Instruments, France) beaded 2 mL tube and 450 µL buffer RLC with 1% 2-mercaptoethanol from the RNeasy Plant Mini-prep kit (Qiagen, Hilden, Germany) was added. Tissue was then homogenized using a PowerLyzer™ (MoBio Laboratories Inc., Carlsbad, CA) for 30 seconds at 1890 RCF. RNA was extracted from the resulting lysate according to the RNeasy Plant Mini-prep kit manual. Any residual DNA was removed from each sample using the TURBO DNA-free kit (Invitrogen). A total of 0.5 µg of RNA was used to synthesize cDNA using iScript™ Reverse Transcription Supermix for qRT-PCR (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Synthesized cDNA was used in qRT-PCR reactions to assess the transcript level of genes related to this study. qRT-PCR reactions were performed with SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). All experiments were normalized with the reference genes *ACTIN-DEPOLYMERIZING PROTEIN 1 (ADF1)* (Castonguay et al., 2015; Guerriero et al., 2014) and *ELONGATION INITIATION FACTOR 4A (EIF4A)* (Guerriero et al., 2014) using the primers ms_ADF1qF/R and ms_EIF4AqF/R, respectively that are stable under numerous abiotic stress conditions (**Appendix A**). Each reaction was performed in triplicate and consisted of 0.5 ng cDNA in water, 0.5 µM forward and reverse gene-specific primers, and nuclease-free water to bring the reaction volume to 10 µL. qRT-PCR primers used to amplify *SPL9*, *CATALASE1 (CAT1)*, *GLUTATHIONE SYNTHETASE* (*GSH*), and *DFR* were LA_MsSPL9-Fq1/Rq1, CAT1-F/R, GSH-F/R, and DFR-F/R, respectively (**Appendix A**). The qRT-PCR program continued as follows:

95ºC for 30 sec, 95ºC for 5 sec, primer specific annealing temperature for 15 sec for 45 cycles with a melt curve starting at 65ºC and increasing in 0.5ºC increments every 5 seconds. The annealing temperature when amplifying *CAT1*, *GSH*, and *DFR* was 58ºC and was 55ºC when amplifying *SPL9*. Transcript analysis was performed using the CFX Maestro™ software (Bio-Rad Laboratories) and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4 Analysis of CRISPR/Cas9 mutagenized alfalfa

Genomic DNA from leaves of putative *SPL9*-CRISPR alfalfa was extracted according to the ChargeSwitch gDNA Plant Kit (Thermo Fisher Scientific) protocol. PCR reactions with DreamTaq (Thermo Fisher Scientific) polymerase were used to amplify *MtU6* promoter and *SpCas9* gene according to the manufacturer's protocol using the primers MtU6-F/R and LH_Cas9_F1/R1, respectively (**Appendix A**). Genomic DNA from positive *SPL9*-CRISPR alfalfa transformants containing the *MtU6* promoter and *SpCas9* gene were used as templates to amplify the *MsSPL9* region of interest including the sgRNA complementary guide sequence according to the Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific) protocol. The resulting amplified *MsSPL9* fragment was cloned according to the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) and its sequence was analyzed by Sanger sequencing to identify potential CRISPR/Cas9-directed mutations.

2.5 Characterization of *SPL9*-RNAi alfalfa

Transcript levels of *SPL9* were determined in *SPL9*-RNAi alfalfa and compared to that of WT alfalfa to determine the level of *SPL9* silencing. Three *SPL9*-RNAi genotypes, R1, R2 and R3, were chosen due to their decreased *SPL9* transcript level compared to WT. Phenotypic traits in *SPL9*-RNAi and WT were examined at two and six months in 5 – 10 replicates for each genotype. Flowering time was determined by determining the number of days from transplanting rooted cuttings to soil until first emergence of flowers.

The fresh biomass weight (FW) of alfalfa shoots was determined by cutting and immediately weighing the shoots. The FW of the roots of these plants was also measured

in the same way after the soil was washed from roots and excess water was removed by pat drying. These tissues were then placed in a 65ºC oven for 5 days to dry and measurement of their dry biomass weight (DW) was undertaken.

The phenotypic traits of shoots were compared in *SPL9*-RNAi and WT alfalfa. To determine the number of branches of each plant, main branches were considered any stem emerging directly from the soil and lateral branches were considered any stem originating from a main branch. Plant height was measured in the tallest main branch, upon which number of nodes was also determined. Average internode length was then calculated from the ratio of the number of nodes to plant height. Stem thickness was measured using a digital caliper (Mitytoyo, Japan) between the second and third nodes of the tallest main branch. The phenotypic traits of roots were also compared in *SPL9*-RNAi and WT alfalfa. Root length was considered as the length of the longest root.

2.6 Evaluation of drought tolerance

Rooted cuttings destined for use in drought trials were grown in 15.24 cm pots after removal from rooting cubes. Five weeks after the transfer to soil, to start the drought trial, each individual plant was given approximately 150 mL of water to normalize the amount of water given to each plant. Plants in the control treatment were watered twice a week for the duration of the trial while plants in the drought treatment had water withheld until all WT plants showed signs of stress by wilting (12 days). *SPL9*-RNAi and WT alfalfa were exposed to the drought and control conditions and compared to assess drought tolerance traits.

General phenotypic traits that were evaluated in plants included: change in plant height, leaf senescence, shoot biomass, and root biomass. Plant shoot growth over the duration of the trial was measured as an indication of the ability of the plants to resist the impacts of drought. The difference between the plant height at the end of the trial, day 12, and initiation of the trial, day 0, was evaluated as plant growth during drought stress. Leaf senescence was also evaluated by determining the ratio of senesced leaves (leaves that were seen to roll or desiccate) to the total number of leaves present on the tallest main branch. DW and FW of the shoots were measured as described in **Section 2.5**. The DW,

FW, and length of roots were measured in alfalfa grown in conetainers (Greenhouse Megastore, Danville, IL) instead of regular pots.

2.7 Assessment of survivability

The ability of alfalfa to survive drought stress was compared in WT and *SPL9*-RNAi in 15.24 cm pots. Ten individual plants of each genotype (R1, R2, R3 and WT) were given approximately 150 mL of water after which they were exposed to drought by withholding water for 14 days. On day 14, when all plants were severely stressed, plants were re-watered, and this was continued twice a week for two weeks. After the two weeks of re-watering, the survival of the plant was assessed. If any new growth was present, the plant was scored as having survived. Alfalfa survival was calculated from the ratio of the number of plants that survived to the number grown. This experiment was independently repeated three times.

2.8 Evaluation of plant water status

The water status of alfalfa shoots was evaluated by measuring relative water content (RWC), midday leaf water potential, and shoot water loss. Alfalfa plants were exposed to either drought or control conditions imposed as described in **Section 2.6**.

2.8.1 Relative water content

Ten trifoliate leaves were cut from each alfalfa plant that was exposed to control or drought conditions. Immediately after cutting, the leaves were weighed (FW) and placed in a small glass jar filled with approximately 20 mL of deionized water and sealed with a screw cap. Before closing the jar, the leaves were completely submerged in the water using forceps. The leaves remained in dark conditions submerged in water for two days. Once the leaves were removed from the water they were pressed once with paper towels to remove excess water and weighed a second time (FTW). The leaves were then placed in an oven set at 65ºC for five days, after which they were removed and weighed a third time (DW). RWC was calculated according to equation 1 (Anderson and McNaughton, 1973; Arshad et al., 2017a; Weatherley, 1950):

Equation 1: $$RWC = \left[\frac{FW-DW}{FTW-DW}\right] \times 100$$

2.8.2 Midday leaf water potential

Around midday, 12 days after initiating drought and control conditions, a razor blade was used to cut a trifoliate leaf from the bulk of the alfalfa shoots. Leaves furthest from the base of the shoots with a petiole long enough to fit in the pressure vessel were used. The leaf was placed in the pressure vessel of the SAPS II Portable Plant Water Status Console (Soilmoisture Equipment Corp., Santa Barbara, CA) with the cut end of the petiole visible when the pressure vessel was closed and the trifoliate end within the vessel. Pressure in the vessel was applied using N_2 gas and the reading on the pressure gauge was recorded when a single drop of moisture was observed with a magnifying glass to emerge from the exposed end of the petiole (Turner, 1988). The more water within the leaf, the less pressure needed to be applied to the system for that water to be visible at the site where the leaf was cut.

2.8.3 Shoot water loss assay

A shoot water loss assay was used to evaluate the capability of the shoots to lose water rapidly (Arshad et al., 2017a). Shoots were cut after 12 days of exposure to the control or drought conditions and the site of the cut was sealed with Parafilm M that was secured with tape. The FW of the shoots were then measured with the first measurement considered as 0 min (FW_{t_0}). After the initial measurement, the shoots were weighed every 30 min (FW_{t_n}) for three hrs. Rapid water loss was calculated using equation 2:

Equation 2: $$\%\ water\ loss = \frac{FW_{t_0} - FW_{t_n}}{FW_{t_0}}$$

2.9 Examination of plant photosynthetic capabilities

After 12 days of exposure to drought and control conditions the ability of alfalfa plants to continue photosynthesis despite the conditions they were exposed to was evaluated by

measuring stomatal conductance and leaf chlorophyll content. Drought and control conditions were imposed as described in **Section 2.6**.

2.9.1 Stomatal conductance

A Leaf Porometer (Decagon Devices Inc., Pullman, WA) was used to measure stomatal conductance on both the adaxial and abaxial leaf surfaces. Stomatal conductance was measured around midday 12 days after initiation of the drought trial. The average stomatal conductance of two leaves were measured in each plant as technical replicates. Four trials of two biological replicates were performed for a total of 8 biological replicates. The largest of the leaves available were chosen to measure stomatal conductance to maximize surface area. Calibration of the device was performed by adding Drierite desiccant (W.A. Hammond Drierite Company, Xenia, OH) to the Porometer head and measuring water saturated filter paper on a calibration plate under greenhouse conditions. The device was considered calibrated after three consecutive measurements were taken within 7.5% of the expected conductance from the calibration plate, 240 mmol $m^{-2}s^{-1}$.

2.9.2 Chlorophyll content

A MC-100 chlorophyll concentration meter (Apogee Instruments, Logan, UT) was used to measure the concentration of chlorophyll in alfalfa exposed to drought and control conditions for 12 days. Measurements were taken midday with the detector facing the abaxial leaf surface and the diode facing the adaxial leaf surface. The concentration of chlorophyll was measured in five leaves in each plant and their average was used as one biological replicate. The five leaves chosen were randomly distributed and five biological replicates were used in total.

2.10 Antioxidant capability analysis

Alfalfa plants exposed to drought or control conditions were evaluated for antioxidant capacity by measuring the following: transcript levels of *CAT1* and *GSH* (**Section 2.3**),

and total antioxidant activity using a trolox equivalent antioxidant assay. Drought and control conditions were imposed as described in **Section 2.6**.

2.10.1 Determination of antioxidant capacity

The exact weight of approximately 100 mg of leaf tissue was recorded and total antioxidants were extracted by homogenizing the tissue in 1 mL of pre-chilled 50% methanol in Precellys Lysing Kit (Bertin Instruments) beaded 2 mL tubes in a PowerLyzer (MoBio Laboratories Inc.). The lysate from the homogenization was centrifuged for 2 min at 16,400 RCF at 4ºC and filtered through a 0.45 μm syringe filter (Chromatographic Specialties Inc., Brockville, ON). These filtered extracts were used when completing the Sigma-Aldrich Antioxidant Assay Kit (CS0790) according to the manufacturer's protocol. The spectrophotometric endpoint absorbance at 405 nm was measured using a Multiskan GO spectrophotometer (Thermo Fisher Scientific) to estimate the ability of the extracts to sequester hydrogen peroxide and prevent the formation of the chromogen 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS•+). Absorbance values of the extract were compared to a Trolox standard curve (**Figure 5**) to determine total antioxidant activity after which each value was normalized by the weight of the starting material.

2.11 Determination of anthocyanin content

To determine anthocyanin content, stem bases were collected from alfalfa plants exposed to drought and control conditions. The exact weight of each sample (FW) was weighed and recorded. Anthocyanins were extracted as described by Neff and Chory (1998). Briefly, samples were ground in liquid nitrogen with a mortar and pestle and transferred to Precellys Lysing Kit (Bertin Instruments) beaded 2 mL tubes. A total of 600 μL of methanol in 1% HCl – referred to as the extraction solution – was added to each tube and homogenized using a PowerLyzer (MoBio Laboratories Inc.). Tubes were left overnight at 4ºC in the dark. Samples were then centrifuged at 16,400 RCF for 5 min after the addition of 200 μL water and 500 μL chloroform. 400 μL of 60% extraction solution was added to the supernatant. Spectrometric measurements were made at 530 nm (A_{530}) and

657 nm (A_{657}) using a Multiskan GO spectrophotometer (Thermo Fisher Scientific). All samples were corrected using 60% extraction solution as a blank and absorbance measurements were calculated according to equation 3:

Equation 3: $$Relative\ anthocyanin\ content = \frac{A_{530} - A_{657}}{FW}$$

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism. Characterization of *SPL9*-RNAi alfalfa was achieved using One-way ANOVA analysis. The Dunnett's test was used to elucidate differences between WT and each *SPL9*-RNAi genotype. The student's t-test was used to determine significant differences in *SPL9* expression of WT plants under control and drought conditions. Evaluation of differences between groups in experiments related to drought was determined using Two-way ANOVA. A Dunnett's test was used to determine which *SPL9*-RNAi genotypes were different from WT considering genotypes within treatments and a Sidak's test was used to determine if each genotype had significant change between drought and control conditions.

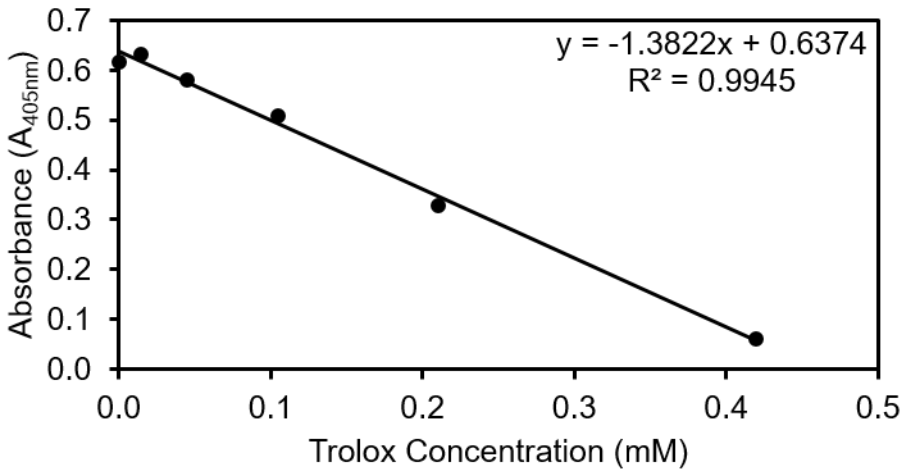

Figure 5. Trolox standard curve

Standard curve of antioxidant assay reaction using trolox as a reference to calculate the antioxidant activity of alfalfa.

Chapter 3

3 Results

3.1 Analysis of *SPL9*-CRISPR transgenic alfalfa

In an attempt to knock-down *SPL9*, a vector containing *SpCas9* and a sgRNA designed to target *SPL9* under the *MtU6* promoter was used in alfalfa *A. tumefaciens*-mediated transformation. Potential CRISPR/Cas9-mutated alfalfa (*SPL9*-CRISPR) plants identified by antibiotic screening were further subjected to analysis by PCR to detect the presence of the *MtU6* promoter and *SpCas9* transgene. A single band of expected size was visible after amplification of 16 different gDNA samples using primers specific to both the *MtU6* promoter (500 bp) and *SpCas9* gene (984 bp) (**Figure 6**). Confirmed *SPL9*-CRISPR plants originated from six independent calli in the tissue culture process. A fragment of the *SPL9* coding sequence containing the sgRNA complementary region was subjected to Sanger sequencing in one representative from each of the six calli to identify potential mutations as a result of the CRISPR/Cas9 system. The representatives from each calli and WT sequences did not differ in the expected sgRNA region therefore CRISPR/Cas9-mediated mutations were not found.

3.2 Molecular analysis of *SPL9m*-OE and *SPL9*-RNAi plants

To characterize the function of SPL9 in development and drought response, alfalfa with altered *SPL9* levels that were developed by Aung (2014) were used. The CaMV 35S promoter was used to drive expression of *SPL9* impervious to miR156 and RNAi to achieve enhanced and reduced *SPL9* levels, respectively. *SPL9* transcript levels were evaluated in potential *SPL9m*-OE plants by qRT-PCR and three with significant increases in *SPL9* transcript levels over WT are shown in **Figure 7A**. Increasing *SPL9* transcript levels were observed in *SPL9m*-OE 1 (OE1) (32-fold), *SPL9m*-OE 2 (OE2) (73-fold), and *SPL9m*-OE 3 (OE3) (133-fold) relative to WT. Similarly, qRT-PCR was employed to investigate *SPL9* transcript abundance in each *SPL9*-RNAi plant. *SPL9*-RNAi 3 (R3) had

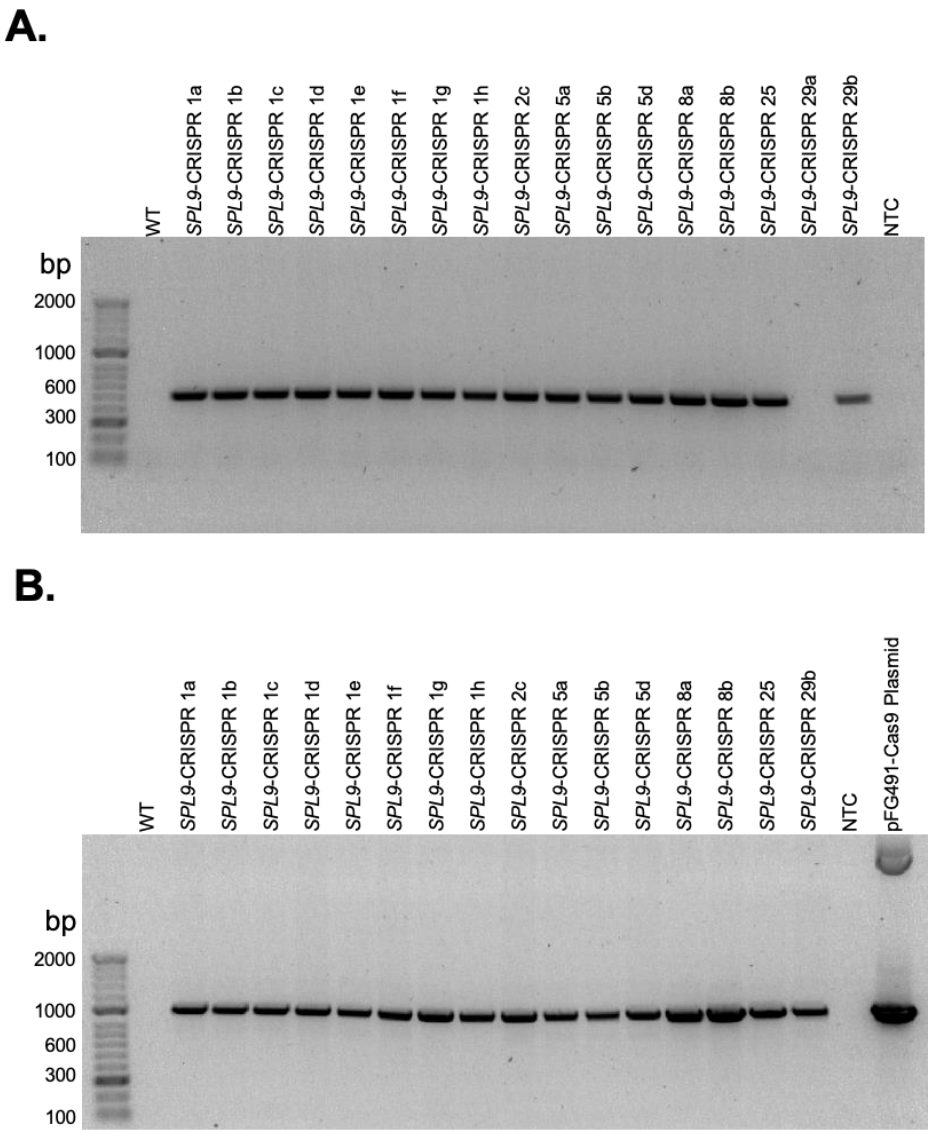

Figure 6. Screening of transgenic alfalfa plants for presence of *MtU6* and *SpCas9*

Primers designed to amplify fragments of **A**. *MtU6* (500 bp) and **B**. *SpCas9* (984 bp) were used in PCR reactions to test for their presence in genomic DNA (gDNA) of potential *SPL9*-CRISPR alfalfa. gDNA from WT alfalfa was used as negative control. A PCR reaction in which genomic DNA was replaced with water was included as the no template control (NTC). In **B**., the pFG491-Cas9 plasmid was included as a positive control for comparison and the presence of *SpCas9* was confirmed by Sanger sequencing. Each *SPL9*-CRISPR number indicates the calli from which each plant was taken. Each letter indicates the individual embryo taken from a single callus.

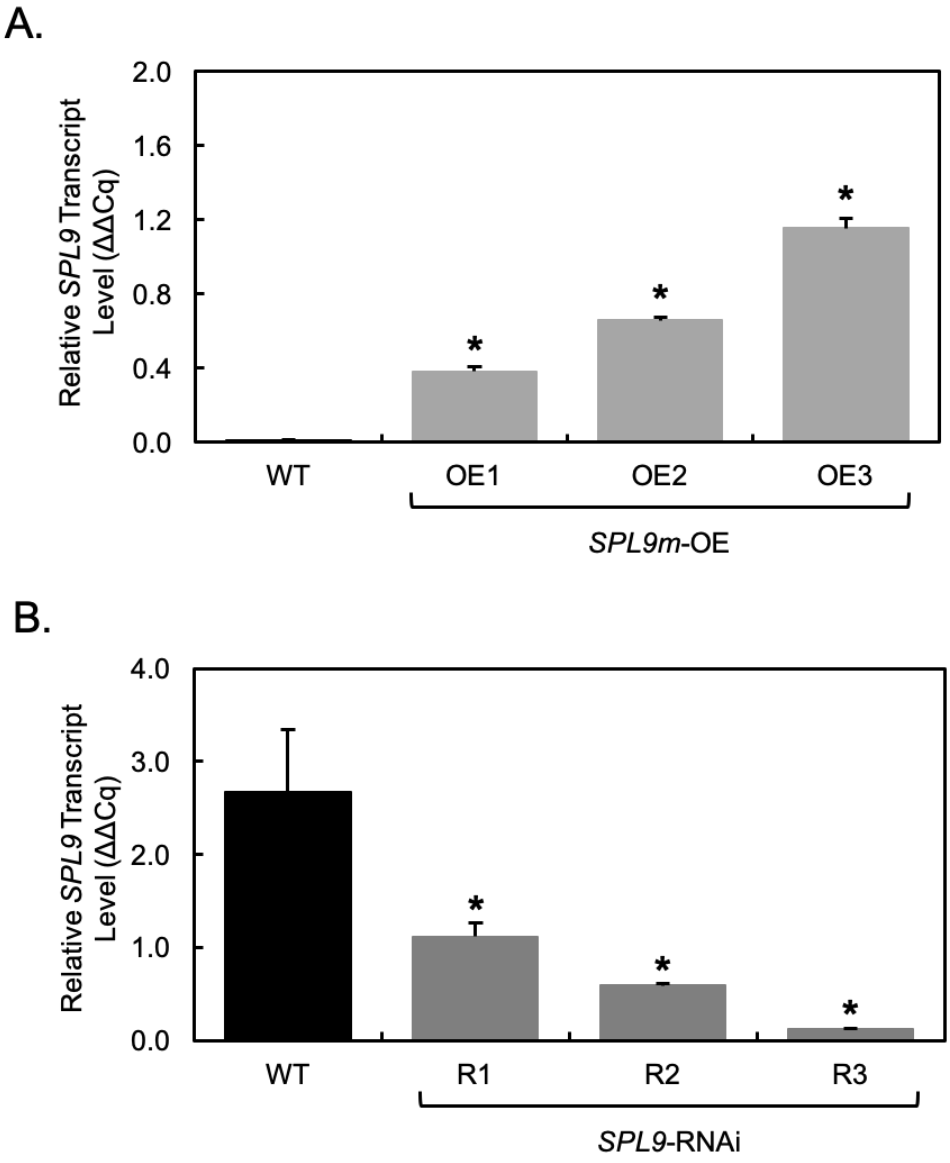

Figure 7. Relative *SPL9* transcript level in *SPL9m*-OE and *SPL9*-RNAi plants

Relative *MsSPL9* transcript levels measured in WT and transgenic alfalfa by RT-qPCR. **A.** *SPL9* transcript levels in WT and *SPL9m*-OE plants. *SPL9m* is impervious to miR156. **B**. *SPL9* transcript levels in WT and *SPL9*-RNAi plants. Asterisks indicate a significant change in transcript levels between transgenic and WT plants ($p < 0.05$, where $n = 3$, One-Way ANOVA, Dunnett's test). Error bars indicate standard deviation.

the greatest level of silencing, followed by *SPL9*-RNAi 2 (R2), and then *SPL9*-RNAi 1 (R1) with the lowest level of silencing relative to WT (**Figure 7B**).

3.3 Phenotypic characterization of *SPL9*-RNAi alfalfa

Given the different levels of *SPL9* silencing in *SPL9*-RNAi R3, R2, and R1 genotypes, these plants were chosen for use in subsequent experiments to determine the extent to which *SPL9* is involved in the regulation of development and to examine drought-related traits that are known to be impacted by miR156. Initially, phenotypic traits were characterized and compared between 2- and 6-month-old *SPL9*-RNAi and WT alfalfa (**Figure 8**).

3.3.1 Characterization of 2-month-old *SPL9*-RNAi alfalfa

Phenotypic traits of *SPL9*-RNAi plants were compared to those of WT plants at the vegetative stage by evaluating 2-month-old plants. Stem diameters in R1, R2 and R3 plants – measured between the second and third nodes of the main branch – were significantly thinner than those of the WT plants (**Figure 9**). However, this was the only trait that was measured in 2-month old plants that had significant differences between *SPL9*-RNAi and WT plants as summarized in **Table 1**. Fresh weight (FW) of roots, FW of shoots, dry weight (DW) of roots, DW of shoots, number of main branches, number of lateral branches, number of total branches, root length, plant height, number of nodes, and average internode length were statistically indistinguishable between WT and *SPL9*-RNAi plants.

3.3.2 Characterization of 6-month-old *SPL9*-RNAi alfalfa

Phenotypic traits of *SPL9*-RNAi were also evaluated at the reproductive stage by measuring traits in 6-month-old plants. At this stage, *SPL9*-RNAi plants were more distinguishable from WT (**Table 2**). Reduced plant height was observed in *SPL9*-RNAi plants, and this trait was maintained in all three *SPL9*-RNAi genotypes. R1 and R3 plants both had increased number of lateral and total branches as well as decreased average internode length compared to WT. R2, however, had decreased number of main branches

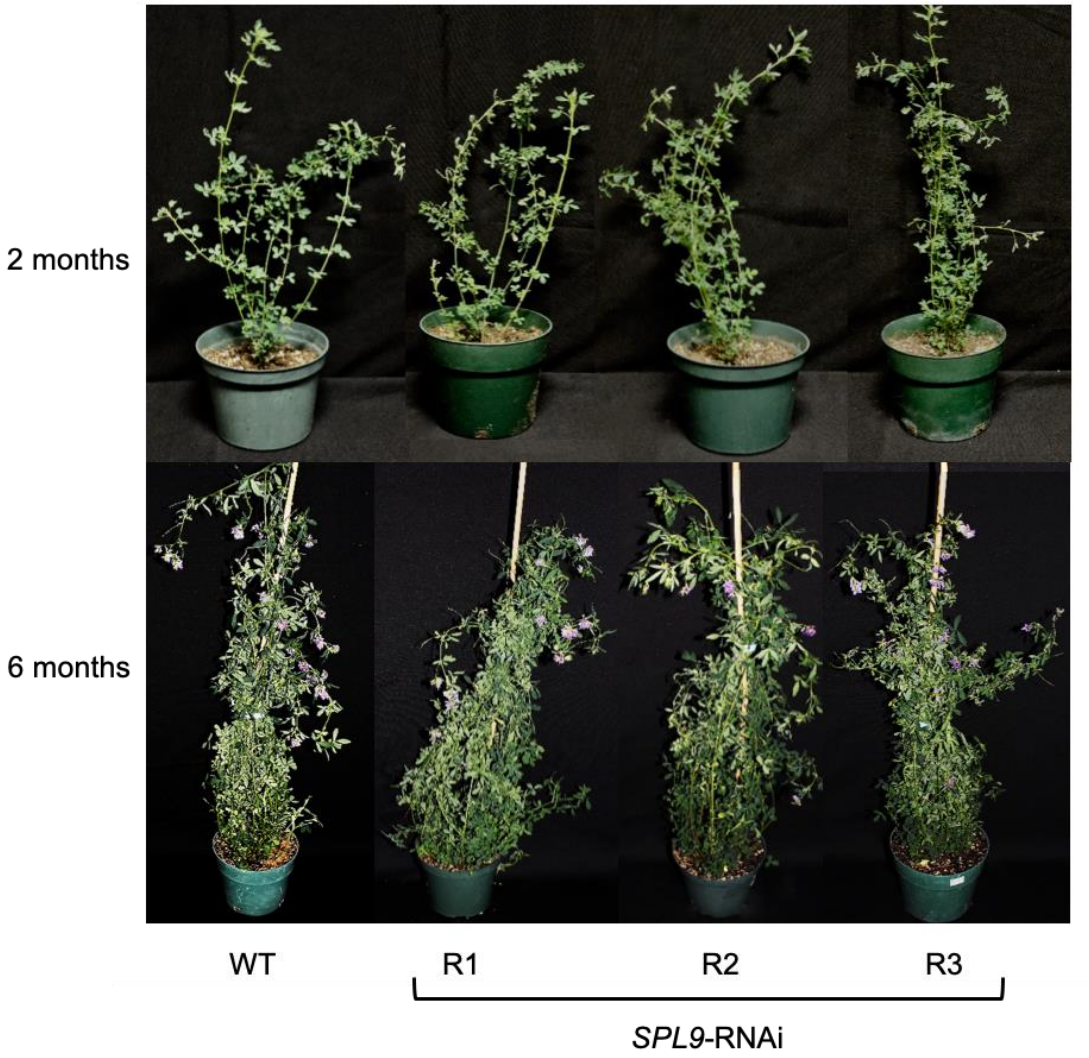

Figure 8. Phenotypic characterization of 2- and 6-month-old alfalfa plants

Representative WT and *SPL9*-RNAi alfalfa grown for two months and six months under normal greenhouse conditions.

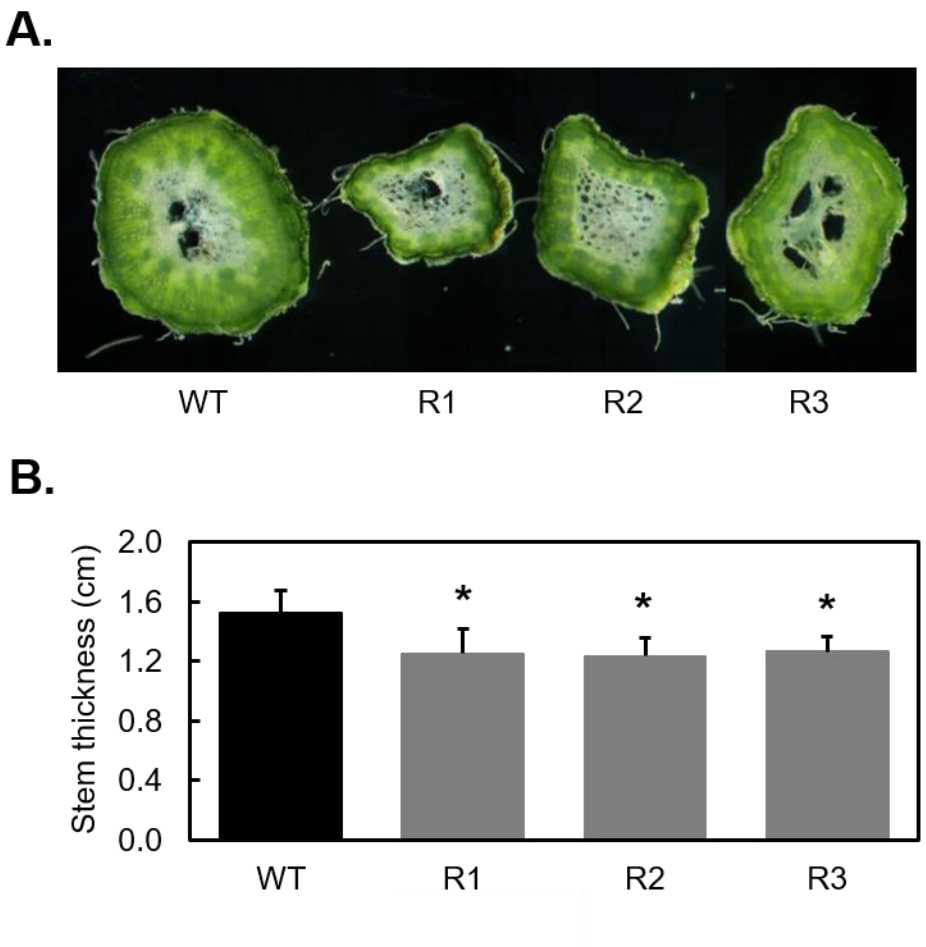

Figure 9. Stem thickness of 2-month-old alfalfa plants

A. Cross section of stems between nodes two and three where stem thickness was measured, and **B.** Stem thickness of 2-month-old *SPL9*-RNAi (R1, R2, and R3) and WT alfalfa. Asterisks indicate a significant change in stem thickness between *SPL9*-RNAi and WT plants ($p < 0.05$, where $n = 10$, One-Way ANOVA, Dunnett's test). Error bars indicate standard deviation.

Table 1. Analysis of phenotypic traits in 2-month-old alfalfa plants

Phenotypic traits of WT and *SPL9*-RNAi alfalfa were measured after 2 months of growth and recorded in the table ± standard deviation. Asterisks indicate significant differences between *SPL9*-RNAi and WT plants ($p < 0.05$, where $n = 10$, One-Way ANOVA, Dunnett test).

Genotype	2-month old alfalfa			
	WT	**R1**	**R2**	**R3**
# Main branches	2 ± 0.82	2 ± 0.67	2 ± 0.47	3 ± 1.03
# Lateral branches	7 ± 4.19	9 ± 5.43	7 ± 4.86	11 ± 3.68
Total branching	9 ± 4.57	11 ± 5.87	9 ± 4.89	14 ± 3.74
# of nodes	10 ± 2.80	10 ± 3.05	10 ± 1.69	11 ± 2.92
Plant height (cm)	32.7 ± 12.79	32.4 ± 7.84	33.3 ± 5.76	36.5 ± 6.56
Average internode length (cm)	3.5 ± 1.61	3.3 ± 1.01	3.5 ± 0.64	3.5 ± 0.95
Fresh weight (g)	3.61 ± 2.60	4.09 ± 2.98	3.23 ± 2.67	4.07 ± 2.48
Dry weight (g)	0.94 ± 0.60	0.99 ± 0.69	0.75 ± 0.56	1.11 ± 0.64
Time to flower (days)	NF	NF	NF	NF
Root length (cm)	37.9 ± 3.84	41.4 ± 9.35	38.8 ± 7.89	40.5 ± 5.01
Root fresh weight (g)	4.78 ± 3.20	6.03 ± 4.58	4.69 ± 3.69	5.94 ± 3.87
Root dry weight (g)	0.97 ± 0.31	0.96 ± 0.40	0.88 ± 0.27	1.30 ± 0.36

Table 2. Analysis of phenotypic traits in 6-month-old alfalfa plants

Phenotypic traits of WT and *SPL9*-RNAi alfalfa were measured after 6 months of growth and recorded in the table ± standard deviation. Asterisks indicate significant differences between *SPL9*-RNAi and WT plants ($p < 0.05$, where n = 5-13, One-Way ANOVA, Dunnett test).

Genotype	6-month old alfalfa			
	WT	**R1**	**R2**	**R3**
# Main branches	27 ± 5.72	24 ± 9.52	15 ± 6.79 *	31 ± 7.78
# Lateral branches	104 ± 39.04	151 ± 31.20 *	113 ± 22.80	182 ± 34.08 *
Total branching	131 ± 40.37	175 ± 36.32 *	128 ± 20.06	213 ± 34.61 *
# of nodes	19 ± 1.48	19 ± 1.55	17 ± 0.00 *	18 ± 0.76
Plant height (cm)	86.7 ± 12.17	70.4 ± 2.75 *	71.8 ± 7.12 *	63.6 ± 3.92 *
Average internode length (cm)	4.6 ± 0.56	3.7 ± 0.22 *	4.2 ± 0.37	3.5 ± 0.18 *
Fresh weight (g)	130.62 ± 22.02	115.41 ± 22.92	101.02 ± 26.08	125.58 ± 21.71
Dry weight (g)	33.46 ± 5.61	28.55 ± 6.30	24.65 ± 6.69	33.79 ± 5.87
Time to flower (days)	114 ± 8.77	113 ± 6.18	146 ± 25.56 *	122 ± 15.55

and number of nodes. However, these changes to alfalfa traits after six months of growth did not result in discrepancies between *SPL9*-RNAi and WT plants in FW or DW of shoots. Also, only R2 had delayed flowering compared to WT plants.

3.4 Investigating the role of *SPL9* in drought response in alfalfa

The miR156-mediated regulation of drought response has been investigated in switchgrass (Sun et al., 2012), *B. juncea* (Bhardwaj et al., 2014), *A. thaliana* (Cui et al., 2014) and alfalfa (Arshad et al., 2017a; Feyissa et al., 2019). SPL9 is a confirmed target of miR156 (Gao et al., 2016) and could play a role in controlling miR156-mediated drought response traits. This study investigated the hypothesis that SPL9 regulates miR156-mediated drought response in alfalfa by comparing the effects of drought in *SPL9*-RNAi and WT alfalfa.

3.4.1 *SPL9* transcript analysis in response to drought

Establishing the extent to which *SPL9* is involved in the response to drought will extend our knowledge of miR156-mediated abiotic stress tolerance in alfalfa that was initially demonstrated by Arshad et al. (2017a) and Feyissa et al. (2019). To investigate the natural response of alfalfa to drought, the transcript level of *SPL9* in WT plants was compared between drought stress and well-watered conditions. *SPL9* transcript levels decreased in drought stressed compared to well-watered WT plants (**Figure 10A**).

3.4.2 Drought survival analysis

Since one of the objectives of this study was to determine if *SPL9* is targeted by miR156 in response to drought in alfalfa, I investigated whether alfalfa with lower levels of *SPL9* are more tolerant to drought than the WT alfalfa. First, the ability of *SPL9*-RNAi to survive drought stress was assessed by exposing alfalfa plants to drought for two full weeks and then re-watering for two weeks. WT plants were the first to show signs of drought stress with wilting and curling leaves after 10 days. After 12 days of withholding water both WT and *SPL9*-RNAi alfalfa exhibited signs of drought stress (**Figure 10B**). Despite the fact that *SPL9*-RNAi plants showed signs of stress later than WT plants, both

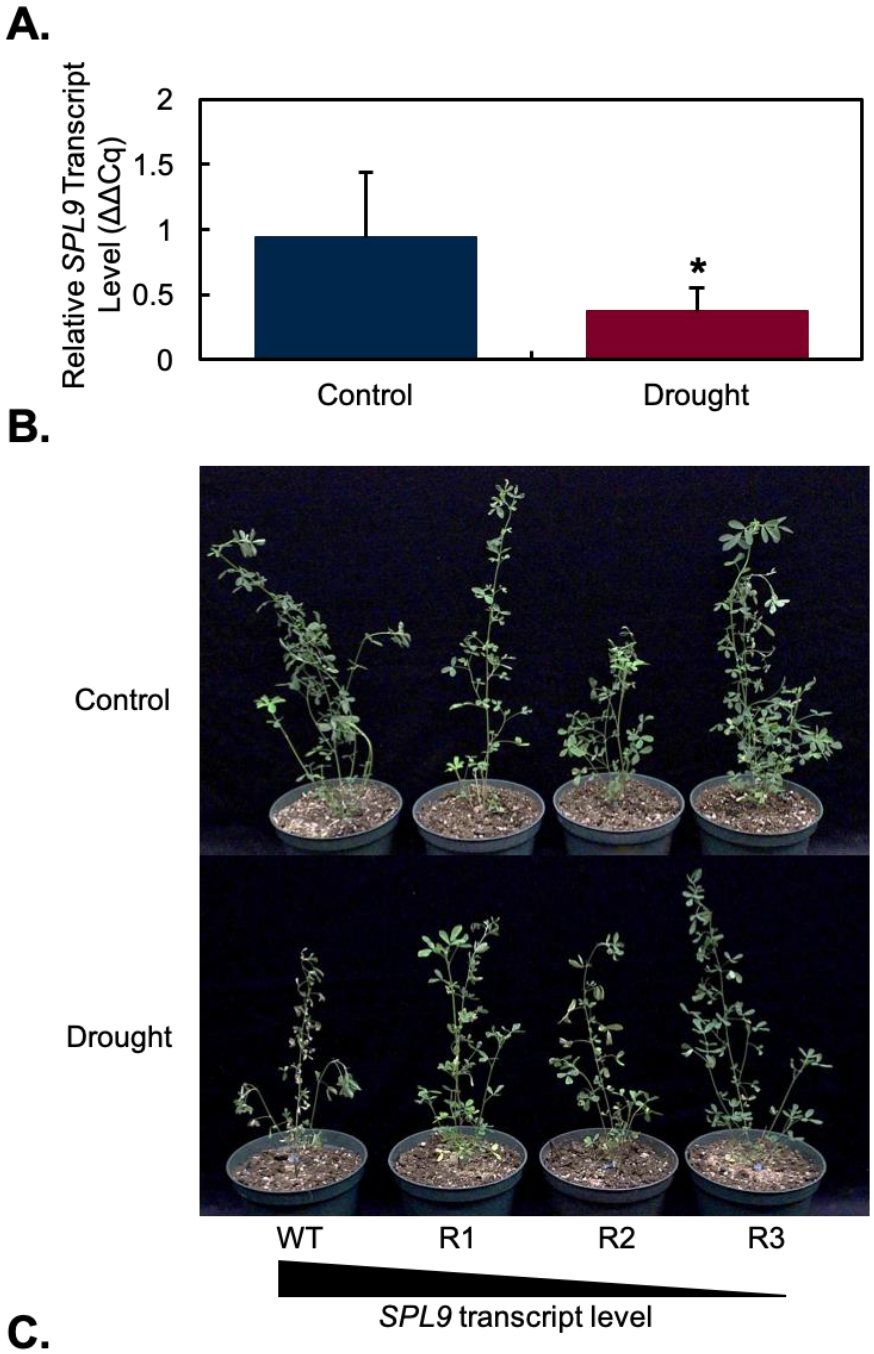
A.
Relative SPL9 Transcript Level (ΔΔCq)
2
1.5
1
0.5
0
*
Control
Drought
B.
Control
Drought
WT
R1
R2
R3
SPL9 transcript level
C.
% Survival
100
80
60
40
20
0
WT
R1
R2
R3

Figure 10. *SPL9*-RNAi response to drought stress

A. Relative *SPL9* transcript levels of WT alfalfa exposed to control and drought conditions. Asterisk indicates significant change between conditions ($p < 0.05$, where n = 9-10, Student's t-test). Error bars indicate standard deviation. **B**. Representative WT and *SPL9*-RNAi plants that were exposed to 12 days of well-watered control or drought conditions. **C**. Survival of alfalfa plants exposed to drought measured by evaluating the ratio of plants capable of growing after two-weeks of recovering under control conditions to the total number of plants in the trial. Asterisk indicate significant change between WT and transgenic plants in three independent experiments where n = 10 ($p < 0.05$, One-Way ANOVA, Dunnett test). Error bars indicate standard deviation.

sets of plants had similar survival ability (**Figure 10C**).

3.4.3 Changes to alfalfa growth in response to drought

In addition to survival, the extent to which miR156-mediated control of *SPL9* is involved in the regulation of drought tolerance was evaluated by measuring phenotypic traits in *SPL9*-RNAi and WT alfalfa under drought. Delayed leaf senescence indicates drought tolerance in plants (Rivero et al., 2007) therefore tolerance to drought was assessed first by evaluating the severity of senescence in response to the stress (**Figure 11A**). All alfalfa, both WT and *SPL9*-RNAi, had more leaf senescence when exposed to drought than their well-watered control counterparts. That being said, when comparing plants exclusively under drought conditions, all three *SPL9*-RNAi genotypes had decreased leaf senescence compared to WT.

Next, overall plant growth, despite the condition to which they were exposed, was examined as a function of the change in plant height (**Figure 11B**). Plant growth within each respective condition did not change between WT and *SPL9*-RNAi plants. However, when each genotype was compared to its counterpart between conditions, those with the highest *SPL9* transcript level, WT and R1, had reduced growth. R2 and R3, the genotypes with the lowest *SPL9* transcript level, had no change in growth between drought and control conditions.

Lastly, overall root growth, a trait observed in the drought tolerant miR156 OE alfalfa (Arshad et al., 2017a; Feyissa et al., 2019), was compared in drought exposed and well-watered WT and *SPL9*-RNAi plants (**Figure 11C**). There was no change in root length between WT and *SPL9*-RNAi plants within their respective conditions nor were there changes between drought and control conditions in each genotype.

3.4.4 Shoot and root biomass assessment in response to drought

Drought can impede plant growth (Daryanto et al., 2016) and as such, evaluating shoot and root growth in response to drought could indicate tolerance. Change in alfalfa biomass in response to drought was examined in *SPL9*-RNAi and WT shoots and roots. Well-watered, *SPL9*-RNAi plants had less shoot FW than WT plants, however, drought-

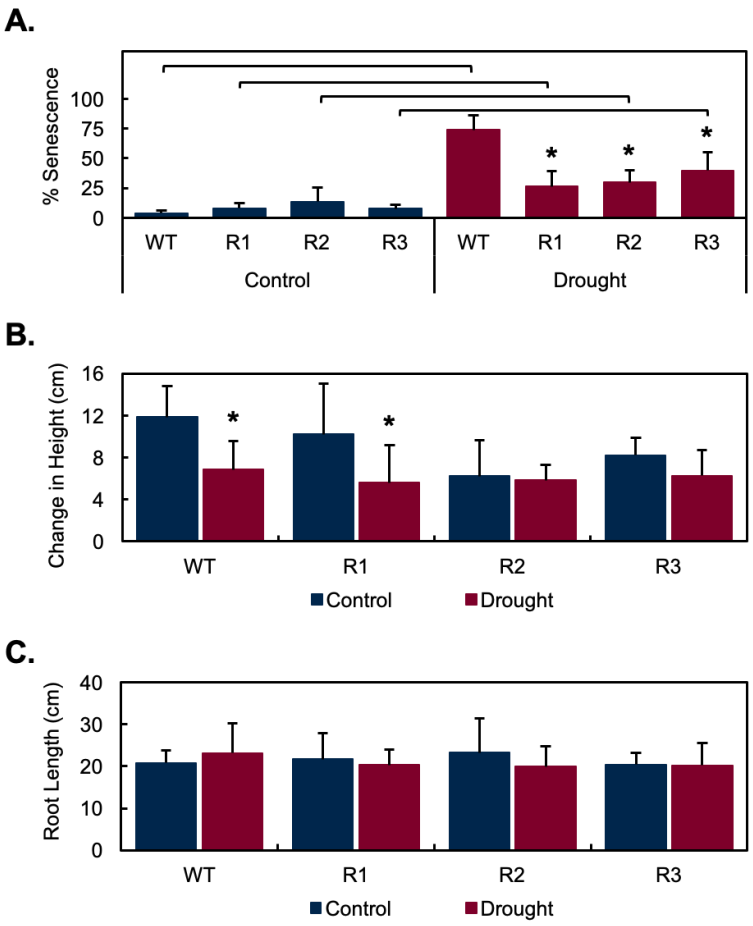

Figure 11. Alfalfa growth in response to drought

A. Proportion of senescing leaves in WT and *SPL9*-RNAi plants under control and drought conditions. Asterisks indicate a significant change within conditions between WT and *SPL9*-RNAi plants (Dunnett test) and bars indicate a significant change between conditions (Sidak test) in a Two-Way ANOVA ($p < 0.05$, where $n = 10$). Error bars indicate standard deviation. **B**. Growth of WT and *SPL9*-RNAi alfalfa in response to drought measured by analyzing the change in plant height during the treatment. Asterisks indicate significant change between conditions ($p < 0.05$, where $n = 10$, Two-Way ANOVA, Sidak test). Error bars indicate standard deviation. **C**. Root length of WT and *SPL9*-RNAi alfalfa under control and drought conditions. Asterisks indicate significant change between conditions ($p < 0.05$, where $n = 10$, Two-Way ANOVA, Sidak test). Error bars indicate standard deviation.

exposed *SPL9*-RNAi and WT shoots showed no difference in FW (**Figure 12A**). In addition, reduced shoot FW between well-watered and drought conditions was seen in WT and R1 but not in R2 and R3. There was no change in root FW between *SPL9*-RNAi and WT plants within each condition, but WT and R1 had decreased root FW in response to drought whereas R2 and R3 saw no change (**Figure 12B**). When examining shoot DW, there was no change in DW between drought and control plants within each genotype however, under drought stress, R1 and R2 plants had decreased shoot DW compared to WT and R2 had decreased shoot DW compared to WT under control conditions (**Figure 12C**). Root DW was indistinguishable both between and within well-watered and drought conditions (**Figure 12D**).

3.5 Evaluation of plant water status in *SPL9*-RNAi alfalfa under drought stress

Alfalfa with miR156 OE was reported to have enhanced water status under water-deficit conditions resulting in plants with tolerance to drought (Arshad et al., 2017a; Feyissa et al., 2019). Therefore, the role of SPL9 in miR156-mediated regulation of water status under drought conditions was evaluated by comparing water loss, relative water content (RWC), and midday leaf water potential in *SPL9*-RNAi and WT alfalfa.

3.5.1 Short term water loss in drought stressed alfalfa

In response to drought, plants release ABA to mitigate water loss (Bauer et al., 2013), and thus evaluating rapid water loss in plants has been used to examine drought tolerance (Arshad and Mattsson, 2014; Arshad et al., 2017a; Dai et al., 2007). To determine if SPL9 is involved in the regulation of drought tolerance, the extent of water lost from *SPL9*-RNAi and WT alfalfa plants that were exposed to drought and well-watered conditions was evaluated over a three-hour period. Comparing *SPL9*-RNAi plants to WT plants under both well-watered and drought conditions revealed that R1, the *SPL9*-RNAi genotype with the highest *SPL9* transcript level, had increased water loss. Under control conditions, R1 was the only *SPL9*-RNAi genotype to show a significant difference in water loss from WT plants starting after one hour (**Figure 13A**). Enhanced water loss

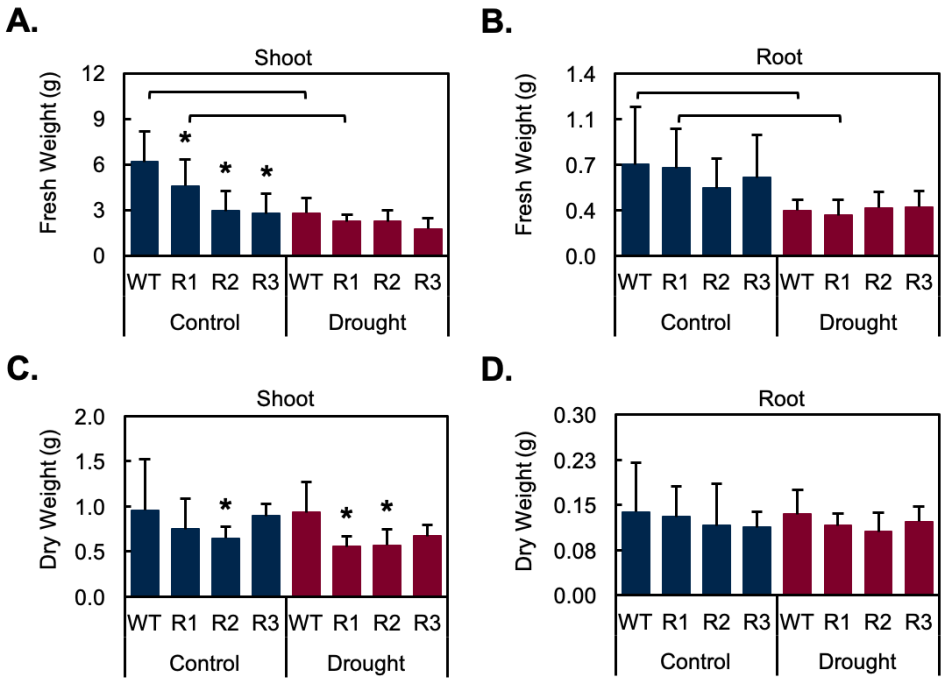

Figure 12. Effect of drought on *SPL9*-RNAi alfalfa biomass

Fresh weight (FW) of shoots (**A**) and roots (**B**) of WT and *SPL9*-RNAi alfalfa under control and drought conditions measured immediately after harvesting the plants. Dry weight (DW) of shoots (**C**) and roots (**D**) of WT and *SPL9*-RNAi alfalfa under control and drought conditions measured after drying. Asterisks indicate significant differences within conditions (Dunnett test) and bars indicate significant differences between conditions (Sidak test) in a Two-Way ANOVA where $p < 0.05$ and $n = 10$. Error bars indicate standard deviation.

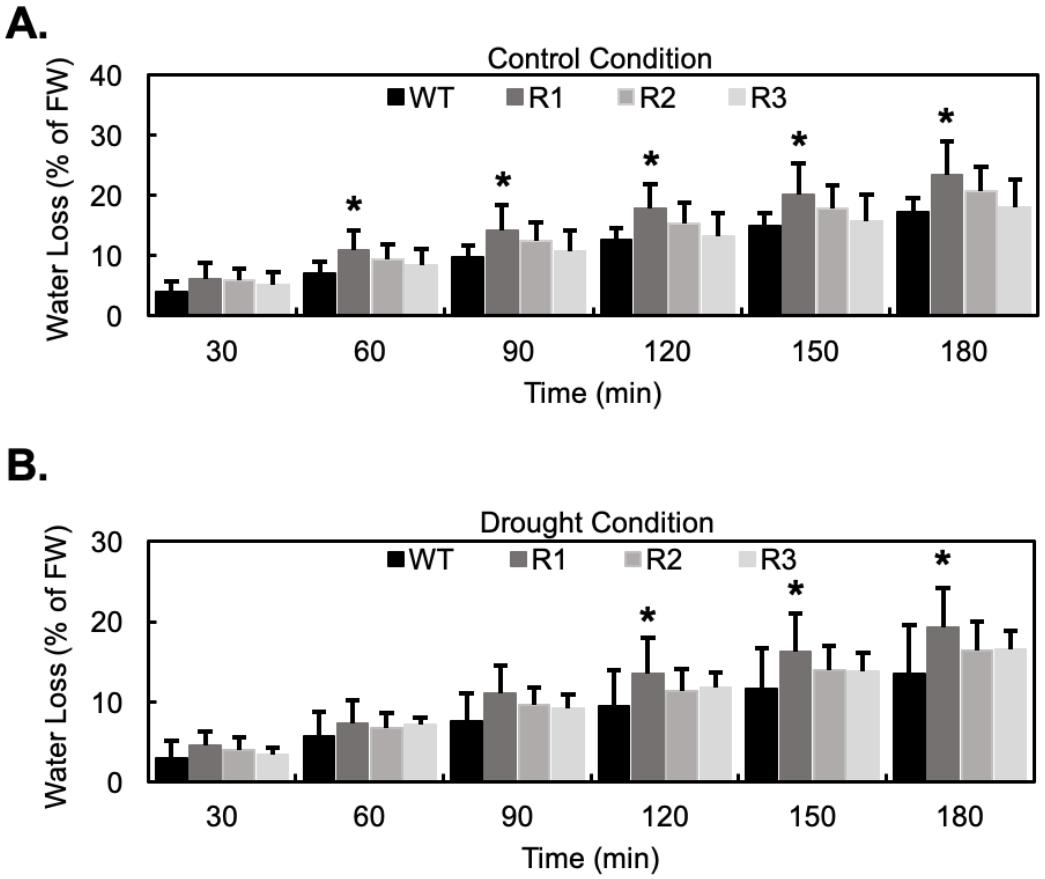

Figure 13. Effect of drought on short term water loss

Water lost from shoots, over three hours, as a function of initial fresh weight (FW) was determined in **A.** well-watered and **B.** drought stressed WT and *SPL9*-RNAi alfalfa. Asterisks indicate significant difference between *SPL9*-RNAi and WT within the time point ($p < 0.05$, where $n = 10$, Two-Way ANOVA, Dunnett test). Error bars indicate standard deviation.

from R1 compared to WT plants was observed after two hours when plants were drought stressed but continued for the rest of the water loss assay (**Figure 13B**). Well-watered and drought stressed R2 and R3 alfalfa did not differ from WT in water loss.

3.5.2 Relative water content of alfalfa exposed to drought

In addition to water loss, RWC can indicate plant water status and is useful for determining the extent of water deficiency a plant is experiencing as a result of abiotic stress conditions (Anderson and McNaughton, 1973; Arshad et al., 2017a; Weatherley, 1950). Therefore, to evaluate the role of SPL9 in the regulation of drought tolerance, RWC of WT and *SPL9*-RNAi plants was compared under control and drought conditions. Consistent with the observation that WT plants were more severely impacted by drought (**Figure 10B**), the RWC of WT plants exposed to drought was significantly decreased compared to well-watered WT plants (**Figure 14A**). On the other hand, *SPL9*-RNAi genotypes had no differences in RWC between the control and drought conditions. In addition, R1 and R2 (but not R3) had increased RWC compared to WT under drought stress, while none of the *SPL9*-RNAi genotypes differed from WT under well-watered conditions.

3.5.3 Effect of drought on midday leaf water potential in alfalfa

Midday leaf water potential, another indicator of plant water status (Turner, 1988), was measured to investigate altered drought tolerance in *SPL9*-RNAi plants relative to WT. Midday leaf water potential was measured in well-watered and drought-exposed WT and *SPL9*-RNAi alfalfa (**Figure 14B**). When comparing WT and *SPL9*-RNAi alfalfa within their respective conditions, the midday leaf water potential of plants was indistinguishable. WT, R2, and R3 plants also showed no difference in water potential between drought and control conditions, but drought-stressed R1 plants had decreased water potential compared to well-watered R1 plants.

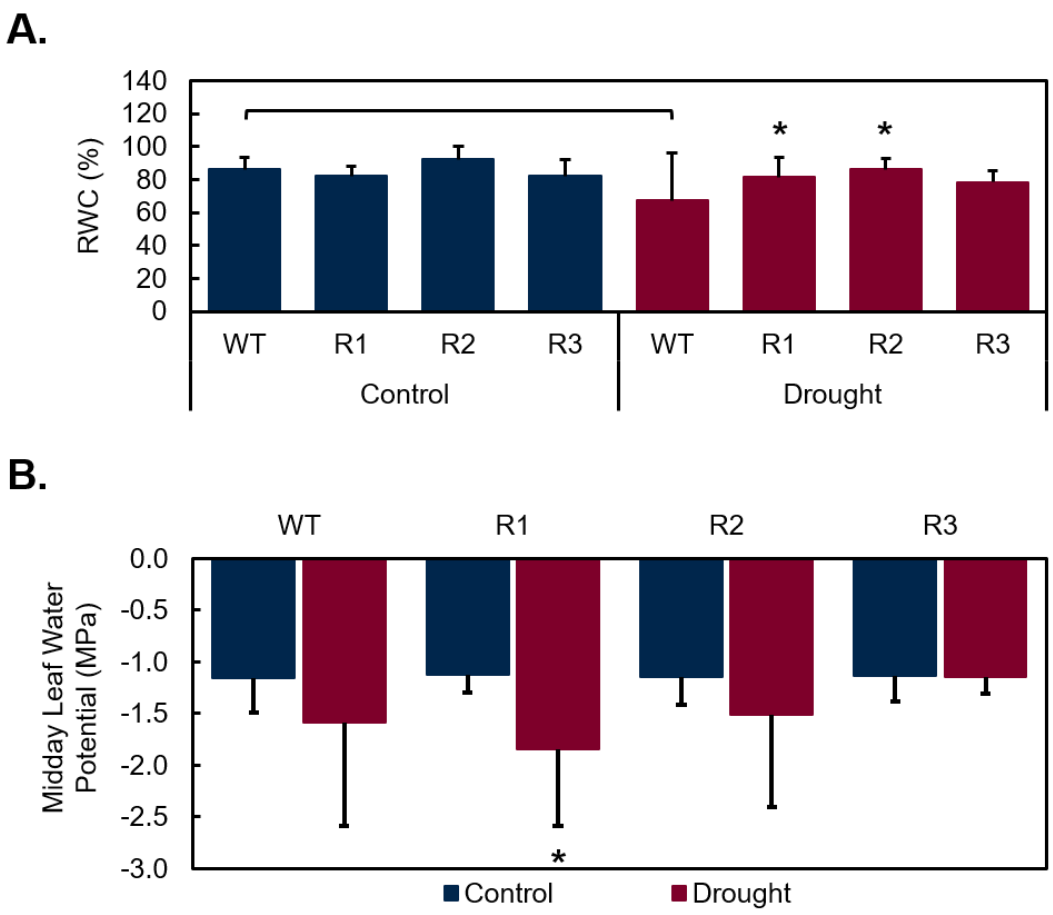

Figure 14. Plant water status in response to drought

A. Relative water content (RWC) of WT and *SPL9*-RNAi alfalfa leaves under control and drought conditions. Asterisks indicate significant differences within treatments (Dunnett test) and bars indicate significant differences between treatments (Sidak test) in a Two-Way ANOVA where $p < 0.05$ and $n = 10$. Error bars indicate standard deviation. **B.** Midday leaf water potential of WT and *SPL9*-RNAi alfalfa leaves under control and drought conditions. Asterisks indicate significant differences between conditions ($p < 0.05$, where $n = 9$-10, Two-Way ANOVA, Sidak test). Error bars indicate standard deviation.

3.6 Effect of drought on photosynthetic capabilities

Water deficiency in plants causes reduced photosynthetic rates (Lamaoui et al., 2018; Sicher et al., 2012). In this study, I used stomatal conductance and chlorophyll content to infer the photosynthetic capabilities of drought stressed alfalfa. Furthermore, to determine if SPL9 regulates the photosynthetic response to drought, stomatal conductance and chlorophyll content of *SPL9*-RNAi and WT plants were compared between well-watered and drought conditions.

3.6.1 Stomatal conductance

Drought causes the release of ABA resulting in stomata closure and consequently a reduction in stomatal conductance (Lamaoui et al., 2018; Sicher et al., 2012; Zhang and Davies, 1989). Stomatal conductance was measured in both the adaxial and abaxial surfaces of leaves of well-watered and drought stressed WT and *SPL9*-RNAi alfalfa to estimate the degree to which stomata were open. Drought-exposed alfalfa regardless of genotype had significantly reduced stomatal conductance compared to well-watered plants of the same genotype except in the abaxial surface of R2 which had no significant differences between drought and control treatments (**Figure 15A**). On both the adaxial and abaxial surface, WT and *SPL9*-RNAi leaves were indistinguishable when they were compared within each condition.

3.6.2 Chlorophyll content

Arshad et al. (2017a) reported that alfalfa with enhanced water status under drought stress also had enhanced chlorophyll content. To determine if SPL9 is involved in the regulation of chlorophyll content in response to water deficiency, chlorophyll content of WT and *SPL9*-RNAi alfalfa were compared under drought conditions (**Figure 15B**). WT, R1, and R3 plants had decreased chlorophyll content in drought-stressed plants compared to their well-watered counterparts. R2 plants were indistinguishable between drought and control conditions. In addition, under control conditions, R2 plants had decreased chlorophyll content compared to WT.

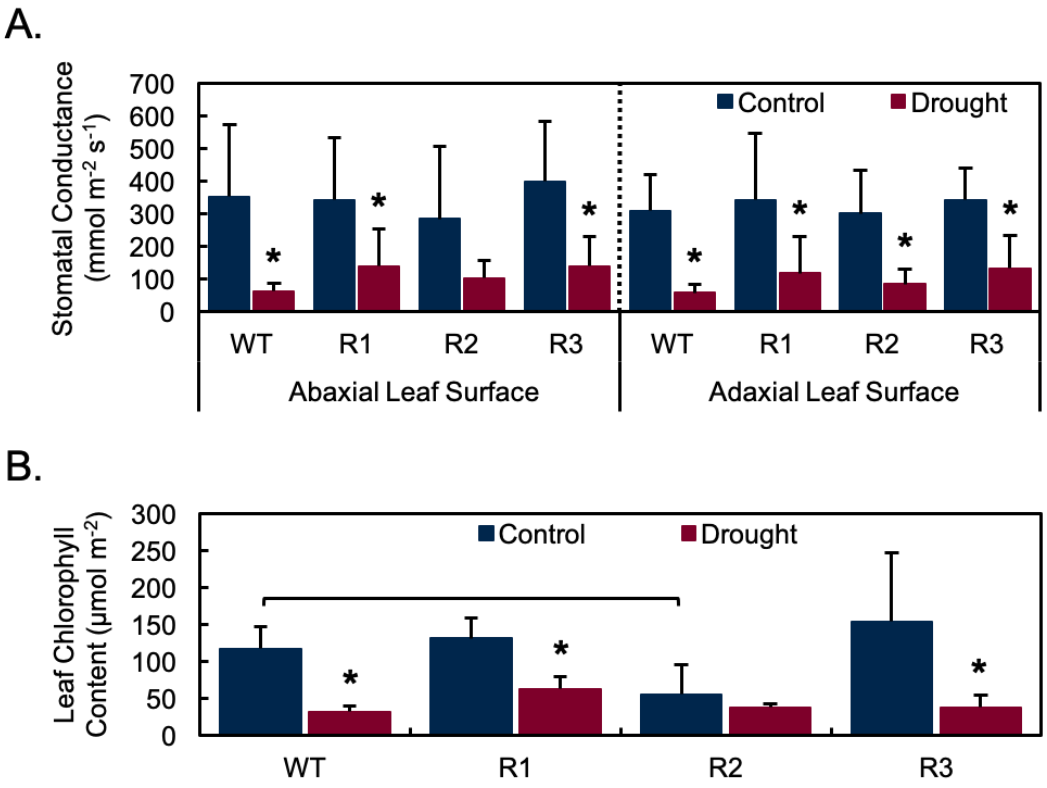

Figure 15. Photosynthetic capability of alfalfa in response to drought

A. Stomatal conductance of WT and *SPL9*-RNAi alfalfa under drought and control conditions measured on the abaxial (left) and adaxial (right) leaf surfaces. Asterisks indicate significant differences between treatments ($p < 0.05$, where $n = 8$, Two-Way ANOVA, Sidak test). Error bars indicate standard deviation. **B.** Chlorophyll content in WT and *SPL9*-RNAi alfalfa leaves under control and drought conditions. Asterisks indicate significant differences between conditions (Sidak test) and bars indicate significant differences within conditions (Dunnett test) in a Two-Way ANOVA where $p < 0.05$ and $n = 5$. Error bars indicate standard deviation.

3.7 Analysis of antioxidant-mediated ROS scavenging capabilities

ROS accumulates in plants exposed to drought, and thus ROS scavenging antioxidants can mitigate ROS buildup resulting in tolerance to the stress (Kang et al., 2020; Sarker and Oba, 2018). To determine if SPL9 regulates drought response by mediating the ability of the plant to scavenge ROS, the antioxidant activity and transcript levels of *GSH* and *CAT1* were evaluated in well-watered and drought-stressed WT and *SPL9*-RNAi alfalfa.

3.7.1 Effect of drought on antioxidant content of *SPL9*-RNAi alfalfa

Enhanced antioxidant activity was reported in miR156 OE alfalfa that had enhanced tolerance to heat and drought stress (Arshad et al., 2017a; Matthews et al., 2019). To investigate the role of SPL9 in miR156-mediated drought response, the ability of WT and *SPL9*-RNAi alfalfa leaf extracts to sequester hydrogen peroxide was evaluated by comparing ABTS•+ production in the presence of the extracts with a Trolox standard curve (**Figure 16A**). The antioxidant activities of WT and *SPL9*-RNAi plants compared both within and between drought and well-watered conditions were indistinguishable.

3.7.2 Effect of drought on *CAT1* and *GSH* expression in *SPL9*-RNAi alfalfa

The effect of drought on expression of antioxidant-related genes in alfalfa was previously reported, with enhanced levels of *CAT1* and *GSH* in miR156 OE alfalfa under drought stress (Arshad et al., 2017a). Transcript levels of *GSH*, involved in biosynthesis of glutathione, and *CAT1*, an enzymatic ROS scavenger, were investigated to indicate potential changes to antioxidant production as a result of altered *SPL9* levels in *SPL9*-RNAi compared to WT. The transcript levels of *GSH* decreased in WT leaves in response to drought (**Figure 16B**). *SPL9*-RNAi leaves were no different from WT leaves under control conditions. Out of the *SPL9*-RNAi plants, only R3 differed from WT under drought conditions in that R3 leaves had increased *GSH* transcript levels. WT leaves had

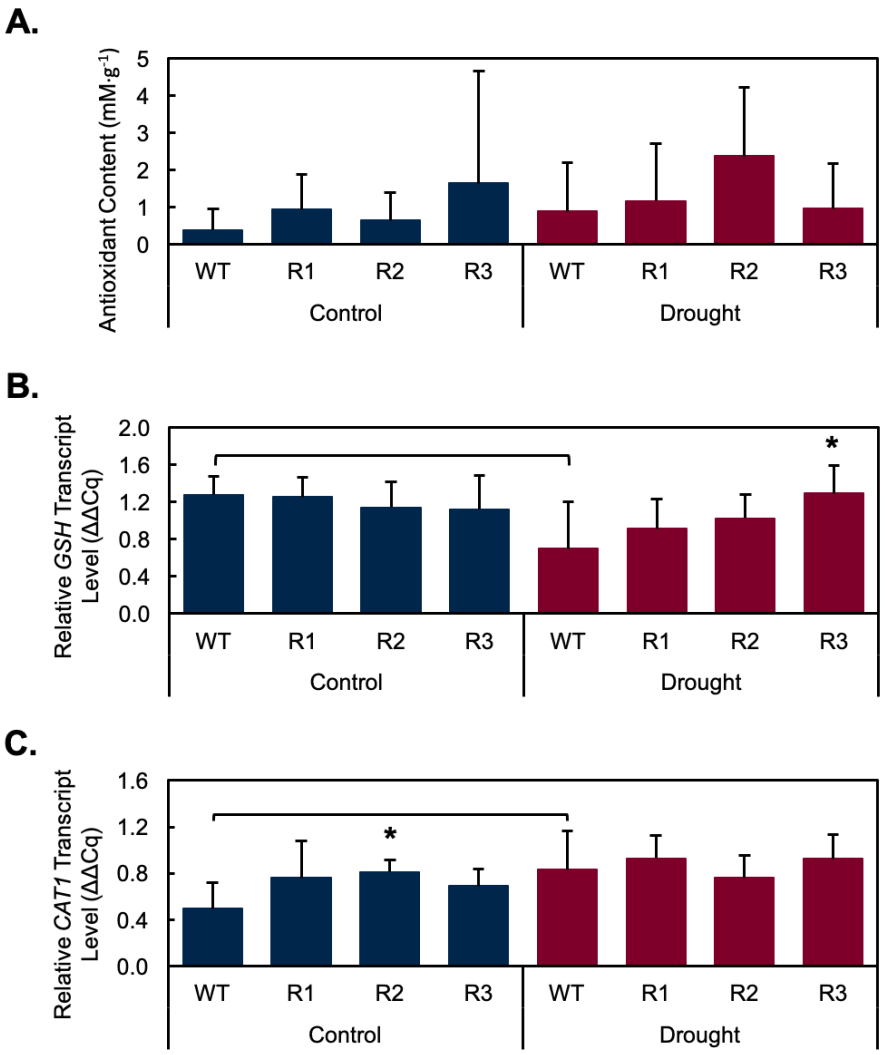

Figure 16. ROS scavenging capabilities of alfalfa in response to drought

A. Antioxidant activity of WT and *SPL9*-RNAi leaf extracts under drought and control conditions. **B**. *GSH* transcript levels in WT and *SPL9*-RNAi leaves under drought and control conditions. **C**. *CAT1* transcript levels in WT and *SPL9*-RNAi leaves under drought and control conditions. Asterisks indicate significant differences within conditions (Dunnett test) and bars indicate significant differences between conditions (Sidak test) in a Two-Way ANOVA where $p < 0.05$ and $n = 8$-10. Error bars indicate standard deviation.

increased *CAT1* transcript levels in response to drought (**Figure 16C**). R2 leaves also had enhanced *CAT1* transcript levels compared to WT under well-watered conditions. WT and *SPL9*-RNAi leaves had indistinguishable *CAT1* transcript levels under drought conditions.

3.8 Regulation of anthocyanin biosynthesis by SPL9 under drought

Anthocyanins, which are capable of scavenging ROS, accumulate in response to drought and in drought tolerant plants (Cui et al., 2014; Feyissa et al., 2019). Transcription of *DFR*, an enzyme involved in the biosynthesis of anthocyanins, is inhibited by SPL9 in *A. thaliana* (Gou et al., 2011). This interaction has also been implicated in anthocyanin biosynthesis in soybean (Gupta et al., 2019). As a result, transgenic *A. thaliana* with miR156-insensitive *SPL9* were sensitive to drought (Cui et al., 2014). To investigate my hypothesis that the miR156-*SPL9*-*DFR* interaction regulates anthocyanin biosynthesis providing control over drought tolerance in alfalfa, the accumulation of anthocyanins and the transcript levels of *DFR* under drought conditions were evaluated in *SPL9*-RNAi and WT plants.

3.8.1 Analysis of *DFR* transcript levels in drought stressed *SPL9*-RNAi alfalfa

In *A. thaliana, DFR* is negatively regulated by SPL9 (Gou et al., 2011), and upregulated in response to drought (Cui et al., 2014). *DFR* transcript abundance was analyzed in leaves and stems of *SPL9*-RNAi and WT plants to investigate if a similar inhibitory role exists for SPL9 in response to drought in alfalfa. In leaves, *DFR* transcript levels were significantly reduced in *SPL9*-RNAi compared to WT when well-watered, but showed no significant difference under drought (**Figure 17A**). In addition, *DFR* transcript levels decreased in WT but not *SPL9*-RNAi leaves when well-watered and drought conditions were compared. In stems, *DFR* transcript levels were reduced in WT and R3 under drought compared to well-watered conditions (**Figure 17B**). Well-watered *SPL9*-RNAi and WT stems did not differ in *DFR* transcript level, but R1 stems had increased *DFR*

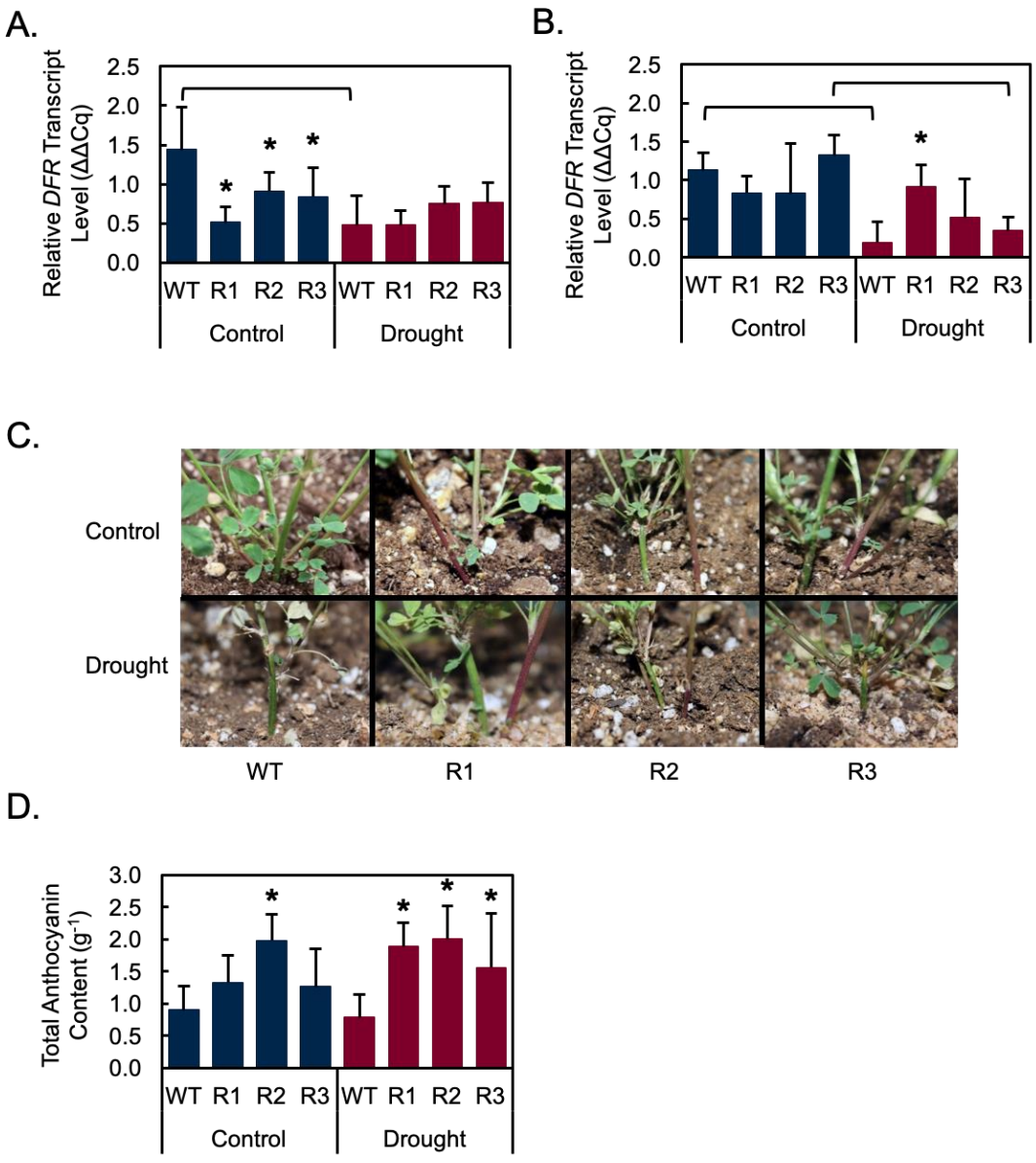

Figure 17. Effect of drought on anthocyanin accumulation in alfalfa

Relative *DFR* transcript levels in leaves (n = 8-10) (**A)** and basal stems (n = 2-5) (**B)** of WT and *SPL9*-RNAi alfalfa under drought and control conditions. **C**. Basal stem of WT and *SPL9*-RNAi alfalfa displaying pigments characteristic of anthocyanin accumulation. **D.** Total anthocyanin content of WT and *SPL9*-RNAi basal stems exposed to drought and control conditions (n = 10). Asterisks indicate significant differences within conditions (Dunnett test) and bars indicate significant differences between conditions (Sidak test) in a Two-Way ANOVA where $p < 0.05$. Error bars indicate standard deviation.

compared to WT under drought.

3.8.2 Effect of drought on anthocyanin content of *SPL9*-RNAi alfalfa

Regulation of anthocyanin accumulation by miR156-mediated silencing of SPL9 under drought was reported in *A. thaliana* (Cui et al., 2014) and suggested in soybean (Gupta et al., 2019). To investigate this regulatory mechanism in alfalfa, total anthocyanin contents of *SPL9*-RNAi and WT stems under control and drought conditions were compared. Stem bases of *SPL9*-RNAi alfalfa under both conditions had enhanced red pigmentation, but this was less apparent in WT plants (**Figure 17C**). While there was no difference in anthocyanin content between WT and *SPL9*-RNAi (except for R2) under control condition, *SPL9*-RNAi stems had significantly increased levels of these compounds under drought (**Figure 17D**).

Chapter 4

4 Discussion

4.1 Overview of research

miR156 is a master regulator of plant development, controlling the expression of many different families of downstream genes (Wu et al., 2009). Expression levels of miR156 decrease as the plant transitions from the juvenile and vegetative phase to the reproductive phase, and functions by silencing its immediate downstream gene targets, some of which belong to the SPL family (Ahsan et al., 2019; Wu and Poethig, 2006; Wu et al., 2009; Xu et al., 2020; Zheng et al., 2019). Aung et al. (2015) described traits in alfalfa associated with the overexpression of miR156, including delayed flowering, decreased plant height, increased branching, and reduced stem thickness, among others. SPL9 was found to have complementary sites to miR156 resulting in decreased expression in response to miR156 overexpression (Gao et al., 2016). SPL9 has also been characterized in *A. thaliana* and was found to be involved in developmental control (Schwarz et al., 2008; Yu et al., 2015). In soybean, SPL9 was found to regulate branching, number of nodes and dry weight (Bao et al., 2019). However, this is not the case in all species as Ahsan et al. (2019) suggested that *SPL9* may not be involved in regulating plant development in mango, avocado and macadamia. With this research in mind, this study operated under the hypothesis that SPL9 will also play a role in the control of miR156-mediated trait development in alfalfa. Over the course of this study, it was found that alfalfa with reduced *SPL9* transcript levels shared some phenotypic traits with alfalfa overexpressing miR156, including plant height, stem thickness, branching and internode length as reported by Aung et al. (2015).

Not only does miR156 regulate developmental control, it is also involved in the response to abiotic stress. Studies of drought, heat, and salinity stress demonstrated that overexpressing miR156 in alfalfa resulted in plants with greater tolerance (Arshad et al., 2017a; Arshad et al., 2017b; Feyissa et al., 2019; Matthews et al., 2019). Abiotic stress

trials in *A. thaliana* provided evidence that SPL9 is regulated by miR156 in response to both drought and salt stress (Cui et al., 2014). SPL9 was found to inhibit the transcription activating complex of *DFR*, preventing the biosynthesis of anthocyanins (Gou et al., 2011). Anthocyanins, being ROS scavengers (Ali et al., 2016; Azuma et al., 2008; Kähkönen and Heinonen, 2003; Rice-Evans et al., 1996), have the potential to mitigate some of the adverse effects of drought stress on the plants (Cui et al., 2014; Feyissa et al., 2019). Thus, control of anthocyanin biosynthesis through the regulation of SPL9 mediated by miR156 provides tolerance to drought in *A. thaliana* (Cui et al., 2014). *SPL13* was confirmed to be a target of miR156 in response to drought stress in alfalfa (Arshad et al., 2017a; Feyissa et al., 2019). Regulation of *SPL13* by miR156 was shown to impact drought tolerance in alfalfa, however, the drought response traits investigated between miR156 overexpression and *SPL13* silencing plants were not identical (Arshad et al., 2017a; Feyissa et al., 2019). Therefore, the hypothesis that SPL9 is involved in miR156-mediated drought tolerance through the miR156-*SPL9*-*DFR* pathway similar to that which was uncovered in *A. thaliana* by Gou et al. (2011) and Cui et al. (2014) was investigated in alfalfa. Alfalfa with a reduced *SPL9* level was able to maintain growth, had reduced leaf senescence, and enhanced RWC under drought stress culminating in greater tolerance to drought. Reduced *SPL9* level was also correlated with increased anthocyanin content and *DFR* transcript level.

4.2 Impacts of *SPL9* silencing on alfalfa morphology

As a consequence of miR156-mediated silencing of *SPL9* (Gao et al., 2016), plants overexpressing (OE) miR156 should share at least some phenotypes with those having decreased expression of miR156-regulated *SPL* genes. In *A. thaliana*, miR156 OE extended the juvenile phase leading to delayed flowering (Schwab et al., 2005; Wu and Poethig, 2006). Enhanced juvenile character is also a consequence of miR156 OE in alfalfa, as miR156 OE resulted in plants with delayed flowering (Aung et al., 2015). In this study, reduced levels of *SPL9* resulted in delayed flowering in R2. R2 had intermediate *SPL9* transcript levels compared to the other two *SPL9*-RNAi genotypes with R1 and R3 having higher and lower *SPL9* transcript levels, respectively. Given that flowering time was unaffected at low and high levels of *SPL9* expression, but could still

be reduced at a certain intermediate level, it could be that this trait requires a certain level of *SPL9* expression that is below that of R1 but above that of R3. Other *SPL*s could also be involved in flowering which would support the variation in flowering time seen between *SPL9*-RNAi genotypes. For example, *SPL3* has been implicated in regulating flowering in *D. catenatum* due to it being the only *SPL* to have enhanced expression in mature leaf and stem tissue (Zheng et al., 2019). A similar result was observed in mature mango plants as *SPL3*, *SPL4*, and *SPL5* were upregulated over time (Ahsan et al., 2019).

While the juvenile traits measured in *A. thaliana* were centered around leaf phenotype (Schwab et al., 2005; Wu and Poethig, 2006; Wu et al., 2009), other vegetative state- and root-associated traits were examined in alfalfa in an effort to improve harvest yield and quality (Aung et al., 2015). miR156 OE alfalfa plants showed reduced stem thickness (Aung et al., 2015). As expected, alfalfa with reduced *SPL9* transcript levels also displayed reduced stem thickness. Unlike flowering time, all three levels of *SPL9* silencing resulted in a reduction of stem thickness. Stem thickness was the only trait measured in this study to be impacted by reduced *SPL9* transcript levels after two months of growth. Similar to stem thickness, plant height was reduced after six months regardless of the level of *SPL9* silencing. However, plant height may be more sensitive to *SPL9* than stem thickness. Traits that were altered after six months but not after two months, such as plant height, may be a result of the greater sensitivity of that trait to *SPL9.* Over time, the levels of miR156 decrease resulting in enhanced expression of *SPL*s in non-transgenic plants (Ahsan et al., 2019; Wu and Poethig, 2006; Xu et al., 2020). Therefore, at later stages of plant development, the difference in the transcript levels between WT and *SPL9*-RNAi plants should increase, making traits with more sensitivity to *SPL9* more apparent. Internode length and branching were also altered in *SPL9*-RNAi compared to WT plants, but only after six months of growth. Increased branching was also observed in soybean plants with mutated *SPL9* (Bao et al., 2019). Therefore, the results of this study further support the role of *SPL9* in plant development.

Especially relevant to the agricultural industry was the observation by Aung et al. (2015) that miR156 OE resulted in alfalfa plants with increased biomass. Bao et al. (2019) also demonstrated that soybean plants with mutated *SPL9* had enhanced dry weight and

concluded that *SPL9* plays a role in miR156-mediated regulation of biomass. Despite branching, internode length, plant height, and stem thickness being impacted by the silencing of *SPL9*, *SPL9*-RNAi alfalfa did not show increased biomass. Both fresh weight and dry weight of *SPL9*-RNAi alfalfa shoots and roots were indistinguishable from WT plants leading to the conclusion that *SPL9* is not targeted by miR156 to impact alfalfa biomass. Seven alfalfa SPL members have so far been reported to have miR156 complementary regions (Aung et al., 2015; Gao et al., 2016) but there are at least 11 in *A. thaliana* (Addo-Quaye et al., 2008; Guo et al., 2008; Shikata et al., 2009; Wang and Wang, 2015; Wu and Poethig, 2006), seven in papaya (Xu et al., 2020), and 10 in tomato (Salinas et al., 2012). Possibly, additional SPLs could be present in alfalfa than have already been discovered that may regulate miR156-mediated traits. To observe changes in biomass, reduction in other alfalfa *SPLs* individually or in combination, may be necessary. In other words, reduction of *SPL9* alone, as is the case in *SPL9*-RNAi alfalfa, may not be sufficient. SPL family members other than SPL9 may also play a role in miR156-mediated developmental control. While it is possible that they could impact the same traits, it is also likely that yet to be characterized SPLs impact miR156-mediated traits different from those that are impacted by SPL9.

4.3 Involvement of SPL9 in alfalfa drought response

In addition to control over developmental traits, miR156 plays a role in abiotic stress response, specifically, miR156 is upregulated in plants exposed to drought (Sun et al., 2012; Bhardwaj et al., 2014; Cui et al., 2014). Arshad et al. (2017a) and Kang et al. (2020) demonstrated that miR156 OE in alfalfa and *N. tabacum*, respectively, displayed traits associated with drought tolerance. Furthermore, *NtSPL9* was downregulated in response to drought (Kang et al., 2020). To confirm the involvement of *SPL9* in alfalfa's response to drought, *SPL9* transcript level was compared between WT plants under drought and control conditions. Similar to observations of *NtSPL9* (Kang et al., 2020), *SPL9* expression was reduced in response to drought in WT plants suggesting that it is targeted by miR156 to impact drought tolerance.

Decreased *SPL9* expression in response to drought in WT plants alone is only enough to conclude that SPL9 is related to alfalfa response to drought. To determine if the degradation of *SPL9* transcript by miR156 is part of the miR156-induced drought tolerance demonstrated in alfalfa, the ability of *SPL9*-RNAi plants to resist the stress was evaluated. First, the number of leaves on the plant that were senescing was measured. Both WT and *SPL9*-RNAi plants experienced signs of drought stress; both had an increased percentage of senescing leaves under drought conditions compared to their control condition counterparts. However, the *SPL9*-RNAi plants had significantly less leaf senescence than WT plants under drought, indicating that *SPL9*-RNAi plants were more tolerant than WT to drought. In fact, WT plants were the first to display signs of drought stress which could be seen after 10 days of withholding water while *SPL9*-RNAi plants lasted 12 days before signs of drought stress were observed. Similar findings were observed by Kang et al. (2020) in which senescence-related genes were downregulated in *N. tabacum* plants with miR156 OE.

Secondly, growth of each plant, regardless of treatment, was evaluated by measuring the change in plant height over the course of the drought trial. Kang et al. (2020) observed that constitutive expression of *Zm*miR156 in *N. tabacum* resulted in plants with better growth under drought conditions. Interestingly, the plants with the highest *SPL9* transcript levels, WT and R1, were not able to maintain growth in response to drought, displaying reduced growth in drought stressed plants compared to well-watered plants. R2 and R3, which had the lowest *SPL9* transcript levels, were able to maintain plant growth despite the drought condition, thus providing further evidence for the conclusion that *SPL9* is downregulated to provide miR156-mediated drought tolerance. In addition, the maintenance of growth in plants with a high level of *SPL9* silencing suggests that tolerance regulated by *SPL9* can only be achieved if *SPL9* expression is maintained below a certain threshold level. This is in contrast with a similar finding reported for SPL13, where only *SPL13*-RNAi alfalfa plants with reduced *SPL13* levels but over a certain threshold showed significant drought tolerance (Feyissa et al., 2019).

Although, *SPL9*-RNAi plants displayed the aforementioned drought resistance traits, this did not translate to increased biomass. R2 had decreased DW compared to WT under

drought and control conditions while R1 had decreased DW under drought conditions. Tolerance to drought in *SPL9*-RNAi plants did not translate to biomass yield perhaps because SPL9 alone is not enough to regulate complete survival under drought. In *B. juncea*, although miR156 was upregulated in response to drought, a *SPL2*-like gene was also upregulated despite having complementarity to miR156 (Bhardwaj et al., 2014). Bhardwaj et al. (2014) suggested that due to the complexity of the miR156-*SPL* interaction, multiple SPLs could be present in *B. juncea* affecting drought response. Arshad et al. (2017a) and Feyissa et al. (2019) also established SPL13 as playing a role in miR156-induced drought tolerance, and therefore, considering *SPL9* is also targeted in response to drought, miR156 must target multiple downstream genes, including those belonging to the SPL family, to regulate drought response. While it was reported that miR156 OE alfalfa had increased survival compared to EV control plants (Arshad et al., 2017a), *SPL9*-RNAi plants survived drought conditions just as well as WT plants. In this study, *SPL9*-RNAi alfalfa had greater drought tolerance than WT plants, but both sets of plants had similar survivability rates.

4.4 Alfalfa water status is impacted by *SPL9*

One of the numerous responses to drought that was demonstrated in miR156 OE alfalfa was enhanced plant water status (Arshad et al., 2017a; Feyissa et al., 2019). Alfalfa plants with miR156 OE had diminished water loss and increased relative water content which was attributed to enhanced survival (Arshad et al., 2017a). Assuming these responses are a result of miR156-mediated silencing of *SPL9*, similar responses would be observable in *SPL9*-RNAi plants as was the case when RWC was examined. WT plants that were exposed to drought had significantly less RWC than well-watered WT plants. *SPL9*-RNAi plants on the other hand were capable of maintaining their RWC between drought and control conditions. In addition, the RWC of R1 and R2 was enhanced compared to WT when exposed to drought further, thus supporting that *SPL9*-RNAi plants were under less drought stress.

However, SPL9 appears, to differ from miR156 in how it regulates water loss. Arshad et al. (2017a) came to the conclusion that miR156 OE plants were able to resist drought as a

result of control over stomata that resulted in a decrease in water loss. In the current study, two of the three *SPL9*-RNAi genotypes were indistinguishable from WT in water loss under both drought and control conditions. Drought-treated R1 did have increased water loss after two hours and control-treated R1 had increased water loss after one hour. Therefore, SPL9 does not regulate drought stress tolerance through the mitigation of water loss. Increased water loss in R1 may explain why this genotype was unable to maintain growth to the same extent as the other two *SPL9*-RNAi genotypes. Although, according to this study, water loss is not regulated by SPL9, SPL13-RNAi plants had reduced water loss compared to EV control plants (Arshad et al., 2017a) supporting the idea that miR156 regulates multiple SPLs to control drought tolerance.

Decreased midday leaf water potential in R1 alfalfa exposed to drought further supports water loss as a cause of R1 deficiency to maintain growth under drought stress. R1 had decreased water potential in drought stressed leaves suggesting water loss. It is worth noting a limitation of the method to measure leaf water potential. I found that finding leaves on the plant with petioles long enough for the pressure chamber was crucial to the consistency of the data. As a result, it can be difficult to standardize which leaves are sampled from each plant, increasing the chances of variability in the measurements. Regardless, increased water loss explains the observation that shoot FW decreased in R1 plants in response to drought. In addition, Feyissa et al. (2019) demonstrated that *SPL13* is involved in miR156-mediated leaf water potential in alfalfa. Thus, as was observed in this study, *SPL9* may not be involved in this process.

4.5 Role of miR156-*SPL9*-*DFR* gene regulatory model in drought tolerance

While maintaining RWC was critical for drought tolerance in *SPL9*-RNAi plants, these plants also had enhanced accumulation of stress mitigating anthocyanins. Anthocyanins have a capacity for ROS scavenging, thereby preventing ROS build-up in cells due to abiotic stress (Ali et al., 2016; Azuma et al., 2008; Kähkönen and Heinonen, 2003; Rice-Evans et al., 1996). Stem tissues of miR156 OE *A. thaliana* were noted to have pigmentation typical of anthocyanin accumulation, whereas *SPL9* OE *A. thaliana* had

reduced anthocyanin content (Gou et al., 2011). Similar to *A. thaliana*, miR156 OE resulted in alfalfa plants with increased anthocyanin content (Feyissa et al., 2019). The spatial pattern of *DFR* expression was, however, inverse to known expression patterns of *SPL9* and *SPL15*; *DFR* levels were higher in basal stem plant tissue and low in apical tissues (Gou et al., 2011). In *A. thaliana*, SPL9 inhibits the formation of the transcriptional activating complex of *DFR* by competing with TT8 for binding to PAP1 (Gou et al., 2011). DFR is an enzyme in the biosynthetic pathway of anthocyanins (Aerts et al., 1999; Dixon et al., 2013; Gonzalez et al., 2008; Gou et al., 2011), and therefore miR156 can increase the biosynthesis of anthocyanins through the negative regulation of *SPL9*, allowing for the formation of the *DFR* transcriptional activating complex (Gou et al., 2011). Similar interactions have also been implicated in anthocyanin biosynthesis in soybean (Gupta et al., 2019). The interplay between miR156, SPL9 and *DFR* has been shown to mediate biosynthesis of anthocyanins to provide plants with increased tolerance to abiotic stress (Cui et al., 2014). *DFR* levels were also increased in miR156 OE alfalfa plants (Feyissa et al., 2019), and therefore a similar pathway could be present in alfalfa. Support for the involvement of miR156-SPL9-*DFR* pathway in alfalfa drought tolerance was immediately evident in the red pigments visible in *SPL9*-RNAi plants under control and drought conditions. The incidence of red tissue was also centered around the basal stem similar to Gou et al. (2011) that observed anthocyanin-related pigments exclusively in *A. thaliana* stems.

Through extraction and quantification of anthocyanins in basal *SPL9*-RNAi stem tissues, the role of SPL9 in regulating anthocyanin biosynthesis was established in alfalfa. Anthocyanin accumulation was increased in stems of all three *SPL9*-RNAi genotypes under drought, and R2 stems even had higher anthocyanin content compared to WT under well-watered conditions. Increased accumulation of anthocyanins in *SPL9*-RNAi stems supports the hypothesis that SPL9 is involved in anthocyanin biosynthesis in alfalfa, and because these plants additionally have increased tolerance to drought, this also indicates that miR156-mediated drought tolerance is due at least partly to the accumulation of anthocyanins.

Whether the interplay between SPL9 and *DFR* in alfalfa mirrors that in *A. thaliana* was investigated. Reduced *DFR* in *SPL9*-RNAi leaves is not unexpected as Gou et al. (2011) reported *DFR* expression in apical tissue was lower than that of basal tissue and anthocyanin pigments were concentrated to stem tissues. Therefore, *DFR* transcript levels were also measured in alfalfa basal stem tissue and due to the maintenance of *DFR* levels between conditions in R1 and R2 stems and increased *DFR* in R1 stems under drought, SPL9 must be involved in *DFR* regulation. Similar *DFR* transcript level patterns between WT and R3 stems suggest that a certain level of SPL9 abundance may be necessary for *DFR* regulation. Another possibility is that there is a SPL paralogue in alfalfa that is functionally redundant with SPL9. Wu and Poethig (2006) demonstrated that in *A. thaliana*, SPL3 acts redundantly with SPL4 and SPL5 and overexpression of any of these SPLs, when miR156 action was prevented, achieved similar phenotypic changes. Also, in *A. thaliana*, SPL15 operates redundantly with SPL9 (Schwarz et al., 2008; Yu et al., 2015). In fact, *spl15/spl9* double mutant has a stronger phenotypic change than single *spl15* and *spl9* mutants individually (Schwarz et al., 2008; Yu et al., 2015). A *SPL15* orthologue has not been identified in alfalfa but a yet to be identified SPL could be expressed in R3 at a higher level than the other *SPL9*-RNAi plants causing a decrease in *DFR* expression in response to drought. Interestingly, Feyissa et al. (2019) found that reduced levels of *SPL13* resulted in alfalfa plants with enhanced levels of *DFR* and anthocyanin content due to the direct interaction between SPL13 and *DFR* promoter.

4.6 SPL9 role in drought response is independent of antioxidant accumulation, root architecture and photosynthetic capability

Arshad et al. (2017a) reported that enhanced tolerance of miR156 OE alfalfa plants to drought was partly due to increased antioxidant activity. In addition to finding an increased level of antioxidant activity in miR156 OE plants, two genes related to antioxidant activity, *CAT1* and *GSH*, were upregulated in response to drought (Arshad et al., 2017a). N. *tabacum* plants with OE of miR156 also had enhanced expression of genes related to antioxidant activity (Kang et al., 2020). The antioxidant and drought traits were examined in *SPL9*-RNAi plants to determine if miR156-mediated antioxidant activity is

regulated through SPL9 in response to drought. Increased *GSH* transcript levels were observed in R3 compared to WT leaves under drought, and while WT leaves had significantly reduced *GSH* between conditions, *GSH* was maintained in *SPL9*-RNAi leaves between conditions. A similar result was seen when *CAT1* transcript levels were examined. WT leaves had enhanced *CAT1* in response to drought while again *CAT1* was maintained between treatments in *SPL9*-RNAi leaves. R2 even had enhanced *CAT1* compared to WT under well-watered conditions. Despite increases in the transcript levels of individual genes related to antioxidant activity, the overall activity of antioxidants was unchanged between *SPL9*-RNAi and WT plants. Therefore, SPL9 does not appear to play a large role in antioxidant regulation via CAT1 and GSH in response to drought stress in alfalfa.

Another trait that was affected in miR156 OE but not in *SPL9*-RNAi leaves was stomatal conductance. *SPL9*-RNAi leaves had the same stomatal conductance as WT plants under both control and drought conditions. Significantly reduced stomatal conductance between the conditions was observed in both *SPL9*-RNAi and WT leaves. As previously discussed, there are other miR156-targeted SPLs that regulate stomatal conductance. For example, Arshad et al. (2017a) demonstrated that *SPL13*-RNAi plants have similar increases in stomatal conductance as miR156 OE plants. Therefore, miR156-regulated drought tolerance may depend on multiple downstream SPL targets, which involve different stress tolerance strategies. Observations of stomatal conductance combined with chlorophyll content indicated enhanced photosynthetic capabilities of *SPL13*-RNAi plants (Arshad et al., 2017a). Similar to the result regarding stomatal conductance, SPL9 does not appear to regulate chlorophyll content under drought. *SPL9*-RNAi plants, except for R2, had decreased chlorophyll content between conditions. Therefore, the enhanced tolerance in *SPL9*-RNAi plants to drought is not a result of increased photosynthetic capabilities.

In contrast to *SPL13*-RNAi plants investigated by Arshad et al. (2017a) and Feyissa et al. (2019), *SPL9*-RNAi had no change in root architecture in response to drought. *SPL13*-RNAi genotypes displayed increased root length (Arshad et al., 2017a) while the root length of *SPL9*-RNAi plants was unaltered in response to drought. *SPL9*-RNAi roots

were also of similar length to WT plants under both control and drought conditions which is in contrast with the findings of Yu et al. (2015) that demonstrated a role for *SPL9* in the regulation of root length in *A. thaliana*. In addition, the DW of roots was unchanged both between and within control and drought treatments. FW of roots was maintained between drought and control conditions in R2 and R3 plants whereas root FW decreased in response to drought in WT and R1 plants. However, the finding that roots had reduced FW but consistent DW in response to drought supports the conclusion drawn from shoot FW; overall, plants with the highest levels of *SPL9* are losing water while those plants with the lowest *SPL9* are capable of mitigating the water deficiency as indicated by RWC.

4.7 Using CRISPR/Cas9 to silence *SPL9*

Successful *A. tumefaciens*-mediated alfalfa transformation was evaluated by searching for the presence of the exogenous *MtU6* promoter and *SpCas9* gene that were expected to be transferred as part of the CRISPR/Cas9 construct to target *MsSPL9* for mutagenesis. PCR amplification of gDNA extracted from potential *SPL9*-CRISPR plants using primers specific to *MtU6* and *SpCas9* were used to confirm the presence of these genes in the alfalfa genome and Sanger sequencing was utilized to determine if CRISPR/Cas9 system resulted in *MsSPL9* mutagenesis. Mutations of *MsSPL9* were not found in the region complementary to the CRISPR/Cas9 sgRNA, although the presence of *Cas9* and *MtU6* were confirmed. Therefore, attempts to produce *SPL9*-CRISPR alfalfa plants were unsuccessful.

Previously, utilizing CRISPR/Cas9 to edit *MsSPL9* was attempted but yielded low alfalfa genome editing efficiency (Gao et al., 2018). This study sought to improve the genome editing efficiency of the CRIPR/Cas9 system in alfalfa. First, while CRISPR-P (Lei et al., 2014) was utilized by Gao et al. (2018), the online tool has been updated and CRISPR-P 2.0 (Liu et al., 2017) was used in this study. As a result of the update, different sgRNAs were deemed ideal choices for CRISPR/Cas9 targeting of *MsSPL9*. Secondly, Gao et al. (2018) utilized the *A. thaliana U6* promoter, but the *Medicago truncatula U6* (*MtU6*) promoter was used in this study in an attempt to improve genome editing efficiency in

M. sativa by using a promoter from species in the same genus. Meng et al. (2017) had greater success in editing *M. truncatula* using its native promoter than Gao et al. (2018) using the non-native *AtU6* promoter. Despite these improvements, alfalfa genome editing was not observed in this study. One of the limitations of using CRISPR-P 2.0 (Liu et al., 2017) is that sgRNAs are designed based on the *M. truncatula* genome. CRISPR-P 2.0 (Liu et al., 2017) restricts sgRNA design to a finite list of reference genomes, which does not include *M. sativa*. Therefore, sgRNAs designed using this tool have to be altered to reflect the *MsSPL9* sequence. Another limitation of this study is that only a single sgRNA was used in the CRISPR/Cas9 design, while Gao et al. (2018) used two sgRNAs. According to the CRISPR-P 2.0 (Liu et al., 2017) sgRNA design rules and literature (Doench et al., 2014; Doench et al., 2016; Hsu et al., 2013; Liang et al., 2016) there was no other sgRNA with a high enough score to use. It is possible that the native *MsU6* promoter used in tandem with multiple sgRNAs designed using software with updated scoring rules would result in alfalfa editing with higher efficiency in the future.

4.8 Conclusion

Abiotic stress has the potential to significantly reduce crop yields (Daryanto et al., 2016), which adds to the growing problems of meeting the demands for an ever-growing human population (Godfray et al., 2010; Tilman et al., 2011). Economically important crops such as alfalfa must be improved in both quality and quantity in the face of these adverse conditions. When increasing the land upon which these crops are grown is not an option, crop yields on pre-existing agricultural areas should be improved (Tilman et al., 2011). A molecular tool to achieve this, miR156 has not only been demonstrated to increase alfalfa yields (Aung et al., 2015) but has also been linked to the regulation of abiotic stress tolerance (Arshad et al., 2017a; Arshad et al., 2017b; Feyissa et al., 2019; Matthews et al., 2019). miR156 functions by downregulating downstream genes to control different stress mitigating strategies (Rajwanshi et al., 2014). These downstream genes have not been fully characterized in alfalfa and present an opportunity to investigate the mechanism by which miR156 not only controls development but also tolerance to abiotic stresses, such as drought.

SPLs are a family of downstream targets of miR156 (Wang and Wang, 2015) and some SPLs, like SPL9, are largely uncharacterized in alfalfa. In this study, the impact of SPL9 on alfalfa developmental control was assessed by comparing *SPL9*-RNAi with WT plants. Since *SPL9*-RNAi plants displayed reduced stem thickness, internode length and plant height and enhanced branching, it can be concluded that SPL9 plays a role in the positive regulation of stem thickness, internode length and plant height and a negative role in branching (**Figure 18**). In addition, plant height, internode length and branching were found to be more sensitive than stem thickness to the level of *SPL9* silencing present, resulting in changes to these traits only being observed at later stages of plant development. miR156 OE plants also had similar traits according to Aung et al. (2015) therefore establishing that, either alone or with other SPLs, reduced levels of *SPL9* mediated by miR156 result in phenotypic changes related to plant development.

Not only does SPL9 impact alfalfa development, but it also regulates response to drought, as plants with silenced *SPL9* showed improved tolerance. Building on the observation by Arshad et al. (2017a) and Feyissa et al. (2019) that miR156 OE plants also had increased tolerance to drought, *SPL9* was reduced in response to drought in WT alfalfa. This study, combined with previous investigations of SPL13 (Arshad et al., 2017a; Feyissa et al., 2019), provided evidence that multiple SPLs are targeted by miR156 in response to drought in alfalfa. SPL9-RNAi plants had reduced leaf senescence under drought conditions, and this resulted in the maintenance of growth despite the adverse drought conditions. Drought trials regarding *SPL9*-RNAi plants revealed that a certain level of silencing is required for improved tolerance to be observed; with the plants with the lowest expression of *SPL9* having the best tolerance. Unlike the case of plants with silenced *SPL13*, which displayed drought tolerance due to enhanced root length, photosynthetic capability, and water retention (Arshad et al., 2017a; Feyissa et al., 2019), drought tolerance in *SPL9*-RNAi plants could be attributed to enhanced anthocyanin content contributing to improved ROS scavenging (**Figure 18**).

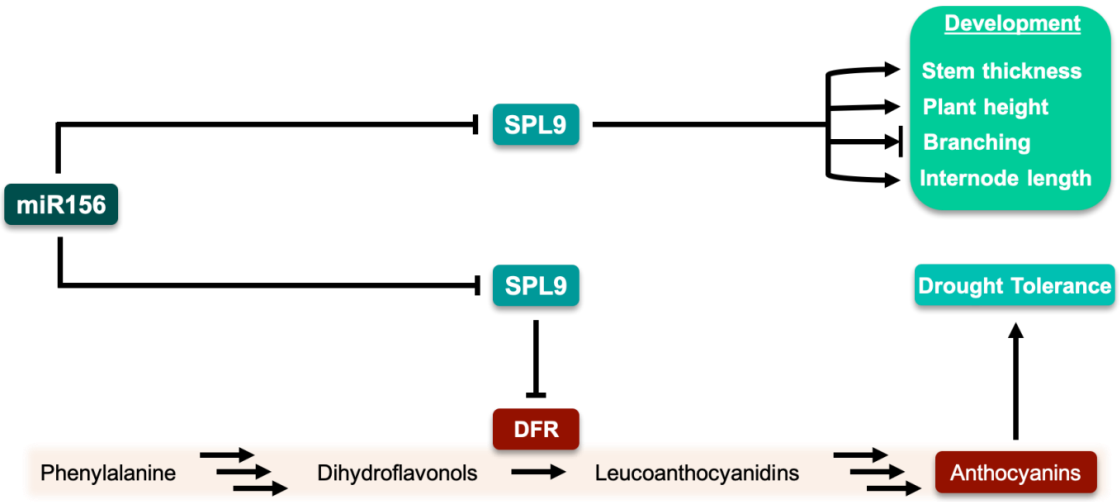

Figure 18. A model for the miR156/SPL9 module in regulating plant development and drought stress

In this study, SPL9 was found to positively regulate plant height, stem thickness, and internode length and negatively regulate branching as part of the miR156 control over plant development. Reduced levels of *SPL9* also resulted in an increase in anthocyanin biosynthesis supporting its negative regulation of *DFR* transcription in alfalfa.

Chapter 5

5 Future Directions

Determination of the role of SPL9 in alfalfa development in this study was based on the evaluation of plants with reduced *SPL9.* Consensus between the three *SPL9*-RNAi plants in phenotypic traits was only seen in stem thickness and plant height. Transgenic alfalfa with increased *SPL9* should also be evaluated. Examining phenotypic traits in *SPL9m*-OE alfalfa could further uncover the role of *SPL9* in alfalfa development.

While drought stress was the focus of this study, miR156 has been found to mediate tolerance to a number of different abiotic stresses in alfalfa, including salinity and heat (Arshad et al., 2017b; Matthews et al., 2019). The involvement of SPL9 in abiotic stress tolerance may not be limited to drought. Thus, the role of SPL9 in miR156-mediated tolerance to other abiotic stresses should be evaluated. In addition, the exact nature of the mechanism through which SPL9 exerts control over anthocyanin biosynthesis should be examined further. Direct interaction between SPL9, PAP1 and *DFR* was reported in *A. thaliana* (Gou et al., 2011), but has not been examined in alfalfa. The upregulation of anthocyanins in alfalfa plants with reduced levels of *SPL9* supports the model investigated by Gou et al. (2011), but further investigation is required to dissect the mode of action in alfalfa.

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Appendices

Appendix A

Primers utilized and the project in which they were used.

Primer Name	Primer Sequence	Primer Use
SPL9RNAi_F	CACCCTCTCTCTTCTGTCAAATCAAACA TGGG	SPL9-RNAi construct cloning
SPL9RNAi_R	TTACAGTGACCATTGAGAAGATTCATA GG	SPL9-RNAi construct cloning
LA-MsSPL9-Fq1	AGATACAGCTCTTGCTACTG	qPCR – *SPL9* transcript level
LA-MsSPL9-Rq1	GTTGAGAATGTTGAACTGAC	qPCR – *SPL9* transcript level
DFR-F	GTTTGTGTCACAGGGGCTTC	qPCR – *DFR* transcript level
DFR-R	TTCAAGTTTTCTGGGTCGCG	qPCR – *DFR* transcript level
CAT1-F	GAAAAGCTTGCCCAATTTGA	qPCR – *CAT1* transcript level
CAT1-R	ATGACGGGTGTCTGAACTCC	qPCR – *CAT1* transcript level
GSH-F	ACGCTTCCCAGCTTTAATGA	qPCR – *GSH* transcript level
GSH-R	CCCCAACAAGAAGACCATTG	qPCR – *GSH* transcript level
ms_ADF1qF	TCAAGGCGAAAAGGACACAC	qPCR (reference gene) - *MsADF*
ms_ADF1qR	AAAACAGCATAGCGGCACTC	qPCR (reference gene) - *MsADF*
ms_EIF4AqF	TGCTAAGTTGCCTGAAACCG	qPCR (reference gene) – *MseIF4A*
ms_EIF4AqR	TGCCCATGTTTTCACCTTGC	qPCR (reference gene) – *MseIF4A*
LH_Cas9_F1	CCAGAGAAAATCAGACCACA	PCR – *SpCas9* amplification
LH_Cas9_R1	CTTGAGGCATAGAGAGAACC	PCR – *SpCas9* amplification
MtU6-F	GCTTAGGCCTTCTAGAATCCAACATTTC ACTTGAGTTAACT	PCR – *MtU6* amplification
MtU6-R	AAACCCTGCTGTTCGTCTAG	PCR – *MtU6* amplification
Scaffold-R	GGCAACGCGTTCTAGAAAAAAAAGCAC CGACTCGGTGC	PCR – *MtU6*:sgRNA amplification

Appendix B

Full length *Medicago sativa SPL9* cDNA (Gao et al., 2016) with the protein coding sequence highlighted in yellow, the miR156 recognition sequence shown in red bolded text, SPL9RNAi_F/R primers labelled with a single underline, LA-MsSPL9-Fq1/Rq1 primers labelled with a double underline, and the portion of the sgRNA used to target *MsSPL9* using CRISPR is shown in black bolded text.

ATACGCTGCACTGCATCACTGCACTGAACTGGGTTCCTGCCAACAATACATATGGCCCAGCAGTGTCAACTC
ACTTCTTCCTTCCACCTCTTCCTCTTTTGTCTTCCACTTCACTTTTCCACTATCATTCACTCTCTTCTTTTT
TTAATGTTTTCACTTTAACCAACAATAACAACCACCACTCCCCAGACCCCCCTCACCTATACTATTCTCTCA
CTCTTACACTCACACTTCACTTCACTTCACTTATCTCTCACCAATGAACACAATCTAGCAACCACCACCAAA
CCTCACACCAATGGATTCAGGAGGCAACTCTTCTTCGGAAGAGTCCTCTCTTAATGGCTTAAAATTTGGCCA
ACGAATCTATTTTGAAGATACAGCTCTTGCTACTGCTGCTGCTGCTACTTCTACCACCATTGCTGCTAGTTC
TTCTTCTTCTTCTGGTTCAAAGAAAGGAAGAGGTGGGTCAGTTCAACATTCTCAACCACCTCGGTGTCAAGT
TGAAGGATGTAAACTAGATCTGACTGATGCTAAAGCTTACTATTCTAGACACAAAGTTTGTAGCATGCACTC
TAAGTGCCCAACTGTTACTGTTTCTGGTCTACAACAAAGGTTTTGTCAACAATGTAGCAGATTTCATCAGCT
TGCTGAGTTTGATCAAGGAAAAAGAAGTTGCCGGAGACGACTAGCTGGTCATAACGAGCGTCGCAGAAAGCC
CCCACCCAGCTCTCTCTTAACCTCACGTTTTGCCAGGCTTTCTTCATCTGTTTTTGGTAACAGTGACAGAGG
TGGCAGCTTCTTGATGGAATTTGCTTCAAACCCAAAACTTAGTCTGAGAAATTCACTTCCACCACCCGGAAA
TCAGACCACAACAATCGGTTGGCCTTGGCCGGGGAACACGGAGTCGCCATCTGACAACCTTTTCTTGCAAGG
TTCGGTGGGTGGGACAAGCTTCCCTGGTGCCAGGCATCCTCCCGAGGAAACTTACACCGGAGTCACAGATTC
AAACTG**TGCTCTCTCTCTTCTGTCAA**ATCAAACATGGGGTTCTCGAAACACAGAACCAAGTCCTGAATTGAA
TAACATGCTGAATTTCAATGGGACATCCATGACACAACATGCTACATCTTCTCATGGTGTAGCCATGCATCA
AATTCCAAACAATTACGAGGTTGTCCCTGATCTTGGTCGGGGTCACATTTCGCAGCCTCTTGGTAGCCA**ACT**
CTCTGGTGAGCTTGATCTGTCGCAGCAGGGAAGGAGGCATTATATGGATGTAGAACATTCCAGGGCCTATGA
ATCTTCTCAATGGTCACTGTAATGCACTTGTTTGCTTTCAGGTTTGTAATAACATGTTTCACAAATATTTGA
ACTCAGGAAAGTGAGAAGTGAACTAAGGCATACTTGATGCTCTTGCTTGTTTTGGTTTGTTTAAACTGTTAG
GCAAGGTGGGGCTAGCCTTGCTTCACTTTGTGGTTTGTAATCTCTTCCTAGTTATTTGAGATTATCATGGTT
TCAAATTTCAGGAAGTTGTTTGATGTGGATTTGGTTGCACCTTTGTAGCATTGTGATTGTGAAAATTGCAAA
TAAATGTTGCAATAGCGGCTTGAATCAATTTTATGTTTGCATTGAATGAT

Curriculum Vitae

Name: Alexandria Hanly

Post-secondary Education and Degrees:
McMaster University
Hamilton, Ontario, Canada
2012-2017 B.Sc.

The University of Western Ontario
London, Ontario, Canada
2018-2020 M.Sc.

Honours and Awards:
McMaster Entrance Scholarship
2012

Related Work Experience
Laboratory Assistant
AAFC Saskatoon Research and Development Centre
2015

Laboratory Assistant
AAFC London Research and Development Centre
2016

Teaching Assistant
University of Western Ontario
2018-2020

Presentations:
Hanly A, Amyot L, Karagiannis J, Hannoufa A. (2019) Characterization of the role of *SPL9* in drought stress tolerance in *Medicago sativa*. Plant Canada; Guelph, Ontario. [Poster].